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TRACE AND ULTRA-TRACE ANALYSIS OF NATURAL SUBSTANCES OF TERPENOID CHARACTER

HABILITATION THESIS

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Your time is limited, so don't waste it living someone else's life. Don't be trapped by dogma, which is living with the results of other people's thinking. Don't let the noise of others' opinions drown out your own inner voice. And most important, have the courage to follow your heart and intuition.

-Steve Jobs-

ACKNOWLEDGEMENT

CONTENT

ABBREVIATIONS

Ab	antibody
ACN	acetonitrile
BR	brassinosteroid
CE	capillary electrophoresis
DART-MS	direct analysis real-time mass spectrometry
DMAPP	dimethylallyl diphosphate
DW	dry weight
EC	ecdysteroid
EI	electron impact
ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light scattering detector
ESI	electrospray ionisation
FA	formic acid
FW	fresh weight
GA	gibberellin
GC	gas chromatography
GGPP	geranylgeranyl diphosphate
IA	indole alkaloid
IPP	isopentenyl diphosphate
LDR	linear dynamic range
LC	liquid chromatography
LOD	limit of detection
LOQ	limit of quantitation
MAO	monoamine oxidase
MEKC	micellar electrokinetic chromatography
MeOH	methanol
MEP	2-C-methyl-D-erythritol 4-phosphate pathway
MIA	monoterpene indole alkaloid
MIP	molecularly imprinted polymers
MS	mass spectrometry

ABBREVIATIONS

MSI	mass spectrometry imaging
MSPE	magnetic solid-phase extraction
MVA	mevalonate pathway
MW	molecular weight
PE	phytoecdysteroid
QQQ	triple quadrupole analyser
RIA	radioimmunoassay
SFC	supercritical fluid chromatography
SPE	solid-phase extraction
Trp	tryptophan
UHPLC	ultra-high performance liquid chromatography

I. INTRODUCTION

This work focuses on using modern separation and detection methods for the quantitative analysis of biologically important, low molecular weight (MW) substances in tissues of plant origin. Expert knowledge and tools from the field of analytical chemistry are applied to biological sciences here. A speciality of this work is determination of natural substances of plant origin that naturally occur in **trace and ultra-trace quantities**. According to the still valid IUPAC definition from 1979, a trace amount is considered a concentration not exceeding 100 ppm, *i.e.,* 100 $\mu g \cdot g^{-1}$ [1], whereas no formal definition has been provided for an ultra-trace amount. However, most of the scientific community in biochemical/ biological research regard trace analysis as the analysis of substances whose content in biological material does not exceed 1 ppm (1 $\mu g \cdot g^{-1}$) [2]. Another common feature of the natural substances detailed in this work is their **terpenoid** character.

Despite a widespread belief among the public that trace/ultra-trace analysis of natural substances is of minimal importance, it is a field of analytical chemistry/biochemistry that significantly contributes to understanding not only the growth and development of plants but also the occurrence of natural substances in various plant sources. In past decades, many materials of plant origin were considered to lack certain compounds due to the limited detection capabilities of the available analytical methods. Notably, for some reason, many substances are still not perceived as natural constituents of plants by a large proportion of the public, *e.g.*, steroid compounds like progesterone, testosterone and oestrogens, described in detail in **Article 1**. Therefore, they are often referred to as "contamination" from animal sources, although plant cells are also capable of their biosynthesis.

One should keep in mind that the plant and animal kingdoms have evolved together hand in hand. Therefore, it is likely that they share many biochemical and other tools for regulation of their development, growth and defence against predators or adverse environments. Every substance synthesised in a eucaryotic cell (plant or animal) plays a certain role at a cellular or higher level. Substances formed in concentrations regarded as trace or ultra-trace have no less biological significance than those produced in larger amounts. As an illustration, we could use the example of cholesterol. Both animal and plant cells are capable of its biosynthesis. It is an important C_{27} molecule of triterpenoid

character that is an indispensable precursor for the synthesis of other substances of steroid character, including hormones and signalling compounds with a steroid skeleton. Its levels are significantly lower in plant cells ($< \mu g \cdot g^{-1}$). Consequently, some biochemistry textbooks contain various false or at least misleading statements, such as "Membranes of plant cells do not contain cholesterol" [3], or "cholesterol is rarely present in plants" [4]. It seems that some authors view the "very small" amount as equating to "zero" [5], which is, objectively speaking, not correct. There are also other cases similar to cholesterol that could be described in a separate publication.

I consider that today's analytical chemists can play an important role in the field of biological sciences by using currently available technically highly advanced tools to obtain new knowledge as well as expanding the available methods. This thesis aims to show my contribution to the study of the biosynthesis, metabolism and occurrence of low-abundance natural constituents of plant cells with terpenoid character.

II. TERPENOIDS

The terpenoids are a huge and structurally diverse group of natural substances formed from five-carbon (C_5) units of isoprene (2-methyl-1,3-butadiene) via "head-to-tail" addition, as shown around 70 years ago by Ruzicka (1953) [6], a Croatian chemist of Czech origin who had already been awarded the 1939 Nobel Prize in Chemistry for his work in this area [7]. Compounds formed by combining isoprene units are now also known as *isoprenoids*. Although isoprene is a naturally occurring substance formed by the decomposition of various cyclic hydrocarbons, it is not itself a direct participant in terpenoid biosynthesis. Its biochemically active and biologically important forms are dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP). Various numbers of these C_5 isoprenoid units can be combined to form linear monoterpenes (C_{10}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes (> C_{40}) [8]. These linear molecules often undergo extensive intracellular modification, typically involving cyclisation, oxidation or rearrangement reactions. Importantly, isoprenoids are found in all living organisms, including eukaryotes (plants, animals, fungi) and prokaryotes (microorganisms). Several thousand isoprenoids have been identified to date, and new family members are regularly discovered. Studies on their biological effects have shown that they play many different roles in nature, acting as attractants, repellents, hormones, growth inhibitors, pigments and electron transport chain components (quinones). The most extensively studied essential isoprenoids are a family of tetracyclic triterpenoids known as sterols, which are found in both the animal and plant kingdoms, *i.e.,* in most eukaryotes. In this thesis, I will focus on a group of terpenoid substances referred to as plant hormones or signalling substances derived from isoprene. An overview article on their biosynthesis and biological significance is enclosed as **Article 2**.

II.I. PLANT HORMONES

Most (if not all) organisms use chemical signals in cell–cell communication. Thus, chemical signalling is extremely ancient. However, the complexity of cell signalling increased considerably when first prokaryotic and subsequently eukaryotic cells began to associate together in multicellular organisms, putatively several billion and one billion years ago, respectively [9]. Following the emergence of multicellularity, cell specialisation increased as tissues and organs with diverse specific functions evolved. Coordination of the growth and development of these cells, tissues and organs, as well as the environmental responses of complex multicellular organisms, required increasingly intricate signalling networks. Many of our current concepts about intercellular communication in plants were derived from similar animal studies in which two main systems evolved, *i.e.,* the nervous and endocrine systems. Plants lacking motility never developed a nervous system, but they evolved hormones as chemical messengers.

A hormone is generally considered to be a signalling molecule secreted by an organism from glands and subsequently transported by the circulatory system to target organs to regulate their physiology and behaviour. However, the term "hormone" is sometimes extended to include compounds produced by cells that subsequently affect either the same cell or nearby cells. In target cells, hormones bind to specific receptor proteins, causing a change in cell function and activation of the entire signalling pathway. Unlike animals, plants have no specific hormone-secreting glands, but small signalling molecules are still produced in them, known as **plant hormones**. They are substances that regulate cellular processes in certain target cells near to their site of production or after transport to other organs where they are supposed to operate. Plant hormones play essential roles in the regulation of a myriad of physiological processes involved in plant growth, development, senescence and responses to environmental stimuli. Until the 1990s, only five types of plant hormones that acted *in vivo* individually or in concert were known: auxins, cytokinins, gibberellins, ethylene and abscisic acid. However, during the last two decades, compelling evidence has led to the discovery of four other classes of substances that exhibit growth and development activity, *i.e.,* brassinosteroids, strigolactones, jasmonic acid and salicylic acid, including their conjugates with amino acids and saccharides, which are collectively referred to as jasmonates and salicylates [10]. Surprisingly, there is unfortunately no official definition of a plant hormone available

in the literature. However, this group generally includes any compound or group of structurally related compounds that meet the following criteria:

1. The compound(s) occurs uniformly in all higher plants (often also lower ones).
2. The receptor for the compound(s) has been identified.
3. The compound(s) occurs in very low concentrations (usually 10^{-15} to 10^{-12} g·g^{-1}).
4. The compound(s) participates in the regulation of physiological processes in plants, such as growth, development, reproduction and others.

II.I.1. DITERPENOID PLANT HORMONES GIBBERELLINS

Gibberellins (GAs) are a group of plant hormones with a tetracyclic *ent*-gibberelane skeleton consisting of 20 carbon atoms (with rings designated A, B, C and D) or with a 20-nor-*ent*-gibberelane skeleton possessing 19 carbon atoms. Therefore, in terms of carbon numbers, GAs can be divided into two groups: C_{19}-GAs (*e.g.,* GA4, GA1) and C_{20}-GAs (*e.g.,* GA12, GA53) - **Fig. 1** [11]. The prefix *ent* indicates that the skeleton is derived from *ent*-kaurene, a tetracyclic hydrocarbon that is enantiomeric to the naturally occurring compound kaurene.
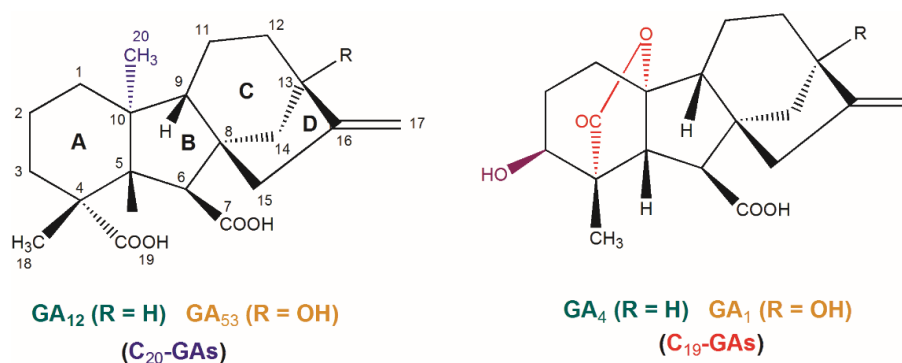

GA$_{12}$ (R = H)   GA$_{53}$ (R = OH)
(C_{20}-GAs)

GA$_4$ (R = H)   GA$_1$ (R = OH)
(C_{19}-GAs)

Figure 1. Structures of C_{19} and C_{20} gibberellins.

All GAs are produced in plant cells from geranylgeranyl diphosphate (GGPP), which is the common C_{20} precursor for all diterpenoids and is formed *de novo* from the basic C_5 isoprenoid diphosphates IPP and DMAPP (see page 19). A detailed description of GA biosynthesis can be found in **Article 2**. The main function of GAs in plants is the regulation of various developmental processes, including stem elongation (internodes),

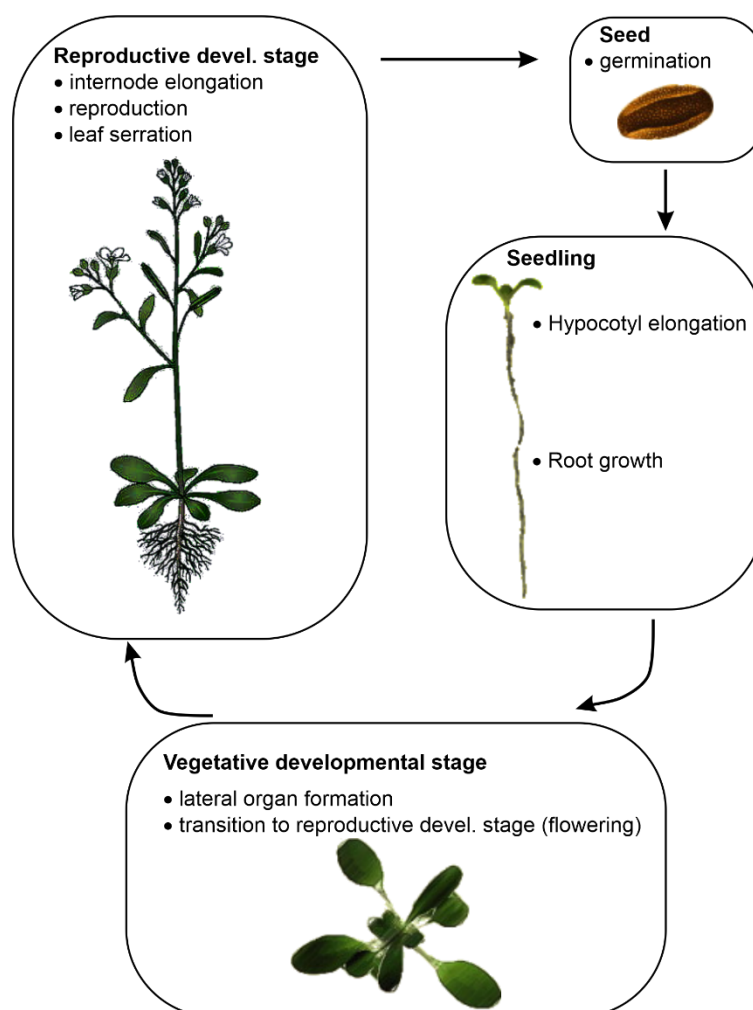germination, vegetative growth, flowering and reproduction – the formation of flowers and fruits – **Fig. 2**.

Figure 2. Scheme of physiological functions of diterpenoid plant hormones gibberellins.

Since their discovery in 1938 [12], 136 gibberellins have been identified in various natural sources to date. The nomenclature for labelling these substances is GA_1 to GA_n, where *n* stands for the order of discovery in natural sources. A database of all compounds in this group is freely available at http://www.phytohormones.info/gibberellins.htm. Interestingly, among all the GAs, only six of them show biological activity [13]. The crucial structural moieties for their biological activity are a hydroxy group on C-3 and a carboxyl group on C-6. These groups enhance binding to receptors through interaction with polar amino-acid residues, whereas a hydroxy group on C-2 decreases the binding affinity.

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Therefore, hydroxylation at C-2 is a main mechanism for GA inactivation to regulate the concentration of bioactive GAs in angiosperms (flowering plants).

Like other classes of plant hormones, concentrations of GAs in plant tissues are usually extremely low (generally $pg \cdot g^{-1}$ fresh weight, FW). Thus, very sensitive analytical methods are required for their detection. However, levels of GAs may vary substantially even within a single plant organ. Vegetative tissues (stems, roots and leaves) typically contain several $pg \cdot g^{-1}$ FW, whereas reproductive organs (such as seeds and flowers) often have three orders of magnitude higher levels (*i.e.,* $ng \cdot g^{-1}$ FW). Despite the hormone definition above, it is important to note that not all GAs are present in all plant species. Some are common to most plants, whereas others are species-specific. For method development of their isolation from plant tissue, including sensitive detection, 20 GAs were selected (**Fig. 3)** that occur in most plant species and *Arabidopsis thaliana,* the main model plant in biology. Therefore, the developed method offers great potential for applications in experimental plant biology.
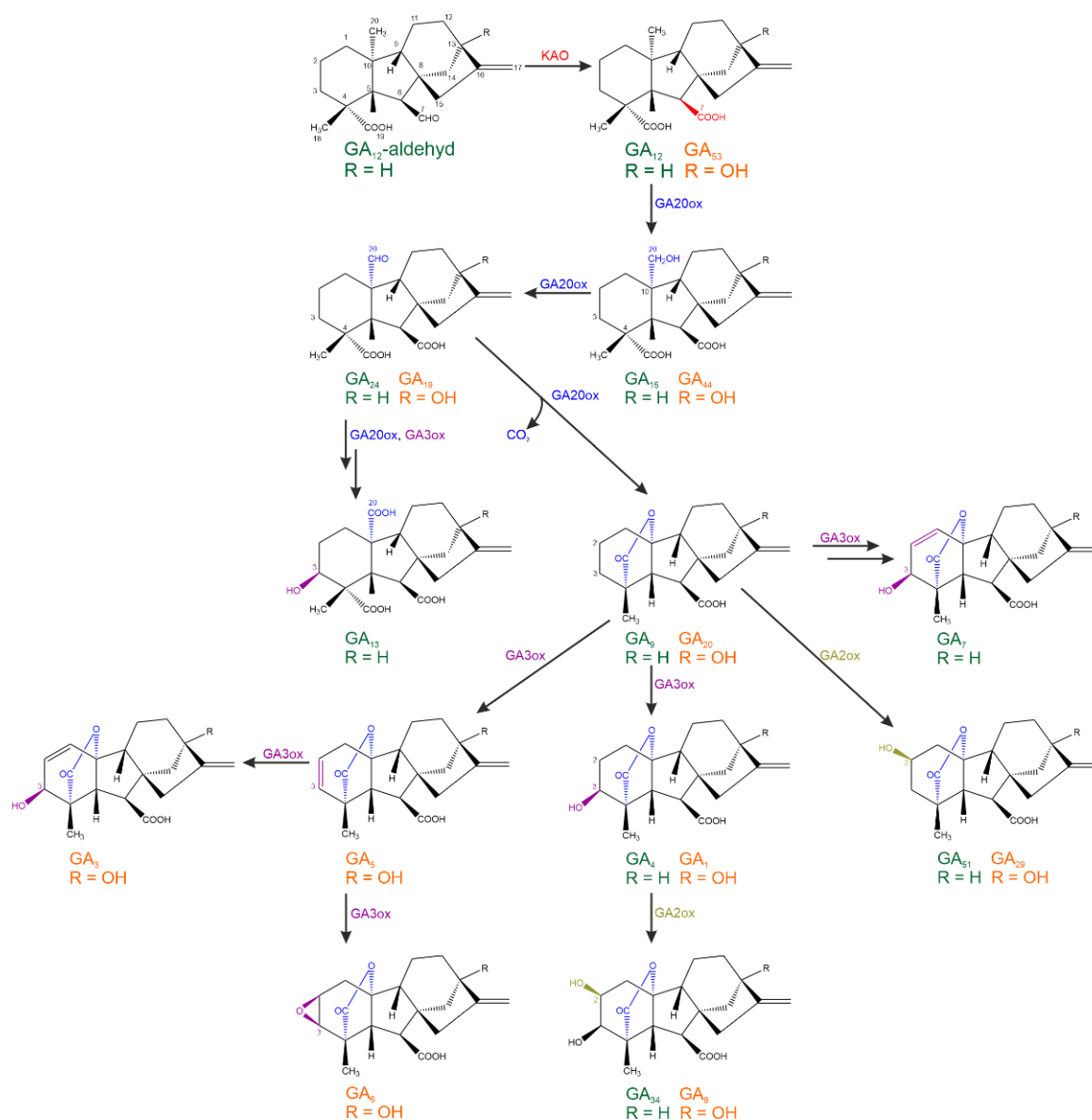
KAO - cytochrome P450 monoxygenase (P450), heme-containing enzyme that catalyses oxidations of a broad range of organic compounds

GA20ox, GA3ox, GA2ox - 2-oxoglutarate-dependent dioxygenases (2ODDs), soluble enzymes catalysing oxidations of various organic compounds and using 2-oxoglutarate as co-substrate

GA20ox catalyses sequential oxidation of C-20 including the loss of C-20 as CO_2 and the formation of γ-lactone. Thus, GA20ox is responsible for the production of C_{19}-GAs using C_{20}-GAs as substrates.

GA3ox converts inactive precursors (GA_9, GA_{20}) into bioactive GAs (GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7)

GA2ox initially identified to use C_{19}-GAs as substrates, including bioactive GAs and their immediate precursors GA_9 and GA_{20} (Thomas et al., 1999); later, a new type of GA2ox that accepts only C_{20}-GAs was reported (Schomburg et al., 2003; Lee and Zeevaart, 2005)

Figure 3. *Chemical structures and biosynthetic relationships of 20 selected gibberellins. KAO - ent-kaurenoic acid hydroxylase; GA20ox - GA 20-oxidase; GA3ox – GA 3-oxidase; GA2ox – GA 2-oxidase.*

Physicochemical properties of gibberellins

GAs cover a wide range of polarities, even within their sub-groups. The only property they share is they contain a carboxyl group, and thus behave as weak organic acids with pKa ≈ 4.0 [14]. Due to the structure of the skeleton, they do not show any spectral characteristics, such as fluorescence or UV absorption (only below 220 nm), that could be used for their detection. Some GAs are highly oxidised molecules with many functional groups and may be relatively labile, especially in aqueous solutions at extreme pH or elevated temperatures. Under alkaline conditions, 3β-hydroxy GAs undergo a reversible retro-aldol rearrangement, resulting in epimerisation, whereas 1,2-dehydro C_{19}-GAs, such as GA_3, isomerise to 19,2β-lactones with a shift of the double bond. This rearrangement may also occur in the heated injection port of a gas chromatograph (GC).

On the other hand, under very acidic conditions below pH 2.5, rearrangement of the C/D rings and saturation of the double bond between carbons C-16 and C-17 can occur. Therefore, all the above facts must be considered when isolating GAs from samples of biological origin. Consequently, the optimal pH range for these procedures is 2.5–8.5 and the temperature, especially of aqueous solutions containing GAs, should not exceed 40 °C. To avoid air oxidation of some GA precursors, extracts are best stored at –20 °C.

Extraction and preconcentration of gibberellins

The extraction efficiency of GAs from plant tissue depends on their polarity, subcellular localisation and extent to which they are associated with other compounds in the tissue, such as phenolics, lipids, pigments and proteins. Such substances, mostly originating from primary metabolism, are usually present in plant tissues in multi-fold excesses compared to trace amounts of GAs, creating a very high chemical background in analysed extracts. Figuratively speaking, attempts to quantify ultra-trace amounts of GAs in a vast array of interferents can seem like looking for a nanosized needle in a skyscraper-sized haystack. This is a common feature of ultra-trace analysis of most plant hormones, as detailed in **Article 3**. Hence, extraction and preconcentration of GAs is a key step for their successful detection using modern instrumental methods. As practice shows, without at least one or two preconcentration steps, it is impossible to quantify natural substances at ultra-trace levels successfully with any modern instrumental method, despite several studies claiming to do so [15-17].

The solvent used in the extraction process must be capable of extracting the hormone efficiently while minimising the extraction of interfering substances. Because GAs are synthesised inside cells, it is necessary to *break the cell walls* as a first step of the sample preparation procedure. This allows the most quantitative transferal of GAs from the biological material to the extraction solvent added in a subsequent step. Homogenisation (breaking cell walls in a sample tissue) is often achieved mechanically with the help of homogenisers working on the principle of ball mills (Article 3). GAs are relatively hydrophobic carboxylic acids. Therefore, aqueous solutions with more than 80 % (v/v) *organic solvent* content, such as methanol (MeOH) or acetonitrile (ACN) containing a small percentage of volatile organic acids (*e.g.,* formic acid (FA) or acetic acid), are best suited for their extraction [11]. By optimising the extraction procedure, it was found that the highest recovery and lowest content of interfering substances, mainly plant pigments like chlorophyll, carotenoids, etc., can be achieved by using an 80 % aqueous solution of ACN with 5 % FA, as described in detail in **Article 4**. After extraction, a preconcentration step is needed. Preconcentration of GAs from a biological sample can in principle be achieved in two ways: isolation of only the target analytes as selectively as possible or the sequential non-selective removal of interferents from the crude extracts, leaving GAs in the sample residue. Both these procedures have pitfalls. For the first procedure, either immunoaffinity chromatography (IAC) with the use of antibodies (Abs) against the observed GAs [18-19] or molecularly imprinted polymers (MIP) in the form of *solid phase extraction (SPE)* cartridges can be applied. Unfortunately, neither IAC- nor MIP-based sorbents are currently available for GAs. The impossibility of preparing an Ab with cross-reactivity against all 20 selected GAs, including both C_{19}- and C_{20}-GAs (earlier prepared Abs were available for C_{19}-GAs only [20]) and the necessity of using animal experiments (immunisation) currently hinders the use of IAC-based procedures. Further, the absence of a template or mixture of templates suitable for the preparation of a polymer with the ability to selectively bind a wide range of GAs complicates the use of MIP.

Thus, GA preconcentration from plant extracts is usually performed by one of two non-selective approaches. The first, a kind of "old fashioned", approach is a very laborious and time-consuming (several days) sequence of several steps, including liquid-liquid extraction (LLE; methanolic extract fractionated to ethyl acetate), ion-exchange chromatography using the strong anion-exchange sorbent QAE Sephadex A-25, reversed-

phase (RP) SPE with C_{18} sorbent in the final step, followed by HPLC fractionation [20,21]. The final analysis of GAs is then performed using GC coupled to a mass spectrometer (MS). Since GAs have low volatility, it is necessary to derivatise the analytes prior to GC-MS analysis. This is achieved in two steps by combining methylation followed by silylation reactions [20,21]. The second GAs preconcentration procedure is similar to the first one, but the initial step of LLE is replaced by two SPE steps, followed by use of a weak anion exchanger (DEAE cellulose). HPLC fractionation is here omitted [22]. A preconcentration design consisting of two SPE steps has also been used by Moritz and Olssen [23], who used a weak anex as the first step (sorbent containing an aminopropyl group), followed by SPE with an RP sorbent C_{18}. The sample at this stage was referred to by the authors as "semi-purified" because it was not subjected to routinely used HPLC fractionation prior to derivatisation and GC-MS analysis. As stated in Article 4, the entire preconcentration procedure can be reduced to only two SPE steps to achieve an average recovery of analytes of > 73%. Considering the relative hydrophobicity and acidic character of GAs, modern mixed-mode polymer-based sorbents containing a combination of a RP and anion exchanger were used to preconcentrate these low-abundance hormones from crude plant extracts. In addition, by using 80 % ACN instead of the previously routinely used 80 % MeOH, the recovery of GAs was increased by about 18 %. Unfortunately, the recovery values of this new preconcentration procedure cannot be compared with the recoveries of earlier procedures, as the authors of these works did not report this value. Unpublished results of the author of this thesis further suggest that it could be possible to shorten the entire procedure even further to one SPE step while maintaining or increasing the recovery of most monitored analytes. This can be achieved by significantly reducing the weight of the tissue sample, with concomitant reduction in the amount of interferents. This work is currently being completed and prepared for publication.

Methods for quantitative analysis of gibberellins

As mentioned above, typical endogenous concentrations of GAs in vegetative plant tissues range from 10^{-15} to 10^{-12} g·g^{-1}. Hence, their analysis falls into the field of **ultra-trace analysis**. The first published methods for the instrumental analysis of GAs were in the 1960s and involved the use of GC-MS combined with the earlier mentioned derivatisation of naturally non-volatile GAs using methylation and trimethylsilylation

[24-26]. During the next 30 years, liquid chromatography (LC) gradually replaced GC because it better suits the non-volatile nature of GAs [27-29]. LC is still mostly used for their separation [11,22, Article 3,4].

A successfully applied principle for the separation of relatively hydrophobic tetracyclic GAs is RP chromatography. Unfortunately, there were only slight differences in hydrophobicity among the 20 studied GAs because their chemical structures were similar. Their MW range was relatively narrow (315 to 377 g·mol^{-1}). Several GAs in this group had the same MW because they were positional *isomers*, *e.g.,* GA3, GA6, GA44 and GA24 (MW 346.38 g·mol^{-1}) or GA20, GA51, GA4 and GA12 (MW 332.4 g·mol^{-1}). The situation was further complicated because the *standard isotope dilution method* was used for their most accurate quantification by LC-MS. Only GA standards labelled with two deuterium atoms, *i.e.,* 17-[^{2}H$_2$], are commercially available for that purpose. Thus, a situation where many more analytes share the same MW value arises, *e.g.,* GA29, [^{2}H$_2$]-GA3, GA1, [^{2}H$_2$]-GA6, [^{2}H$_2$]-GA44, GA34, GA53 and [^{2}H$_2$]-GA24, which all have MW = 348.4 g·mol^{-1}. Since each GA had a different biological role, the concentration was expected to indicate a different biochemical event in a given tissue. Therefore, it was important to achieve separation of the entire group of 20 GA substances with the highest possible resolution (R_s). Due to the availability of an instrument for *ultra-high performance liquid chromatography (UHPLC)*, which enables separation with high efficiency (increased sensitivity, short analysis time by allowing a high throughput of samples), the separation was optimised on this device. A wide range of sorbents for UHPLC separations in RP mode are available on the market, with the C18 type being the most often chosen for GA separation [22, 30]. C18 sorbents are generally suitable for all compounds with a certain degree of hydrophobicity. However, for the separation of numerous GA isomers in a mixture, further modification of a RP sorbent was of interest to enable greater resolution of co-eluting or closely eluting analytes. Preferably, the sorbent should offer greater interaction with the polycyclic structure of the analysed substances. Therefore, RP sorbents with positively charged functional groups (*e.g.,* Acquity UPLC CSH C18, Waters or Luna Omega PS C18, Phenomenex) and others bearing a cyclic structure, such as phenyl (*e.g.,* Ascentis Express Phenyl-Hexyl, Sigma Aldrich or Acquity UPLC CSH Phenyl-Hexyl, Waters), were included in the narrow group of LC sorbents tested for the optimisation of separation of acidic GAs. A positive effect on GA separation was obtained with one of the positively charged LC sorbents (column Acquity UPLC CSH C18, 2.1×50 mm, 1.7 μm; Waters), as shown in Article

4. This column gave a better peak shape and peak-to-peak resolution compared to separation using an uncharged LC sorbent (Acquity UPLC BEH C$_{18}$, Waters). Using the Acquity UPLC CSH C$_{18}$ column, 16 of the 20 studied GAs were fully resolved.

The second modification of the C$_{18}$ sorbent (introduction of cyclic groups for the separation of polycyclic GAs) proved to be unsuitable since there was a greater number of co-elutions and a prolonged time of analysis by approx. 7 min (**Fig. 4**).
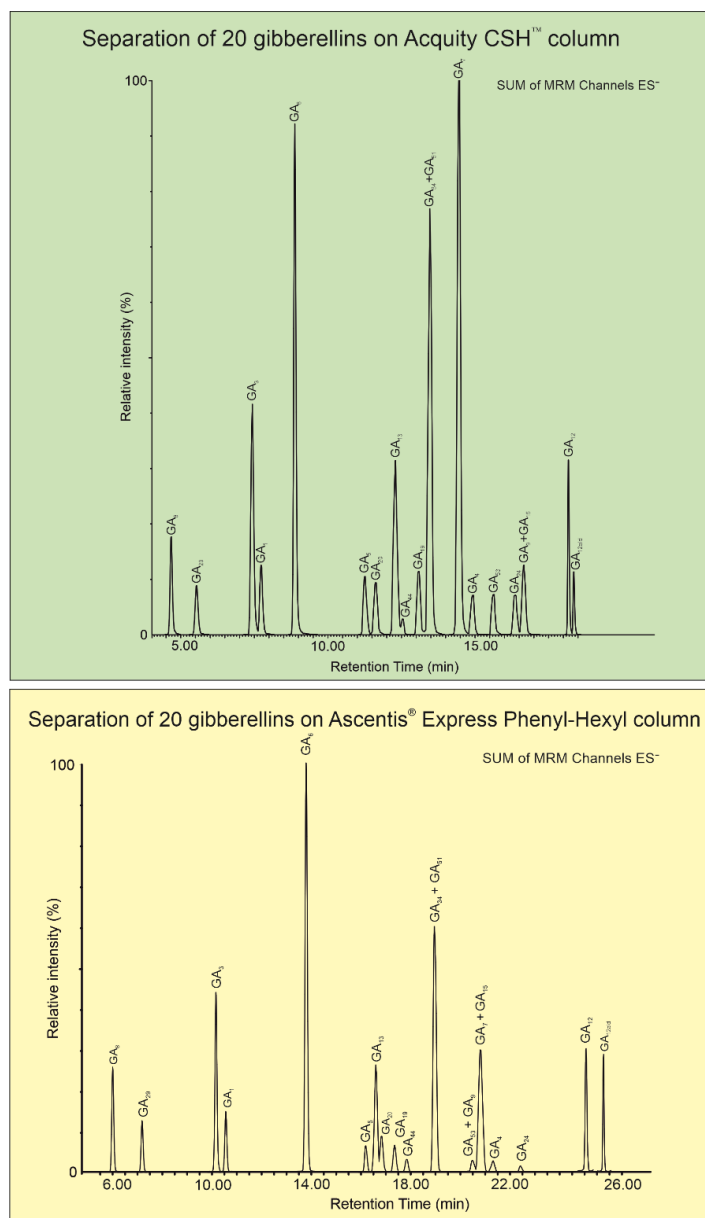

Figure 4. *Separation of 20 gibberellins by ultra-performance liquid chromatography (UPLC). The signal intensity corresponds to 10 pmol/injection. Mobile phase: methanol vs. 10 mM formic acid in water; column temperature 40 °C. The program for gradient elution is listed in Article 4.*

A longer analysis time for the column containing cyclic hydrocarbon groups confirmed the assumption that a more significant interaction between the analytes and sorbent increased their retention on this type of sorbent. On the other hand, for some pairs of peaks (GA$_1$ *vs.* GA$_3$, GA$_{12}$ *vs.* GA$_{12}$ald), there was a significant improvement in the R_s value compared to separation on the CSH sorbent.

However, the possibility of GA separation using other separation techniques, such as capillary electrophoresis (CE) [31], must not be neglected. Since native GAs do not contain a chromophore that could allow them to be detected by UV VIS, MS detection must be used instead. However, this detection type is incompatible with the separation of GA anions using a cationic surfactant, such as cetyltrimethylammonium bromide [32]. Therefore, a volatile formate buffer with the addition of ACN has been used for CE separation of eleven GA anions in a capillary coated with a polymer carrying a positive charge (commercial name SMILE(+), Nacalai Tesque, Kyoto, Japan) [33]. Unfortunately, the method's limit of detection (LOD) was found to be unacceptable at 0.31–1.02 µM. In an attempt to decrease the LOD, derivatisation of GAs has been performed, *e.g.,* by introducing a fluorescent label that enables detection based on laser-induced fluorescence (LIF). For instance, 6-oxy-(acetyl piperazine) fluorescein was used for GA$_3$ as a representative of GAs and six other "acidic" hormones (possessing a carboxyl group). Using this approach, the LOD was reduced to 1.6–6.7 nmol·l^{-1} [34]. However, this value may also be insufficient for some types of real samples. In addition, the method would have to be extended to a larger number of GAs to gain significantly more information about GA biosynthesis and catabolism in a tissue of interest. Modification of the analyte to give it a permanent positive charge instead of using a positive charge inside the capillary has also been tested. This method was demonstrated on a group of ten GAs with LOD in the range 0.34–4.59 ng·ml^{-1} [35]. Nevertheless, according to the small number of papers published in subsequent years, this method does not seem to have been used in practice for routine analyses of biological samples. This is also true of other CE-based methods.

Detection of low-abundance GAs in the form of free carboxylic acids is most often performed using a tandem mass spectrometer (MS/MS with a triple quadrupole analyser, QQQ) equipped with an electrospray operating in the negative mode ((-)ESI) - Article 4. However, this mode is generally perceived as less sensitive than the positive mode ((+)ESI). Therefore, a relatively large effort has been made recently to develop methods for the derivatisation of GAs that enable their separation by UHPLC followed by (+)ESI-

MS/MS detection [36-39]. The main motivation is that in some studies focused on the GA biosynthesis and distribution within a relatively small segment of a tissue of a selected organ (flower stamens, millimetre parts of a root or stem, *etc.*), the LODs for native GAs analysed by (-)ESI-MS/MS were deemed insufficient [40]. To objectively evaluate this, the author of this thesis derivatised selected GA standards using three different methods (see the reactions outlined in **Fig. 5**) published in recent years [22, 37, 39]. The LODs obtained during analysis using UHPLC-(+)ESI-MS/MS (QQQ analysers; Waters) for selected GAs are listed in **Table 1**. LOD values were also measured on the same instrument in the (-)ESI-MS/MS mode for comparison.
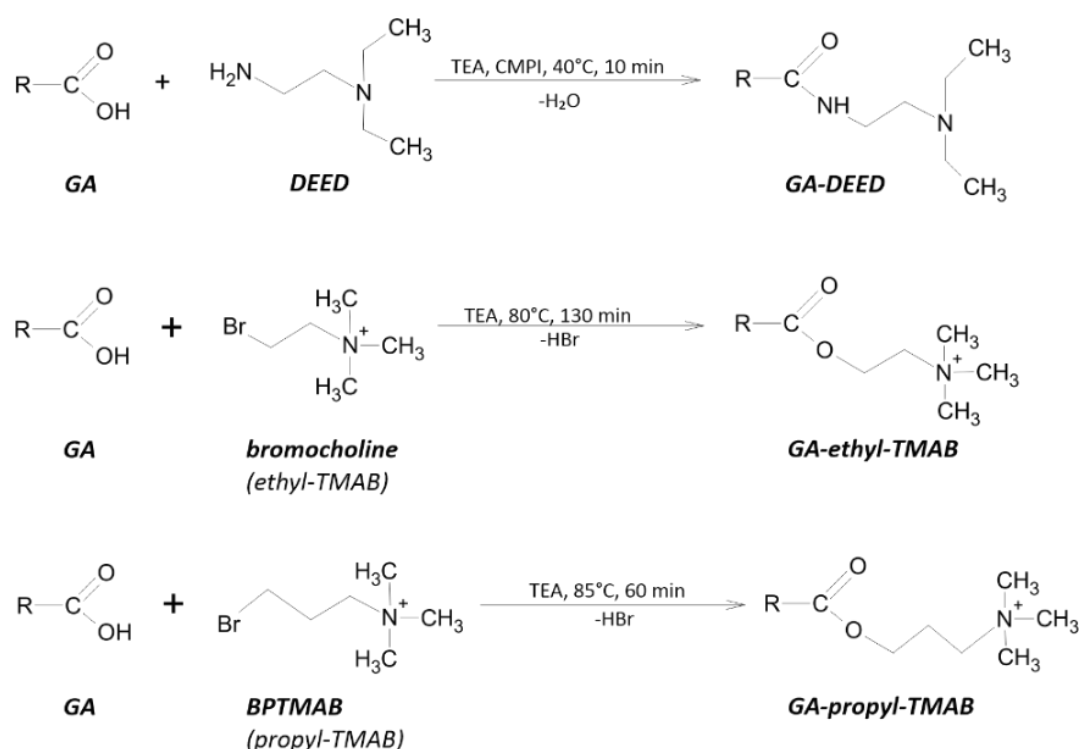

Figure 5. *Chemical reactions used for the derivatisation of GAs for their detection by (+)ESI-MS/MS. DEED - N,N-diethylethylenediamine; TMAB - trimethylammonium bromide; TEA - triethylamine; CMPI - 2-chloro-1-methylpyridinium iodide.*

Table 1. Limits of detection (LOD) for selected GA standards analysed by UHPLC-MS/MS in the multiple reaction monitoring (MRM) mode.

	LOD‡ (fmol/Inj.)							
	(-)ESI-MS/MS		**(+)ESI-MS/MS**					
	without derivatisation		*DEED*		*bromocholine*		*BPTMAB*	
	[1]LGR_{2013} (Article 4)	[2]LGR_{2020}	[2]LGR	Ref. [39]	[2]LGR	Ref. [22]	[1]LGR	Ref. [37]
GA_1	NM	7.72	3.85	0.02	10.86	NM	5.24	0.02
GA_3	10	11.57	180.21	0.02	4.71	NM	0.19	0.04
GA_4	40	1.89	1.65	0.05	2.05	NM	0.92	0.03
GA_9	NM	23.57	35.86	0.01	489.39	NM	216.99	0.01
GA_{15}*	60	4.30	0.55	0.01	437.82	NM	54.35	NM
GA_{19}*	NM	7.03	13.22	0.05	66.12	NM	17.41	0.07
GA_{24}*	60	9.50	70.69	NM	26.12	NM	216.61	NM
GA_{53}*	NM	2.30	1.21	NM	1.64	NM	3.65	NM

‡ calculated as S/N =3; LGR – values determined by the author of this thesis; *GAs with two COOH groups; [1] Xevo TQ MS (Waters; installation in 2009); [2] Xevo TQ XS (Waters; installation in 2019); Ref. [22] - Quattro Premier XE (Waters); Ref. [37] - Agilent 6460 (linear dynamic range–LDR– 0.02-20 pg); Ref. [39] - Shimadzu MS-8050 (LDR 0.02-100 pg); NM – not mentioned; DEED-*N,N*-diethylethylenediamine; BPTMAB- (3-bromopropyl)trimethylammonium bromide.

Table 1 shows that, using our instruments, it was impossible to achieve LOD values as low as those reported by other authors [36-40]. The LOD values obtained using our LC-MS instrument installed in 2020 were lower or comparable with the LOD values obtained after derivatisation reactions. Furthermore, it was found that ghost peaks occurred in the sample after derivatisation, which often interfered with detection of the endogenous GAs of interest and contaminated the quadrupole rods in the MS analyser. For these reasons, we decided to avoid derivatisation reactions for routine analyses of endogenous GAs. Thus, we continue to quantify native GAs in the form of free acids using (-)ESI-MS/MS detection. This method gives satisfactory results and is routinely used for various biological studies, as **Articles 5-7** demonstrate. The method also allowed detection of extremely low levels of GAs in segments of roots and leaves of wheat, an agriculturally important cereal (**Article 8**).

Currently, some efforts are being made to analyse plant hormones at the site of their action, *i.e.,* without their extraction and isolation from the tissue, followed by subsequent quantification by one of the modern instrumental methods. For this purpose, biosensors [41] or mass spectrometry imaging (MSI) have been proposed. In the former case, it may be impossible to construct a single biosensor capable of analysing several tens to hundreds of substances of a hormonal nature and apply it for *in situ* measurements in a given tissue of an experimental plant. Even if it is possible to develop a biosensor for only

one group of hormones (*i.e.,* no more than 20 substances), the size of such a biosensor would need to be limited to the dimensions of a microchip, and even at this size, it would be unusable for some millimetre-sized tissues. So far, only one biosensor has been prepared for measuring a GA, namely GA_3 [42-44]. That sensor was not intended to detect low endogenous levels of this substance in plant tissues but to detect significantly higher exogenous levels in brewing samples where it is used to speed up malt production. It has been stated that 8-10 days are needed to produce malt for top-fermented beers and a little less for bottom-fermented lagers. Two to three days can be reportedly saved if 25-500 µg of GA_3 is added for each kilogram of barley [45]. Monitoring of GA_3 in beer may be necessary because it has been suggested to have a negative effect on human health. In the USA, limits for the maximum permitted amount in food products have already been set [46,47]. In 2012, the European Food Safety Authority (EFSA) published a preliminary study [48], which stated that GA_3 is one of the 295 substances that are in the fourth phase of the review program covered by the Commission Regulation (EC) No. 2229/2004 as amended by Commission Regulation (EC) No. 1095/2007. According to this preliminary study, GA_3 poses only a low risk to mammals: "Low acute toxicity was observed when GA_3 was administered orally, dermally, and by inhalation." Regarding the analytical methods needed to determine this substance in biological fluids, the report states: "Methods for body fluids and tissues are not necessary because the active substance is not classified as toxic or highly toxic". However, studies from the Asian continent have indicated a certain degree of toxicity for rats and mice [49-51]. The only study on human cell toxicity that can be found in the literature was published in Russian and reported an increase in chromosomal aberration (two- to three-fold) caused by GA_3 [52].

MSI is the second potentially applicable method for monitoring substances of interest at their site of action. In plant sciences, the method is used to visualise the spatial distribution of low and high MW substances in plant tissues. So far, this method has not yet been presented in the literature for GAs. Among other plant hormones, application of MSI has been demonstrated only for those formed in much (several thousand-fold) higher concentrations than those of GAs [53]. This includes so-called stress hormones, such as jasmonic acid, salicylic acid, abscisic acid and indole-3-acetic acid, which are produced in higher quantities in plant tissue, especially under biotic/abiotic stress conditions.

II.I.2. TRITERPENOID PLANT HORMONES BRASSINOSTEROIDS

Another group of substances with a terpenoid structure and the nature of plant hormones [54] are brassinosteroids (BRs). These are tetracyclic compounds whose basic skeleton consists of 27-carbon (C$_{27}$) 5α-cholestane (**Fig. 6**).
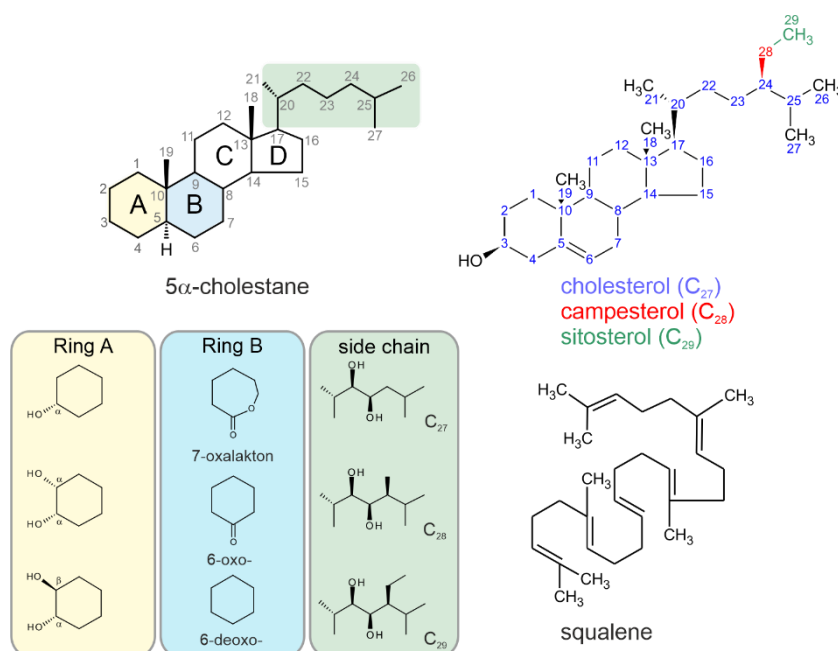

Figure 6. *Structure of 5α-cholestane and its structurally derived natural steroid compounds with 27 to 30 carbons. All possible structural modifications of naturally occurring BRs at ring A are highlighted in yellow and at ring B in blue. Various structural variants of the BR side chain are shown in the green shaded box.*

Like many other plant sterols (phytosterols), BRs are chemically steroid alcohols. To some extent, they structurally resemble cholesterol, which is the C$_{27}$ dominant steroid alcohol (OH group at C-3 position, Fig. 6) in the animal kingdom. Depending on the substitution of the side chains (*i.e.,* according to the type of alkyl at C-24), BRs and other phytosterols can contain 27, 28 or 29 carbon atoms (Fig. 6). The most widespread in nature are C$_{28}$-BRs [55], among which brassinolide (BL), which has a 7-oxalactone ring B, has the highest biological activity [56]. Similarly to the GAs, the BR structure affects their biological activity. Any modification of the B ring leads to a significant reduction in the biological activity of BRs. Thus, lactone BRs (6-oxo-7-oxa configuration) exhibit the highest activity, 6-oxo type BRs (castasterone, CS) have lower activity and BRs without oxygen on ring B (6-deoxo) show no biological activity. Another condition of biological activity is the presence of vicinal diol groups at C-2 and C-3 and the orientation of hydroxy groups at C-22 and C-23 in the side chain, where the *22R,23R*-orientation leads to higher

activity compared to the *RS*- or *SS*- orientation, resp. [56]. All steroid substances, including BRs, are formed in plant cells from squalene ($C_{30}H_{50}$; Fig. 6), a common C_{30} precursor for all triterpenoids. They are formed *de novo* from the basic C_5 isoprenoid diphosphates IPP and DMAPP in plant cells (Article 2). The complete plant sterol biosynthetic pathway involves a sequence of over 30 enzyme-catalysed reactions, leading to the Δ^5-sterols sitosterol, cholesterol and campesterol (CR), the latter of which is the first BR-specific biosynthetic precursor for C_{28} BRs bearing a methyl group at C-24.

BRs are important components of the hormonal network controlling important physiological processes during the plant life cycle (**Fig. 7**). During the reproductive phase of plant development, BRs are involved in germination, flowering and male fertility. In the vegetative growth phase, they regulate organ elongation, the timing of senescence and plant tolerance to abiotic stresses (*e.g.,* salinity, temperature, water) [57].
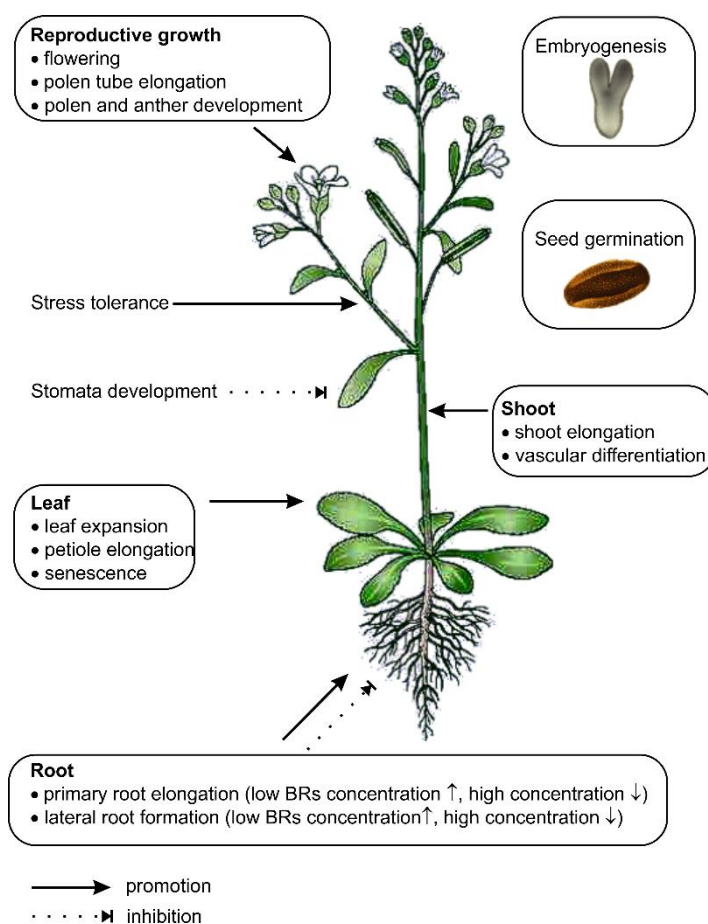

Figure 7. The physiological effects of brassinosteroids.

Since the discovery of BL, the first BR, in 1979 [58], more than 70 naturally occurring BRs have been identified to date [59]. Like GAs, they tend to be relatively abundant in reproductive plant tissues, such as pollen, flowers and immature seeds. However, their levels are extremely low in vegetative tissues, even compared to those of other plant hormones (fg-pg·g^{-1} FW). Thus, analysis of these substances also falls into the ultra-trace analysis category and requires very precise sample preparation and sensitive analytical instruments. A group of 22 substances, including C$_{27}$ BR biosynthetic precursors, the main C$_{28}$ BRs and C$_{29}$ (28-homoBRs), was studied in our laboratory to develop an analytical method to determine BR representatives. Their structures are shown in **Fig. 8**. The method development details are described below.
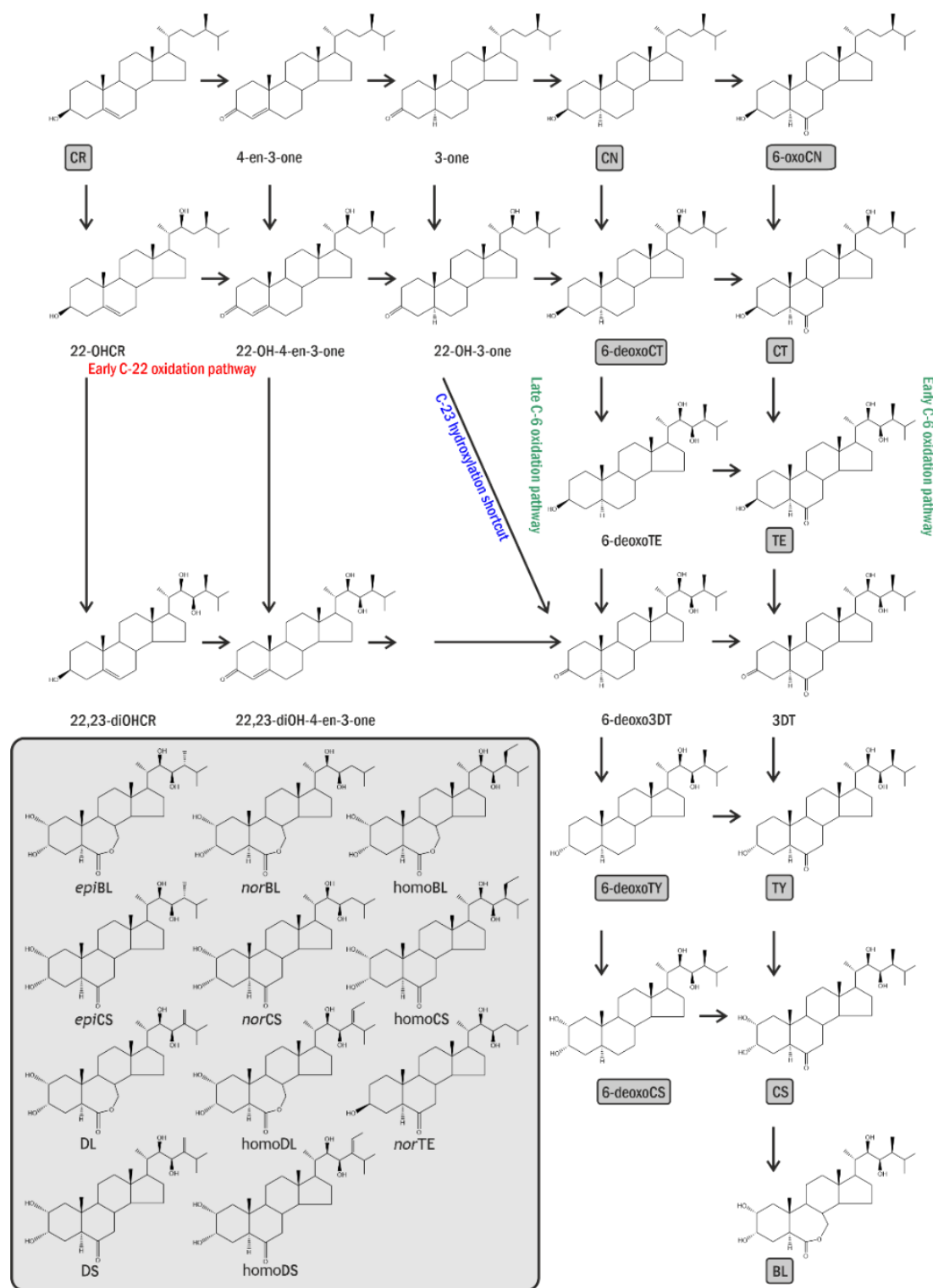
Figure 8. *Structures and biosynthetic relationships of brassinosteroids. The abbreviations of compound names are explained in **Article 9.** They are omitted here for the sake of brevity. The grey boxes show the 22 BRs selected for developing the method for sensitive analytical determination.*

Physicochemical properties of brassinosteroids

Due to their structure, BRs are relatively non-polar substances that, unlike GAs, do not have an ionic character. Therefore, ion exchange chromatography cannot be used for their isolation. Like GAs, naturally occurring BRs do not show any spectral characteristics, such as fluorescence or UV absorption (only at around 204 nm [60]), that could be used for their detection. They are relatively stable compared to GAs and have fewer requirements during sample preparation with regard to, *e.g.,* pH of the environment or type of solvents. Maintaining the temperature of extracts and semi-purified samples between -20 °C and +4 °C is only recommended to prevent enzymatic conversion of the monitored analytes (details are provided in Article 3).

Extraction and preconcentration of brassinosteroids

As already mentioned for GAs, the selection of a suitable extraction agent and method of preconcentration of BRs are very important. Again, the extreme complexity of the plant sample matrix and ultra-trace amounts of BRs, which co-occur with a huge number of interfering substances (*e.g.,* pigments, lipids, proteins) at far greater concentrations, play a key role here. The removal of interferents from extracts is usually achieved in most published protocols using very lengthy and time-consuming procedures, all of which work on the principle of hydrophilic/hydrophobic interactions and most include the following sequential steps: LLE with chloroform, hexane or ethyl acetate, column chromatography (Sephadex LH-20), SPE (diethylaminopropyl and C_{18} sorbents) and finally fractionation using RP-HPLC [61-65]. This entire procedure, in addition to being very time-consuming and unsuitable for a large series of samples, also requires a sample mass of the order of units to hundreds of grams of FW so that, even with large losses at each preconcentration step, the BR content remains at a detectable level in the final sample. Nevertheless, there is a desire to reduce the amount of sample needed because in many biological experiments, it is impossible to obtain large samples for analysis. Thus, there are two options. The first involves reducing the sample mass to an optimal level where the amount of interferents is relatively low but the BR levels are still quantifiable. The preconcentration focuses on the primary removal of interferents, while the target analytes are not retained on the sorbent. This approach was used by the author of this thesis when developing a BR preconcentration method, *i.e.,* interferents were

retained on a Supelco (USA) column (Discovery® DPA-6S, 50 mg/1 ml) and the substances of interest passed through the column without retention. More details can be found in Article 9. The second option is based on increasing the selectivity of the workflow, as discussed above in the chapter "Extraction and preconcentration of gibberellins". There are various ways to increase the selectivity during sample preparation. The longest used is derivatisation of native BRs bearing a C-22,23 diol group. This group reacts selectively with boric acid to form cyclic esters (**Fig. 9**). This reaction was first used in the 1960s for the analysis of naturally non-volatile steroid substances by GC-MS [66, 67].
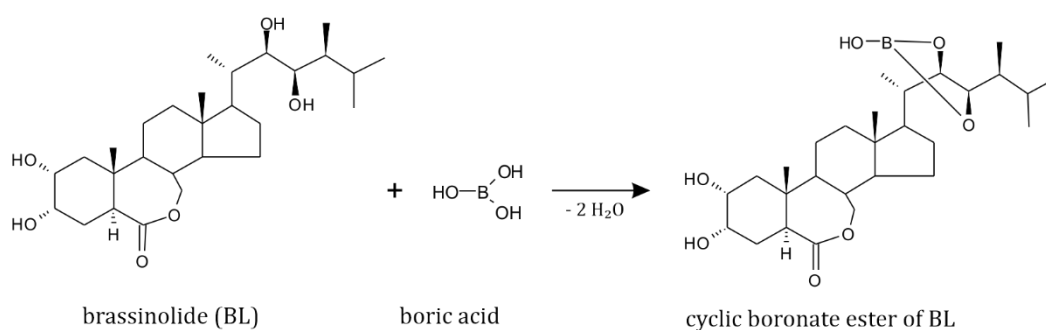

brassinolide (BL)   boric acid   cyclic boronate ester of BL

Figure 9. *Formation of cyclic esters of boric acid with brassinosteroids - an example of the reaction with brassinolide (BL).*

Later, after the discovery of BRs, the principle of this reaction was used for their derivatisation prior to HPLC separation. The main goal was to introduce a chromophore into the molecule to enable UV detection at wavelengths of ~280 nm [68]. For this purpose, 1-naphthyl boronic acid was used, which reacts with BRs in a basic environment (pyridine) to form cyclic mono- (reaction with the diol at the C-22,23 position) and bis-derivatives (reaction with the diol at C-2,3 and C-22,23). After acidification, the resulting ester decomposes into the starting compounds, *i.e.,* the reaction is reversible, pH controlled and can be used for the preconcentration of BRs. In the 1990s, a gel material with immobilised boric acid appeared on the market. It has been used for the selective isolation of BRs bearing a vicinal C-22,23 diol group [69]. However, diols at C-2,3 do not react under these conditions [70]. BR boronate derivatives have since been used countless times in various modifications, *e.g.,* as a sorbent for microextraction in the form of a polymer monolith bearing poly(3-acrylamidophenyl) boric acid [71]. The sorbent was

prepared by polymerisation inside the capillary of a syringe, and the recovery of this procedure varied between 79 and 109 % when using 1 g of plant tissue.

Furthermore, a preconcentration method based on the principle of *in situ* derivatisation (ISD) has been reported. In this experimental setup, the sorbent is freely dispersed in an extract. Centrifugation or removal using magnetic forces for sorbents with magnetic properties (so-called magnetic SPE, MSPE) is then used for its isolation. MSPE-ISD has been applied for the isolation of BRs from an acetonitrile extract based on hydrophilic interactions (hydroxyl groups of BRs) with TiO_2-coated silica magnetic hollow mesoporous particles [72]. Under these conditions, the lowest matrix effect was found at sample weights of 100 mg FW. For loadings <100 mg, some BRs were found below the limit for quantitation (LOQ). A similar procedure can be followed when using magnetic Fe_3O_4 nanoparticles carrying naphthyl boric acid [73]. Surprisingly, shortly after the publication of this highly sophisticated method, the same team of authors published a preconcentration procedure based on the use of non-selective "general purpose" mixed-mode sorbents (MAX and MCX, Waters, Ireland) containing an RP sorbent in combination with an ion-exchange sorbent [74]. Due to the non-ionic character of BRs, use of such expensive SPE columns (approx. 300 CZK/piece) with a large amount of sorbent (500 mg bed size) is puzzling. The authors explained that they assumed that interfering substances of an acidic nature are "strongly retained on the MAX column based on the anion ion exchange mechanism". However, this claim is not supported by any data. The " flow-through" fraction containing BRs in 95 % MeOH obtained from the MAX column was diluted to obtain a sample containing only 10 % MeOH. At this stage, the sample was loaded into the MCX column. No details were provided of the solvents used for activation and conditioning of both SPE columns. After loading the sample, washing steps were performed, first with 5 % MeOH with 5 % FA and then with 5 % MeOH with 5 % ammonia (removal of basic interferents). BRs were eluted from the MCX column with 80 % MeOH. The total recovery of the entire two-step SPE protocol for 1 g of plant tissue was not given. In contrast to this work, a method for BR microextraction with a C_{18} sorbent placed inside a pipette tip (in-tip SPE or micro-SPE) was published three years later [75]. The author of this thesis has tested commercially available in-tips (Supel-Tips C_{18}, 10 μl, Supelco, USA) for BR preconcentration from plant extracts. Two sets of samples were prepared: 1. a set of three samples containing the extraction solvent without plant tissue, and 2. a set of three samples containing the same solvent with 2 mg of FW tissue (rapeseed flowers).

Three tritium-labelled BR standards ([5,7,7-^{3}H]homocastasterone, [5,7,7-^{3}H]*epi*-castasterone and [5,7,7-^{3}H]epi-brassinolide) were added individually to each of the six samples to determine the recovery of the in-tip procedure. As shown in **Fig. 10**, the recovery of ^{3}H-BRs ranged from 50 to 57 % for extracts without plant matrix and 24-34 % for samples with matrix. These results were evaluated as unsatisfactory, and the in-tip SPE procedure is no longer used. Similarly low recovery values of BRs from C_{18} sorbents were found by Swaczynova et al. [76]. Therefore, in the study published in **Article 9**, sorbents with lower hydrophobicity, namely C_1, C_2 and C_4 (Isolute, Biotage, Sweden) were tested. Among these, the C_4 sorbent was finally selected because it showed recovery values of ^{3}H-BRs in samples with 50 mg FW of the biological matrix and after purification through the DPA-6S sorbent in the range of 56-68 %.
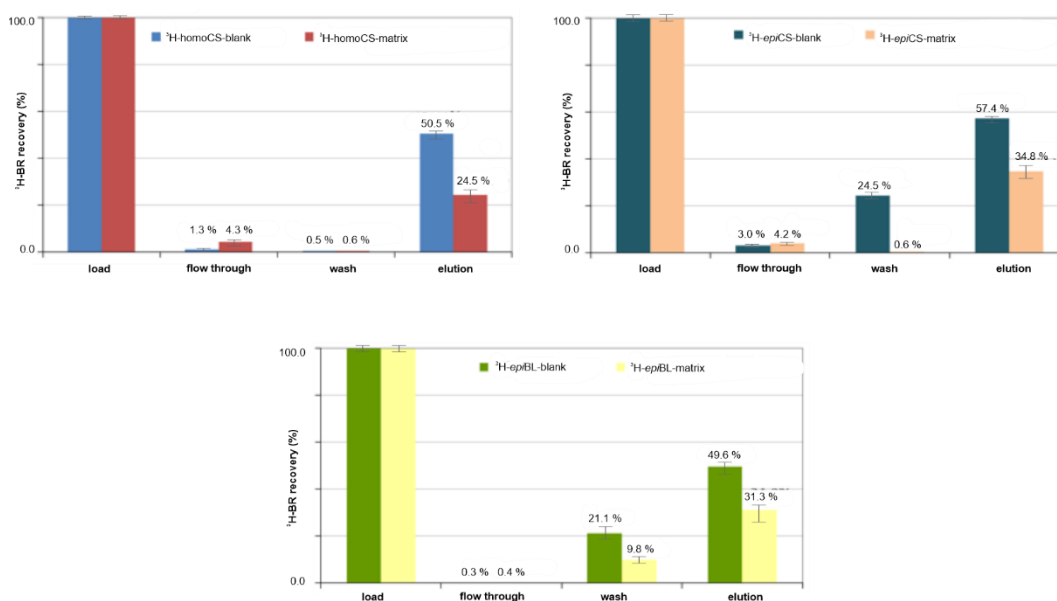

Figure 10. *Barcharts of ^{3}H-BR recovery from in-tip SPE with C_{18} sorbent. Load - 10 μl of extract in 10 % ACN; flow through – the fraction of sample going through the tip after sample loading; wash - the fraction obtained after washing the sorbent tip with 10 μl of 0.1 % TFA (trifluoroacetic acid) in 5 % of ACN; elution - 10 μl of 95 % ACN with 0.1 % FA. The sorbent in the pipette tip was activated with 10 μl of 50 % ACN and equilibrated with 10 μl of 0.1 % TFA before sample loading. The sample loading was performed in 10 consecutive cycles (10x aspirated and 10x pushed out from the tip).*

Another way to selectively preconcentrate BRs is to use the IAC approach. For this purpose, we prepared anti-BRs monoclonal antibodies (mAb) whose cross-reactivity against BRs containing a C-2,3 diol group was over 94 % (**Article 10**). BRs bearing only

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one hydroxyl at the C-3 position (teasterone, TE; typhasterol, TY) showed minimal cross-reactivity. Structurally similar sterols commonly found in plant tissues, such as sitosterol and stigmasterol, did not show any cross-reactivity with this mAb. The Ab was subsequently immobilised on a suitable carrier, then placed into a polypropylene cartridge and used for the isolation of BRs from plant extracts. The reproducibility of the procedure was verified using tritium-labelled BRs and found to be >85 %. The increased selectivity of analyte isolation led to an increased signal-to-noise ratio (S/N) in the subsequent UHPLC-MS/MS analysis, as can be seen in **Fig. 11** for BL and CS.
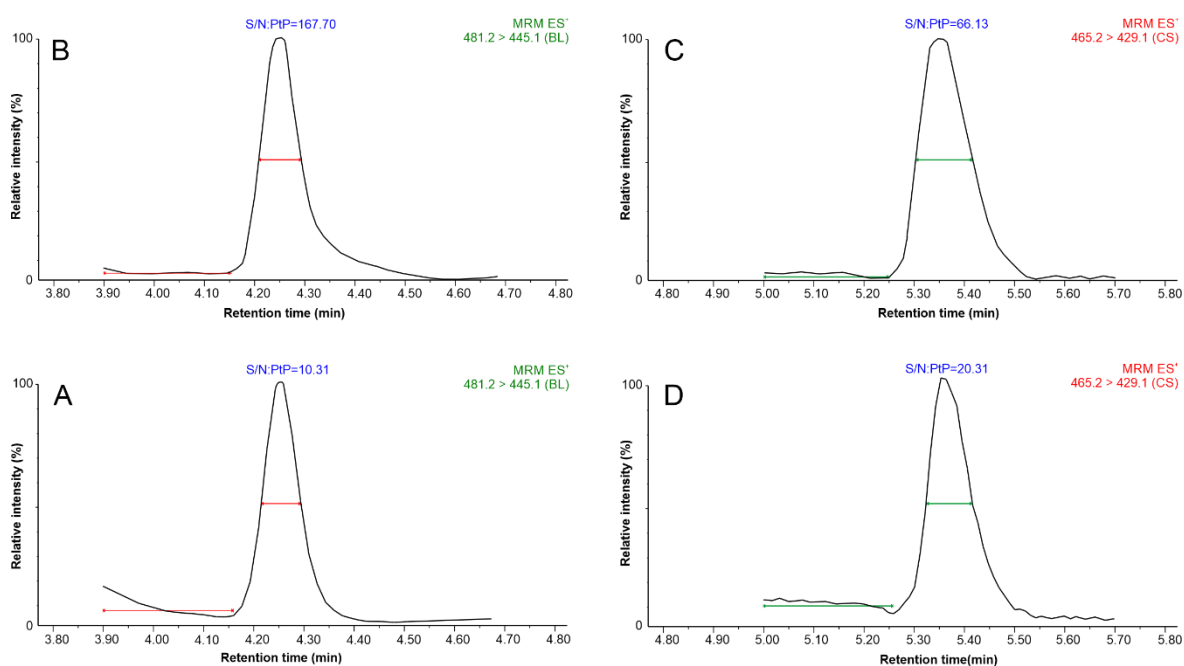

Figure 11. MRM chromatograms of endogenous brassinolide (BL) and castasterone (CS) in 50 mg FW Brassica napus extracts demonstrating the increased sensitivity of UHPLC-MS/MS analysis achieved using immunoaffinity chromatography. (A) and (D) show chromatograms of samples purified by an SPE column only, whereas (B) and (C) show chromatograms for samples purified by a combination of SPE and immunoaffinity chromatography. The calculated signal-to-noise ratio (S/N) is displayed as a peak-to-peak (PtP) function.

Methods for quantitative analysis of brassinosteroids

As mentioned at the start of this chapter, endogenous concentrations of BRs in samples of plant origin are extremely low in the ppt to ppq range (10^{-15} to 10^{-12} g·g^{-1}), *i.e.*, roughly a thousand times lower than the previously described GAs (Article 9). For the successful detection of such low levels, it is necessary to select an appropriate sample size,

take care to remove substances causing a high chemical background and use an analytical detection method that enables a sufficiently low LOD/LOQ. The first methods used for the detection of BRs were bioassays in the early 1980s [77,78]. They allowed measurement of a relatively low amount of BRs ($1 \cdot 10^{-13}$ to $2 \cdot 10^{-11}$ mol). However, their main disadvantage was that they could only detect bioactive BRs (see the structural requirements for BR bioactivity above). Non-active BR biosynthetic precursors and BR metabolites cannot be detected using this approach. Later on, bioassays were used for testing the biological activity of new synthetic analogues. Bioassays have since been replaced by various types of immunoassays, mainly radioimmunoassays (RIA) [79] and enzyme-linked immunoassays (ELISA) [80], which have LODs of about 10^{-12} mol. Unfortunately, the mAbs used for ELISA showed high cross-reactivity with plant sterols other than BRs (sitosterol, ecdysone). This problem was resolved using polyclonal Abs. Correct running of ELISA was independently confirmed by parallel HPLC-MS analysis [77].

Among the instrumental techniques, mass spectrometry also came to the fore for BRs. MS detection was first coupled to GC separation, for which it was necessary to derivatise the analytes due to their non-volatility. The standard derivatisation reaction involved the preparation of bis-esters of bis-methane boronic acid (BSB) with BRs bearing vicinal diols [70, 81]. The principle of the reaction is similar to that shown in Fig. 9. Therefore, BRs that do not contain these vicinal diols, such as biosynthetic precursors located in the biosynthetic pathway before the C-22 and C-23 hydroxylation step (campesterol, CR; campestanol, CN; 6-oxoCN; cathasterone, CT; 6-deoxoCT) cannot be determined using this method (for structures see Fig. 8). The method can be used for both the quantitative analysis (LOD at the sub-ng limit) and identification of BRs in biological samples based on their electron impact (EI) spectrum.

With the gradual introduction of LC, it is now possible to analyse naturally non-volatile BRs without derivatisation. However, owing to the absence of a chromophore, fluorophore or other structural properties that could facilitate detection using available instrumental methods, derivatisation remains the method of choice for laboratories with no access to MS. In addition to the UV detection of boronate derivatives at 210 nm [69], methods based on fluorescence [82-84] and electrochemical detectors [85] have also been reported. For BL, the most bioactive compound of the BR family, the LOD for UV detection was found to be ~100 μg, whereas fluorescence detection gave a much lower LOD at the

fg level. A LOD of 25 pg was reached using electrochemical detection of BL after its derivatisation with ferroceneboronic acid (Fig. 12).
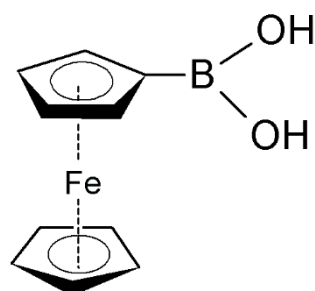

Figure 12. *Structure of ferroceneboronic acid.*

This organometallic compound has been known since 1959 [86], while the ferrocene itself was first prepared in 1951 [87]. Ferroceneboronic acid is very suitable as a redox-active marker for the electrochemical detection of substances containing *cis*-diols that have no redox centre in their native form. Without derivatisation and using an evaporative light scattering detector (ELSD), four BRs were analysed, but only at exogenous levels, as the method only allowed a LOD of 0.12–0.17 µg [88].

However, the most frequently applied detection method for determining trace amounts of BRs remains MS during LC separation, often using an electrospray ion source operating in the positive mode ((+)ESI). In addition to this ionisation technique, a paper describing the use of atmospheric pressure chemical ionisation (APCI) has been published [89]. The method was applied for the determination of BRs in seeds (reproductive tissue) in the form of naphthalene boronates using the selected ion monitoring mode (SIM), achieving a LOD of 2 ng for BL. However, the paper fails to discuss the sample size, extraction and purification procedure specifications. By applying SIM in combination with (+)ESI, a derivatisation method allowing detection of up to 125 attomoles of BL has also been published [90]. Dansyl-3-aminophenylboronic acid was used as the derivatisation agent, which was previously applied as fluorescent label for BRs in their detection at femtomolar levels in tomato cell cultures [91]. However, the extraction and preparation of the sample were unsuitable for routine analysis of a large series of samples as they required 57 g (!) of plant tissue. The remaining part of the sample preparation cannot be characterised as high throughput either: three LLE steps, thin layer chromatography (TLC) followed by RP-HPLC fractionation.

Other LC-MS methods were also based on the preparation of BR boronate derivatives [92-95], but the number of analytes in all these methods did not exceed 13. The method published by us (Article 9) enabled determination of 22 BRs without the need for derivatisation using RP-UHPLC with (+)ESI-MS/MS detection. It has been successfully applied in various biosynthetic studies (**Article 11-13**). **Table 2** shows a comparison with other published methods.

Table 2. Comparison of different methods for the determination of brassinosteroids.

Method	Sample amount	LOD	Derivatisation	No. of analytes	Ref.
UHPLC-(+)ESI-MS/MS	50 mg	0.2-40 pg	✗	22	Article 9
UHPLC-(+)ESI-MS/MS	50 mg	1-50 pg	✗	15	Article 10
UHPLC-(+)ESI-MS/MS	100 mg	1.9-5.2 ng/l	✓	5	[72]
HPLC-(+)ESI-MS/MS	50 mg	0.8-5.7 ng/l	✓	5	[94]
HPLC-(+)ESI-MS/MS	100 mg	n.m.	✓	13	[95]
HPLC-(+)ESI-MS/MS	57 g	0.1 pg	✓	3	[90]
HPLC-UV/(+)APCI-MS	n.m.	2 - 4 ng	✓	4	[89]
HPLC-fluorescence	15 kg	n.m.	✓	6	[91]
GC-MS	10 g	n.m.	✓	6	[61]
GC-MS	541 g	n.m.	✓	4	[96]

n.m. – not mentioned

Let us briefly mention some details about the separation of BR using LC. Due to the hydrophobic nature of BRs, RP-chromatography using C_{18} sorbent is usually the method of choice. As part of our work, the separation was optimised on three RP sorbents with the aim of selecting one that achieved separation with the highest possible R_s value, especially for two pairs of positional isomers BL and 24-*epi*BL and castasterone (CS) and 24-*epi*CS. These isomers differ greatly in their biological activity, and therefore it is desirable to obtain quantitative data on each of them separately. Three sorbents, *i.e.*, BEH C_{18}, CSH C_{18} (both Waters, Ireland) and Phenyl-Hexyl (Ascentis® Express column, Supelco, USA), were tested for this purpose. The mobile phase consisted of 100 % ACN and 10 mM FA, the combination of which achieved the highest ionisation efficiency of the analysed substances in (+)ESI-MS (see Article 9). The highest resolution was achieved on

the last-mentioned column (**Fig. 13** E, F), which was recommended by the manufacturer, especially for separating heterocyclic compounds.
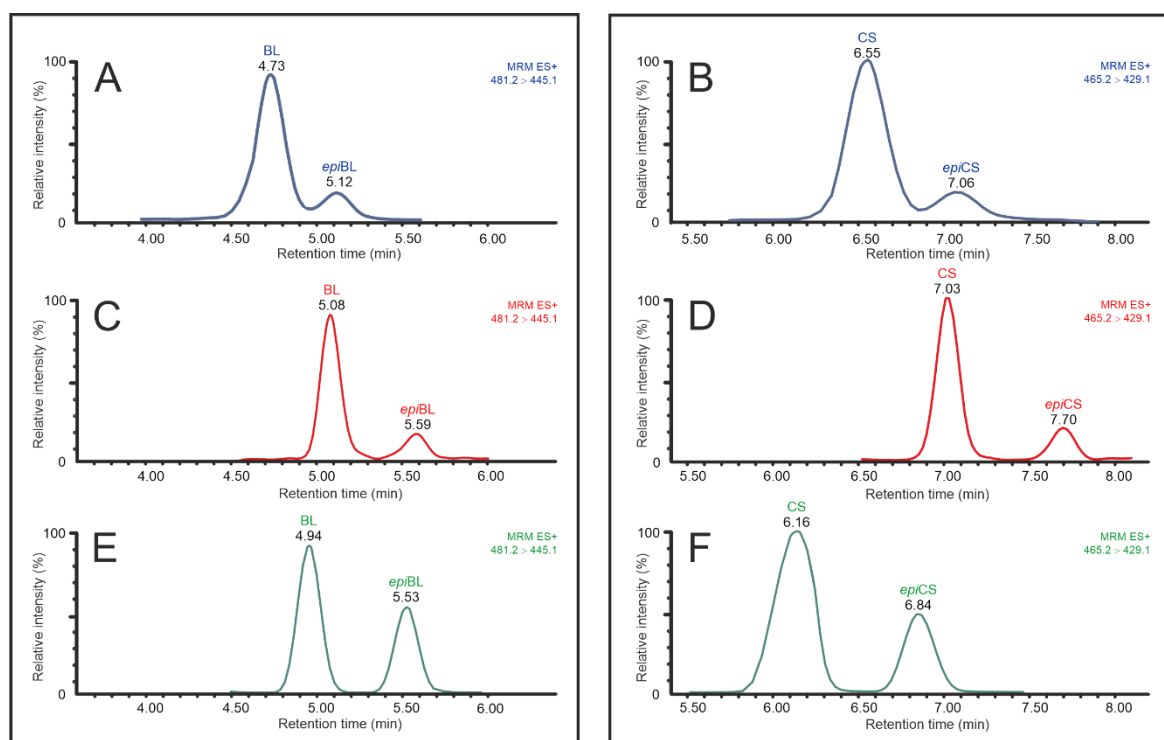

Figure 13. *Comparison of peak shape and peak-to-peak resolution of two pairs of BRs epimers BL vs. epiBL and CS vs. epiCS on Acquity UPLC®BEH C_{18} (A, B), Acquity UPLC® CSH™ C_{18} (C, D) and Ascentis® Express Phenyl-Hexyl (E, F) columns. Specification of the columns: both Acquity UPLC columns were 2.1×50 mm, 1.7 µm and the Ascentis® Express column was 2.1×100 mm, 2.7 µm. T_c = 40 °C, flow-rate 0.3 ml·min^{-1}.*

However, unfortunately, this column failed to impress when evaluating the peak width because it was up to two times higher than the peak widths obtained on BEH and CSH sorbents. Therefore, the compromise solution for RP-LC separation of these BRs isomers was the CSH column (Fig. 13 C, D), for which the other 11 analytes from the end of the biosynthetic pathway showed optimal chromatographic characteristics (Article 9). The remaining seven substances (BR biosynthetic precursors CR, CN, CT, 6-oxoCN, 6-deoxoCT, 6-deoxoTY and 6-deoxoCS – see Fig. 8) from the selected group of 22 BRs could not be separated on the C_{18} sorbent because of their high hydrophobicity. Their high degree of interaction with the C_{18} sorbent did not allow them to be successfully eluted using common RP-LC eluents, such as ACN. Thus, the retention of this group of substances was tested on C_4 sorbent (Acquity UPLC® Protein C4, Waters, Ireland), for which a lower level

of interaction was expected. The same type of sorbent was successfully used in a SPE format for the preconcentration of these substances from biological samples. UHPLC separation of the seven BR precursors was successfully achieved on a column with C$_4$ sorbent and a 40-100 % ACN *vs.* 0.1 % FA gradient in 10 min (**Fig. 14**, Article 9).
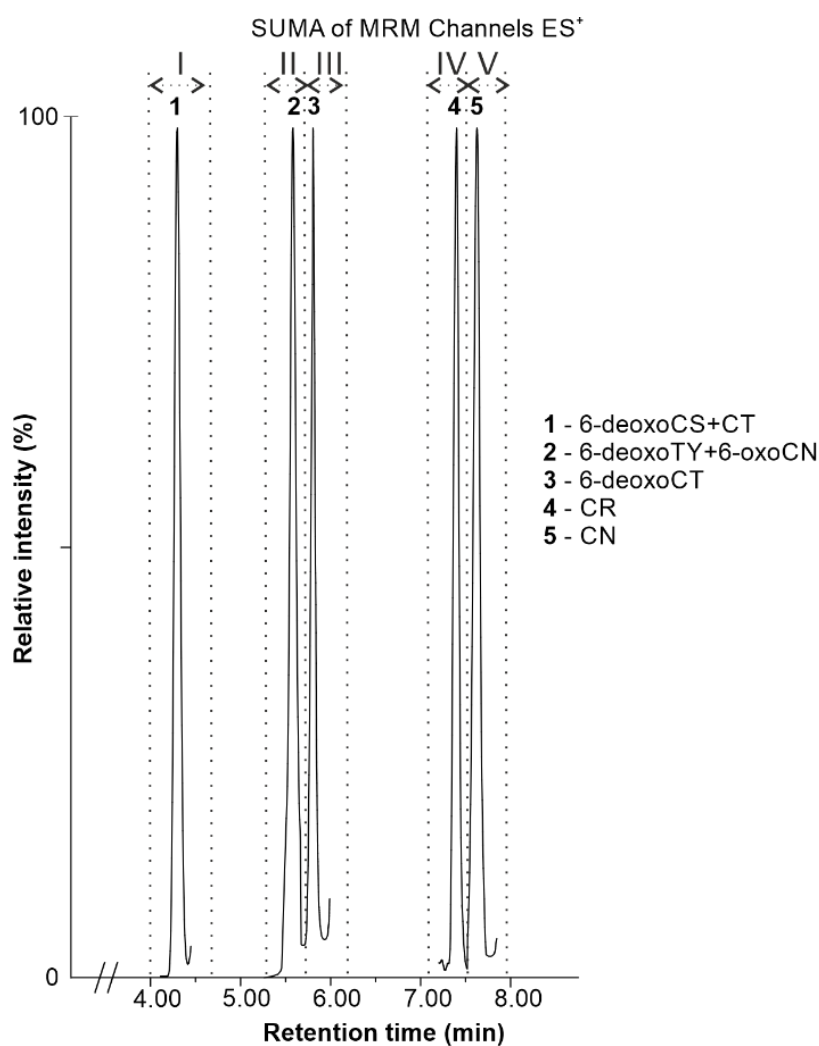

Figure 14. *Separation of seven brassinosteroids (group B) by ultra-high performance liquid chromatography (UHPLC). The UHPLC–MS chromatogram of a BR standard mixture is divided into five MRM channels (I–V) comprising 10 pmol of each BR per injection.*

II.II. OTHER NATURAL SUBSTANCES OF TERPENOID CHARACTER

II.II.1. PHYTOECDYSTEROIDS AND OTHER STEROID COMPOUNDS OF PLANT ORIGIN

As mentioned in chapter III.I., unlike animals, plants did not evolve an endocrine system that secretes chemical messengers needed for intercellular communication to regulate their growth, development and defence against various environmental cues. However, they have evolved other chemical (and physical - *e.g.,* thorns, etc.) mechanisms that fulfil these functions instead. The first two chapters of this thesis dealt with plant hormones. This chapter focuses on low MW substances that plants synthesise *de novo* as part of a chemical defence against predators, such as insects, nematodes, herbivores, etc. Conversely, high MW substances (proteins) are activated by cells within the plant's immune system during attack by pathogens [97]. Low MW substances known as antifeedants, insecticides and predator growth regulators, also known as endocrine disruptors, may be produced when a plant is attacked by predators. Substances of this type include ***phytoecdysteroids (PEs)***. Like BRs, PEs are polyhydroxylated natural steroids. Their biological effects and relations to plant hormones are covered in detail in **Article 14**. Most PEs possess a C_{27} carbon skeleton of cholest-7-en-6-one (**Fig. 15**), derived biosynthetically from cholesterol. Some PEs are derived from C_{28} and C_{29} phytosterols (alkylated at C-24), and thus possess C_{28} and C_{29} skeletons. For details about their biosynthesis and relationship with other plant sterols, including BRs, see Articles 2 and 14 and the summary in **Fig. 16.**
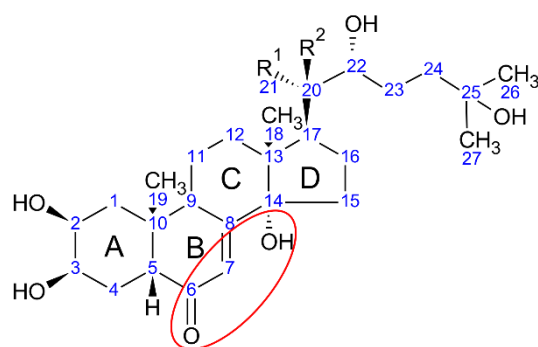

Figure 15. *Chemical structure of cholest-7-en-6-one. For the most common phytoecdysteroid 20-hydroxyecdysone (20E), R^1 = CH_3, R^2 = OH. The red oval indicates the chromophore 14α-hydroxy-7-en-6-one.*

All naturally occurring PEs have CH_3 groups at C-10 and C-13 in the β-configuration. Another common feature is *cis* orientation of the A/B ring junction, whereas the B/C- and C/D-ring junctions are always *trans* [98]. Most PEs contain an OH group in the 14α-position. Free electron pairs on the oxygen of this group are part of a conjugated system (Fig. 15) that gives PEs a characteristic ultraviolet absorption with λ_{max} at 242 nm in methanol. This spectral property sets them apart from BRs, which do not contain a chromophore. Other spectral properties described in the literature are fluorescence induced by sulfuric acid or an aqueous ammonia solution [99]. Typical values for excitation and emission wavelengths are in the region of 380 and 430 nm, respectively [100]. The presence of hydroxyl groups in PE molecules is manifested by strong absorption of infrared light in the 3340-3500 cm^{-1} region [101, 102].
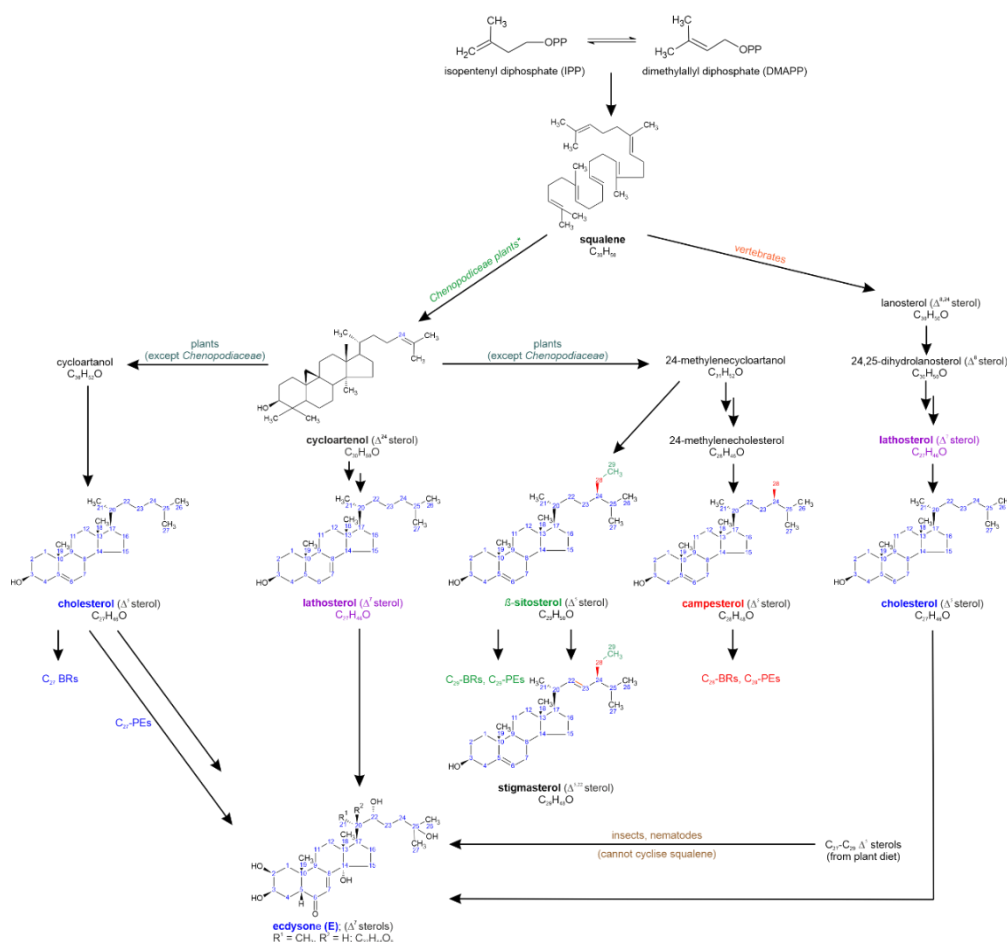

Figure 16. *Simplified plant biosynthetic pathway for ecdysone (E), a representative of phytoecdysteroids (PEs). Chenopodiceae plants* - spinach Spinacia oleracea (Adler and Grebenok, 1995; doi 10.1007/BF02537830). This pathway was also confirmed for Ajuga reptans belonging to the Lamiaceae family (Ohyama et al., 1999; doi 10.1016/S0968-0896(99)00243-6).*

Development and optimisation of a method for preconcentration and trace analysis of these substances is part of a forthcoming publication. Therefore, they are not discussed in detail here. A small part of the results obtained from application of this microscale method is presented in **Fig. 17**. Nevertheless, it is noteworthy that the technical advances in analytical instrumental techniques have significantly contributed to the detection of PEs, even in plant species previously considered PE-free species. One such species is watercress *Lepidium sativum*, which is dealt with in **Article 15,** which reports a study of the interaction of these substances with BRs.
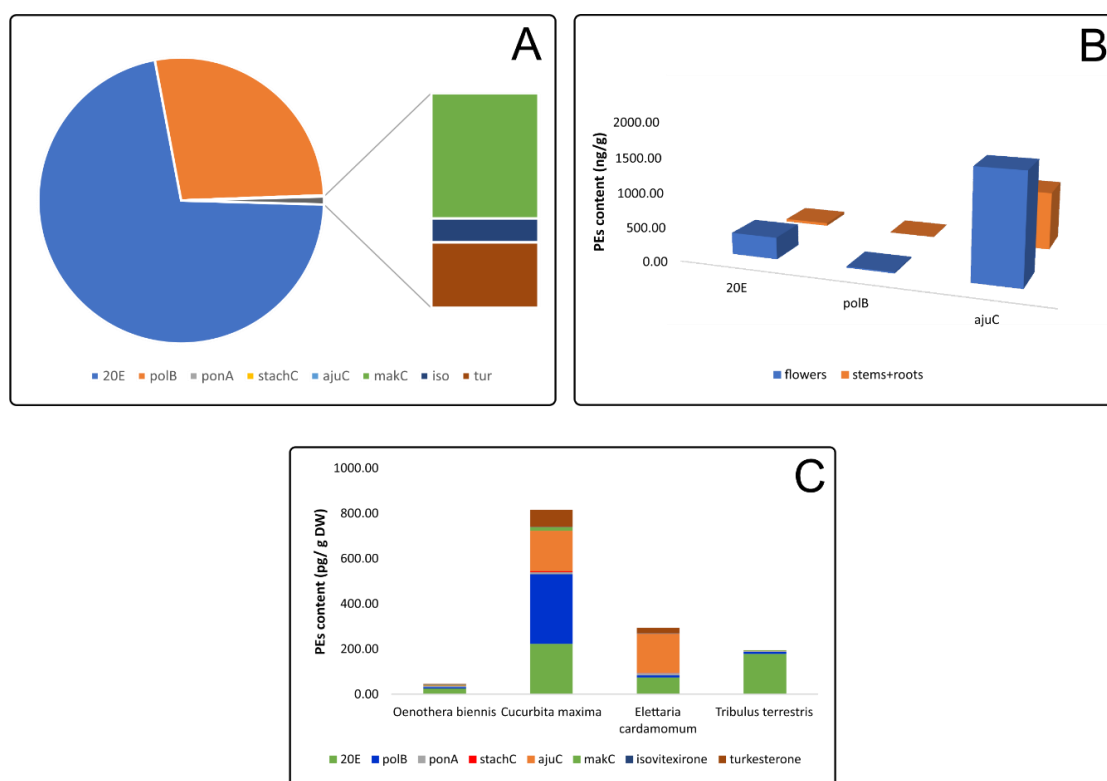

Figure 17. *Profile of phytoecdysteroids in 10 mg samples from various plant species. Seeds of Leuzea carthamoides seeds (A), Ajuga reptans (B) and another four species (C). Abbreviations of PEs: 20E - 20-hydroxyecdysone; polB - polypodine B; ponA - ponasterone A; stachC - stachysterone C; ajuC - ajugasterone C; makC - makisterone C. Tarkowská, in preparation.*

From a physiological perspective, PEs are not classed as plant hormones because they do not occur in all plant species. Their concentrations are often higher than those typical of plant hormones and their receptors have also not yet been identified in plants. The group of PEs is relatively large and includes around 300 substances identified in more than 100 species of terrestrial plants [103]. As mentioned above, their biological function is

probably the defence of plants against attack by invertebrate predators trough reducing their appetite (antifeedant properties) or disrupting their development by interfering with their endocrine system (endocrine disruption), which can reportedly result in the death of the invertebrate individuals [104-106]. In addition to the effect of PEs on invertebrates, the effect of these substances on the health of mammals, including humans, is relatively well-studied. The human diet contains many plant-based foods rich in PEs, and people may also consume preparations from medicinal plants with a range of PE content. Currently, they are most often encountered as adaptogens [107], *i.e.,* substances that help the body to strengthen its stress resistance and adapt to stressors. Most of the data published so far focus on the anabolic effect of PEs, especially in the 1970s and 1980s, after it was shown that the most frequently occurring PE in plants, 20-hydroxyecdysone (20E), causes increased proteosynthesis in mice [108]. The anabolic effect increases muscle mass owing to a sufficient supply of proteins, and hence increases physical performance without training. Interestingly, this effect was reached without the negative side effects known for other steroid substances, as demonstrated in various animal models (rats, mice, pigs, etc.) [109-111]. Among the other proven effects of PEs on mammals, antiglycemic, antioxidative, antimicrobial, antiosteoporotic and other effects have been reported [112]. The plants richest in PEs often belong to the genus *Chenopodium*, such as spinach *Spinacia oleracea* or quinoa *Chenopodium quinoa* [113, 114]. Several medicinal plants are also rich in PEs, such as Maral root *Leuzea carthamoides* [115], *Ajuga reptans* [116] and many others [117] - Fig. 17.

Two other groups of steroid substances of plant origin with the ability to modulate the development of plant predators are **phytoestrogens** and **phytoandrogens**. They generally affect the fertility of predators (most commonly insects and vertebrates such as herbivores) to reduce their cumulative attacks on plants. The success of such a defence depends on the direct synthesis of reproductive hormones or the synthesis of substances that mimic the function of vertebrate reproductive hormones [118]. From this point of view, the above-discussed (chapter II.I.1.) diterpenic gibberellic acid GA_3 can also be classified as a phytoestrogen, which, in addition to the earlier mentioned stimulating effect on the growth and differentiation of plant cells, has been observed to have a stimulating effect on the growth and proliferation of uterine cells in experimental mammals (rats) [119]. Many studies have also reported its androgenic effects (cress growth in male chickens, increased sexual activity in male rabbits) [120, 121].

But back to the topic of phytoestrogens. As the name implies, phytoestrogens act like oestrogens in the animal kingdom, *i.e.,* they control female fertility [122]. Phytoandrogens act similarly and are thought to be synthesised in the plant cell primarily to suppress the fertility of male predators. Unlike the relatively well-studied occurrence of phytoestrogens, there is very little information about phytoandrogens. Most often, both groups are mentioned in connection with human health, as plants rich in them are successfully used in herbalism, *i.e.*, using plants for therapeutic and preventive purposes. The ability of plants to synthesise these substances is often ignored, and it is usually assumed that these substances are found only in the animal kingdom, as outlined in the review attached to this thesis as Article 1. This is partly because our knowledge of their biosynthesis in plant cells is relatively limited. Previously, it was assumed that C_{18}-C_{21} steroid compounds are formed in the cytosol of plant cells from cholesterol by the same processes as has been observed in animals, where the sex hormones are formed from progesterone (PRG; $C_{21}H_{30}O_2$) by successive oxidation steps, both at C-17 and at C-19 [123]. However, studies with ^{3}H and ^{14}C-labelled precursors showed that dominant higher plant C_{29} sterols β-sitosterol (24α-ethylcholesterol) and its Δ^{22}-analogue stigmasterol, as well as C_{28} campesterol, could also serve as PRG precursors [124,125]. A simplified scheme of the biosynthetic relationships between C_{18}-C_{21} steroids in plants is shown in **Fig. 18**.
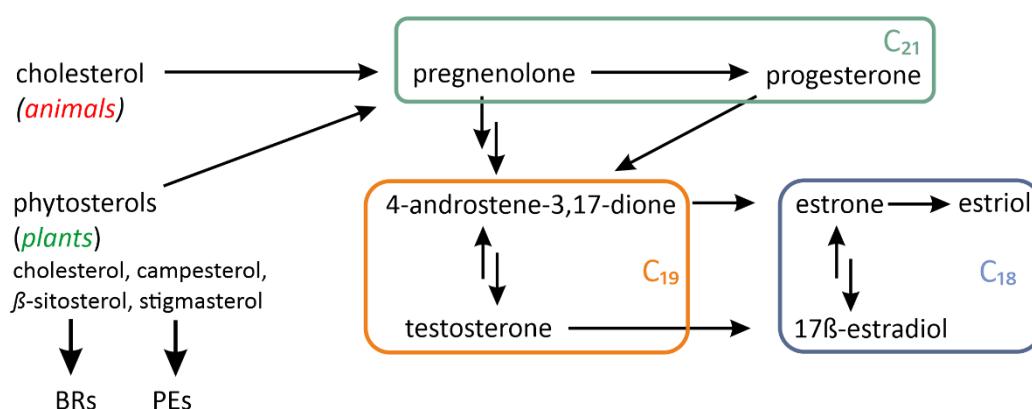

Figure 18. *Simplified scheme of biosynthetic relationships between plant sterols and C_{18}-C_{21} steroid compounds. BRs – brassinosteroids; PEs – phytoecdysteroids.*

This is also demonstrated in **Fig. 19**, which shows the levels of common phytosterols (ng·g^{-1} DW), PRG, oestrogens and androgens (pg·g^{-1}DW) in seeds of various plant species. The data were obtained by two methods developed for microscale preconcentration and analysis of these substances in plant sources (Tarkowská et al., unpublished).
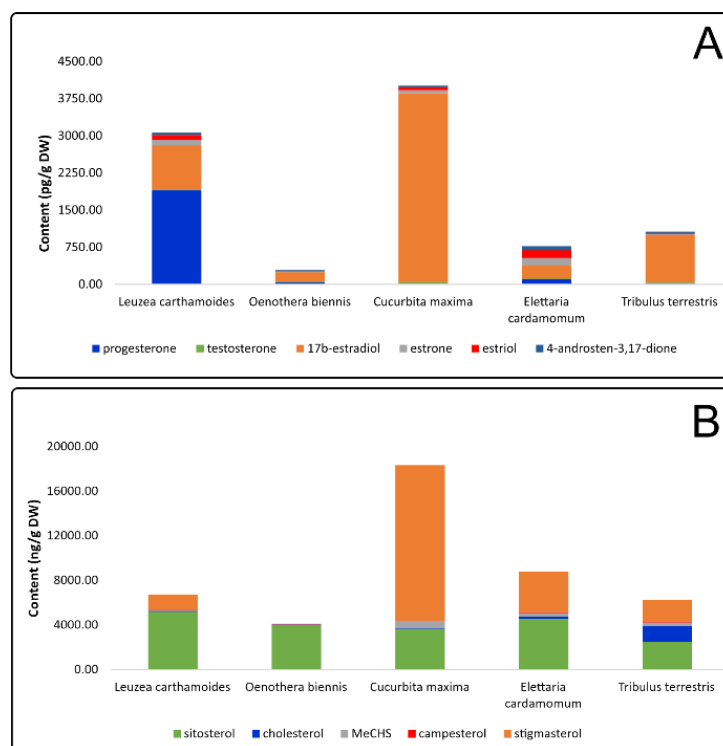

Figure 19. *Profiles of C$_{18}$-C$_{21}$ steroids (A) and phytosterols (B) in 10 mg of seeds of various plant species (Tarkowská et al., in preparation). MeCHS, 24-methylenecholesterol.*

Phytoestrogens are often used in herbal preparations to help alleviate troublesome symptoms in women, especially during the menopausal period and the so-called premenstrual syndrome (PMS) [126]. In both cases, problems arise due to an unfavourable ratio of oestrogens to other steroid hormones. In the case of PMS, levels of oestrogen and prolactin are increased *vs.* decreased progesterone levels in the second half of the menstrual cycle. In the case of the menopause, levels of oestrogen and progesterone decrease owing to the suppression of ovarian function. An insufficiently high oestrogen level is then manifested by symptoms such as hot flashes, depression, mood changes, sleep disorders, vaginal dryness and joint pain [127]. Primarily, these symptoms can be alleviated by hormone replacement therapy. However, epidemiological data have shown that a diet rich in phytoestrogens, a rich source of which is soy, chickpeas and other legumes [128], reduces the number of hot flashes, the incidence of cancer and moderates

other symptoms associated with a lack of oestrogens [128, 129]. For this reason, women are increasingly consuming foods rich in phytoestrogens and seeking phytoestrogen herbal preparations as an alternative therapy [130]. The fact that hormone replacement therapy can lead to a slight but significant increase in the risk of developing breast and endometrial cancer has also played a role [130-133].

II.II.2. INDOLE ALKALOIDS

Alkaloids are another group of low MW substances that are assumed to be synthesised *de novo* by plants as part of a chemical defence against attack, especially by herbivores and pathogens. Considering that plants spend a relatively large amount of energy on their synthesis and at the same time must possess highly specialised enzymes for this, it might be assumed that alkaloids in plants fulfil another, as yet unexplained, function. During the 200 years since the discovery of the first alkaloid morphine (1806), around 20,000 compounds of this type have been isolated in roughly 20 % of plant species [134]. However, the isolation of a given alkaloid has not always been coincident with elucidation of its structure until the beginning of the development of physicochemical methods in the 1950s. For example, 162 years passed from the isolation of caffeine to the discovery of its structure.

The group of alkaloids is structurally very diverse. Still, as their name indicates, they are all substances of an alkaline character. Their common structural feature is a heterocycle with at least one incorporated nitrogen atom, which originates biosynthetically from one of the following amino acids – ornithine, lysine, phenylalanine, tyrosine, tryptophan or histidine. For the work detailed in **Article 16**, a group of alkaloids derived from tryptophan (Trp) was selected - **Fig. 20**. In the literature, they are also called ***indole alkaloids (IAs)***, as the basis of their structure is the indole skeleton (marked in blue in Fig. 20).
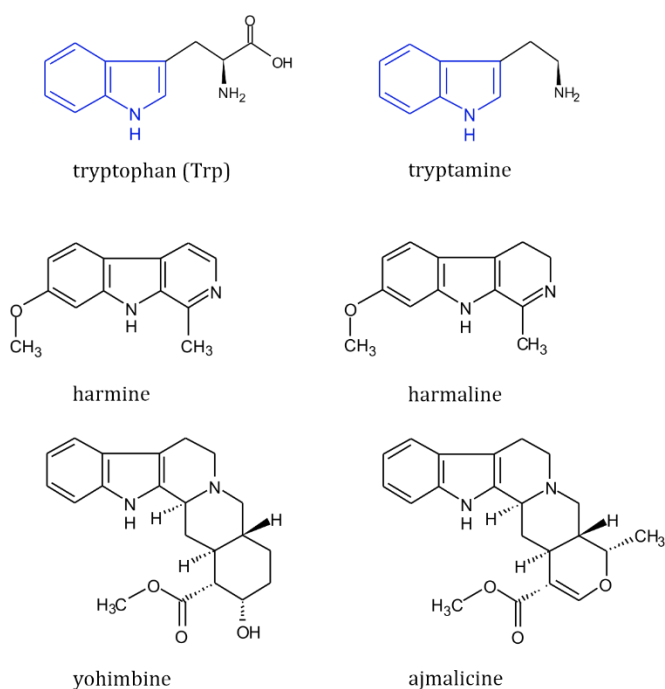
Figure 20. *Chemical structures of tryptophan (Trp) and its structurally derived indole alkaloids. The indole skeleton is marked in blue.*

Most IAs also contain isoprenoid building blocks formed via both known isoprenoid pathways—the mevalonate pathway (MVA) and the non-mevalonate pathway, also called the 2-*C*-methyl-D-erythritol 4-phosphate pathway (MEP) – see Article 2. According to the structure of the terpene unit, IAs can be classified as *simple IAs* (possessing one C_5 unit) or *terpene IAs* (possessing two C_5 units, *i.e.,* containing 9–10 carbons). Simple IAs are derivatives of tryptamine (Fig. 20), a decarboxylation product of Trp [135]. Well-known members of this group are psilocin and psilocybin, alkaloids isolated from the *Psilocybe* genus species. The subjects of interest of this thesis are terpene IAs formed from tryptamine and a C_{10} terpene unit, commonly called ***monoterpenoid indole alkaloids (MIAs)***. It is the largest group of alkaloids in the plant kingdom, consisting of over 2,000 compounds of the 10,000 alkaloids identified so far [136]. In addition to the above-discussed significance of alkaloids for plants, these substances have been shown to have certain pharmacological effects in mammals, which is why these secondary metabolites have been isolated from various medicinal plants. Among MIAs, ***harmala alkaloids*** are the most important representatives – Fig. 20. They are named after the plant *Peganum harmala,* from which they were isolated for the first time [137]. The group includes harmine, harmaline (see Fig. 20), harmalol, harmol and tetrahydroharmine. They are potent monoamine oxidase inhibitors (MAO, EC 1.4.3.4), *i.e.,* they can decrease the

degradation rate of monoamine neurotransmitters, enabling them to be used as antidepressants [138]. They are also present in hallucinogenic preparations, such as the psychedelic drink ayahuasca, which is brewed in some countries to enhance the activity of amine hallucinogenic drugs. However, the ingestion of plant preparations containing harmala alkaloids may result in toxic effects, namely visual and auditory hallucinations, nausea, vomiting, confusion, agitation and low blood pressure [139]. Two other MIAs of interest in this thesis, yohimbine and ajmalicine (Fig. 20), are present in many plant species, especially *Rauvolfia* and *Catharanthus* genera. Their main therapeutic effects are antihypertensive and antiarrhythmic [140,141]. Some studies have also shown an antitumor effect [142,143].

Although the occurrence of MIAs is relatively well-known, approaches for microscale extraction and sample pretreatment prior to their determination by sensitive analytical methods are limited. Moreover, the relative distribution and content of alkaloids in plants are often inconsistent or not well-established. Therefore, our work aimed to establish a fast, reliable and high-throughput method for the determination of four pharmacologically significant substances of the MIA family for rapid testing of their content in food supplements of plant origin. In many countries, products of this type are not thoroughly controlled for active and other substances. Thus, these natural products may be dangerous to some individuals. By increasing the selectivity of the preconcentration procedure by reducing the sample size, using SPE with a mixed mode sorbent (mixture of C_{18} and cation-exchanger) and introducing selective MS detection (MRM mode), we were able to detect these compounds in previously unpublished plant materials.

Extraction and preconcentration of indole alkaloids

The first chapter of this thesis concerned representatives of secondary metabolites with an acidic character (gibberellins), the next chapters discussed neutral substances (steroid alcohols), and this chapter is dedicated to plant terpenoid secondary metabolites with a basic character. The dissociation constants (*pKa*) of four selected compounds from the MIA group ranges from 6.31 (ajmalicine) to 9.55 (harmaline) [144,145]. Therefore, a basic reagent is suitable for the extraction of these substances. The reagent should also contain an organic solvent so that the hydrophobicity of the reagent matches the hydrophobicity

of the indole skeleton (Fig. 20) of the extracted compounds. Most studies have used ethanol [146], methanol [147,148], 60 % (v/v) aqueous methanol solution [149] or chloroform [150,151]. The basic component is often added to the extract in the form of ammonium hydroxide NH_4OH. Either the plant material is wetted with it before extraction with the organic solvent [149, 150] or it is added afterwards [147, 148, 151]. In our experiments, 60 % MeOH containing 0.25 % NH_4OH was found to be most appropriate (Article 16). For the purification of crude extracts containing MIAs, a mixed-mode solid phase extraction (SPE) sorbent, Bond Elut Plexa PCX, with cation-exchange properties was used to effectively retain MIAs on the sorbent, while substances with acidic properties and neutral substances were removed from the sample matrix. Using the extraction and preconcentration procedure described in Article 16, the recoveries of monitored alkaloids from the PCX sorbent ranged from 51.1 % to 87.7 % (harmine).

Methods for quantitative analysis of indole alkaloids

Several chromatographic methods are suitable for the qualitative or quantitative analysis of MIAs. LC has been performed in a HPLC [152,153] or high-performance thin layer chromatography (HPTLC) mode [154,155]. Among LC-based methods, LC-UV is the simplest and most feasible way for detecting indole derivatives since they exhibit absorption in the range 200-400 nm, showing two to four absorption bands depending on the substituent of the indole ring [156] - **Table 3**.

Table 3. UV maxima for selected indole alkaloids.

Compound	λ_{max} (nm)
harmine	241, 301, 336
harmaline	218, 260, 344, 376
ajmalicine	226, 291
yohimbine	226, 291

Another option is the derivatisation of MIAs to incorporate a fluorescent centre into the molecule that can be used for its detection. For example, derivatisation with acetic acid anhydride leading to the formation of acetoxy derivatives with fluorescent properties has been reported for β-carbolines (harman, harmol and their nor-counterparts) [157]. In the last decade, the LC-MS approach has been the predominant technique for the detection of

indole derivatives [154,158-161]. It offers many advantages for the analysis of these types of compounds, mainly speed and sufficient sensitivity.

A GC-MS approach has also been used for the analysis of IAs, either after derivatisation using pentafluorobenzyl derivatives [156] or without a derivatisation step [153].

Several methods have also been developed for the analysis of MIAs based on CE or micellar electrokinetic chromatography (MEKC) separation with UV or MS detection [162-165]. Moreover, direct analysis in real time coupled to mass spectrometry (DART-MS) has been successfully applied to monitor the distribution of MIAs in plant tissue [166,167]. DART is a plasma-based ambient ionisation technique that enables the rapid ionisation of small molecules with high sample throughput. Its coupling to MS could potentially be a sensitive tool for the analysis of small molecules (LOD < pmol), with acquisition times of up to 60 s.

Among electrochemical methods, voltammetry has been used for the analysis of ajmalicine [168]. The procedure involved a glassy carbon electrode modified with gold nanoparticles (GCE/AuNPs). Under the experimental conditions, ajmalicine provided a well-defined oxidation peak at a potential of +0.8 V *vs.* Ag/AgCl. The method showed a dynamic range for concentrations from 5 to 50 μM with a LOD of 1.7 μM. The authors reported that the effect of possible interferents (yohimbine, reserpine) appeared to be negligible. Further, a boron-doped diamond electrode has been used for the determination of harmaline in natural food products [169]. Under optimised experimental conditions, a LOD of 0.2 μM was obtained.

In our work, a fast, reliable and robust method of determination of four selected alkaloids with an indole skeleton, *i.e.,* harmine, harmaline, yohimbine and ajmalicine, based on UHPLC-MS-MS was developed. Details of the method are described in Article 16. The method was shown to be suitable for fast screening of the mentioned compounds in plant tissue extracts as well as natural foodstuffs and beverages (often associated with fermentation processes, such as in the production of wines, beer, soya bean products and vinegar [170]). For LC separation of the four MIAs of interest, two RP columns were tested: a UHPLC column with a sorbent containing a phenyl-hexyl group that was designed for selective retention of polyaromatic compounds through π–π interactions (Acquity UPLC® CSH™ Phenyl–Hexyl, 2.1 × 50 mm, 1.7 μm; Waters, Ireland) and a UHPLC column with C_{18} particles made by technology offering a superior peak shape for basic compounds under weak ionic strength mobile-phase conditions (Acquity UPLC® CSH™,

2.1 × 50 mm, 1.7 μm; Waters, Ireland). A critical pair of compounds for separation were harmine and harmaline, as they differ by only one double bond (see Fig. 20). The phenyl-hexyl CSH column offered better resolution of this pair and was used for further experiments to examine the influence of the mobile phase pH and composition. The best ionisation efficiency was obtained when using ACN and 10 mM ammonium acetate (AA) as mobile phase components. Because of the high pK_a of harmaline (9.55 - see above), it was necessary to keep the pH of 10 mM AA above this value to ensure an acceptable peak symmetry. Indeed, the best peak symmetry was achieved when using 10 mM AA adjusted to pH 10.0 (Article 16). The other three studied substances also showed satisfactory peak shapes under these conditions – **Fig. 21**.
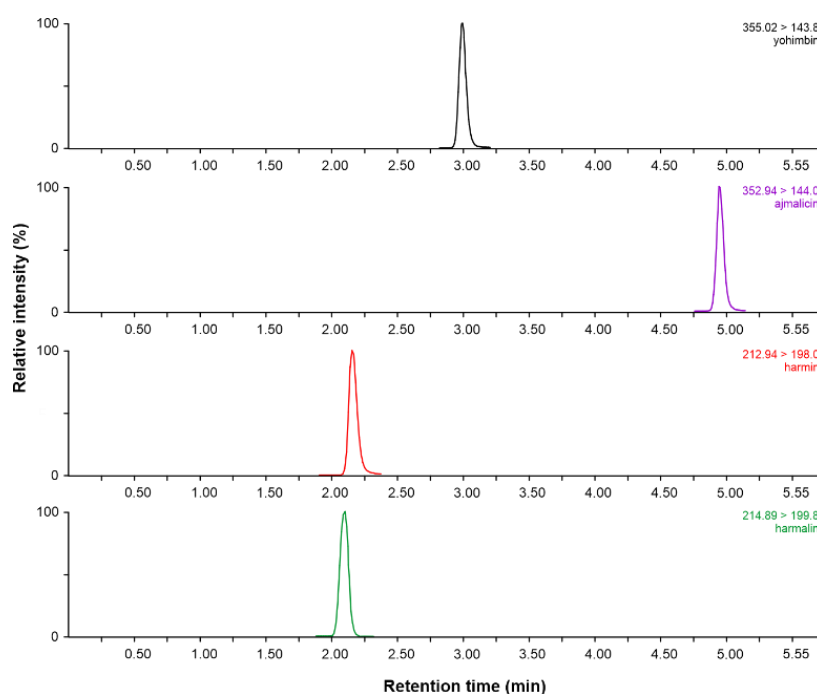

Figure 21. *UHPLC–MS/MS chromatogram of a four-indole-alkaloid standard mixture on an Acquity UPLC® CSH™ Phenyl–Hexyl column using acetonitrile and 10 mM ammonium acetate; pH 10.0.*

Detection of the analytes was performed using (+)ESI-MS in the MRM mode. The lowest LOD/LOQ was found for yohimbine (0.01/0.05 pg), whereas the most basic harmaline showed the highest values (0.31/1.04 pg). A comparison of the newly developed method parameters with those published earlier, mainly the LOD and amount of tissue needed for analysis, is provided in **Table 4**.

Table 4. Comparison of different methods used for the determination of indole alkaloids.

Instrumental method	Amount of tissue	Type of tissue	Limit of detection	Reference	Capacity of the method
UHPLC–(+)ESI–MS/MS	5 mg	whole plant	0.01–0.31 pg	method presented	4 IAs
HPLC–UV	n.a.	whole plant	0.77–56 mg·g^{-1}	[138]	2 IAs
HPLC–UV	0.1 g	roots	6–8 µg·ml^{-1}	[152]	3 IAs
HPTLC	n.a.	seeds	n.a.	[171]	4 IAs
HPLC–UV	1 g	seeds	0.01-0.05 µg·ml^{-1}	[172]	2 IAs
HPLC–UV	n.a.	cell culture	n.a.	[173]	22 IAs
UPLC/IM–QTOF–MS *	0.1 g	yohimbe bark	n.a.	[149]	55 IAs
MEKC–UV/LIF	n.a.	none	n.a.	[163]	6 IAs
HPLC–(+)ESI–QTOF–MS/MS	50 g	roots	n.a.	[146]	47 IAs
HPLC–UV–MS	0.5 g	aphrodisiac products	3-60 ng·ml^{-1}	[174]	1 IA
HPLC–UV	2 g	roots	0.05-0.39 µg·ml^{-1}	[153]	5 IAs
HPLC–UV	5 g	roots	n.a.	[175]	6 IAs

*ultra-performance liquid chromatography/ion mobility quadrupole time-of-flight mass spectrometry; LIF, laser-induced fluorescence; IAs, indole alkaloids; n.a., not available.

The applicability of the method was demonstrated for tobacco and *Tribulus terrestris* plant tissue, seeds of *Peganum harmala* and extract from the bark of the African tree *Pausinystalia johimbe*. The experimentally obtained data on the occurrence of individual alkaloids in each tissue agreed with the available literature, except for the presence of ajmalicine and yohimbine in *Tribulus terrestris*, which was detected in this plant species for the first time.

Furthermore, although there are no reports indicating that MIAs are present in tobacco *Nicotiana tabacum*, the newly developed UHPLC-MS/MS method detected these substances in this plant. In the experiment, 30-day-old tobacco plants were divided into ten parts – leaves at different developmental stages, roots, stem and flowers (**Fig. 22**). Analysis of the individual tissue types revealed that the highest content was that of yohimbine, representing 89.6 % of the total amount of all four detected IAs (**Fig. 22**).
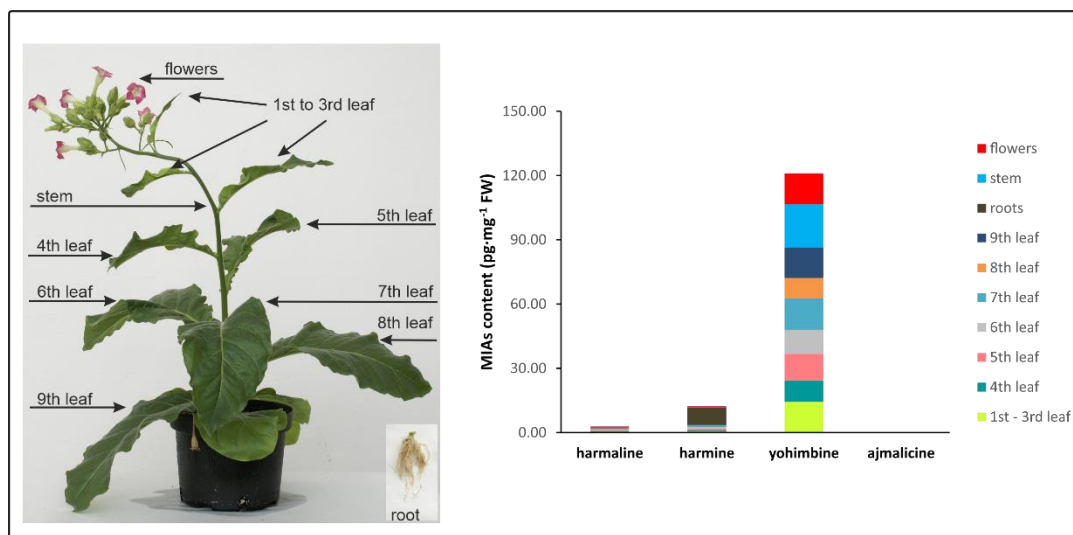
Figure 22. Left – *30-day-old tobacco plant Nicotiana tabacum cv. Petit Havana SR1, wild type. Scheme of division into individual parts according to type and age of tissue before extraction and analysis by LC-MS/MS.* *Right* - *distribution of individual indole alkaloids in 30-day-old Nicotiana tabacum plants. The data represent the mean of three independent determinations.*

The analysis of tobacco showed that this plant species has biosynthetic apparatus capable of synthesizing IAs *de novo*. This was the second new finding related to MIAs that had not previously been published. It is interesting, especially as several studies have described the inhibition of MAO activity in tobacco smokers [176,177]. Exposure to cigarette smoke or saliva from smokers was shown to inhibit MAO in different tissue preparations [178,179]. The authors suggested that MAO inhibition is likely due to the direct action of inhaled smoke [180]. According to the results related to the above-described endogenous levels of MIAs, it can be hypothesised that MIAs in inhaled smoke from tobacco products might participate in the observed inhibition of MAO activity.

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II.III. ROLE OF THE MATRIX IN TRACE ANALYSIS OF SAMPLES OF BIOLOGICAL ORIGIN

As mentioned above, the composition of the matrix of biological samples plays an extremely important role influencing the success of detection of trace substances. For plant samples, it is also important to consider both the plant species and type of tissue being analysed. Indeed, different plant species can have a significantly different matrix composition (different ratios of interferents such as lipids, saccharides, etc.). In the same plant species, different tissues may also have a different matrix composition, *e.g.,* shoot tissues *vs.* roots. Hence, it is important to optimise each preconcentration procedure for a given plant species and modify it when analysing another plant species. As part of these optimisation steps, it is necessary to include an experiment to optimise the sample tissue size *vs.* response of endogenous substances and internal standards. Without their use, trace analysis does not provide reliable and accurate results, even when using the most modern analytical devices. The factor that mathematically describes the effect of the biological matrix on the determination of analytes is called the **matrix effect** (ME). In MS analysis, it causes changes in the ionisation efficiency of the analyte in the ion source due to the presence of interfering substances originating from the biological matrix of the sample [181]. ME manifests as the detection of lower amounts of analytes than is actually the case, which significantly affects the results of quantitative analysis. Therefore, in the case of plant hormones, as well as other substances occurring in trace quantities in plant tissue, a key step in sample preparation is optimizing the weight of biological material (plant tissue) needed for the quantification of these substances. The aim is to determine the amount of tissue that produces the maximal response of individual analytes in the LC–MS analysis. **Figure 23** shows how the situation may vary even for two analytes from the same plant hormone family (GAs). For instance, in 2 mg of tissue, some GAs show a relatively high response (GA$_{19}$, Fig. 23A), whereas others are close to the LOQ (GA$_{29}$, Fig. 23B). This figure also demonstrates the significant influence of the tissue water content, *i.e.*, whether the plant material is extracted as fresh (FW) or dry weight (DW; freeze-dried). The internal standard recovery is more affected for the DW than for the FW (Fig. 23 C, D).
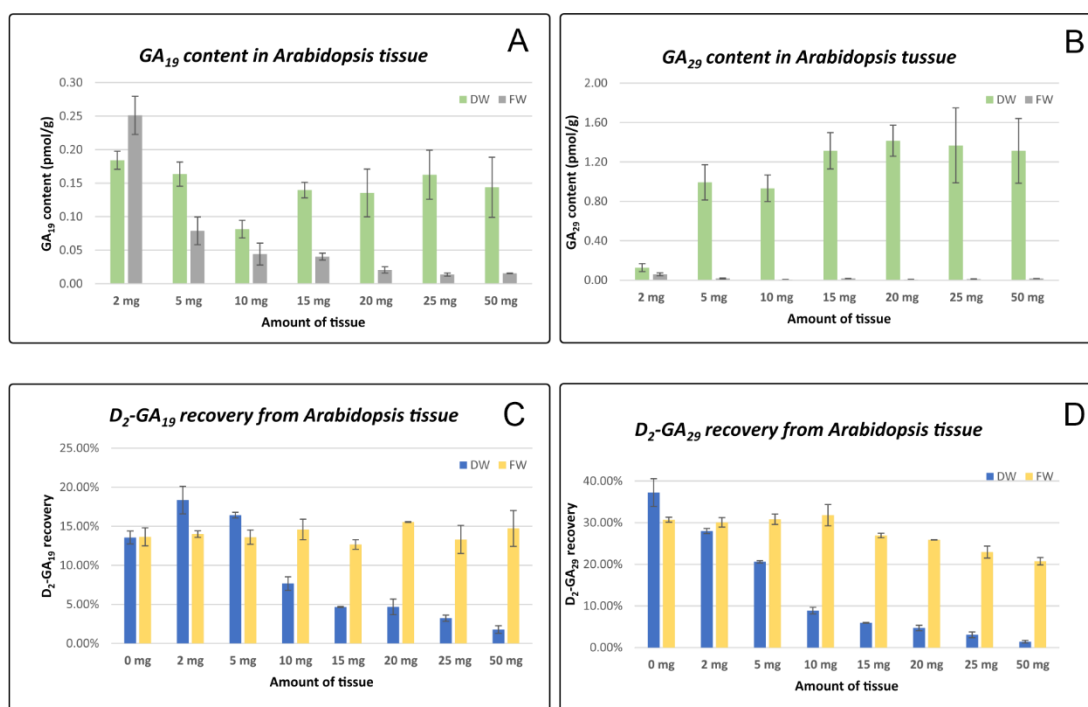
Figure 23. *Demonstration of the matrix effect - dependence of the endogenous hormone response (A, B) and recovery of deuterium-labelled gibberellin internal standards on the amount of biological tissue (30-day-old Arabidopsis thaliana, ecotype Colombia) – C, D. The data/error bars represent the mean/standard deviation of three independent determinations.*

A similar situation can be observed for selected BRs (**Figure 24**) or MIAs (**Figure 25**). For BRs appearing at the level of fmol·g^{-1} in vegetative tissue, it was found that extraction using the DW was better than using the FW because the low quantities of BRs were significantly "diluted" by a high content of water and appeared close to the LOQ (Fig. 24 A, B). On the other hand, the amount of DW should be in the range of 2-5 mg to ensure an acceptable internal standard recovery (Fig. 24 C, D). Only in that case is the final concentration of hormone close to the correct value (response $R = \frac{A_e}{A_{IS}} \cdot c_{IS}$ where A_e is a peak area of endogenous substance, A_{IS} is a peak area of internal standard, and c_{IS} is a concentration of internal standard).
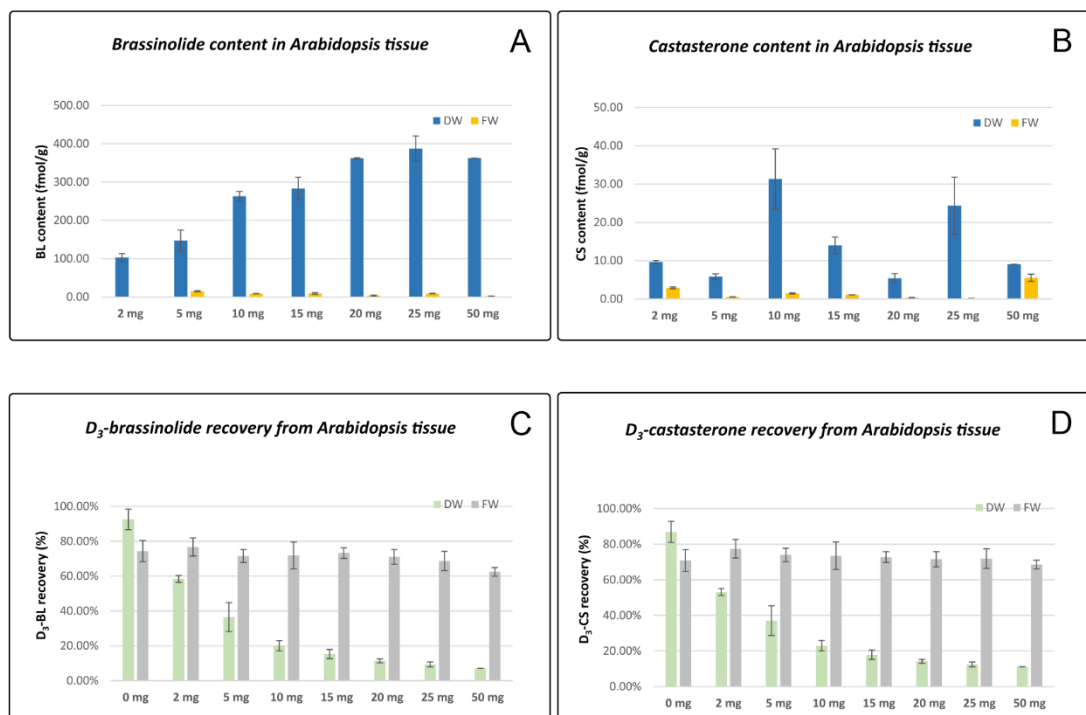
Figure 24. *Demonstration of the matrix effect - dependence of the endogenous hormone content (A, B) and recovery of deuterium-labelled brassinosteroid internal standards on the amount of biological tissue (30-day-old Arabidopsis thaliana, ecotype Colombia) – C, D. The data/error bars represent the mean/standard deviation of three independent determinations.*

MIAs occur in plant tissue at far higher concentrations than those of plant hormones. Some of them have been found at levels of $\mu mol \cdot g^{-1}$ (Fig. 25 A), whereas others were at $nmol \cdot g^{-1}$ (Fig. 25 B). Harmine was detected in yohimbe bark tissue but at levels about fifty times lower than those of ajmalicine, and therefore are not shown. Harmaline was not detected in this type of tissue. As evident from Fig. 25 A, B, the optimal DW for extraction determined to be about 5 mg to ensure that the recovery of the internal standard was not significantly affected by the sample matrix and the levels of endogenous substances were acceptable.
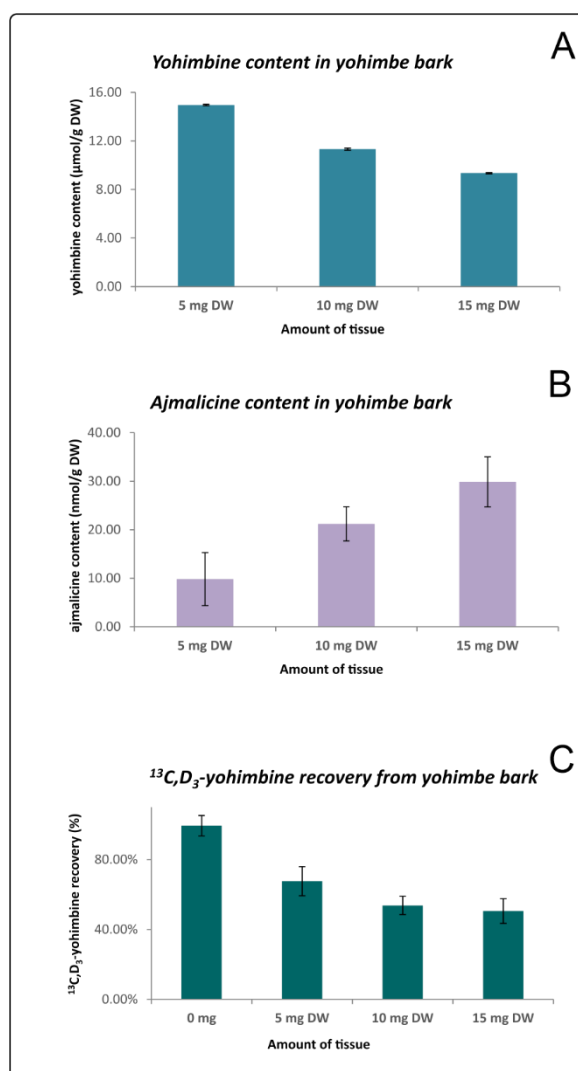
Figure 25. *Demonstration of the matrix effect - dependence of the endogenous indole alkaloid content (A, B) and recovery of methyl-^{13}C, D$_3$-yohimbine internal standard (C) from biological tissue (yohimbe bark). The data/error bars represent the mean/standard deviation of six independent determinations.*

The above examples clearly show that the slogan "more sample, greater chance of successful detection" does not apply in trace analysis.

III. CONCLUSIONS AND OUTLOOK

The sample preparation and trace analysis of natural signalling molecules are challenging because of their wide variety of chemical properties and very different biological roles in the tissues of origin. Among the instrumental analytical techniques used in this research area, LC-MS has become the most versatile, rapid and sensitive technique for the detection of these small molecules in the last two decades. The LC-MS methodologies presented in this thesis represent an improvement or extension to previously established methods or the development of completely new methods. Emphasis is placed on the most efficient method for preconcentration of low-abundance analytes from a complex biological matrix, the high throughput capabilities of the procedure and highly accurate quantification using the isotope dilution method. The approaches presented in this thesis could serve as powerful tools in biological and biochemical studies to elucidate the biosynthesis and metabolism of the mentioned substances and their mechanisms of action. Some of the methods have been designed for applications in food quality control.

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ARTICLES

ARTICLE 1

Plants are capable of synthesising animal steroid hormones

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Contribution: review outline, literature research, manuscript writing

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ARTICLE 1

molecules

Review

Plants are Capable of Synthesizing Animal Steroid Hormones

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Abstract: As a result of the findings of scientists working on the biosynthesis and metabolism of steroids in the plant and animal kingdoms over the past five decades, it has become apparent that those compounds that naturally occur in animals can also be found as natural constituents of plants and vice versa, i.e., they have essentially the same fate in the majority of living organisms. This review summarizes the current state of knowledge on the occurrence of animal steroid hormones in the plant kingdom, particularly focusing on progesterone, testosterone, androstadienedione (boldione), androstenedione, and estrogens.

Keywords: natural sterols; plants; animals; steroid hormones; estrogens; progesterone; testosterone; boldenone; boldione; androstenedione

1. Introduction

The plant and animal kingdoms are not two completely separate worlds coexisting on this planet, but, on the contrary, they are two worlds whose evolution has taken place simultaneously, hand in hand with each other. It is therefore more than obvious that some substances being synthesized in nature for a particular purpose can occur in both plant and animal organisms. Certain compounds that regulate growth and development in plants may also control cellular growth and differentiation processes in animals, and vice versa. An example of such compounds may be sterols, i.e., steroid alcohols. These tetracyclic substances belong to isoprenoids, a large group of naturally occurring compounds formed in a cell by combining six units derived from the five-carbon molecule dimethylallyl diphosphate (DMAPP) and its isomer isopentenyl diphosphate (IPP) [1]. It is worth mentioning that sterols can be found in all eukaryotes (plants as well as animals), where they play many irreplaceable roles, including maintaining membrane semi-permeability, regulating their fluidity, serving as biosynthetic precursors for steroid hormones, and acting as important signaling molecules [1–4]. A typical representative of a natural sterol produced by both plant and animal cells is cholesterol (Figure 1).
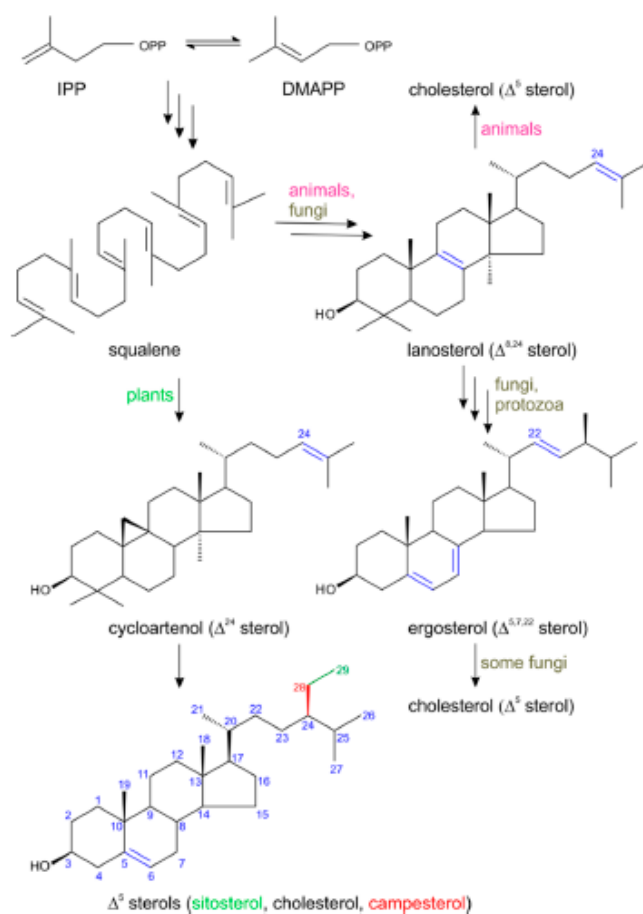
Figure 1. A simplified biosynthetic scheme for selected natural sterols [5–8].

This C_{27} sterol serves as a precursor of steroid plant signaling molecules (brassinosteroids, phytoecdysteroids) [1] as well as androgen- and estrogen-type sex hormones in animals [9]. When looking for the plant/animal origin of the latter hormone group, it seems that estrogens were first discovered in plants (in 1926) when Austrian medical doctor Otfried Otto Fellner, known today as a pioneer in gonadal endocrinology, was able to demonstrate estrogenic activity in oatmeal and rice [10]. At the same time, Dohrn, together with Faure, Poll, and Blotevogel, at the Anatomical Institute in Hamburg showed estrogenic activity also in other plant species including sugar beet and potato, as well as in yeast [11]. Steroid hormones with estrogenic activity were detected in the animal kingdom (in humans) about three years later by Butenandt and Doisy [12,13]. One may ask the question here if the chicken or the egg comes first, i.e., if chemical messengers, hormones, originate from plants or animals, regardless of the order in which they were discovered. Interestingly, some findings on the evolution of the neuroendocrine system in animals suggest that the hormones regulating morphogenesis and reproduction in invertebrate animals have their phylogenetic origin in more primitive multicellular organisms [14].

A recent topic of often heated discussion is the presence of the steroid hormones progesterone, testosterone and their derivatives in human diets of plant as well as animal origin. The reason for this is that a majority of these substances (except progesterone) are listed on the annually updated Prohibited list of The World Anti-Doping Agency (WADA) in the category of anabolic agents (S1) [15]. Therefore, their levels are monitored in the biological fluids of athletes and in the preparations they consume. Besides their presence in the WADA list, all the above mentioned steroids are also prohibited substances that cannot be contained in food products in European countries (according to Regulation (EC) No. 178/2002 of the European Parliament laying down the general principles and requirements of food legislation, and procedures in matters of food safety). However, the legislation does not specify for each prohibited substance at what concentration it is possibly harmful to human health, although since the sixteenth century we know, thanks to Paracelsus (1493 to 1541), that "the dose makes the poison" (Sola dosis facit venenum in Latin), i.e., a substance can cause the harmful effect associated with its toxic properties only if it reaches a biological system in a sufficiently high concentration. Therefore, regardless of their endogenous origin, once they are detected in any food product (vegetable, fruit, herb, meat) in any concentration, the seller is sanctioned for breaking the existing legislation and the food/nutritional supplement is withdrawn from sale.

The aim of this article is to point out that, in addition to well-known animal resources, steroids are also natural and integral components of plants, where they are synthesized de novo as chemical messengers for cell–cell communication, required for the regulation of physiological processes related to growth, development, and reproduction [16–19]. For this reason, it is therefore evident that steroids can be detected in plant-derived animal feed as well as in human phytosterol-rich food, and consequently in products of their secretion. This is very important information, especially for health authorities.

2. Biosynthesis of Plant Sterols with Respect to Steroid Hormone Formation

As mentioned in the Introduction, plant sterols (phytosterols) are a very important family of natural substances that have many biological functions in plants. It has also been known since the 1950s that they are very beneficial for humans as dietary phytosterols are able to lower levels of serum cholesterol via the inhibition of its absorption and the compensatory stimulation of its synthesis, when consumed at intake levels over 1 g per day [20–22]. Phytosterols may also act as precursors for the de novo biosynthesis of steroid hormones. In general, sterols are tetracyclic C_{30} terpenoid (isoprenoid) substances belonging to a group of triterpenoids [23], formed by the condensations of basic five-carbon (C_5) building units of isoprene in the form of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) [1]. In higher plants, two pathways coexist to produce terpenoids—the mevalonate pathway (MVA) and the 1-deoxy-D-xylulose 5-phosphate pathway (DOXP), both named according to the first intermediate formed [1]. DOXP is also called the 2-C-methyl-D-erythritol 4-phosphate pathway (MEP) after the DOXP reduction/isomerization product in this pathway. To our current knowledge, MVA operates in the cytosol of plant cells, while the MEP pathway takes place in plastids in most eukaryotic photosynthetic organisms, but not in animals [24]. In the case of phytosterols, it has been shown that their skeleton is derived from IPP made up exclusively from acetate units via MVA in the cytosol [8,25]. The plant sterol pathway consists of a sequence of more than 30 enzyme-catalyzed steps, all of which are located in plant membranes [26,27]. The key essential intermediate in plant sterol biosynthesis is the linear C_{30} hydrocarbon squalene, which directly undergoes a cyclization to yield the C_{30} Δ^{24} sterol cycloartenol in photosynthetic plants [18]. Fungi without photosynthetic apparatus convert squalene into lanosterol and finally ergosterol [7]. Subsequent alkylation reactions of cycloartenol in photosynthetic plants lead to the synthesis of the first phytosterols (Δ^5 sterols) such as cholesterol (C_{27}), campesterol (C_{28}, i.e., 24-methyl Δ^5 sterol), and sitosterol (C_{29}, i.e., 24-ethyl Δ^5 sterol) [22] (see Figure 1). These phytosterols are starting points for the biosynthesis of the plant steroid signaling molecules phytoecdysteroids and the plant steroid hormones brassinosteroids [1], as well as progesterone, testosterone and its derivatives.

3. Progesterone

Progesterone (PRG; Figure 2) has been described for decades predominantly, as a mammalian gonadal hormone. Its presence in plants was reported for the first time in 1964 [28], but this finding has been questioned for many years, with claims that the methods by which it was detected in plants were non-specific and cannot be trusted [29].
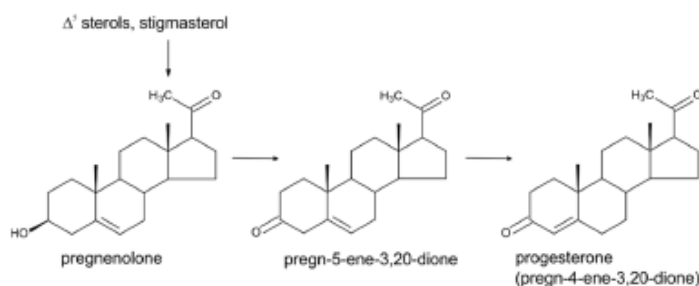
Figure 2. The scheme of progesterone biosynthesis in higher plants.

At that time, thin-layer chromatography, gas chromatography, or immunoassays, were commonly used methods for the detection of natural substances in biological matrices with none or an insufficient sample purification of the crude extract [28,30,31]. However, later, with the gradual introduction of modern analytical instrumental methods (mass spectrometry), it was shown conclusively that PRG is definitely naturally present in plants [29,32,33], where it serves as a precursor in the biosynthesis of androstanes and estranes [33,34]. Numerous studies with ^{3}H and ^{14}C-labelled precursors showed that sitosterol, a predominant sterol in higher plants, and the less abundant sterol cholesterol can serve as precursors of PRG in plants [35–38] (see Figure 2). There are also some studies that suggest that the precursor of PRG could be campesterol and stigmasterol (C_{29}, i.e., 24-ethyl $\Delta^{5,22}$ sterol), as has been well reviewed by Janeczko [19]. There is a relatively poor understanding of the biological importance/physiological functions of PRG in plants. The majority of studies have been conducted by exogenously applying PRG to various plant systems, *i.e.*, either seedlings or plant cell cultures of various plant species. Experiments of this design suggest that this substance has a certain regulatory activity in plant growth and development, influencing both vegetative and reproductive development. For instance, shoot and root growth of the common model plant Arabidopsis thaliana [29] and of sunflower [39] were demonstrated to be influenced by PRG in a dose-dependent manner. The acceleration of flowering was observed in A. thaliana and wheat exposed to micromolar concentrations of PRG [40,41]. The involvement of PRG in reproduction processes in plants has been further indicated by the PRG stimulation of tube growth of mature tobacco pollen [42], and by increasing levels of endogenous PRG during the germination of kiwifruit pollen [43]. It is worth mentioning that the authors of that study observed detectable levels of endogenous PRG and 17β-estradiol (member of the estrogen-type sex hormone family) in ungerminated kiwifruit pollen, but could not detect endogenous testosterone, while during germination (tube organization phase and subsequent elongation), the levels of PRG and 17β-estradiol dramatically increased and testosterone levels comparable with those of PRG were detected. The studies mentioned above are examples of a number of studies documenting the natural occurrence and function of these steroid substances in plants. It is, however, important to note that from the perspective of plant physiology, these compounds are not considered plant hormones by the plant science community. According to the accepted definition, for a substance to be included in the list of plant hormones, it must meet three fundamental criteria: 1. the protein–receptor for its perception must be known, 2. the compound should be omnipresent in the plant kingdom, and 3. the compound should influence physiological processes at a low concentration [44].

Some recent molecular studies dealing with steroid perception in plant cells revealed the presence of a plant membrane-localized steroid binding protein in Arabidopsis that can bind to multiple steroid molecules with different affinities, but the highest affinity was to PRG [45]. It was further shown that this protein negatively regulates cell elongation (transgenic plants overexpressing this gene have a short hypocotyl) and stimulates root gravitropism [46]. The gene for a similar binding protein from rice (*Oryza sativa* L.) was later cloned and its abundant expression described [29]. Using radioligand binding analysis, specific binding sites for PRG have been located within the cytoplasm and cell membrane of wheat [47]. A relatively widespread occurrence of PRG, together with estrogens and androgens, was demonstrated by Simon and Grinwich [31], who screened 128 plant species from over 50 families by radioimmunoassay. They found that PRG was present in about 80% of investigated species, testosterone and its derivatives in about 70% of species, and estrogens (estrone and 17β-estradiol) in about 50% of species. Interestingly, androgens (testosterone and dihydrotestosterone) were detected in the seeds of all species tested. The mass spectrometry-based reports of Iino et al. [29] and Simerský et al. [33] provided evidence that PRG, testosterone, and its derivatives are present in plant tissues in very low concentrations (pg·g^{-1} fresh weight, FW; Table 1). Thus, it may be, therefore, that all three fundamental criteria for PRG to be considered as plant hormone are, to some extent, fulfilled. Nevertheless, additional studies, including the application of PRG biosynthesis inhibitors and mutants with impaired PRG biosynthesis, are needed to understand more deeply the role of PRG and its mechanism of action in planta.

Table 1. The results of quantitative analysis of selected steroids in plant material.

Steroid	CAS Number	Mol. Formula	Mol. Weight (g·mol^{-1})	Amount	Origin	Ref.
progesterone	57-83-0	$C_{21}H_{30}O_2$	314.46	0.08 µg·g^{-1}	*Pinus nigra*	[48]
				3–1600 ng·g^{-1}	31 plant species	[31]
				6–1540 ng·kg^{-1}	8 plant species	[29]
				1.19–15.5 µg·g^{-1}	*Pinus taeda*	[32]
				0.66 ng·g^{-1}	*Inula helenium*	[33]
				17.4 ng·g^{-1}	*Nicotiana tabacum*	[33]
				18.5 ng·g^{-1}	*Digitalis purpurea*	[33]
				0.02–15.39 ng·g^{-1}	*Tribulus terrestris*	Tarkowská, unpublished
testosterone	58-22-0	$C_{19}H_{28}O_2$	288.42	0.08 µg·g^{-1}	*Pinus silvestris*	[49]
				0.09 µg·kg^{-1}	wheat	[50]
				0.05 µg·kg^{-1}	corn oil	[50]
				0.21 µg·kg^{-1}	safflower oil	[50]
				0.01–0.02 ng·g^{-1}	*Tribulus terrestris*	Tarkowská, unpublished
androsta-1,4-diene-3,17-dione (boldione)	897-06-3	$C_{19}H_{24}O_2$	284.40	not quantified	*Pinus halepensis*	[51]
				0.1–2.7 pg·g^{-1}	*Tribulus terrestris*	Tarkowská, unpublished
androst-4-ene-3,17-dione	63-05-8	$C_{19}H_{26}O_2$	286.41	0.59 µg·g^{-1}	*Pinus silvestris*	[49]
				0.09 µg·g^{-1}	*Pinus nigra*	[48]
				0.08 µg·g^{-1}	*Pinus nigra*	[52]
				0.05 ng·g^{-1}	potato	[50]
				0.48 ng·g^{-1}	wheat	[50]
				2.20 ng·g^{-1}	*Nicotiana tabacum*	[33]
				3.20 ng·g^{-1}	*Inula helenium*	[33]
				0.01–0.05 ng·g^{-1}	*Tribulus terrestris*	Tarkowská, unpublished
estrone	53-16-7	$C_{18}H_{22}O_2$	270.37	2.5–4.5 µg·kg^{-1}	pomegranate	[53]
				5.13/5.25 µg·g^{-1}	*Haphaene thebaica*	[54]
				0.04 µg·ml^{-1}	corn oil	[54]
				33.75 µg·g^{-1}	*Olea europea*	[55]
				28–420 ng·g^{-1}	20 plant species	[31]
17β-estradiol	50-28-2	$C_{18}H_{24}O_2$	272.38	2–10 µg·kg^{-1}	*Phaseolus vulgaris*	[56]

4. Testosterone

If there is some reluctance to admit that steroid hormones occur in higher plants, then it is especially true for testosterone (4-androsten-17β-ol-3-one; TS; Figure 3) and its derivatives. Usually it is because people associate the effects of steroid hormones with the endocrinology of animals.
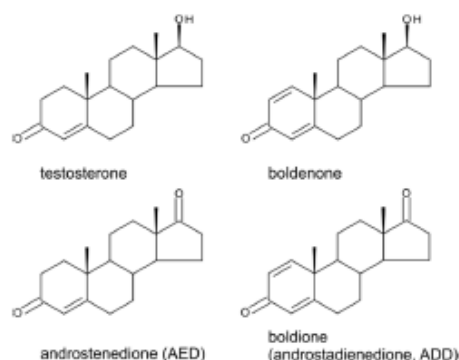
Figure 3. The structures of testosterone and structurally related androstanes.

TS together with epitestosterone (4-androsten-17α-ol-3-one) and androstenedione (4-androsten-3,17-dione) was isolated for the first time from plant sources by Šaden-Krehula et al. in 1971 [49]. The authors used pollen from Scotch pine *Pinus silvestris* and later showed the presence of all these substances as well as PRG in the pollen of *Pinus nigra* [48]. It was confirmed that *Pinus* species are a rich source of testosterone since it was further detected in *P. tabulaeformis* and *P. bungeana* and in the reproductive organs of other plant species, including ginkgo (*Ginkgo biloba*) and lily (*Lilium davidii*) [57]. Although our knowledge of the distribution of TS in plants is still fragmentary, and scientific studies dealing with its isolation and/or determination from plant sources are published very rarely, some traceable information in the literature indicates that TS and dihydrotestosterone occur in twenty plant species, including maize, barley, and rhubarb [31]. Furthermore, Hartman et al. described the natural occurrence of steroid hormones in food, demonstrating the presence of TS in potatoes, soybeans, haricot beans, and wheat, where its levels ranged between 0.02 and 0.2 $\mu g \cdot kg^{-1}$ [50] (Table 1). The authors reported that this androgen also occurs in native oils used in human nutrition, such as olive oil, corn oil, and oil made from safflower (*Carthamus tinctorius*) seeds. Interestingly, safflower oil has been shown to lower lipids and lipoproteins in human serum due to its high content of linoleic acid (73% to 77%) [58], which belongs to the highly important omega-6 unsaturated fatty acids group and is an essential fatty acid in the human diet. Linoleic acid is also known as vitamin F. Safflower is also valuable for beekeepers as a melliferous plant with a high nectar content in late summer as well as being a promising source for biodiesel production, i.e., one of the renewable energy sources [59]. The phytosterol content of safflower is relatively high, ranging between 2000 and 4500 $\mu g \cdot g^{-1}$ in seeds, in which β-sitosterol accounts for the largest percentage (50% to 70%) of the total phytosterol content [60].

From a biochemical standpoint, the biosynthesis and biological function of TS in plants seem to be similar to those in animals [16,61,62]. This C_{19} steroid is formed via the MVA pathway in the cytosol of plant cells from cholesterol by the sequential action of multiple enzymatic reactions. These reactions include the side chain cleavage of cholesterol to the C_{21} steroid pregnenolone, followed by a transformation into androstenedione and finally into TS (Figure 4). The latter enzymatic step was confirmed by feeding experiments with ^{14}C-androstenedione, which was converted to TS in pea and cucumber seedlings and in cultured cells of *Nicotiana tabacum* [63–65]. In contrast to the animal kingdom, where TS and other androgen substances act only as sex hormones, it has been shown in plants that they affect not only their reproductive development (especially flowering and floral sex determination), but also vegetative development [17,18].

cholesterol

pregnenolone

androst-4-ene-3,17-dione
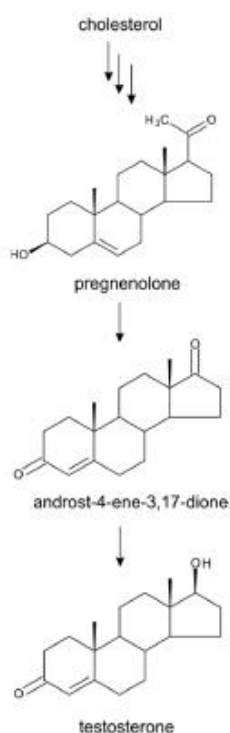
testosterone

Figure 4. A simplified biosynthetic pathway of testosterone in plants.

5. Boldenone, Boldione, and Other Testosterone Derivatives

Boldenone (Bol) is the trivial name for 1-dehydrotestosterone or androsta-1,4-diene-17β-ol-3-one (Figure 3). Androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (AED) are closely related metabolites—Figure 3. ADD (androstadienedione) is recognized as a Bol precursor in various animal species including humans and is trivially named as boldione, whereas AED is considered as a TS precursor [66] and can be found in the literature often as androstenedione rather than its full name or AED. Both Bol and boldione are prohibited anabolic steroids in European Union Member States. As in the case of estrogens, PRG and TS, there are still discussions about whether these substances are detected in surveillance laboratories as a result of illegal direct administration or whether they are of endogenous origin. These discussions concern both animal and human samples as well as plant-based food supplements, in which their positive detection attracts special attention. In the vast majority of cases, a positive sample of a nutritional supplement is considered to be harmful to health because it is assumed to be deliberately enriched with a forbidden anabolic steroid. There are, however, scientific studies that admit that both weak androgenic substances of interest (Bol and boldione) can be formed endogenously from phytosterols and may thus occur naturally in plants [67]. If we look for plant sources where either ADD and/or AED was detected, we find that AED was determined in pine pollen of *P. sylvestris* ($0.59 \ \mu g \cdot g^{-1}$) and *Pinus nigra* ($0.08 \ \mu g \cdot g^{-1}$), using various analytical methods, as far back as in 1971, 1979, and 1983 [48,49,52]. Later, in 1998 relatively significant amounts of AED were quantified in the important foodstuffs – wheat ($0.48 \ ng \cdot g^{-1}$) and potato ($0.05 \ ng \cdot g^{-1}$) [50]. Quantitation data are summarized in Table 1. Trace quantities were further observed in soybeans, haricot beans, mushrooms,

olive oil, and safflower oil, as well as in wine and beer. With increasing sensitivity, i.e., the ability to reach lower limits of detection (LOD)/limits of quantitation (LOQ), AED was determined in other plant species such as tobacco (*Nicotiana tabacum*; 2.20 ng·g^{-1} FW) and the native European herb elecampane (*Inula helenium*; 3.20 ng·g^{-1} FW) [33]. AED and ADD together with PRG and TS were unequivocally detected in the annual creeping herbaceous plant *Tribulus terrestris* by very sensitive and precise analysis based on ultra-high performance liquid chromatography–tandem mass spectrometry (Tarkowská, unpublished). *T. terrestris* is widespread globally and it is used in folk medicine, mainly for the treatment of cardiovascular and eye diseases and for high blood pressure. In Europe and the USA, food supplements containing *T. terrestris* extracts are on sale as regenerative/adaptogenic agents similar to ashwagandha (indian ginseng; *Whitania somnifera*) or ginseng (*Panax ginseng*) [68]. The highest concentrations of the four investigated steroids in *T. terrestris* were found for PRG, with levels in plant tissues ranging from 0.01 to 0.015 μg·g^{-1} of dry weight. TS and AED were present at approximately the same level and reached about 0.25% of PRG levels. AED levels were an order of magnitude lower than TS levels. The lowest levels were found for ADD, which generally was present at one tenth to one hundredth of the concentration found for AED (Table 1). Recently, phytochemical analysis of the crude extracts isolated from *Pinus halepensis* needles revealed the presence of ADD [51]. Thus, taking into account all the above-mentioned facts, it is clear that these androgen substances, which were assumed to be animal-derived, may also have a plant origin.

The effect of androgens on physiological processes has been best investigated in humans and other animals. To investigate their functions in plants, similar experimental designs have been applied to several plant species. Plant growth was affected in Arabidopsis and winter wheat (*Triticum aestivum*), where the TS precursor AED stimulated the proliferation of callus tissue and promoted the germination and growth of immature embryos, respectively [69,70]. However, it is unfortunate that these data are difficult for the general scientific community to access. There is also evidence that AED can influence developmental processes associated with plant reproduction, such as flowering. In some plant species (Arabidopsis, wheat), AED treatment caused a significant increase in the percentage of plants reaching the reproductive stage [40,41]. Experiments examining the metabolism of androgens in higher plants (pea and cucumber) showed that ^{14}C-labeled AED added to leaves was transformed to TS [63,64]. These results are in agreement with those found in animal cells. Therefore, it seems that plant cells might have a similar enzymatic apparatus for the biosynthesis and metabolism of androgen substances as that of animal cells.

6. Estrogens

Estrogens (Figure 5) belong to steroid-type substances commonly referred to as animal sex hormones since they are produced by ovaries in adult females of higher vertebrates, in which they are responsible for the development and regulation of the female reproductive system.
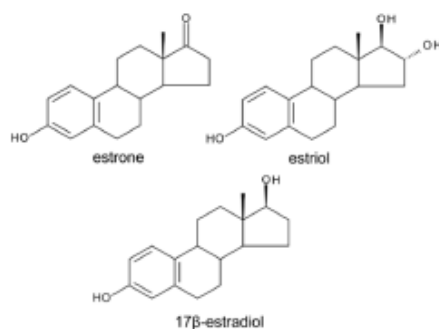
Figure 5. The structures of selected estrogens.

However, the observation of estrogenic activity was first made in plant extracts (late 1920s) even before structure elucidation of the endogenous animal estrogens [11,71]. Estrogenically active substances were subsequently isolated in crystalline form from palm kernels [72] and willow catkins [73]. Various authors later confirmed the presence of steroidal estrogens in many plant species including date palm, bean, pomegranate, and the species of the *Prunus* genus [53,56,74–78]. Although numerous authors have described the presence of steroidal estrogens as well as their biosynthetic pathway, some authors have, historically, disputed their presence in plants [79,80]. Nevertheless, in 2001, estrogen receptor-like proteins were isolated from various plant organs and shown to be localized in the nucleus [81]. Furthermore, there are certain indications that steroidal estrogens affect growth and reproduction in plants [14,18]. For instance, they have been shown to stimulate embryo and seedling growth in pea [82,83], sunflower [39], and tomato [84]. Analogously to their reproductive function in animal kingdom, steroidal estrogens were reported to also influence plant reproductive development such as flowering and sex determination of flowers. Already in 1937, Chouard demonstrated the flower promoting effect of estrogens by watering the plants of *Callistephus sinensis* with 17β-estradiol (Figure 5) solution [85]. Later, the induction of flowering by estrogens was achieved also in very diverse plants growing under non-inductive conditions. In common chicory *Cichorium intybus*, the inductive cold period could be substituted by 17β-estradiol or estrone (Figure 5) treatment [86]. Similarly, the flowering of scarlet sage *Salvia splendens*, which is naturally initiated under long day conditions, was induced by 17β-estradiol and by unidentified estrogen-like substance isolated from flowering *Salvia* plants under short day conditions [87]. According to some scientific reports, the application of estrogens can considerably affect sex determination of flowers of plants that have both male and female flowers, i.e., dioecious plants. The ratio of female to male flowers was significantly affected by the application of estrogens (estrone, estriol, 17β-estradiol) when the percentage of female flowers increased by 66% in *Ecballium elaterium* L. while the total number of flowers was enhanced by 18% to 35% [88]. 17β-estradiol can be used to modulate the development of flowers to male or female, which has been shown in cucumber *Cucumis sativus*. The treatment of cucumber plants with 0.1 mg of 17β-estradiol induced a formation of an increased number of female flowers by about 20% [89].

Considering the above-mentioned findings, it is not surprising that the levels of endogenous steroidal estrogens are highest in reproductive parts of the plant, such as flowers, pollen grains, fruits, and seeds, whereas vegetative organs (stem, leaves, roots) are poorer sources of these substances [14]. As mentioned above, estrogens were detected in 50% of 128 species screened [31] so they can be considered as widespread naturally occurring substances—see Table 1.

Regarding their formation in cells of higher plants, it is assumed that the precursor of steroidal estrogens is cholesterol formed via the MVA pathway, i.e., the biosynthetic pathway of estrogens in plants is similar to that known in animals [16]. This hypothesis has been supported by experiments with radiolabeled precursors, from which it was observed that 17β-estradiol was formed from $[2\text{-}^{14}C]$ mevalonic acid in bean seedlings [56]. The same authors demonstrated the conversion of estrone to 17β-estradiol in the same plant species [90]. They proposed that enzymes capable of this conversion were located in leaf tissue.

7. Conclusions

The data discussed in this review provide evidence of the ability of plants to convert sterols into steroid hormones. This applies both to the plant steroid hormones brassinosteroids, as well as to other steroid substances, such as phytoecdysteroids, estrogens, progesterone, and androgenic substances of the testosterone type, including testosterone precursors and derivatives. Although it was reported here that all classes of animal steroids have been found in plants, this does not necessarily mean they are hormonally active.

Conflicts of Interest: The author declares no conflict of interest.

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ARTICLE 2

Isoprenoid-derived plant signaling molecules: biosynthesis and biological importance

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ARTICLE 3

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Quo vadis plant hormone analysis?

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Contribution: review outline, literature research, manuscript writing

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REVIEW

Quo vadis plant hormone analysis?

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Abstract Plant hormones act as chemical messengers in
the regulation of myriads of physiological processes that
occur in plants. To date, nine groups of plant hormones
have been identified and more will probably be discovered.
Furthermore, members of each group may participate in the
regulation of physiological responses *in planta* both alone
and in concert with members of either the same group or
other groups. The ideal way to study biochemical processes
involving these signalling molecules is 'hormone profiling',
i.e. quantification of not only the hormones themselves, but
also their biosynthetic precursors and metabolites in plant
tissues. However, this is highly challenging since trace
amounts of all of these substances are present in highly
complex plant matrices. Here, we review advances, current
trends and future perspectives in the analysis of all cur-
rently known plant hormones and the associated problems
of extracting them from plant tissues and separating them
from the numerous potentially interfering compounds.

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Abbreviations

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
BRs	Brassinosteroids
CE	Capillary electrophoresis
CKs	Cytokinins
GAs	Gibberellins
GC-MS	Gas chromatography-mass spectrometry
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
JA	Jasmonic acid
JAs	Jasmonates
LC	Liquid chromatography
MS	Mass spectrometry
SA	Salicylic acid
SLs	Strigolactones
UHPLC-MS/MS	Ultra-high performance liquid chroma-tography-tandem mass spectrometry

Introduction

Most (if not all) organisms use chemical signals in cell–
cell communication. Thus, chemical signalling is extremely
ancient. However, the complexity of cell signalling leapt
when first prokaryotic and subsequently eukaryotic cells
began to associate together in multicellular organisms,
putatively several billion and one billion years ago, respec-
tively (Parfrey et al. 2011). Following the emergence of
multicellularity, cell specialisation increased as tissues
and organs with diverse specific functions evolved. Co-
ordination of the growth and development of these cells,

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tissues and organs, as well as the environmental responses of complex multicellular organisms, required increasingly intricate signalling networks. Many of our current concepts about intercellular communication in plants have been derived from similar studies in animals, in which two main systems evolved: the nervous system and endocrine system. Plants, lacking motility, never developed a nervous system, but they did evolve hormones as chemical messengers. Plant hormones play essential roles (individually and in concert) in the regulation of myriads of physiological processes involved in plants' growth, development, senescence and responses to environmental stimuli. Until the 1990s, there were just five known types of plant hormone: auxins, cytokinins, gibberellins, ethylene and abscisic acid. However, during the last two decades compelling evidence has emerged that four other classes of substances (brassinosteroids, jasmonates, salicylic acid and most recently strigolactones) act as signalling molecules and probably have growth-regulating activities.

Plant hormones, also known as 'phytohormones', are usually present at extremely low concentrations in plant tissues, generally pg/g fresh weight (FW), while substances that interfere with their analysis are present in far greater concentrations. This is the major problem associated with plant hormone analysis. Thus, sound knowledge of the analytical and chemical principles underlying the extraction, purification, identification and quantification of plant hormones is essential for their accurate and precise determination. In this review, we summarise current understanding of these principles, methodologies for plant hormone analysis, factors that complicate their extraction and isolation from the highly complex matrices of plant and other tissues (which contain thousands of substances) and future perspectives.

Extraction and purification

Prior to extraction, plant material must be homogenised to break the cell walls in the tissues (Harrison 2011) and thus allow any hormones present to migrate to an appropriate extraction solvent. This can be done by grinding freeze-dried or fresh plant tissue (gram amounts) in a mortar with a pestle under liquid nitrogen then adding an appropriate solvent to the ground material. Alternatively, very small amounts of plant material (mg) can be ground in 1.5–2.0 ml plastic microtubes with a selected extraction solvent and tungsten carbide or zirconium oxide beads in a homogenizer for an appropriate time at a selected frequency. The most effective devices use multi-directional motions to transmit high kinetic energy to the beads and are capable of grinding tens of samples simultaneously. To avoid enzymatic or chemical degradation of the hormones, the plant material should be kept cold during the entire homogenisation process. The

efficiency of extraction of a target hormone from a plant tissue will depend on its polarity, its subcellular localisation and the extent to which it is associated with other compounds in the tissue such as phenolics, lipids, pigments and proteins (Hillman 1978). The solvent used must be capable of extracting the hormone efficiently, while minimising extraction of interfering substances. Methanol, acetonitrile, mixtures of these solvents with aqueous solutions of organic acids (generally formic or acetic) or buffers adjusted to neutral pH are usually used as extraction solvents for isolating plant hormones (Kowalczyk and Sandberg 2001; Nordström et al. 2004; Novák et al. 2008; Kojima et al. 2009; Urbanová et al. 2013). Analyte losses during the sample purification procedure can be accounted for by adding internal standards (usually labelled with stable isotopes) to the plant extracts. This procedure also provides a measure of the percentage recovery of target metabolites throughout the purification procedure. Ideally, recovery markers should be included for every plant hormone metabolite that is being quantified. However, in many studies only a few internal standards have been used, often added at late stages during the extraction process, or even just before quantitative analyses (Witters et al. 1999; van Rhijn et al. 2001). Clearly, all the current methodologies could be further improved by sophisticated internal standardisation of some of the missing labelled standards, mainly to cover the enormous variations in chemical properties of the substances, even within each phytohormone group. Dissimilar chemical nature of endogenous and internal substances subsequently leads to errors in their determination.

The ideal extraction duration depends on the target plant hormone group and (to a lesser degree) the specific target hormones. Generally, it should be long enough to allow quantitative migration of the analytes into the extraction medium and isotopic equilibration between the endogenous compounds and added internal standards. Decomposition of the endogenous hormones during prolonged extractions can be minimised by performing the extraction at low temperature (between −20 and 4 °C) and adding an appropriate antioxidant (for instance diethyldithiocarbamic acid; Pěnčík et al. 2009) to the extraction solvent.

The optimal purification method depends on the chemical nature of the target hormones, the type of analysis to be performed and choice of analytical instrument. In addition, appropriate separation procedures must be applied to reduce levels of interfering compounds in the extracts while maximising recoveries of the hormones in each purification step (Ljung et al. 2004). The first step is often liquid–liquid extraction combined with solid-phase extraction (SPE). SPE columns are packed by the manufacturers with solid sorbents that bind plant hormones (and other compounds, to varying degrees), usually via hydrophobic, polar or ionic interactions (often sorbents with hydrocarbon groups, graphitized carbon-based material and ion-exchange matrices,

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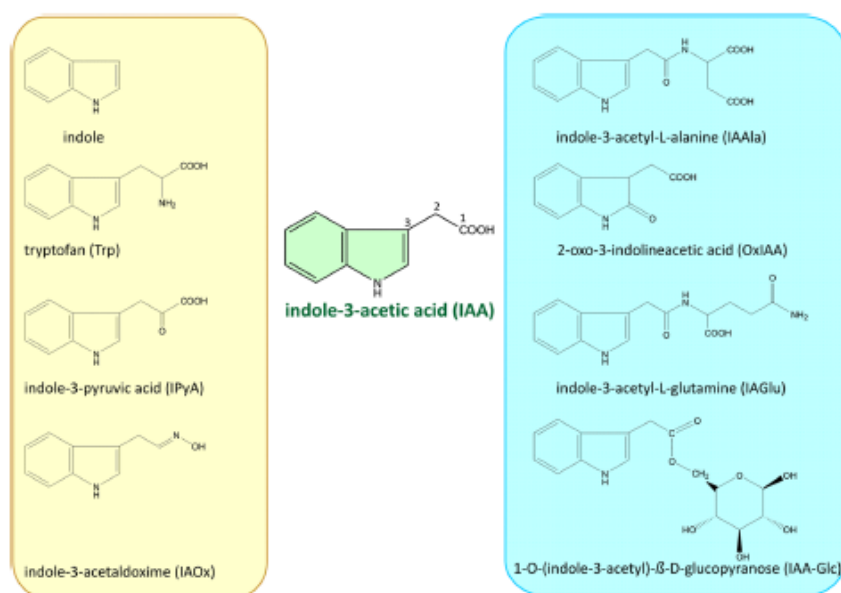
Fig. 1 Structures of auxins

respectively). Interfering substances are removed by washing the column with a suitable solvent and hormones are then eluted using a solvent that disrupts the bonds formed by the interactions between the hormones and the sorbent in the column. "Mixed-mode" SPE columns, packed with a mixture of two types of sorbent, are also available and have become very popular recently (Nordström et al. 2004; Dobrev et al. 2005; Novák et al. 2008; Kojima et al. 2009; Urbanová et al. 2013) due to their ability to reduce the number of required purification steps (since more than one separation mechanism can be exploited using a single column), while maintaining high sample clean-up efficiency. SPE allows high throughput of samples when combined with automatic systems, SPE robots, which are capable of purifying tens of sample simultaneously (Nordström et al. 2004; Kojima et al. 2009). However, no miniature mixed-mode purification system capable of handling extracts from mg FW samples has been developed yet.

Auxins

Auxins were the first discovered family of plant hormones. In the earliest recorded inference, Charles and Francis Darwin concluded that plant growth is regulated by a signal transported from one part of the plant to another where the physiological growth response occurs (Darwin and Darwin 1880). This "signal" was subsequently called auxin (from the Greek word "auxein" meaning "to grow") and identified as indole-3-acetic acid (IAA; Kögl and Kostermans 1934; Went and Thimann 1937). IAA (Fig. 1) is the major auxin involved in a plethora of physiological processes in plants. Its activities include induction of cell division and elongation in stems, and regulation of cell differentiation, various tropisms, abscission, apical dominance, senescence and flowering (Woodward and Bartel 2005; Teale et al. 2006). Two major IAA biosynthesis pathways have been postulated in plants: the tryptophan (Trp)-independent and Trp-dependent pathway (Normanly 2010; Mano and Nemoto 2012). After synthesis, IAA may be deactivated by catabolic oxidation (decarboxylative or non-decarboxylative), or conjugation to sugars and amino acids (Normanly 2010; Ljung 2012).

To obtain complete understanding of IAA metabolism in a given biological sample, information on levels of free hormone, its major metabolites and biosynthetic precursors is highly important. Accurate estimation of these substances requires the detection and quantification of minute amounts of analytes in plant extracts containing huge numbers of other substances at far higher concentrations.

Therefore, it is essential to use methodology that offers low detection limits and high selectivity, i.e. methods that are minimally sensitive to impurities. Several methods have been described for detecting free IAA, including HPLC with fluorescence detection (Crozier et al. 1980; Sundberg et al. 1986; Mattivi et al. 1999; Dobrev et al. 2005) or chemiluminescence detection (Xi et al. 2009), with or without enhancement by immunoaffinity-based purification techniques (Pengelly et al. 1981; Sandberg et al. 1985; Cohen et al. 1987; Marcussen et al. 1989). However, the most commonly employed method for quantifying IAA in plant tissues seems to be gas chromatography–mass spectrometry (GC–MS) with electron impact ionisation (Chen et al. 1988; Dunlap and Guinn 1989; Edlund et al. 1995; Ribnicky et al. 1998; Perrine et al. 2004; Barkawi et al. 2010). A drawback of this approach is that IAA is not volatile so it must be derivatised (usually by methylation or trimethylsilylation). Several methods for preparing derivatives of IAA precursors for GC–MS analysis have also been developed, including acylation of tryptamine, trimethylsilylation of indole-3-ethanol, and methyl chloroformate derivatisation of tryptophan (Quittenden et al. 2009; Liu et al. 2012a, b, c). Samples can be purified by reversed-phase SPE (Barkawi et al. 2008), mixed-mode SPE (Dobrev et al. 2005) or immunoaffinity extraction (Sundberg et al. 1986; Pěnčík et al. 2009). To avoid preparation of antibodies in animals, selective binding in a polymer matrix with a "molecular imprint" (MIP) of auxin can be used (Zhang et al. 2010). A miniature system for purifying IAA and its biosynthetic precursors using SPE tips has been developed (Liu et al. 2012a, b, c), and the best currently available analytical technology is based on liquid chromatography-tandem mass spectrometry (LC–MS/MS), which is capable of determining both IAA and its amino acid conjugates (Kowalczyk and Sandberg 2001; Pěnčík et al. 2009). However, this requires a much more intricate procedure than measurements of IAA alone, mainly because levels of IAA conjugates in plant extracts are significantly lower. However, all IAA metabolites except indole-3-pyruvic acid (IPyA, Fig. 1) can be analysed without any derivatisation prior to their MS detection in positive or negative electrospray mode (Kai et al. 2007a, b; Sugawara et al. 2009; Mashiguchi et al. 2011; Novák et al. 2012). Recently, IPyA (the most labile auxin precursor) has been identified as an important intermediate in the Trp-dependent IAA biosynthesis pathway in Arabidopsis (Mashiguchi et al. 2011; Stepanova et al. 2011). Tam and Normanly (1998) described a simple, rapid method for its reliable quantification based on derivatisation of the carbonyl group by hydroxylamine to form the oxime. Other methods, such as derivatisation of IPyA by 2,4-dinitrophenylhydrazone (Mashiguchi et al. 2011), cysteamine (Novák et al. 2012)

or sodium borodeuteride (Liu et al. 2012a, b, c) have also been developed.

Cytokinins

Cytokinins (CKs) are endogenous N^6-substituted adenine derivatives with the well-known primary ability to induce cell division activity in plant callus cultures (Skoog and Miller 1957). However, they also have a very wide spectrum of other physiological effects on various plants and tissues, notably they can delay senescence, inhibit root growth and branching, increase resistance to environmental stresses and initiate seed development (Richmond and Lang 1957; Mok 1994). As shown in Fig. 2, CKs can be divided into two subgroups based on their chemical structure: isoprenoid CKs (ISCKs), which bear an isoprenoid side chain at position N-6 and include zeatin, isopentenyl and dihydrozeatin forms; and aromatic CKs (ARCKs), which bear a side chain of aromatic (benzyl or furfuryl) origin. From a physiological perspective, there are four main types of CK metabolism: interconversion, hydroxylation, conjugation and oxidative degradation. However, the
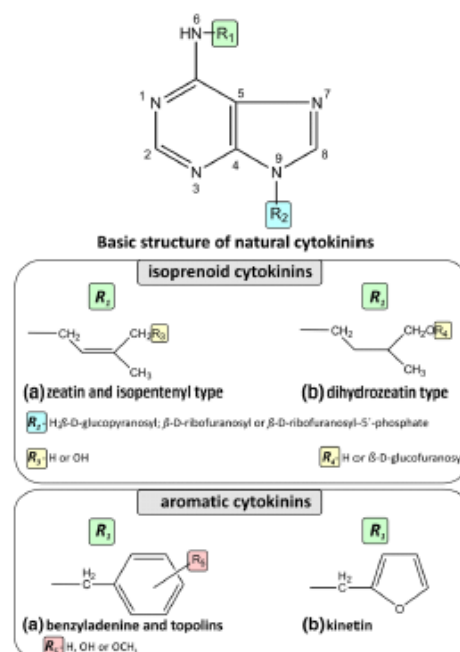

Fig. 2 Structures of cytokinins

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major CK metabolic processes are interconversions of CK bases, nucleosides and nucleotides (Chen 1981), as rates of CK nucleoside and nucleotide conversions to bases (the biologically active forms) reportedly control CK activity in plant cells (Kurakawa et al. 2007). Side chain modifications of ISCKs include stereospecific hydroxylation of the isopentenyl side chain, yielding zeatin (Takei et al. 2004), and reduction of the zeatin side chain, yielding dihydrozeatin (Mok and Martin 1994). Zeatin occurs naturally as two geometric isomers: *trans*- and *cis*-zeatin. In general, *trans*-zeatin (*tZ*) is considered as a cytokinin with high activity, compared to the little or no active *cis*-zeatin (*cZ*) (Kudo et al. 2012). Early investigators postulated that *tZ* was the predominant form, while the *cis*-isomer was much less abundant *in planta* (Schmitz and Skoog 1972; Mok et al. 1978). However, there are growing indications that *cZ* is the dominant cytokinin species in various plants, such as rice (Takagi et al. 1985), maize (Veach et al. 2003; Vyroubalová et al. 2009), potatoes (Suttle and Banowetz 2000) and several species of legumes (Emery et al. 1998, 2000; Quesnelle and Emery 2007). Interestingly, relative levels of zeatin stereoisomers can also differ substantially during a plant's lifecycle, *cZ*-type CKs generally predominate in tissues exposed to various stresses (drought, heat or biotic stress), while *tZ*-type CKs are often more abundant in unstressed tissues (Havlová et al. 2008; Pertry et al. 2009; Vyroubalová et al. 2009; Dobra et al. 2010). Common modifications of ARCK side chains are regiospecific hydroxylations, leading to formation of either *meta*- or *ortho*-derivatives called topolins (Kamínek et al. 1987; Strnad 1997). *Meta*-position of hydroxyl functional group increases CK activity of the parent compound, while at the *ortho*-position leads to its decrease (Holub et al. 1998). Glycosylation, leading to the formation of *N*- or *O*-glycosides of CKs, also occurs in many plant species (Entsch et al. 1979). *N*-glycosides lack CK activity in bioassays, indicating that their formation is a form of irreversible inactivation (Laloue 1977). In contrast, *O*-glycosides are considered inactive storage forms that play important roles in balancing CK levels (McGaw and Burch 1995). Free CK bases and nucleosides with unsaturated N^6-side chains may be irreversibly degraded by cleavage of the side chain catalysed by cytokinin oxidase/dehydrogenase, yielding adenine or adenosine and the corresponding side chain aldehyde (Galuszka et al. 2001).

CK metabolites have significantly differing chemical properties that must be considered in analyses. Notably, their ionic forms are dependent on pH, which thus strongly influences their behaviour on ion-exchange columns. For instance, at a pH of ca. 2, CK nucleotides are zwitterionic (uncharged), while CK bases and several metabolites (including 9-ribosyl and 3-, 7- and 9-glycosyl metabolites) are cationic. In addition, nucleotides are more polar and

thus less hydrophobic than glycosides, which in turn are more polar and less hydrophobic than CK bases and ribosides. Thus, CK metabolites' chromatographic properties vary widely, which complicates their analysis. In the 1960s, during the GC boom, both GC–MS and GC–ECD techniques were introduced for CK analysis. However, chemical modification of hydrogen-binding functional groups was essential for converting CKs (which are not volatile; Horgan and Scott 1987) into volatile derivatives suitable for GC. Various derivatisation approaches have been published, including trimethylsilylation (TMS, Most et al. 1968), permethylation (Morris 1977) and trifluoroacetylation (TFA, Ludewig et al. 1982). However, these procedures are associated with a number of technical difficulties, such as requirements for extremely water-sensitive reagents, inappropriate and time-consuming preparation, the extreme sensitivity of some derivatives (TMS and TFA) to moisture, and the need for high temperatures to elute permethylated derivatives. To avoid the problems arising from CK derivatisation for GC, attention has focused on LC–MS. The first LC–MS method for CK analysis, involving the separation of underivatised cytokinins using a frit-fast atom bombardment interface, was published by Imbault et al. (1993). The sensitivity of this method was subsequently improved, to low femtomolar detection limits, by derivatising 10 ISCKs using propionyl anhydride to form CK propionyl derivatives (Åstot et al. 1998; Nordström et al. 2004). In addition, atmospheric pressure ionisation (APCI, Yang et al. 1993) and electrospray ionisation (ESI, Prinsen et al. 1995; Witters et al. 1999; Novák et al. 2003) interfaces have been used for CK determination, affording picomolar to low femtomolar detection limits in analyses of 0.1–1 g FW samples of plant tissue. Nowadays, ESI is the only MS interface routinely used for quantitative analysis of CKs that offers sufficient ionisation efficiency not only for CK but also for the majority of plant hormones (Novák et al. 2008; Svačinová et al. 2012; Farrow and Emery 2012; Dewitte et al. 1999). Since CKs strongly absorb UV light (in the 220–300 nm region), several LC–UV methods have been earlier applied for quantitative analysis of CKs (Campell and Town 1991; Chory et al. 1994). UV detection can be further advantageous for analyses of immunoaffinity-purified cytokinin samples (Nicander et al. 1993) and separation of CKs by capillary electrophoresis (CE; Pacáková et al. 1997; Béres et al. 2012). In some cases, CE has been found to have distinct advantages over ultra-high performance liquid chromatography (UHPLC) in terms of separation efficiency, costs and simplicity, while maintaining comparable sensitivity to MS detection (Ge et al. 2006).

As substituted purine derivatives CKs also have typical electroactive properties, so they can be detected by electrochemical reduction or oxidation using appropriate electrodes (Hernández et al. 1995; Hušková et al. 2000;

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Tarkowská et al. 2003). However, these methods are more useful for screening purposes than routine analysis of endogenous cytokinin levels in plant tissues.

Similarly to other plant hormones, numerous attempts have been made to increase the sensitivity, peak capacity and speed of analyses of the trace quantities of CKs present in small amounts of various plant tissues (e.g. apical roots, stem regions, seeds and buds) or even individual cell organelles. Such requirements can be fulfilled by UHPLC in combination with tandem MS. However, extremely careful attention must also be paid to the efficiency of CK extraction and isolation from plant matrices, which (as mentioned) are very complex and typically contain thousands of substances. SPE followed by a high-throughput batch immunoextraction step and subsequent UHPLC separation has proved to be highly valuable for this, allowing for example the separation of 50 CKs—including bases, ribosides, 9-glycosides, *O*-glycosides and nucleotides—from several milligrams of poplar (*Populus × canadensis* Moench, cv *Robusta*) leaves (Novák et al. 2008).

Recently, miniature sample pretreatments based on hydrophilic interaction liquid chromatography (HILIC) combined with MS/MS have also been used for CK analysis (Liu et al. 2010, 2012a). Further improvements allowing reductions of starting amounts of tissue while maintaining sensitivity have been achieved by miniaturisation of SPE apparatus from polypropylene columns to pipette tips, so-called stop-and-go-microextraction or StageTip purification, which affords attomolar detection limits using 1–5 mg FW of *Arabidopsis* seedlings (Svačinová et al. 2012). In addition, magnetic solid-phase extraction techniques, involving use of magnetic or magnetizable adsorbents with high adsorption ability and superparamagnetism, have been introduced for effective sample enrichment and purification of CKs prior to HILIC combined with tandem mass spectrometry (Liu et al. 2012b). This approach was applied to analyse CKs in 200 mg FW extracts of rice roots (*Oryza sativa*) and *Arabidopsis thaliana* seedlings with pg/mL detection limits. Another approach for improving sample enrichment is to selectively bind CKs from plant extracts using molecularly imprinted polymers (MIPs) prior to LC–MS/MS analysis. This method was developed and applied to estimate levels of two 2 ISCKs and two ARCKs in 5 g FW extracts of tobacco, rape and soybean leaves with pg/mL detection limits (Du et al. 2012).

Gibberellins

Gibberellins (GAs) are a class of diterpenoid carboxylic acids that include biologically active compounds produced by various microbes (fungal and bacterial) and lower as well as higher plants, where they are endogenous growth regulators. To date, 136 naturally occurring GAs from diverse natural sources have been characterised (http://www.plant-hormones.info/gibberellins.htm). The most prominent physiological effects of bioactive GAs (e.g. GA_1, GA_4, Fig. 3) include for instance induction of flowering and germination, stimulation of stem elongation and delay of senescence in leaves and citrus fruits (Hedden and Thomas 2012).

All naturally occurring GAs possess a tetracyclic *ent*-gibberellane skeleton consisting of 20 carbon atoms (with rings designated A, B, C and D; Fig. 3), or a 20-nor-*ent*-gibberellane skeleton (in which carbon-20 is missing, so there are only 19 carbon atoms). Therefore, in terms of carbon numbers, GAs can be divided into two groups: C_{19}-GAs (e.g. GA_4, GA_1) and C_{20}-GAs (e.g. GA_{12}, GA_{53}). The prefix *ent* indicates that the skeleton is derived from *ent*-kaurene, a tetracyclic hydrocarbon that is enantiomeric to the naturally occurring compound kaurene. Like other classes of plant hormones, concentrations of GAs in plant tissues are usually extremely low (generally pg/g FW). Thus, very sensitive analytical methods are required for their detection. However, levels of GAs may vary substantially even within a plant organ. Vegetative tissues (stems, roots and leaves) typically contain several pg/g FW, while reproductive organs (such as seeds and flowers) often have three orders of magnitude higher levels (i.e. ng/g FW). The chemical nature of GAs also varies substantially, notably

Fig. 3 Structures of C_{19} and C_{20} gibberellins

GA$_{12}$ (R = H)   GA$_{53}$ (R = OH)
(C$_{20}$-GAs)

GA$_4$ (R = H)   GA$_1$ (R = OH)
(C$_{19}$-GAs)

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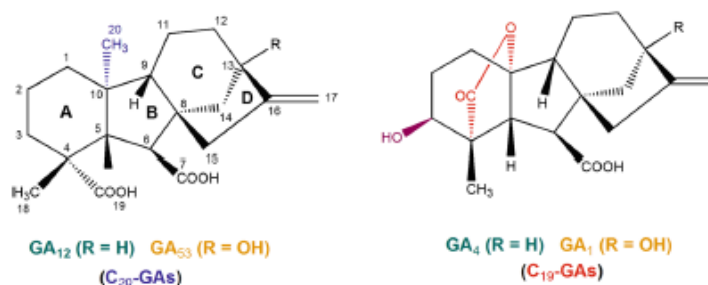
they cover a broad range of polarities and the only properties they share are that they behave as weak organic acids, with dissociation constants (pK_a) around 4.0 (Tidd 1964), and have no spectral characteristics such as fluorescence or UV absorption (except below 220 nm) that could easily distinguish them from other organic acids. The first methods for GA analysis, based on GC–MS determinations of volatile methyl ester trimethylsilyl ether derivatives, were introduced in the 1960s (Pryce et al. 1967; MacMillan and Pryce 1968; Binks et al. 1969). This approach is still used in some laboratories for quantifying and identifying GAs as it is highly sensitive (Mauriat and Moritz 2009; Magome et al. 2013). However, LC–MS is becoming more popular for quantitative analysis of GAs, mainly because it avoids derivatisation requirements. For instance, Varbanova et al. (2007) published a method for analysing 14 GAs in extracts of *Arabidopsis* mutants within 16 min by LC–MS/MS (after a laborious five-step purification procedure). The quantification procedure involved addition of deuterium-labelled internal standards before purification followed by isotope dilution analysis, as generally recommended for precise quantification (Croker et al. 1994). LC–MS/MS-based analysis has also been successfully used to determine endogenous GAs in Christmas rose (*Helleborus niger* L.) during flowering and fruit development (Ayele et al. 2010). Most recently, a rapid, sensitive method based on a two-step isolation procedure followed by UHPLC-MS/MS analysis has been published (Urbanová et al. 2013). This methodology is capable of quantifying 20 naturally occurring biosynthetic precursors, bioactive GAs and metabolic products from extracts of 100 mg FW plant tissues with low femtomolar detection limits.

Abscisic acid

Abscisic acid (ABA) is an optically active C_{15} terpenoid carboxylic acid (Fig. 4) that was discovered during the early 1960s, when it was found to be involved in the control of seed dormancy and organ abscission (Liu and Carns 1961; Ohkuma et al. 1963; Cornforth et al. 1965). Later, it was shown that the role of ABA in regulating abscission is minor and its primary role is in regulating seed dormancy and stomata opening (Patterson 2001). ABA plays important roles in many other numerous physiological processes such as seed maturation, adaptive responses to abiotic stress (Nambara and Marion-Poll 2005), shoot elongation, morphogenesis of submerged plants (Hoffmann-Benning and Kende 1992; Kuwabara et al. 2003), and root growth maintenance (Sharp and LeNoble 2002). It is a non-volatile, relatively hydrophobic substance containing a carboxylic group (Fig. 4). Therefore, commonly applied approaches for its extraction and purification include liquid–liquid extraction (Liu et al. 2002; Schmelz et al. 2003; Durgbanshi et al. 2005), liquid–liquid–liquid microextraction (Wu and Hu 2009; Bai et al. 2012), SPE (Dobrev and Kamínek 2002; Chiwocha et al. 2003; Zhou et al. 2003; Dobrev et al. 2005) and solid-phase microextraction (Liu et al. 2007). Like other phytohormones, it was initially determined by bioassays based on its physiological properties (Sembdner et al. 1988). The naturally occurring form is S-(+)-ABA, and the side chain of ABA is in 2-*cis*, 4-*trans* configuration by definition (Addicott et al. 1968). Due to this optical property, ABA was also previously determined by polarimetry (Cornforth et al. 1966). However, specific rotation is often influenced by numerous other substances

Fig. 4 Structures of stress-related plant hormones

(+)-Abscisic acid (ABA)

Salicylic acid (SA)

(-)-Jasmonic acid (JA)

12-oxo-phytodienoic acid (OPDA)
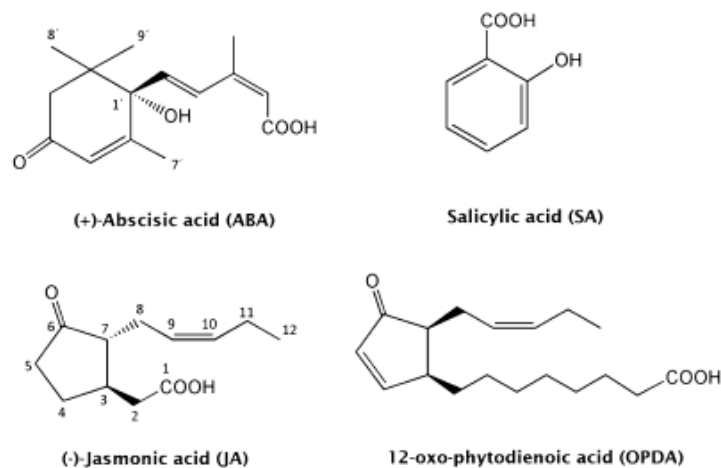
in plant extracts, thus such determination is very inaccurate. The compound also strongly absorbs ultraviolet (UV) radiation, maximally at about 260 nm (due to the presence of chromophores, chemical groups capable of absorbing light, resulting in the colouration of organic compounds), which allows its detection in HPLC eluates by monitoring their UV absorption (Ciha et al. 1977; Cargile et al. 1979; Mapelli and Rocchi 1983). HPLC has also been used to determine two metabolites of ABA: phaseic acid (PA) and dihydrophaseic acid (DPA) (Durley et al. 1982; Hirai and Koshimizu 1983).

Immunological methods such as radioimmunoassays (RIAs) (Weiler 1979, 1980; Walton et al. 1979; Mertens et al. 1983) and enzyme-linked immunosorbent assays (ELISAs) based on competitive binding between free and alkaline phosphatase-labelled ABA (Daie and Wyse 1982; Weiler 1982) have also been successfully used for estimating ABA levels and are still highly recommended for estimating free ABA levels in plant tissues. A method based on immunoaffinity chromatography (IAC) in combination with LC–MS has also been recently described (Hradecká et al. 2007). Early analytical methods for measuring levels of ABA metabolites employed GC coupled to electron capture detection (GC–ECD; Harrison and Walton 1975; Zeevaart and Milborrow 1976) or flame ionisation detection (FID; Watts et al. 1983) systems. These methods were capable of quantifying PA, DPA, *epi*-DPA and ABA glucose ester (ABAGE) in plant tissues at levels of about ng/g FW. Boyer and Zeevaart (1982) also developed a method for measuring ABAGE, as its tetraacetate derivative, by GC–ECD. In addition, several methods for quantifying ABA and ABAGE by GC–MS in selected ion monitoring (SIM) mode (Netting et al. 1982; Duffield and Netting 2001) and multiple ion monitoring (MIM) mode (Neill et al. 1983) have been published. However, GC–MS is limited to the analysis of volatile compounds, thus methylation of these analytes with diazomethane prior to the analysis is required. Regarding detection techniques following GC separation, ECD permits quantitative analyses of ABA in much smaller samples of plant material than FID. When GC coupled with MS in SIM mode, even much higher sensitivity is then achieved. Further, methods for determining ABA by CE have been published (Liu et al. 2002, 2003). CE has advantages for analysing ABA (as a trace substance in complex plant extracts), but it suffers from low sensitivity in combination with UV detection. This problem can be overcome using either micellar electrokinetic capillary chromatography (MECC; Liu et al. 2002) or laser-induced fluorescence (LIF) detection, both of which provide high sensitivity, but again require derivatisation because ABA is not fluorescent. Therefore, in the second cited study by Liu et al. (2003), ABA was labelled with 8-aminopyrene-1,3,6-trisulfonate via reductive amination in the presence of

acetic acid and sodium cyanoborohydride. The resulting conjugate was quantified, with fmol detection limits, and the method was used to analyse ABA in crude tobacco extracts. Recently, LC coupled to MS with soft ionisation techniques (ESI, APCI) has proved to be very powerful for analysing substances in plant extracts since they are often polar, non-volatile, thermally labile and (hence) inappropriate for GC analysis. Due to its high selectivity and sensitivity, LC–MS in multiple reaction monitoring mode (MRM) has also become increasingly popular for analysing ABA and its metabolites (Gómez-Cadenas et al. 2002; López-Carbonell and Jáuregui 2005; Chiwocha et al. 2007; López-Carbonell et al. 2009). Another technique that has been used for quantifying ABA and ABAGE is LC–MS in SIM mode, either directly (Hogge et al. 1993; Schneider et al. 1997) or following several purification steps (Vilaró et al. 2006). Further improvements in the analysis of ABA metabolites have been obtained through use of a UHPLC-based MS method, which is faster, affords higher throughput and is more sensitive than conventional LC–MS (Turečková et al. 2009). The detection limits of the technique were found to be at low picomolar levels for ABAGE and ABA acids in negative ion mode, and femtomolar levels for ABAGE, ABAaldehyde, ABAalcohol and the methylated acids in positive ion mode.

Ethylene

Ethylene is a flammable unsaturated gaseous hydrocarbon (Fig. 5) with a molecular weight of 28.05 g/mol. It has been indirectly used for thousands of years to ripen fruits, for instance via the ancient Egyptian practice of gashing figs (Galil 1948). It seems to have been first described by Becher (1669, Physica Subterranea), identified as a natural plant product by Gane (1934) and shown to influence plant growth and development by Crocker et al. (1935). It is formed essentially in all cells, but often most abundantly in fruits and wounded tissues, diffuses through tissues and is finally released into the surrounding atmosphere. The levels of ethylene produced by plants are low and of the same order as those of other phytohormones. Thus, sensitive methods are essential for its determination. The first methods for ethylene detection, like those for other plant hormones, were based on certain bioassays, mainly because of the lack of instrumental methods at the time. The first was the 'triple response' etiolated pea plant bioassay based on measurement of reductions in stem elongation (Nejlubow 1901) and several others were subsequently developed (Crocker et al. 1932; Addicott 1970; Kang and Rat 1969). However, all the bioassays lack specificity (for instance, propylene, acetylene and butylenes can induce similar responses, albeit at up to a thousand times higher

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Fig. 5 Structure of ethylene and its biosynthetic precursors ACC and MACC
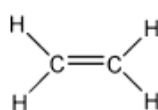
Ethylene
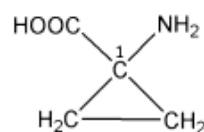
1-aminocyclopropane-1-carboxylic acid (ACC)
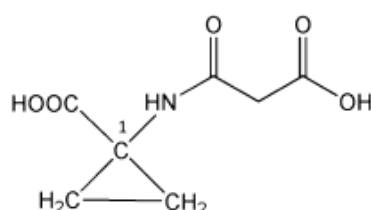
1-(malonylamino)cyclopropane-1-carboxylic acid (MACC)

concentrations than ethylene) and thus are rarely used now. The development of chromatographic (especially gas chromatography) techniques allowed the identification (by coupling to MS) and quantification of low molecular weight hydrocarbons including ethylene and its biosynthetic precursors 1-aminocyclopropane-1-carboxylic acid (ACC) and 1-(malonylamino)cyclopropane-1-carboxylic acid (MACC), for structures see Fig. 5. All of the mentioned substances of biological interest can be clearly distinguished from other low molecular weight hydrocarbons with high accuracy at approximately 10^{-12} m^3 dm^{-3} in a 1 cm^3 volume (ppb level) of air. The first GC method for ethylene determination was applied to measure this substance from apples (Burk and Stolwijk 1959; Huelin and Kennett 1959). The major drawback of this approach based on thermal conductivity detection (TCD) was a relatively high detection limit of 10–100 μL/L. The introduction of flame ionisation detection (FID) and the photoionisation detector (PID) in 1980s significantly improved the detection limit of ethylene to tens of nL/L levels (Bassi and Spencer 1985; Bassi and Spencer 1989). At the beginning, the ethylene sampling procedure and its subsequent injection into the GC column have been done manually with a gas-tight syringe, which was filled with gas from the headspace of a closed cuvette, in which the plant was enclosed for a few hours (Abeles et al. 1992). For low reproducibility and high time consumption of this system, it has been later replaced by automatic samplers based on concentric rotary valves (Cristescu et al. 2013). To achieve better sensitivity, GC systems can be equipped with preconcentration devices that enable to store the emitted ethylene (Segal et al. 2000). The plants are placed in closed cuvettes and continuously flushed with air. Ethylene is trapped inside a tube containing an appropriate adsorption material (e.g. carbon molecular sieve) and is then released into a smaller volume by heating the adsorbent. In addition to GC and GC–MS (Smets et al. 2003) approaches, photo acoustic laser spectrophotometry (PALS; Cristescu et al. 2008), LC–MS (Petritis et al. 2003) and CE–LIF (Liu et al. 2004) methods for ethylene (or ACC) determination have been published. PALS offers higher detection sensitivity (ppt level) than GC and is highly selective for particular substances. This is disadvantageous in some respects, as the equipment has much narrower applications than GC. However, before use of the GC–MS and CE–LIF methods, the analytes in plant extracts must be modified by derivatisation, which greatly increases time consumption, and the derivatisation procedure has poor reproducibility when concentrations of ACC are low. Recently, methodology based on in vitro measurement of the activity of two key biosynthetic enzymes, 1-aminocyclopropane-1-carboxylate synthase (ACS) and 1-aminocyclopropane-1-carboxylate oxidase (ACO), as well as ethylene itself, has been reported (Bulens et al. 2011).

Brassinosteroids

Brassinosteroids (BRs) are relatively young group of naturally occurring triterpenoid plant growth substances with hormonal function (Caño-Delgado et al. 2004). More than 70 BR analogues have been identified so far in nearly 60 plant species (Choe 1999). Their common structural feature is a 5α-cholestane skeleton (Fig. 6) and they are divided

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Fig. 6 Structure of 5α-cholestane and biologically active naturally occurring brassinosteroids

5α-cholestane

brassinolide (BL)   castasterone (CS)
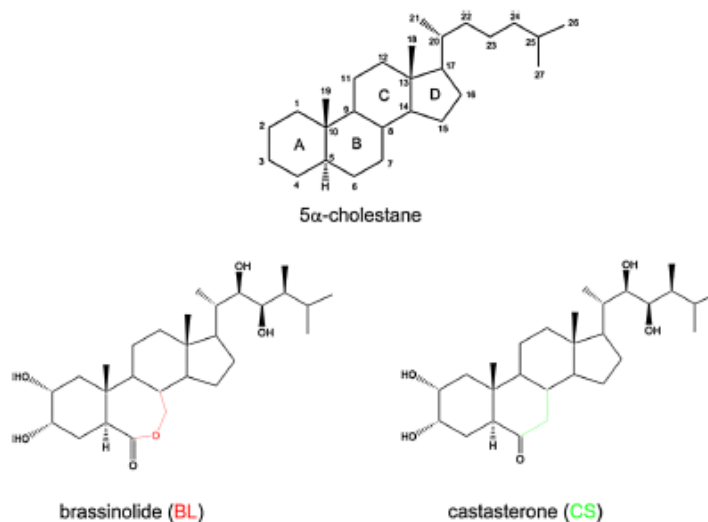
into different categories depending on the side chain structure and modifications of the A and B rings. Physiologically, BRs participate with other plant hormones in the regulation of numerous developmental processes, including shoot growth, root growth, vascular differentiation, fertility and seed germination (Fujioka and Sakurai 1997). BRs also have anti-stress effects, i.e. they participate in ameliorative responses to various stresses, such as low and high temperature, drought and infection. Like GAs, they tend to be relatively abundant in reproductive plant tissues, such as pollen, flowers and immature seeds, but their levels are extremely low in vegetative tissues, even compared to those of other plant hormones (fg-pg/g FW).

Initially, immunoassays and bioassays were mainly used for detecting BRs (Takatsuo and Yokota 1999). Some of the bioassays have good sensitivity and are still used for testing the biological activity of BRs, particularly the bean second-internode bioassay (Mitchell and Livingston 1968) and rice lamina inclination bioassay (Maeda 1965), which provide 2×10^{-11} mol and 1×10^{-13} mol detection limits, respectively (Thomson et al. 1981; Wada et al. 1984). Immunological methods such as RIAs and ELISAs have also been used for exploring the distribution of BRs in plant tissues (Horgen et al. 1984; Yokota et al. 1990). RIA was found to be useful for detecting the two most common bioactive BRs, castasterone (CS) and brassinolide (BL) (Fig. 6), with approximately 0.3 pmol detection limits. However, ELISA based on a mouse monoclonal antibody against 24-epibrassinolide (epiBL) was shown to respond not only to epiBL but also to other, non-BR phytosterols

(sitosterol, ecdysone). So, this method could not be used for analysing BRs. Swaczynová et al. (2007) subsequently improved the ELISA method using selective polyclonal antibodies against 24-epicastasterone (epiCS) and successfully detected this substance in Brassica napus and Arabidopsis tissues. These antibodies cross-reacted with BL and epiBL, but not with non-BR plant sterols. Thus, the method was applied for determining BR levels in extracts of tissues from several plant species. In addition, good agreement was found between results obtained using the ELISA method and a simultaneously developed HPLC–MS approach.

Several hyphenated (GC–MS and LC–MS) techniques were also gradually introduced. Since BRs are not volatile they must be derivatised prior to GC–MS analysis (Takatsuo et al. 1982). The standard derivatisation procedure is based on formation of bis-methaneboronates (BMBs) of BRs with vicinal diol groups (e.g. BL and CS). Thus, a disadvantage of this approach is that it cannot be used to analyse BRs lacking this conformation (e.g. teasterone and typhasterol). The detection limits of BMB derivatives are at the sub-ng level. GC–MS has also been used to elucidate structures of new BRs and BR biosynthesis pathways (Fujioka and Sakurai 1997). Liquid chromatography is generally suitable for non-volatile compounds, therefore, BRs can be advantageously analysed using this technique without derivatisation. However, although several LC methods have been published, only one can be used for direct determination of free BRs (Swaczynová et al. 2007). The others still require derivatisation. Gamoh et al. (1996) developed a method based on preparation of naphthaleneboronates,

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which is also applicable to teasterone and typhasterol (unlike BMB derivatisation). It has a reported detection limit of 2 ng and was applied to analyse BRs in *Cannabis sativa* seeds. Another LC method provides 125 attomole detection limits for dansyl-3-aminophenylboronate derivatives of BRs in highly laboriously purified extracts (57 g) of 24-day-old *Arabidopsis* plants grown in vitro. (Svatoš et al. 2004). Recently, two other LC–MS methods for preparing and analysing boronate derivatives of BRs have been reported (Huo et al. 2012; Ding et al. 2013), but the starting amount of tissue (*Arabidopsis*) used in the cited studies was still extremely high: 1 or 2 g FW.

Jasmonates

Jasmonic acid (JA) and its metabolites, collectively called jasmonates (JAs), are cyclopentanone compounds (Fig. 4) that share remarkable structural and functional properties with prostaglandins found in animals (Wasternack and Kombrink 2010). In the 1990s, JAs were proposed to be stress-related compounds (Farmer and Ryan 1990; Parthier 1991) that accumulate in plants in response to various stresses, such as wounding or pathogen attack (Creelman et al. 1992), in plant tissues or cell cultures treated with fungal elicitors (Müller et al. 1993), and tissues subjected to abiotic stressors such as UV radiation, low and high temperatures, osmotic stress and ozone exposure (Parthier et al. 1992). JAs seem to occur in most organs of most plant species (Wasternack and Hause 2013). Their *in planta* concentrations, which can be determined by various methods, are comparable to those of other plant hormones, ranging from ng to µg/g FW, depending on the plant tissue, species, developmental stage and both environmental and physiological conditions (Wilbert et al. 1998). The major physiologically active jasmonates are reportedly (−)-JA, methyl jasmonate (MeJA), and conjugates of (−)-JA with the amino acids isoleucine (JA-Ile), valine (JA-Val), and leucine (JA-Leu) (Sembdner and Parthier 1993). JA-amino acid conjugates are constitutively produced in plant tissues and their levels increase upon osmotic stress (Kramell et al. 1995). In plant–herbivore interactions, JA-amino acid conjugation is necessary for JA activation, and (−)-JA-Ile is the bioactive form of the hormone (Staswick and Tiryaki 2004; Fonseca et al. 2009). MeJA plays important roles as a fragrant volatile compound, particularly in plant–plant interactions, in which it acts, in concert with other volatile substances emitted from the plants, as an aerial signal for communication with their environment (Pichersky and Gershenzon 2002). Its synthesis is induced by various external stimuli, such as adverse weather conditions, and attacks by herbivores or pathogens (Paré and Tumlinson 1999). Another JA metabolite, which is highly active

in plant–insect interactions, is *cis*-jasmone (Birkett et al. 2000; Bruce et al. 2008).

Key considerations in the analysis of jasmonates are that JA and its conjugates are non-volatile, while MeJA and *cis*-jasmone are volatile. GC–MS is the most frequently used approach for quantifying JA, but as JA is not volatile it must first be derivatised, for instance by preparation of pentafluorobenzyl ester derivatives (Müller and Brodschelm 1994). This provides high sensitivity, but requires an elaborate preconcentration procedure. Another GC–MS-based technique for JA quantification has been described (Engelberth et al. 2003), in which the only purification step is collection of derivatised JA on a polymeric adsorbent. Nevertheless, these time-consuming steps still seriously limit the number of samples that can be processed per day. MeJA can be successfully quantified directly by GC with FID or MS detection after concentration by solid-phase microextraction (SPME) on fused silica fibre coated with a polymeric sorbent (Meyer et al. 1984). The reported detection limit of this method is 1 ng/injection, sufficiently low for detecting MeJA in plant tissues at levels between 10 and 100 ng/g DW (Müller and Brodschelm 1994; Wilbert et al. 1998).

Due to the polarity and non-volatility of JA most researchers use LC-based methods for its analysis. Anderson (1985) described an HPLC assay for the simultaneous determination of ABA and JA in plant extracts, following derivatisation (of both growth regulators) with a fluorescent hydrazide to obtain stable fluorescent products—dansyl hydrazones. This procedure allows detection of both hormones at low pmol levels. The method was demonstrated using extracts of several different tissues of soybean (*Glycine max*), snap beans (*Phaseolus vulgaris*), lima beans (*Phaseolus lunatus*) and broccoli (*Brassica oleracea*). However, only 20 % of the JA was converted to the corresponding ester during the derivatisation procedure. Fluorescent labelling usually affords great sensitivity for detecting the resulting derivatives (approx. 10^{-17} mol) and thus prompted other researchers to optimise this kind of derivatisation to introduce fluorophores into the chemical structure of JA to monitor it after separation by either HPLC (Kristl et al. 2005; Xiong et al. 2012) or CE (Zhang et al. 2005). However, the highest selectivity and sensitivity for JA determination can be currently achieved using MS/MS in MRM mode (Tamogami and Kodama 1998; Wilbert et al. 1998; Segarra et al. 2006). Another of JA's key physicochemical properties is amenability to oxidation, which was recently exploited for its electrochemical detection following LC (Xie et al. 2012). The method was successfully applied to analyse endogenous JA in wintersweet flowers and rice florets with a detection limit of 10^{-8} mol/L. To study physiological process in plants under various stresses, many researchers also monitor levels of precursors in the

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JA biosynthetic pathway (especially 12-oxo-phytodienoic acid, OPDA) and JA metabolites. For these studies, LC–MS/MS is the method of choice (Radhika et al. 2012; Van-Doorn et al. 2011). Similarly, stress resistance investigators (profiling the main stress response actors such as JAs, ABA and salicylic acid) generally use LC–MS/MS methods, which have been accelerated by coupling UHPLC, rather than conventional HPLC, systems to tandem mass spectrometers (Flors et al. 2008; López-Ráez et al. 2010; Balcke et al. 2012). This strategy also increases sensitivity, allowing successful quantification of target stress hormones in milligram quantities of plant tissue samples.

Salicylic acid

Salicylic acid (SA; Fig. 4) is an endogenous signalling molecule that is predominantly active in plant immune responses to avirulent pathogens, but like other phytohormones it is also involved in the regulation of several developmental processes, especially flowering (Singh et al. 2013). In most plants, pathogen attack, insect feeding and other kinds of physical wounding trigger both local and systemic resistance, mediated by the accumulation of defence-related proteins at sites of infection/damage and healthy tissues, respectively (Hammond-Kosack and Jones 2000). On the molecular level, accumulation of SA in cells leads to the release of NPR1 protein, activation of TGA1 and TGA2 transcription factors and expression of pathogenesis-related proteins (Pieterse and Van Loon 2004). To elucidate their signalling roles, SA and its metabolites (including methylsalicylate, salicylic acid glucoside and salicylic acid glucosylester) must be precisely quantified in plant tissues by robust, sensitive analytical methods. HPLC with fluorescence detection has been successfully used for quantifying SA, following a complex purification procedure (Meuwly and Metraux 1993), in cucumber (*Cucumis sativus* L.) seedlings infected by *Pseudomonas lachrymans* on the first leaf. Free SA contents increased locally in the infected leaf and systemically in the second leaf to 33-fold and 4.2-fold higher than detection limits, respectively, while remaining undetectable in controls. Recently, another HPLC method with fluorescence detection has been reported for quantitative analysis of SA in tobacco leaf tissues (Verberne et al. 2002). This methodology increased SA extraction recovery from plant tissues by reducing SA sublimation during purification via the addition of a small amount of HPLC eluent, resulting in recoveries in the range of 71–91 % for free SA and 65–79 % for acid-hydrolysed SA.

However, fluorometric analysis cannot fully distinguish SA and its metabolites from other plant components, particularly simple phenolics and phenylpropanoids, which might be present in infected plants and participate in disease resistance. Partly for this reason, methods allowing their precise, accurate determination based on analyses of their molecular masses and specific daughter fragments (tandem MS) or other analyte-specific approaches have been developed. Initially, a method based on electrospray tandem MS coupled to capillary LC was introduced to detect SA (together with JA and MeJA) in extracts of fresh poplar leaves (Wilbert et al. 1998). In addition, a bacterial biosensor that is highly specific for SA, methyl-SA and the synthetic SA derivative acetylsalicylic acid was recently shown to be suitable for quantifying SA in crude plant extracts (Huang et al. 2005). Following increases in its throughput, this approach was successfully applied for genetic screenings for SA metabolic mutants and characterising enzymes involved in SA metabolism (Defraia et al. 2008; Marek et al. 2010). Another LC–MS/MS approach has been applied to study SA and JA levels in cucumber cotyledons under biotic stress induced by the necrotrophic pathogen *Rhizoctonia solani* (Segarra et al. 2006). An LC–MS method has also been compared to a capillary electrophoresis (CE) technique, and used to study SA and related phenolics in wild-type *Arabidopsis* plants and two lines with mutations affecting SA accumulation in response to two avirulent bacterial strains (Shapiro and Gutsche 2003). Furthermore, in efforts to elucidate SA metabolism Pastor et al. (2012) developed an LC–MS/MS method that enabled them to identify two conjugates: salicylic glucosyl ester (SGE) and glucosyl salicylate (SAG). Their results also revealed that SA and its main glucosyl conjugates accumulate in *Arabidopsis thaliana* in a time-dependent manner, in accordance with the up-regulation of SA-dependent defences following *Pseudomonas syringae* infection. In addition to SA signalling, Belles et al. (1999) found that gentisic acid, a product of SA hydroxylation, is a complementary pathogen-inducible signal that is essential for accumulation of several antifungal pathogenesis-related proteins in tomato. Both gentisic and salicylic acids were quantified in SA-treated chamomile by a rapid UPLC-MS/MS method originally developed for analysing hydroxy-benzoates and hydroxycinnamates in beverages (Gruz et al. 2008; Kovacik et al. 2009). SA was accurately quantified using deuterium-labelled internal standards of salicylic $(3,4,5,6-[^2H_4])$ and 4-hydroxybenzoic $(2,3,5,6-[^2H_4])$ acids to account for ESI–MS signal trends, matrix effects and potential extraction losses.

GC–MS has also been used to quantify SA, after derivatisation (to enhance volatility and sensitivity) of the carboxylic acid with diazomethane to form SA methylester (Scott and Yamamoto 1994). The disadvantage of this method is that it requires elaborate sample preparation, from ca. 1 g FW of tissue, including anion exchange and preparative HPLC. In 2003, Engelberth and co-workers introduced a method for SA and JA analysis based on collecting

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Fig. 7 Natural strigolactones— basic structure and the examples

Basic structure of natural strigolactones

(+)-Strigol   **Orobanchol**
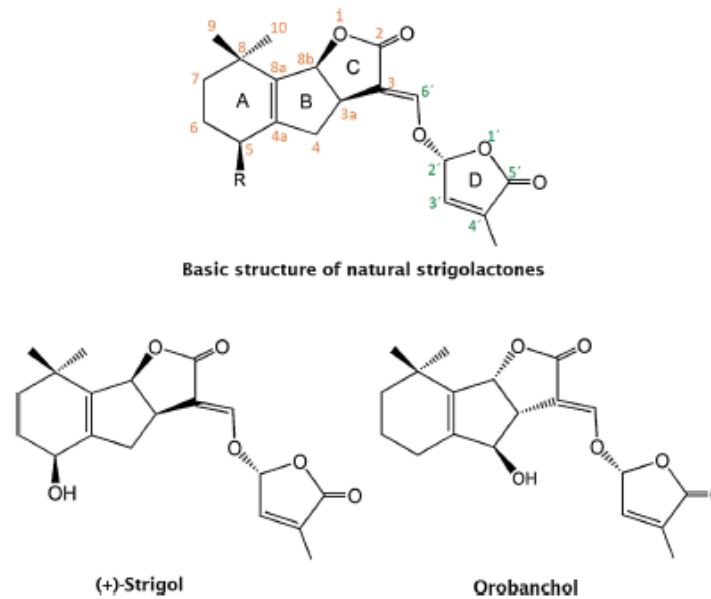
derivatised and volatilised compounds on a polymeric adsorbent and GC-positive ion chemical ionisation-MS using only milligrams of plant tissue. This method was later used to explore changes in the metabolic profiles of SA, cinnamic acid, JA, IAA, ABA, unsaturated C18 fatty acids, 12-oxo-phytodienoic acid, and the phytotoxin coronatine in *Arabidopsis* infected by *P. syringae* (Schmelz et al. 2004). However, this approach also requires both purification and derivatisation steps.

Strigolactones

Strigolactones (SLs) are the most recently described class of plant hormones. They were originally regarded as a family of carotenoid-derived plant secondary metabolites (Xie et al. 2010) with roles in signalling between organisms (allelochemicals). Initially, they were of interest due to their action as seed germination stimulants of the root parasitic allelopathic weeds *Orobanche* and *Striga* (Cook et al. 1966, 1972). Seeds of these parasites germinate only when they perceive chemical signals produced by and released from the roots of other (host or nonhost) plants. This germination-inducing activity of SLs was the basis of a sensitive SL bioassay using *Striga* and *Orobanche* seeds (Joel et al. 1995), and very recently a fast convenient method for determining the germination rate of parasitic weeds

seeds has been reported (Pouvreau et al. 2013). In 2008, SLs were classified as a new group of plant signalling molecules with endogenous hormonal activity due to involvement in the inhibition of shoot branching (Gomez-Roldan et al. 2008; Umehara et al. 2008). Earlier it was also found that they are able to stimulate hyphal branching in mycorrhizal fungi (Akiyama et al. 2005). To date, more than 20 compounds in this family of sesquiterpene lactones have been identified from root exudates of various plant species (Tsuchiya and McCourt 2009; Xie et al. 2010). The most well-known SLs are strigol and orobanchol (Fig. 7). Structurally, all natural SLs contain a tricyclic lactone skeleton (cycles A, B and C) bound to a butenolide moiety (ring D) via an enol ether bond. The A and B rings bear various substituents (Zwanenburg et al. 2009).

A pioneering GC–MS approach for analysing SLs in plant samples was introduced by Yokota et al. (1998). However, this method required time-consuming purification of extracts and TMS derivatisation. Furthermore, some SLs partially decomposed during the procedures. An approach for analysing strigol heptafluorobutyrate derivatives obtained from extracts of *Striga asiatica* host plants (maize and proso millet) was subsequently developed (Siame et al. 1993), based on electron impact (EI) and both positive and negative chemical ionisation (CI) mass analyses of HPLC fractionated samples. This method avoided some of the previous problems, but the purification procedure

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was still extremely tedious and labour intensive. Another two approaches for analysing SLs, based on LC–MS/MS, have been published, with detection limits (in MRM mode) ranging between 0.1 and 1 pg/μL of SLs (Sato et al. 2003, 2005). Most recently, Xie et al. (2013) reported an LC–MS/MS procedure allowing structural elucidation of 11 SLs in root exudates of tobacco and detection of four SLs in rice. Notably, no SL profiling has been reported to date.

Hormone profiling

Since it is becoming increasingly evident that hormones do not act separately, but have highly interactive physiological effects and mutually affect each other's biosynthesis and metabolism, there is increasing interest in 'hormone profiling', i.e. analysing hormones of several classes (together with their precursors and metabolites) in the same plant tissue simultaneously. This greatly increases analytical complexity as it requires methodology capable of quantitatively detecting chemically and structurally diverse substances rather than a single targeted group of plant hormones. Since many plant hormones are acidic, published methods have often focused on these classes of compounds (Müller et al. 2002; Schmelz et al. 2003; Durgbanshi et al. 2005). Hormone profiling was first successfully applied, by Chiwocha et al. (2003), in a study of thermodormancy where four CKs and 10 acidic plant hormones (IAA, ABA, ABAGE, 7′OH-ABA, PA, DPA and four GAs) in 50–100 mg DW extracts of lettuce seeds were all measured by LC–MS, using a single purification step and a 40 min chromatographic gradient. Pan et al. (2008) subsequently developed an LC–MS/MS technique (requiring no purification or derivatisation) for simultaneous quantification of 17 plant hormones including auxins, CKs, ABA, GAs, JAs, salicylates and corresponding methyl esters in crude extracts of samples (50–100 mg) of Arabidopsis plants that had been mechanically wounded or challenged with the fungal pathogen *Botrytis cinerea*. Limits of quantification reportedly ranged from 0.01 to 10 pg/g FW. In addition, a fast LC–MS/MS method combined with an automatic liquid handling system for SPE was recently used for simultaneous analysis of 43 plant hormone substances, including CKs, auxins, ABA and GAs in the rice GA-signalling mutants *gid1-3*, *gid2-1* and *slr1* to study relationships between changes in gene expression and hormone metabolism (Kojima et al. 2009). To enhance sensitivity, a nanoflow-LC–MS/MS approach has also been used to detect 14 plant hormones, following a two-step purification procedure, in extracts of *Arabidopsis* and tobacco seedlings (Izumi et al. 2009). The limit of detection was found to be in the sub-fmol range for most studied analytes. However, this method failed for the acidic plant hormones, especially GAs. Capillary electrophoresis

with laser-induced fluorescence detection (CE–LIF) has also been applied in profiling, to simultaneously determine plant hormones containing carboxyl groups, including GA_3, IAA, indole butyric acid (IBA), 1-naphthalene acetic acid (NAA), 2,4-dichloro-phenoxy acetic acid (2,4-D), ABA and JA in crude extracts of banana samples (500 mg) without further purification (Chen et al. 2011). Finally (for this summary), 20 plant growth substances (including IAA, ABA, CKs and structurally related purines) have been determined in single chromatographic runs (Farrow and Emery 2012). The methodology involved extraction from 100 mg samples of *Arabidopsis thaliana* leaves, purification and analysis by conventional HPLC with a fused core column. QTRAP mass analyzer has been utilised here for detection of selected analytes. Reported detection limits ranged from 2 pmol for zeatin-9-glucoside to 750 pmol for IAA. High-resolution MS (HR-MS) is not yet widely used for quantitative analysis of plant hormones, but will probably soon be employed for their routine quantification. An HR-MS approach has already been applied to identify and quantify a large number of endogenous phytohormones in tomato fruits and leaf tissues (Van Meulebroek et al. 2012). The cited authors selected eight phytohormones—GA_3, IAA, ABA, JA, SA, zeatin (not mentioned if *trans-* or *cis-*), BAP and *epi*BL—as representatives of the major hormonal classes, and applied a simple extraction procedure followed by UHPLC-Fourier Transform Orbitrap MS separation and detection for hormonally profiling 100 mg FW samples of the tomato tissues. The samples were extracted in methanol:water:formic acid (75:20:5) over 12 h, ultra-centrifuged, then injected directly into an UHPLC system equipped with C_{18} column of 2.1×50 mm diameter (particle size 1.8 μm) and coupled to a benchtop Orbitrap mass spectrometer, equipped with a heated electrospray ionisation source (HESI), operating in both positive and negative modes. This technique provided detection limits of the analytes ranging from 0.05 to 0.42 pg/μL. Moreover, full mass scans by the Orbitrap MS provided a dataset including information on hundreds of matrix compounds. Therefore, this metabolomic profiling approach might lead to the discovery of compounds with no previously known hormonal activities or roles in plants. However, although HR-MS provides opportunities to use narrow mass windows to exclude interfering matrix compounds and selectively analyse substances (Kaufmann 2012), Van Meulebroek et al. (2012) used a relatively broad mass window of 5 ppm, to ensure that no compounds would be completely undetected.

Conclusion and perspectives

Analysis of plant hormones is very challenging because they have extremely wide ranges of physicochemical

properties, and plant tissues contain trace quantities of hormones together with thousands of other substances at far higher levels. However, there have been great advances in analytical techniques used in diverse "life sciences" during the last decade. LC–MS has become the most versatile, rapid, selective and sensitive technique available for identifying and quantifying small molecules (Pan et al. 2008; Van Meulebroek et al. 2012). Thus, it is replacing all other approaches in plant hormone analysis. New technologies based on a unique ion transfer device designed to maximise ion transmission from the source to the mass analyzer, could further improve the sensitivity (typically the primary concern) of phytohormone measurements. Typical gains obtained using such device include generally 25-fold increases in peak areas and 10-fold increases in signal-to-noise ratios, which are highly valuable for phytohormone quantifications. The next challenge could be to develop robust techniques for extending the breadth of profiling, including more phytohormone precursors and metabolites, as well as those of other signalling molecules in plants. LC–MS/MS methods may be particularly useful for this as they afford capabilities for simultaneously quantifying metabolites with diverse properties of both single and multiple phytohormone groups.

Even with further anticipated advances it will be extremely challenging to quantify all phytohormones and related compounds in a single LC–MS/MS run due to their high chemical diversity and the inherent difficulty in distinguishing numerous metabolites that may have very similar chromatographic properties, share the same mass and yield very similar fragments. The extremely high levels of matrix compounds typically present in plant extracts compound the problem. However, additional orthogonal separation techniques have been recently introduced that provide high selectivity, further improve spectral quality, enhance the quality of acquired datasets and facilitate their interpretation, thus surmounting some of the difficulties. Notably, ion mobility separation (Eugster et al. 2012) and MS^E have been combined in a powerful new approach called high definition mass spectrometry (Sotelo-Silveira et al. 2013), which could generate more precise datasets from explorations of endogenous phytohormone levels and their changes during developmental processes in plants. The recent progress in analytical MS technologies could also enable tissue- and cell-specific quantifications as well as analyses of levels of multiple hormones in single plant cells or subcellular compartments.

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ARTICLE 4

Analysis of gibberellins as free acids by ultra-performance liquid chromatography–tandem mass spectrometry

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Contribution: review outline, literature research, manuscript writing

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Analysis of gibberellins as free acids by ultra performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

A robust, reliable and high-throughput method for extraction and purification of gibberellins (GAs), a group of tetracyclic diterpenoid carboxylic acids that include endogenous growth hormones, from plant material was developed. The procedure consists of two solid-phase extraction steps (Oasis® MCX-HLB and Oasis® MAX) and gives selective enrichment and efficient clean-up of these compounds from complex plant extracts. The method was tested with plant extracts of *Brassica napus* and *Arabidopsis thaliana*, from which total recovery of internal standards of about 72% was achieved. A rapid baseline chromatographic separation of 20 non-derivatised GAs by ultra performance liquid chromatography is also presented where a reversed-phase chromatographic column Acquity CSH® and a mobile phase consisting of methanol and aqueous 10 mM-ammonium formate is used. This method enables sensitive and precise quantitation of GAs by MS/MS in multiple-reaction monitoring mode (MRM) by a standard isotope dilution method. Optimal conditions, including final flow rate, desolvation temperature, desolvation gas flow, capillary and cone voltage for effective ionisation in the electrospray ion source were found. All studied GAs were determined as free acids giving dominant quasi-molecular ions of [M−H]$^-$ with limits of detection ranging between 0.08 and 10 fmol and linear ranges over four orders of magnitude. Taking advantage of highly effective chromatographic separation of 20 GAs and very sensitive mass spectrometric detection, the presented bioanalytical method serves as a useful tool for plant biologists studying the physiological roles of these hormones in plant development.

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1. Introduction

Gibberellins (GAs) are a class of diterpenoid carboxylic acids present in plants and some bacterial and fungal species, and which include compounds that act as endogenous growth regulators in higher plants. The main physiological effects of biologically active GAs include the induction of germination and flowering, stimulation of stem elongation through enhanced cell division and elongation, parthenocarpic (seedless) development of some fruit in the absence of fertilisation and delay of senescence in leaves and citrus fruits [1–3].

Structurally, GAs possess either the *ent*-gibberellane (containing 20 carbon atoms), or a 20-nor-*ent*-gibberellane (containing only 19 carbon atoms) carbon skeletons. The simplest examples of C_{20}- and C_{19}-GAs are GA_{12} and GA_9, respectively, the latter being formed biosynthetically from the former by loss of C-20 (Fig. 1). The C_{19}-GAs include the biologically active forms, which must contain an hydroxyl group on C-3β, a γ-lactone between C-4 and C-10 and a free carboxyl group on C-6 for optimal binding to the GID1 receptor, while hydroxylation on C-2β causes loss of binding and biological activity [4,5]. Mechanisms for GA inactivation, which as well as 2β-hydroxylation include epoxidation on C-16–C-17, methylation of the carboxylic group and glucosylation, are necessary for regulating the size of the pool of active hormone [6].

Gibberellins are present in plant tissues at very low concentrations, normally in the range 10^{-9}–10^{-15} mol g^{-1} fresh weight, depending on the tissue. Therefore very high enrichment of the GAs is essential prior to their detection by standard analytical techniques. The extraction and preconcentration steps are often tedious, labour intensive and time-consuming, comprising usually liquid–liquid extraction, solid-phase extraction (SPE) using general

Fig. 1. Structures and biosynthetic relationship of the 20 GAs including in this study, showing the enzymes responsible for each reaction (above the arrow). KAO: *ent*-kaurenoic acid oxidase; GA20ox: GA 20-oxidase; GA3ox: GA 3-oxidase and GA2ox: GA 2-oxidase.

purpose sorbents, ion-exchange and high performance liquid chromatography (HPLC). Ideally, the extraction solvent should extract the GAs efficiently, whilst the quantity of interfering substances extracted should be as low as possible. The extraction efficiency of GAs depends on the extent to which they are associated with such as phenolics, lipids, pigments or proteins. The purification of plant extracts is then usually achieved by one of two main approaches: either a combination of solvent partitioning between water and ethyl acetate, strong anion-exchange chromatography and C_{18} solid phase extraction (SPE) followed by HPLC fractionation [7,8], or liquid–liquid extraction can be replaced by SPE step [9]. The low concentrations of GAs necessitate very

sensitive analytical tools, the most common approaches involving gas or liquid chromatography combined with mass spectrometry (GC–MS, LC–MS). Pioneering work on GA analysis by GC–MS originates already from the 1960s [10–12] and this method is still widely used for both the identification and quantitation of GAs as volatile methyl ester trimethylsilyl ether derivatives. The quantitative analysis of GAs is usually achieved by isotope dilution giving accurate determination [13]. Highly selective multiple reaction monitoring (MRM) can be used to provide sufficient selectivity in quantitative analysis, which needs to overcome problems arising from the occurrence of many interfering compounds [14,15]. Since the early 1990s, LC–MS is slowly replacing GC–MS as the routine

Table 1

Tabulated negative-ESI precursor ion mass spectra of authentic gibberellin and deuterium labelled gibberellin standards with spectra normalised to the most abundant product ion (bold entries).

Compound	Precursor ion [M−H]−	Diagnostic fragment ions: m/z (relative intensity, %)
GA9	363	321(40), **275(100)**, 257(45), 119(55)
[2H2]-GA9	365	**277(100)**, 321(70), 229(50), 158(50)
GA29	347	**303(100)**, 259(50), 163(70)
[2H2]-GA29	349	305(50), **261(100)**, 219(20), 173(10)
GA3	345	301(30), **239(100)**, 143(95)
[2H2]-GA3	347	**241(100)**, 143(15)
GA1	347	303(50), **259(100)**
[2H2]-GA1	349	**261(100)**, 231(50), 143(5)
GA6	345	301(35), 257(80), 239(40), 161(40), **119(100)**
[2H2]-GA6	347	259(90), 241(70), 161(80), **119(100)**
GA5	329	285(40), 241(20), 223(25), **145(100)**
[2H2]-GA5	331	**287(100)**, 269(90), 243(80), 225(45), 145(70)
GA20	331	**287(100)**, 250(10), 225(10), 219(15), 173(50), 147(30)
[2H2]-GA20	333	**289(100)**, 271(75)
GA13	377	**359(100)**, 303(45), 259(5), 215(15)
GA44	345	**301(100)**, 273(60), 187(50), 111(40)
[2H2]-GA44	347	**303(100)**, 257(50)
GA19	361	317(70), **273(100)**, 255(45), 229(45), 203(60), 133(40)
[2H2]-GA19	363	**275(100)**, 232(70), 185(60), 147(80)
GA34	347	303(25), **259(100)**, 241(35), 199(20)
[2H2]-GA34	349	305(10), **261(100)**, 243(20), 201(10)
GA51	331	**287(100)**, 243(75), 219(60), 182(20)
[2H2]-GA51	333	**289(100)**, 261(50)
GA7	329	287(5), **223(100)**, 211(15), 168(10)
[2H2]-GA7	331	287(20), **225(100)**, 195(10)
GA4	331	313(50), **287(100)**, 257(75)
[2H2]-GA4	333	289(50), **259(100)**, 245(50), 213(30)
GA53	347	**329(100)**, 303(75), 233(35), 189(70)
[2H2]-GA53	349	**331(100)**, 305(80), 233(60)
GA24	345	301(90), **257(100)**, 213(60), 135(35)
[2H2]-GA24	347	302(20), 284(30), **259(100)**, 219(40),16(30)
GA9	315	**271(100)**, 253(20), 121(10)
[2H2]-GA9	317	**273(100)**, 257(70)
GA15	329	285(40), **257(100)**, 220(15), 185(50), 123(20)
[2H2]-GA15	331	287(15), **259(100)**, 187(70), 123(30)
GA12	331	**313(100)**, 270(50), 287(80), 201(20)
[2H2]-GA12	333	**315(100)**, 289(60), 220(50)
GA12-ald	315	**271(100)**, 227(7), 163(30), 121(10)
[2H2]-GA12-ald	317	**273(100)**, 257(10), 165(25), 135(20)

analytical method for quantitative analysis of GAs [16–19] and is now the most common analytical method [20], usually utilising electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS) [9,21–24]. A separation method for GAs based on capillary electrophoresis coupled to MS has also been described [25]. Importantly however, none of these methods has dealt with the analysis of more than ten GAs in one chromatographic run although from physiological perspective, there are at least 23 GAs already identified in the most studied model plant *Arabidopsis thaliana* (Arabidopsis), while 12 GAs in rice (*Oryza sativa*) and 14 in oilseed rape (*Brassica napus*) have been identified in these important crop plants [26]. Although it is essential to know the concentrations of the bioactive forms of GAs (such as GA1, GA3 and GA4), knowledge of the concentrations of their precursors and metabolites provides important information on GA metabolism and its regulation by, for example, genetic or environmental factors. In the present study, we report on the development of a method for extraction and pre-concentration of 20 GAs as free acids (Table 1), and their quantitation by ultra performance liquid chromatography (UPLC) followed by (−)ESI-MS/MS with limits of detection ranging between 0.08 and 10 fmol. The structures and biosynthetic relationship of the analysed GAs are shown in Fig. 1. The method was successfully applied for the quantitation of GAs in *B. napus* flowers by isotope dilution analysis [27].

2. Material and methods

2.1. Chemicals and reagents

Authentic gibberellins (GA1, GA3, GA4, GA5, GA6, GA7, GA8, GA9, GA12 and GA12-aldehyde, GA13, GA15, GA19, GA20, GA24, GA29, GA34, GA44, GA51 and GA53) and their corresponding 17-^{2}H2-labelled analogues were purchased from OlChemIm (Olomouc, Czech Republic). Tritium-labelled GAs ([1,2-^{3}H2]GA1, [1,2-^{3}H2]GA4 and [1,2,3-^{3}H3]GA20) were produced as custom synthesis by Amersham Radiolabelling Services (Cardiff, UK) and in the case of [^{3}H]GA1 and [^{3}H]GA4 were generous gifts from Professor Makoto Matsuoka (Nagoya University, Japan). Formic acid (FA) and methanol (MeOH, HPLC grade) were purchased from Merck (Darmstadt, Germany). Deionised (Milli-Q) water obtained from a Simplicity 185 water system (Millipore, Bedford, MA, USA) was used to prepare all aqueous solutions. All other chemicals (analytical grade or higher purity) were from Sigma-Aldrich Chemie (Steinheim, Germany).

2.2. Biological material

Arabidopsis thaliana Columbia-0 (Arabidopsis) was grown for 4 weeks on soil at 22 °C, with an 18-h photoperiod, photon density 130 μE m^{-1} s^{-1}. The winter cultivar of oil seed rape plants *Brassica napus* (L.) var. napus f. biennis was grown in the field from August to May when fully expanded flowers were harvested at different times during the day. Detached flowers of *B. napus* in 50 mL Falcon tubes and whole shoots of Arabidopsis weighing 100 mg in 1.5 mL Eppendorf tubes were immediately immersed in liquid nitrogen after harvesting, and then stored at −80 °C until preparation.

2.3. Extraction and purification of gibberellins

Frozen plant tissues (100 mg) were ground to a fine consistency using a MM 301 vibration mill at a frequency of 30 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany) after adding 1 mL of ice-cold 80% AcCN containing 5% FA as extraction solution and 3-mm tungsten carbide beads (Retsch GmbH & Co. KG, Haan, Germany). Internal standard mixture containing 50 pmol each of ^{2}H2-labelled GAs was also added to the samples. For optimisation of the purification procedure, ^{3}H- together with ^{2}H2-labelled GAs were used. For ^{3}H-labelled standards we used 555 kBq each of [^{3}H2]GA1, [^{3}H2]GA4 and [^{3}H3]GA20. The samples were extracted for 12 h at 4 °C using a benchtop laboratory rotator Stuart SB3 (Bibby Scientific Ltd., Staffordshire, UK). The homogenates were then centrifuged (14 000 rpm, 10 min, 4 °C; Beckman Avanti™ 30) and the resultant pellets were re-extracted in the same way for 60 min with rotation at 4 °C. The combined extracts were evaporated to the water phase *in vacuo* (CentriVap® Acid-Resistant benchtop concentrator, Labconco Corp., MO, USA) and purified using joint Oasis® MCX and Oasis® HLB cartridges (150 mg and 60 mg, respectively, both Waters, Milford, MA, USA) activated with MeOH and pre-equilibrated with 5% aqueous MeOH (v/v). The evaporated samples were dissolved in 5% aqueous MeOH (3 mL), loaded onto the joint cartridges, which were washed with 5.75 mM FA (pH 3) and 5% aqueous MeOH (each 9 mL). The coupled columns were then run to dryness, disconnected and GAs were eluted from the HLB cartridge with MeOH/diethyl ether (20:80 v/v) (3 mL). The eluates were evaporated to dryness under a stream of nitrogen using evaporation system TurboVap® LV (Caliper Life Sciences, Hopkinton, MA, USA). The sample was dissolved in
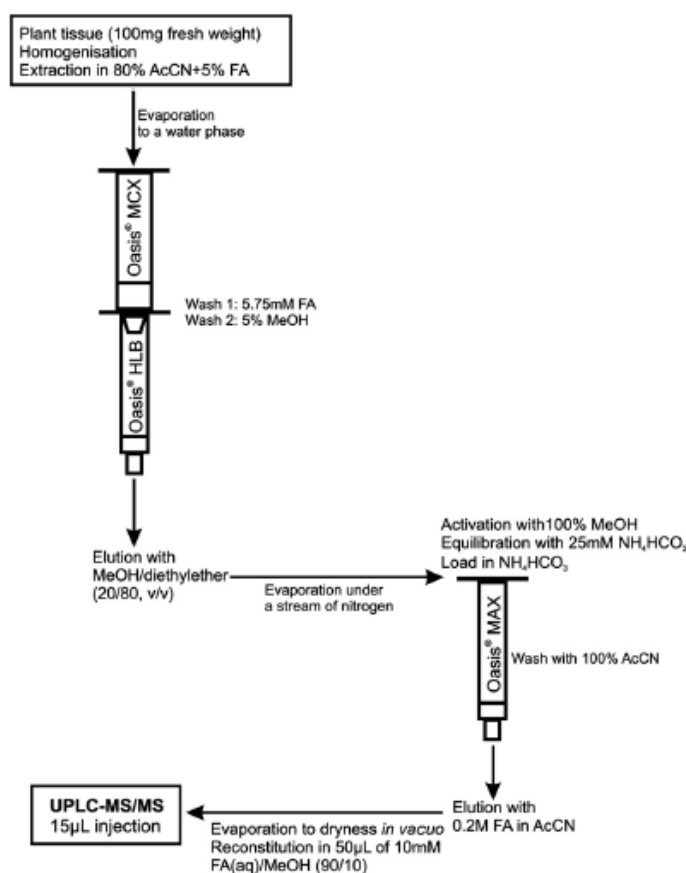
Fig. 2. Scheme of extraction and purification procedure for GAs from plant tissues.

50 μL of MeOH by vortexing and ultrasonication for 5 min and made up to 3 mL with 25 mM NH₄HCO₃ before loading onto Oasis® MAX polymer-based mixed mode columns. The sorbent in these columns was first activated with MeOH (3 mL) and then equilibrated with 25 mM NH₄HCO₃ (6 mL) before loading the sample. The MAX columns were then washed with AcCN (3 mL) and GAs eluted with 0.2 M FA in AcCN (3 mL), which was evaporated to dryness in vacuo. The Visiprep™ Solid Phase Extraction Vacuum Manifold (Supelco®, Bellefonte, PA, USA) was used for the purification stated above. If not analysed immediately, the evaporated samples were stored in a freezer (−20 °C) until UPLC–MS/MS analysis.

The radioactivity of ³H-labelled GA standards during the purification procedure optimisation was measured after addition of a 50 μL aliquot from 3 mL sample to 3 mL of liquid scintillation cocktail Ultima Gold™ using a multi-purpose scintillation counter LS 6500 (both Beckman Coulter, Brea, CA, USA).

2.4. LC–MS/MS apparatus

An Acquity UPLC™ System (Waters, Milford, MA, USA) consisting of a Binary solvent manager and Sample manager coupled to a Xevo® TQ MS triple-stage quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with electrospray (ESI) interface and the collision cell—ScanWave™ was utilised for GA quantitation. The entire LC–MS system was controlled by Masslynx™ Software (version 4.1, Waters, Manchester, UK).

2.5. UPLC–ESI-MS/MS conditions

The dried samples were reconstituted in 50 μL of mobile phase (initial conditions), and 15 μL of each sample was then injected onto the reversed-phase UPLC column (Acquity CSH®, 2.1 mm × 50 mm, 1.7 μm; Waters) coupled to the ESI-MS/MS system. Gibberellins were analysed in negative ion mode as [M–H]⁻, the product and precursor ions for each GA and ²H-labelled internal standard are listed in Table 1. The compounds of interest were separated in a linear gradient of MeOH (A) and 10 mM FA (B) at a flow rate of 0.25 mL min⁻¹, from 10:90 A:B (v/v) to 60:40 (v/v) over 15 min. Finally, the column was washed with 100% MeOH (0.75 mL) and equilibrated to initial conditions (10:90 A:B, v/v) for 2.5 min. For retention time of each GA studied see supplementary Table S1.
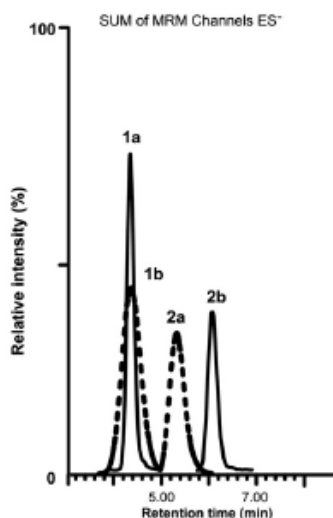
Fig. 3. Comparison of peak shape and peak-to-peak resolution of two selected GAs (GA₉-1a, 1b and GA₂₉-2a, 2b) on column Acquity UPLC® CSH (solid line) and Acquity UPLC®BEH column (dashed line).

The column was kept at 40 °C. Tandem mass spectra of all GAs included in this study were acquired by continuous infusion of 10^{-3} mol L^{-1} solution in MeOH at a flow rate of 15 μL min^{-1}. Capillary voltage, cone voltage, collision cell energy, and ion source temperatures were optimised for each individual compound using the same setup. The MS settings were as follows: capillary voltage 15 kV, cone voltage 30 V, source temperature 150 °C, desolvation gas temperature 650 °C, cone gas flow 2 L h^{-1}, desolvation gas flow 650 L h^{-1} and collision cell energy 25 eV. MS data were recorded in the multiple reaction monitoring (MRM) mode, the dwell time of each channel calculated to provide 16 scan points per peak, with an inter channel delay of 0.1 s. All data were processed by the Masslynx™ Software (ver. 4.1, Waters).

3. Results and discussion

3.1. Extraction and purification procedure

In order to quantify GAs, their precursors and metabolites in plant tissue, the samples have to be firstly homogenised and extracted with a suitable solvent under the conditions under which these substances are stable and enzymatic degradation is mini-mised. The extraction and purification procedures should be per-formed in solutions between pH 2.5 and 8.5 to avoid rearrangement of the C/D rings and hydration of the 16, 17-double bond (under acidic conditions) and a reversible retro-aldol rearrangement result-ing in epimerisation of the 3β-hydroxyl when present, and rearran-gement of 1,2-dehydro C₁₉-GAs (such as GA₃) to the 19,2β-lactones with a shift of the double bond (under alkaline conditions). Furthermore, the solutions containing GAs (especially aqueous ones) should not exceed 40 °C. The extraction of plant tissues with an aqueous solution of organic solvent with higher water content might be preferred due to the relatively low pigment content of crude plant extracts, but more hydrophobic GAs (i.e. GA₉, GA₁₅, GA₁₂ and GA₁₂-aldehyde for instance) may not be efficiently extracted under these hydrophilic conditions. We compared extraction of GAs from homogenised biological material using ice-cold MeOH or acidified (5% FA) AcCN, both with different water content, with rotation to achieve high extraction efficiency. In order to determine the content of the most abundant plant pigments in the extraction solution, the levels of chlorophylls a (chl$_a$) and b (chl$_b$) were measured using a previously described standard spectrophoto-metric method [28]. Extracts of 100 mg shoots from 3-week old Arabidopsis were prepared in triplicate for each extraction solution. In extracts prepared in 0–80% MeOH, the level of chl$_a$ ranged between 2.78 and 22.07 mg L^{-1}, while that of chl$_b$ between 5.10 and 17.90 mg L^{-1}. When performing the same experiment with 0–80% AcCN as the extraction solvent, the equivalent ranges were 1.5–22.26 mg L^{-1} for chl$_a$, 2.68–15.13 mg L^{-1} for chl$_b$. Thus, 80% AcCN extracted about 15% less interfering plant pigments than 80% MeOH as the more polar chlorophyll, chl$_b$, was extracted relatively less efficiently by 80% AcCN as compared to chl$_a$. Furthermore, recovery of ^{3}H-labelled GA standards after purification from extracts of green plant material with acidified 80% AcCN was on average >73%, compared with only 55% recoveries when the solvent was 80% MeOH (data not shown). Comparing acidified 80% AcCN and 80% AcCN without addition of acid, we obtained similar losses during purification for all GAs tested except the most hydrophobic, for which recoveries of 80% were achieved after extraction with AcCN with 5% FA compared to non-acidified AcCN (cca 60% recoveries). Therefore, this latter solvent was used for tissue extraction in all subsequent optimisation procedures.

In general, the supernatants after centrifugation of the crude extracts require further purification before analysis by standard methods for the reasons mentioned above and also for prevention of contamination and overloading the UPLC column. Gibberellin isolation can be usually achieved by SPE in one or two steps when interfering compounds are not retained on the sorbent in a pre-packed cartridge or they are washed out before the GAs are eluted. Commercial C₁₈ cartridges of different optional format and bed sizes are suitable for purification of aqueous methanolic extracts to effectively separate nonpolar impurities such as chlorophyll and carotenoids from the GAs depending on sample weight and volume of extract [29]. Recently, polymer based hydrophilic–lipophilic-balanced (HLB) reversed-phase SPE columns and mixed mode cartridges have become increasingly popular [10,30,31].

We tested different purification schemes for our extracts in acidified 80% AcCN. All purification procedures were examined for the matrix effect and the recoveries of the endogenous GAs, their deuterium-labelled as well as tritium-labelled analogues in extracts with and without biological background (100 mg 3-week old seedlings of Arabidopsis 3 replicates). At first, we tested recovery of GAs (free acids) on the cartridge commercially prepacked with polymer-based mixed mode anion-exchange sorbent (Oasis MAX®, Waters). The extracts where evaporated to the water phase and diluted in 25 mM NH₄HCO₃. GAs form negatively charged ions in this alkaline medium that would interact with the polymeric MAX sorbent bearing positively charged tertiary amine groups (pK_a >18). Under these experimental conditions, the recovery mean for GAs/[^{2}H₂]-GAs was found to be about 70% except for the more hydro-phobic GAs (GA₅₃, GA₂₄, GA₉, GA₁₅ and GA₁₂) for which recoveries varied between 20% and 57% (Table S2).

In case of GA₁₂-ald, only 16% was recovered. It is probably due to the fact that this very hydrophobic compound is not fully dissolved in aqueous NH₄HCO₃ as loading medium. When using [^{3}H]GA₁, [^{3}H]GA₄ and [^{3}H]GA₂₀, the recoveries from the MAX column ranged between 97 and 100%. Finally, we evaluated GA recoveries on MAX sorbent to be satisfactory and suitable for further optimisation of the purification procedure. Since the sample after MAX purification was not sufficiently pure for the subsequent UPLC–MS/MS analysis (visible presence of some plant pigments residue), we decided to examine some additional purification step prior to MAX. In the first

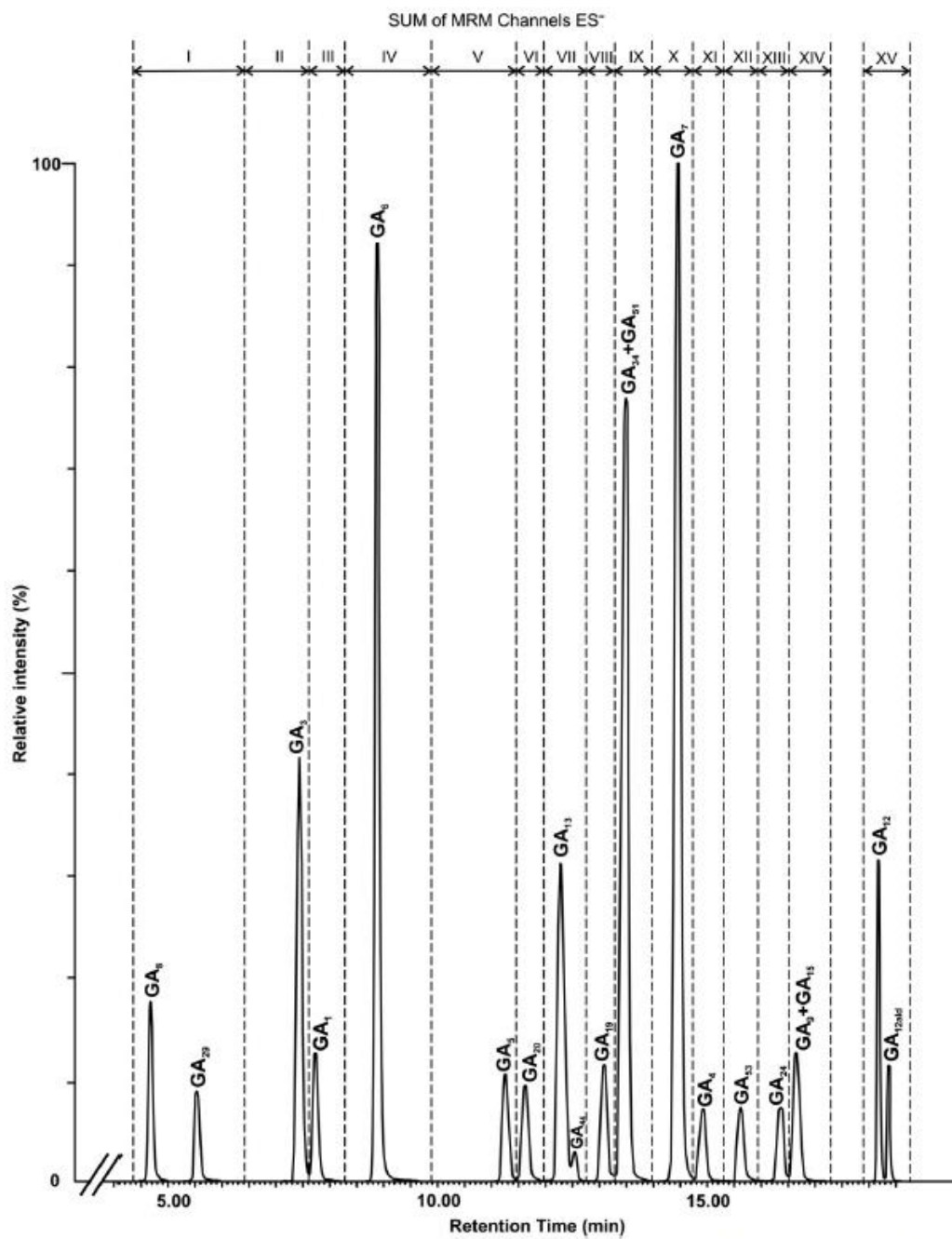T. Urbanová et al. / Talanta 112 (2013) 85–94

Fig. 4. Separation of 20 GAs by ultra-performance liquid chromatography (UPLC). UPLC–MS chromatogram of free acids standard mixture divided into 15 MRM channels (I–XV) containing 10 pmol of each GA per injection.

Table 2
Optimised MS conditions for each of the analysed gibberellins.

Compound	Diagnostic transition	Confirmation transition*	Cone voltage (V)	Collision energy (V)	Dwell time (s)	Retention time window (min)	Channel
GA_8	363 > 275	363 > 119	25	18	0.150	4.00–5.50	I
$[^2H_2]$-GA_8	365 > 277	–	25	18	0.150		
GA_{29}	347 > 259	347 > 303	30	15	0.150		
$[^2H_2]$-GA_{29}	349 > 261	–	30	15	0.150		
GA_3	345 > 239	345 > 143	25	14	0.290	6.80–7.40	II
$[^2H_2]$-GA_3	347 > 241	–	25	14	0.290		
GA_1	347 > 259	347 > 303	32	18	0.290	7.15–7.70	III
$[^2H_2]$-GA_1	349 > 261	–	32	18	0.290		
GA_6	345 > 119	345 > 257	30	26	0.250	8.30–8.80	IV
$[^2H_2]$-GA_6	347 > 119	–	30	28	0.250		
GA_5	329 > 145	329 > 285	28	24	0.127	10.78–11.25	V
$[^2H_2]$-GA_5	331 > 287	–	25	18	0.127		
GA_{20}	331 > 287	331 > 173	32	19	0.335	11.00–11.65	VI
$[^2H_2]$-GA_{20}	333 > 289	–	32	19	0.335		
GA_{13}	377 > 359	377 > 303	25	21	0.190	11.70–12.50	VII
GA_{44}	345 > 301	345 > 273	32	23	0.190		
$[^2H_2]$-GA_{44}	347 > 303	–	32	23	0.190		
GA_{19}	361 > 273	361 > 317	32	27	0.240	12.65–13.00	VIII
$[^2H_2]$-GA_{19}	363 > 275	–	32	27	0.240		
GA_{34}	347 > 259	347 > 241	30	17	0.120	12.95–13.50	IX
$[^2H_2]$-GA_{34}	349 > 261	–	30	17	0.120		
GA_{51}	331 > 287	331 > 243	33	18	0.120		
$[^2H_2]$-GA_{51}	333 > 289	–	33	18	0.120		
GA_7	329 > 223	329 > 211	25	18	0.300	13.95–14.40	X
$[^2H_2]$-GA_7	331 > 225	–	25	18	0.300		
GA_4	331 > 257	331 > 287	33	24	0.300	14.30–15.00	XI
$[^2H_2]$-GA_4	333 > 259	–	33	24	0.300		
GA_{53}	347 > 329	347 > 303	35	26	0.300	15.20–15.60	XII
$[^2H_2]$-GA_{53}	349 > 331	–	35	26	0.300		
GA_{24}	345 > 257	345 > 301	35	25	0.300	15.85–16.35	XIII
$[^2H_2]$-GA_{24}	347 > 259	–	35	25	0.300		
GA_9	315 > 271	315 > 253	32	23	0.150	16.10–16.70	XIV
$[^2H_2]$-GA_9	317 > 273	–	32	23	0.150		
GA_{15}	329 > 257	329 > 185	37	22	0.150		
$[^2H_2]$-GA_{15}	331 > 259	–	37	22	0.150		
GA_{12}	331 > 313	331 > 287	35	23	0.107	17.95–18.90	XV
$[^2H_2]$-GA_{12}	333 > 315	–	35	23	0.107		
GA_{12ald}	315 > 271	315 > 163	32	27	0.056		
$[^2H_2]$-GA_{12}-ald	317 > 273	–	32	24	0.056		

* Optimised MS conditions (cone voltage, collision energy and dwell time) are not shown.

instance, we decided to test GA recoveries on C_{18} SPE cartridges (Bond Elut® C18, Agilent Technologies). Silica-based C_{18} columns were activated with 100% MeOH and equilibrated by ultra pure water. After a washing step (ultra pure water), GAs were eluted with 100% MeOH. This procedure gave overall recoveries of about 46%. In order to improve recoveries of GAs, polymeric StrataX sorbent (Phenomenex, Torrance, CA, USA) was examined as an alternative reverse-phase medium to C_{18}. Functionalized polymeric sorbent of StrataX allows generally strong retention even under high organic wash conditions required when unwanted contaminants including phospholipids and diverse pigments need to be removed, while C_{18} sorbent offers strong hydrophobic retention with negligible secondary polar interactions from active silanol groups. However, about 53% of GAs were washed out from the StrataX sorbent when washing the cartridge with 5.75 mM FA and 5% MeOH, with only 33% recovered in the elution fraction. The conclusion is that this polymeric reversed-phase sorbent is not suitable for GA purification from plant extracts. C_{18} SPE cartridges were evaluated also as unsuitable.

Finally, we tested a combination of polymer-based cation-exchange SPE (Oasis MCX®, Waters) directly coupled to the last generation reversed-phase SPE (Oasis HLB®, Waters). Gibberellins are not retained on the negatively charged MCX matrix, which would remove alkaloids and other basic interfering substances from plant extracts. HLB is a polymeric reversed-phase sorbent containing both hydrophilic and lipophilic ligands and would be expected to retain GAs with a wide range of polarities.

After evaporation of the extraction solution to the water phase, the sample was loaded in 5% MeOH onto the MCX column pre-equilibrated with the same solvent and coupled directly with HLB column prepared in the same way. The columns were disassembled after the washing step and the sample containing GA standards was obtained after washing the Oasis® HLB cartridge with 5.75 mM FA followed by 5% MeOH and eluting with an appropriate organic solvent. MeOH and MeOH/diethyl ether (20/80, v/v) were investigated for their efficacy in eluting GAs from the HLB cartridge. MeOH/diethyl ether gave about 17% higher recoveries than MeOH alone. Moreover, when used with plant extracts, elution with MeOH/diethyl ether rather than MeOH improved overall recoveries of the tested GAs in the system MCX-HLB followed by MAX, from 37% to 72% (data not shown). The MCX-HLB procedure itself gave a recovery of about 75% with only a small reduction (about 10%) when tested in the presence of plant extracts. Thus, the combined Oasis® MCX-HLB columns were judged to provide an effective and convenient enrichment of GAs from extracts and were chosen as a first purification step before anion exchange on Oasis® MAX columns (Fig. 2).

3.2. Liquid chromatography

A solution containing a mixture of all unlabelled GA standards (20 substances) and their deuterium-labelled analogues (19 compounds) was prepared to find the optimal LC separation conditions for all 20 analytes in a single chromatographic run. For this purpose,

Fig. 5. MRM chromatograms of GA$_{15}$ and GA$_{24}$ standards (1A, B, C and 3A, B, C) and of the endogenous compounds in an extract of 100 mg *Brassica napus* flowers (2A, B, C and 4A, B, C) in the presence of ^{2}H$_2$-labelled internal standards. MS spectra were recorded under optimised conditions for standard of GA$_{15}$ (1D), [^{2}H$_2$]GA$_{15}$ (1E), GA$_{24}$ (3D) and [^{2}H$_2$]GA$_{24}$ (3E).

Table 3
Method validation—selected parameters of the UPLC-MS/MS method for 50 pmol of selected GAs tested.

Compound	Diagnostic transition	LOD[a] (pmol)	LOQ[b] (pmol)	Recovery (%)	Content (pmol mg^{-1})	Content SD[c]	n	Analytical precision (%)	Analytical accuracy (%)
GA$_{15}$	329 > 257	0.06	0.19	80	0.05	0.01	6	2.33	116.11
GA$_{24}$	345 > 257	0.06	0.20	95	0.01	0.00	6	1.12	111.07
GA$_4$	331 > 287	0.04	0.14	78	0.04	0.20	6	3.68	119.32
GA$_3$	345 > 239	0.01	0.02	68	0.5×10^{-3}	0.02×10^{-3}	6	3.82	107.34

[a] Limit of detection LOD; $LOD = (3 \times S_b)/k$, where S_b is standard deviation of calibration equation and k is its slope.
[b] Limit of quantification (LOQ; $LOQ = (10 \times S_b)/k$), where S_b is standard deviation of calibration equation and k is its slope.
[c] Standard deviation.

two reversed-phase UPLC (RP-UPLC) columns (2.1 × 50 mm, 1.7 μm), Acquity UPLC® BEH (Ethylene Bridged Hybrid) C$_{18}$ and Acquity UPLC® CSH (Charged Surface Hybrid) (both from Waters) were tested. The peak shape and the ionisation efficiency were found to be acceptable when using MeOH and 10 mM HCOOH as solvents A and B, respectively. Under these conditions, the Acquity UPLC® CSH column gave better peak shape and peak-to-peak resolution compared to the BEH column (Fig. 3).

The retention times ranged between 4.3 min (GA$_8$) and 18.3 min (GA$_{12}$-aldehyde) - Table S1. As expected, each unlabelled analyte/deuterated internal standard couple co-eluted with very close retention times, usually the deuterated analogue eluted earlier than the unlabelled standard (data not shown) due to the chromatographic isotope effect [32]. Sixteen of the 20 GAs studied were fully resolved under the RP-UPLC conditions (Fig. 4).

Only members of the pairs GA$_{34}$ (m/z 347)/GA$_{51}$ (m/z 331) and GA$_9$ (m/z 315)/GA$_{15}$ (m/z 329) were not resolved and co-eluted completely, while it was not possible to achieve baseline separation of GA$_{13}$ and GA$_{44}$. However, these GAs can be distinguished by the MS detector. Under our optimised conditions the stability of the retention times had a coefficient of variation between 0.60% and 0.82% ($n = 20$) which is satisfactory. The mean of chromatographic peaks width of the studied compounds was 0.2505 min, which corresponds to a dwell time of about 0.93 s to reach the minimum required 16 data points per peak as a data sampling rate suitable for reproducible integration. Dwell time values for

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appropriate analytes are listed in Table 2. Further, due to low loading capacity, it was possible to achieve excellent peak shape for all the analytes in selected low ionic strength mobile phase and rapid mobile-phase re-equilibration.

3.3. MS/MS detection

Solutions containing mixtures of standards, comprising the unlabelled GAs and their respective deuterium-labelled internal standards were used to identify the appropriate precursor-to-product ion transition for each compound using (−)ESI–MS/MS. All investigated GAs provided the background-subtracted ESI⁻ spectra exhibiting $[M–H]^-$ as base peaks, but varied considerably in their fragmentation pattern. Nevertheless, some fragmentation rules could be found. Twelve of the 20 GAs studied have 19 carbons and 10 of these C_{19}-GAs with no internal double bonds give the diagnostic or confirmation losses of 88 (COO^- plus $CO–O$ from ring A) or 44 (COO^-) mass units from their precursor ions $[M–H]^-$ (see Table 2). On the other hand, two C_{19}-GAs containing double bond (GA_3 and GA_7) give the diagnostic transition corresponding to the loss of 106 mass units, reflecting the loss of water (18 m/z), in addition to the 88 m/z fragment cleavage. In the case of the C_{20}-GAs, only three GAs (GA_{13}, GA_{53} and GA_{12}) from eight show the same fragmentation pattern, losing water from the precursor ion $[M–H]^-$. In half of the C_{20}-GAs (GA_{13}, GA_{44}, GA_{24} and GA_{15}), the loss of a CH_3 group from C-4 occurs as the second most significant fragmentation route. The C_{20}-GAs GA_{19} and GA_{24} behave in the mass spectrometer as C_{19}-GA, i.e. give the ions 273 and 257 as the most abundant fragments after loss of m/z 88 from the precursor ions $[M–H]^-$ 361 and 345, respectively. It is possibly due to the presence of the aldehyde functional group at C-10 that might stabilise the gibberellane skeleton and allow the cleavage of both carboxyl groups from the molecule. The double deuterium-labelled counterparts at C-17 position show no difference in fragmentation pattern compared to their unlabelled analogues. Based on the mass spectra obtained, the quasi-molecular ions $[M–H]^-$ and the most intensive fragment ions were selected for mass spectrometric detection in MRM mode (Table 1). The entire chromatographic run was then divided into 15 retention windows (channels I–XV), each characterised by defined MRM functions for appropriate analyte (Fig. 4). Examples of mass spectra of the diagnostic product ions are shown in Fig. 5. Interestingly, the intensities of GA product ions are lower than those of their deuterated GA counterparts (see Fig. 5–1D vs 1E and 3D vs 3E). To our knowledge, there is no such effect described in the literature. We might speculate that higher intensity of product ion is related to lower stability of precursor ion containing deuterium.

3.4. Method validation and application

The newly developed UPLC–MS/MS method was tested by analysing the levels of endogenous GAs in samples of different biological origin: shoots of A. thaliana and flowers of field-grown B. napus. To create calibration curves, eight solutions containing varying amounts of each unlabelled GA and a known, fixed amount of the corresponding deuterium-labelled internal standard (IS) were prepared as follows: 0.098/15; 0.5/15; 1/15; 1.5/15; 3/15; 5/15; 7.5/15 and 10/15 (endogenous/internal standard; values in pmol/15 μL). The resulting calibration curves from four separate injections were established to be linear in the selected concentration range for all 20 endogenous compounds investigated (correlation coefficient R^2 values appeared in the interval 0.9965 to 0.9995, see Table S1). For GA_{13}, in the absence of a deuterium-labelled analogue, $[^2H_2]_2GA_{44}$ was found to be an appropriate internal standard on account of their very close chromatographic behaviour and linearity of the $[^2H_2]GA_{44}/GA_{13}$

calibration curve. The linear range for all calibration curves was shown to be four orders of magnitude. The limit of detection (LOD) was evaluated using the approach based on the standard deviation s_b of the calibration curve and the slope k of a regression curve (LOD $= 3 \times s_b/k$) [49]. The LODs for selected GAs are summarised in Table 3. The limit of quantitation (LOQ) was evaluated using the standard-deviation/slope ratio approach (LOQ $= 10 \times s_b/k$) [33] and it is indicated for 4 selected GAs in Table 3.

We tested different concentrations of IS (10–100 pmol) added into the extraction media and found 50 pmol to be the most appropriate for all GAs investigated in tissues containing chlorophyll and other plant pigments. Addition of 10 pmol of GAs IS gave also satisfactory recoveries (cca 80%), however only in case of biological material without plant pigment present (seeds, data not shown). Therefore, 50 pmol of each IS was added to the samples before purification. The plant extracts were purified by SPE (MCX-HLB followed by MAX), concentrated in vacuo and the GAs were quantified by LC–MS as described above.

The analytical accuracy of the UPLC–MS/MS method was evaluated by spiking sample aliquots (100 mg of plant tissue in 1 mL of extraction solution, 6 replicates) with known amounts of individual compounds (50 and 75 pmol $GA_{15}/[^2H_2]$-GA_{15}, $GA_{24}/[^2H_2]$-GA_{24}, $GA_4/[^2H_2]$-GA_4 and $GA_3/[^2H_2]$-GA_3) prior to sample purification. The assessment of analytical accuracy ranged within 107 and 120% of the true amounts value (Table 3). The analytical precision was determined in the range of 1.1 and 3.8% for the 4 above-mentioned GAs selected (Table 3).

4. Conclusion

In this report, we describe a method for the simultaneous analysis of 20 GAs, which have been identified in Arabidopsis, the most frequently used model plant. The developed UPLC–ESI–MS/MS method is based on effective chromatographic separation combined with a suitable extraction and purification procedure for plant samples. The solvent extraction process followed by two solid-phase based procedures, allows these 20 GAs to be isolated effectively and rapidly from plant material. This method was then successfully applied to the analysis of biologically active GAs, their metabolites and precursors in B. napus flowers and Arabidopsis shoots. The developed LC–MS/MS method offers a fast separation, high chromatographic resolution, with sufficient selectivity and a satisfactory sensitivity for studying the distribution of GAs and their physiological roles in plants.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.03.068.

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ARTICLE 5

Arabidopsis NAC transcription factor JUB1 regulates GA/BR metabolism and signalling

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Contribution: analysis of plant hormones, data evaluation, manuscript writing

ARTICLE 6

Hormone profiling and the root proteome analysis of *itpk1* mutant seedlings of barley (*Hordeum vulgare*) during the red-light induced photomorphogenesis

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Contribution: analysis of gibberellins, data evaluation, manuscript writing

ARTICLE 7

Domestication has altered the ABA and gibberellin profiles in developing pea seeds

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Contribution: analysis of gibberellins, data evaluation, figures preparation, manuscript writing

ORIGINAL ARTICLE

Domestication has altered the ABA and gibberellin profiles in developing pea seeds

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Abstract

Main conclusion We showed that wild pea seeds contained a more diverse combination of bioactive GAs and had higher ABA content than domesticated peas.

Abstract Although the role of abscisic acid (ABA) and gibberellins (GAs) interplay has been extensively studied in *Arabidopsis* and cereals models, comparatively little is known about the effect of domestication on the level of phytohormones in legume seeds. In legumes, as in other crops, seed dormancy has been largely or entirely removed during domestication. In this study, we have measured the endogenous levels of ABA and GAs comparatively between wild and domesticated pea seeds during their development. We have shown that wild seeds contained more ABA than domesticated ones, which could be important for preparing the seeds for the period of dormancy. ABA was catabolised particularly by an 8′-hydroxylation pathway, and dihydrophaseic acid was the main catabolite in seed coats as well as embryos. Besides, the seed coats of wild and pigmented cultivated genotypes were characterised by a broader spectrum of bioactive GAs compared to non-pigmented domesticated seeds. GAs in both seed coat and embryo were synthesized mainly by a 13-hydroxylation pathway, with GA_{29} being the most abundant in the seed coat and GA_{20} in the embryos. Measuring seed water content and water loss indicated domesticated pea seeds′ desiccation was slower than that of wild pea seeds. Altogether, we showed that pea domestication led to a change in bioactive GA composition and a lower ABA content during seed development.

Keywords Desiccation · Legume · Maturation · Phytohormones · Pigmentation · Seed-coat

Abbreviations

DAP   Days after pollination
GAs   Gibberellins
NCED   9-*cis*-Epoxycarotenoid dioxygenase

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Introduction

The seed consists of an embryo and an endosperm (resulting from double fertilization), which are enclosed in a maternally derived seed coat. In legumes, the seed coat and endosperm develop first, followed by the development of embryo (Weber et al. 2005). The endosperm is present only during early seed development and provides nutrients for the developing embryo. However, by approximately 17 days after pollination (DAP), the expanding embryo consumes the endosperm, and most of the seed comprises the growing embryo (Ribalta et al. 2017; Zablatzká et al. 2021). Nevertheless, to develop successfully, these three components must communicate with each other to coordinate their growth (Ochatt and Abirached-Darmency 2019). Growth is the result of a balance between many promoting and inhibiting factors, including plant hormones.

The seed development comprises three phases: histodifferentiation, maturation and desiccation, interlaced with

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two lag phases (Hedley and Ambrose 1980). During histodifferentiation, the embryo is produced through cell division and differentiation. After these events, cell expansion and deposition of stored reserves take place (maturation). Seed maturation is a physiological process accompanied by changes in levels of certain plant hormones, mainly gibberellins (GAs) and abscisic acid (ABA). Early embryo growth is mainly maternally controlled, and the transition into maturation indicates a switch to filial control. Finally, seed development terminates during maturation, and the seed enters a quiescence associated with a rapid decline in seed water content (Weber et al. 2005).

In legume seeds, physical dormancy develops during the later period of seed maturation. Physical dormancy (hardseededness) is characteristic of an impermeable seed coat, which does not allow water and gases to enter the seed. The mechanism underlying this phenomenon is not yet fully explained. However, it was shown that not only the seed coat thickness but also seed coat texture and biochemical and chemical composition are crucial components of this complex seed trait (Hradilová et al. 2017; Janská et al. 2019; Zablatzká et al. 2021). Unlike physiological dormancy, physical dormancy is not based on ABA and GAs balance. However, the model legume *Medicago truncatula* exhibits both physical and physiological dormancy (Ochatt and Abirached-Darmency 2019).

ABA is a sesquiterpene influencing many aspects of the plant lifecycle, including seed morphogenesis as well as germination. In the seed, the ABA hormone level results from transportation from the mother plant through the phloem and synthesis in the seed itself (Ali et al. 2022). Karssen et al. (1983) showed that ABA is synthesized especially during seed maturation, first in the seed coat and then at lower levels in the embryo and endosperm. The level of ABA decreases during desiccation and becomes relatively low in dry mature seeds.

ABA action in the seed has been shown to be the result of ABA synthesis, catabolism, transport and sensing (Ali et al. 2022). In plants, ABA is synthesized from a C_{40} carotenoid precursor via oxidative cleavage. The first oxygenated carotenoid precursor, zeaxanthin, is converted by zeaxanthin epoxidase (ZEP) into all-*trans*-violaxanthin and then to *trans*-neoxanthin, which are isomerized into the required *cis*-forms (North et al. 2007). The subsequent reaction leading to the next ABA precursor, xanthoxin (C_{15}), is catalyzed by 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), which are key regulators of ABA synthesis. Xanthoxin is then converted to abscisic aldehyde by a short-chain dehydrogenase/reductase (SDR1), and finally, the abscisic aldehyde is oxidized to ABA by abscisic aldehyde oxidase (AO) (Nambara and Marion-Poll 2003). Inactivation of ABA can be achieved by its oxidation at the C-7′, 8′ or C-9′ positions (Schwartz and Zeevaart 2010). The hydroxylation at the C-8′ position

produces unstable 8′-hydroxy-ABA (8′-OH-ABA) that isomerizes spontaneously to phaseic acid (PA) and can be further reduced to dihydrophaseic acid (DPA). Hydroxylation at the 7′ and 9′ positions gives 7′-hydroxy-ABA (7′-OH-ABA) and 9′-hydroxy-ABA (9′-OH-ABA), respectively. 9′-OH-ABA cyclized to neophaseic acid (neoPA) (Zhou et al. 2004). ABA may also be inactivated by conjugation to glucose, producing a stored/transport form, the ABA glycosyl ester (ABA-GE) (Schwartz and Zeevaart 2010). ABA-GE can be converted back to free ABA by β-glucosidases (BGs) (Lee et al. 2006).

Both ABA and its cross-talk with other hormones and signalling molecules are crucial for seed development (Ali et al. 2022). GAs are known especially as growth-promoting plant hormones. Moreover, it was shown that bioactive GAs act as key mediators in growth responses to environmental cues (for example, light and temperature).

GAs are a family of diterpenoid carboxylic acids consisting of either 19 or 20 carbon atoms (Tarkowská and Strnad 2018). Among more than 130 known GAs, only a few are endogenous bioactive substances that control a wide range of plant growth and developmental processes, including seed germination. The major biologically active GAs are GA_1, GA_3, GA_4, GA_5, GA_6 and GA_7 (Yamaguchi 2008). The other GAs are either their inactive biosynthetic precursors or catabolites. Thus, the concentration of bioactive GAs is determined by the balance in their de novo biosynthesis and deactivation. In higher plants, GA_{12} is a precursor for all GAs which is produced by the oxidation of GA_{12}-aldehyde by the enzyme ent-kaurenoic acid oxidase (KAO) (Hedden and Thomas 2012). There are two biosynthetic pathways leading to bioactive GAs: the 13-hydroxylation pathway (leading to the production of GA_1 and GA_3) and non-13-hydroxylation (leading to the production of GA_4 and GA_7) (Reinecke et al. 2013). The enzymes GA 20-oxidase and GA 3-oxidase are responsible for subsequent conversions of GA precursors and the production of bioactive GAs, respectively. The maintenance of the level of bioactive GAs is regulated by the action of GA 2-oxidase (Hedden and Thomas 2012).

Although the phytohormones stand behind the semidwarf varieties developed during the Green Revolution, their role in the early stages of the domestication process has not been studied so far. Since secondary metabolites pathways have often been altered (Alseekh et al. 2021), it is highly likely that phytohormone levels were also changed. Domestication affected many aspects of the plant lifecycle (Smýkal et al. 2018), including seed development. Particularly seed dormancy has been largely or entirely removed to allow the crop's rapid establishment. This is also the case with legumes (Smýkal et al. 2014), but its effect on the hormone profiles of developing seeds of wild and cultivated pea (legume) genotypes remains unknown. The aim of the present study was to investigate the role of ABA and GAs

in embryo and seed coat development in wild and domesticated pea seeds. This might shed new light on understanding the differences in seed development in these accessions and provide a picture of the dynamics of pea seed development.

Materials and methods

Plant material

In these experiments, wild *Pisum elatius* M. Bieb. (JI1794, Israel origin), cultivated *Pisum sativum* L. (JI92, Afghan landrace) acquired from John Innes Pisum Collection (Norwich, UK), and the cultivated *P. sativum* cv. Cameor from INRAe France, used for the pea reference genome (Kreplak et al. 2019), were used. In addition, for seed water content and water loss measurements, wild JI64 pea (Turkey origin) was used instead of JI1794. Plants were cultivated and sampled as described in Zablatzká et al. (2021). The embryo and seed coat samples were collected at four time points: 13, 17, 23 (mid-development) and 28 DAP (mature seed), which were marked as developmental stages 1, 2, 3 and 4, respectively. At developmental stage 1, embryos of JI92 and JI1794 were not available in sufficient amounts and were thus not analyzed. Respective developmental stages were collected on several different days (i.e., the biological replicates). The seed coats and embryos of the same age (i.e., at the same developmental stage) but collected on different days were combined into one sample to cover the biological variation between different individuals and days of collection. Then, the sample was divided into three aliquots (replicates) for measurements.

Isolation and analysis of ABA and its metabolites

Samples were extracted, purified and analysed according to a method described in Turečková et al. (2009). Briefly, 20 mg of plant tissue per sample was homogenized using a bead mill (27 Hz, 10 min, 4 °C; MixerMill, Retsch GmbH, Haan, Germany) and extracted in 1 ml of ice-cold methanol/water/acetic acid (10/89/1, by vol.) and internal standard mixtures, containing (-)-7′,7′,7′-$^2H_3$-phaseic acid; (-)-7′,7′,7′-$^2H_3$-dihydrophaseic acid; (-)-8′,8′,8′-$^2H_3$-neophaseic acid; (+)-4,5,8′,8′,8′-$^2H_5$-ABA-GE; (-)-5,8′,8′,8′-$^2H_4$-7′-OH-ABA (National Research Council, Saskatoon, Canada) and (+)-3′,5′,5′,7′,7′,7′-$^2H_6$-ABA (OlChemIm, Olomouc, Czech Republic). After 1 h of shaking in the dark at 4 °C, the homogenates were centrifuged (20 000 g, 10 min, 4 °C), and the pellets were then re-extracted in 0.5 ml extraction solvent for 30 min. The combined extracts were purified by solid-phase extraction on Oasis® HLB cartridges (60 mg, 3 ml, Waters, Milford, MA, USA), then evaporated to dryness in a Speed-Vac (UniEquip, Planegg, Germany) and finally analysed by UHPLC-ESI(-)-MS/MS (Waters, Manchester, UK).

Isolation and analysis of gibberellins

The sample preparation and analysis of GAs were performed according to the method described in Urbanová et al. (2013) with some modifications. Briefly, tissue samples of about 5 mg (fresh weight, FW) were ground to a fine consistency using 2.7-mm zirconium oxide beads (Retsch GmbH) and a MM 400 vibration mill (Retsch GmbH) at a frequency of 27 Hz for 3 min with 1 ml of ice-cold 80% acetonitrile containing 5% formic acid as extraction solution. The samples were then extracted overnight at 4 °C using a benchtop laboratory rotator Stuart SB3 (Bibby Scientific Ltd., Staffordshire, UK) after adding internal gibberellins standards ([$^2H_2$]GA$_1$, [$^2H_2$]GA$_3$, [$^2H_2$]GA$_4$, [$^2H_2$]GA$_8$, [$^2H_2$]GA$_9$, [$^2H_2$]GA$_{19}$, [$^2H_2$]GA$_{20}$, [$^2H_2$]GA$_{24}$, [$^2H_2$]GA$_{29}$, [$^2H_2$]GA$_{34}$, [$^2H_2$]GA$_{44}$, [$^2H_2$]GA$_{51}$ and [$^2H_2$]GA$_{53}$) purchased from OlChemIm. The homogenates were centrifuged at 36 670 g and 4 °C for 10 min, with the corresponding supernatants further purified using mixed-mode SPE cartridges (Waters, Milford, MA, USA) and analyzed by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS; Micromass, Manchester, UK). GAs were detected using a multiple-reaction monitoring mode of the transition of the ion [M–H]$^-$ to the appropriate product ion. Masslynx 4.2 software (Waters, Milford, MA, USA) was used to analyze the data, and the standard isotope dilution method (Rittenberg and Foster 1940) was used to quantify the GAs levels.

Measurement of seed water content and water loss

Pea seeds were collected at five different developmental stages (13, 17, 23, 28, and 33 DAP, labeled as 1–5). Water loss experiments were performed according to Ranathunge et al. (2010) with few modifications. At the beginning of the experiment, FW of each seed was determined. Ten seeds of each stage were placed on a plate (Suppl. Fig. S1) and put into a glass desiccator filled with 400 g of freshly dried silica gel. Relative humidity in the desiccator was 4.9% ± 4.4%, measured with a humidity logger (Comet System, Rožnov pod Radhoštěm, Czech Republic) during the whole experiment. Seeds were weighed every hour for 5 h. All measurements were averaged and converted into percentages of seed FW. After water loss analysis, all seeds were dried in an oven at 103 °C for 17 h and dry weight (DW) was measured. Seed water content was calculated as the difference between seed FW and DW. The water loss rate was calculated as an average water loss per hour in % of FW. Data were calculated from 2 to 3 runs (measured in 2020 and 2021), each composed of 20–40 replicates per developmental stage. Experiments were performed at room temperature (around 23 °C).

RNA sequencing

Frozen seed coats and embryos collected at four developmental stages 13, 17, 23 and 28 DAP (labelled as 1, 2, 3 and 4, respectively) were ground to a fine powder with liquid nitrogen, and total RNA was isolated using PureLink™ Plant RNA Reagent (Thermo Fisher Scientific, Waltham, MA, USA). Residual DNA was removed by Baseline-ZERO DNase (Epicenter, Madison, WI, USA) treatment followed by phenol/chloroform extraction. The RNA integrity of the samples was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). RNA sequencing was performed using Illumina NovaSeq platform performed by Novogene Ltd. (Cambridge, UK). The bioinformatics analysis of RNA-sequencing data was done as described in Balarynová et al. (2022). The expression level was normalized as FPKM (Fragments Per Kilobase Million).

Statistical analysis

Since the data were not normally distributed, a distribution-independent test was used. Statistical analysis was performed by the Kruskal–Wallis test with the following post hoc non-parametric multiple comparisons (Siegel and Castellan 1988) at a 0.05 significance level using R 4.0.2. (R Core Team 2020).

Results

ABA is detected especially in the embryo, and dihydrophaseic acid is its predominant degradation product in pea seeds

The level of ABA was determined in the dissected seed coats and embryos of cultivated (Cameor, JI92) and wild (JI1794)

pea genotypes, as shown in Fig. 1. The ABA contents in Cameor and JI92 seed coats were quite stable during development, while the ABA level in the seed coat of JI1794 (the wild pea) was higher than those of Cameor and JI92, and it decreased with the developmental stage. On the contrary, in the embryo, the ABA level increased with development, particularly in Cameor. Interestingly, the amount of ABA in JI1794 embryos decreased markedly after the 3rd developmental stage, while for JI92, the ABA content decreased constantly and progressively as the embryos matured. In our growth conditions, seeds were completely mature at around 28 DAP (4th developmental stage).

To monitor the changes in ABA level in a broader context, the profiles of ABA metabolites were analysed during pea seed development as well (Fig. 2, Suppl. File S1). We found that DPA, which is produced from PA by 8'-hydroxylation pathway (Fig. 2g), was the major catabolic product in both seed coats and embryos, whereas neoPA, the product of the 9'-OH pathway, was the least abundant. Moreover, the levels of DPA correspond with levels of ABA in both tissues of all genotypes (except seed coats of JI92, which did not contain DPA at the 3rd and 4th developmental stages). In seed coats, PA was detected in the 1st and 2nd developmental stages of Cameor and JI64, while in JI92 it was found only in the latter stage. Similarly, in embryos, PA was identified in all studied developmental stages of Cameor and JI64, while in JI92 it was found only in the last developmental stage. On the other hand, 7'-hydroxy-ABA (7'-OH-ABA) was not detected in JI92 seed coat.

GA$_1$ and the gibberellins of the 13-hydroxylation pathway prevailed in the developing pea seeds

As mentioned above, not only ABA but also GAs play an important role during seed development. For this reason, the level of bioactive GAs, their biosynthetic precursors and
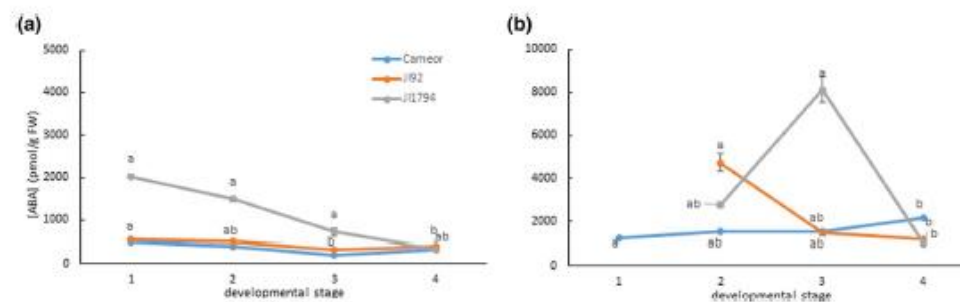

Fig. 1 ABA levels in seed coats (a) and embryos (b) of cultivated (Cameor and JI92) and wild (JI1794) pea genotypes during the seed development. Data expressed means±SD of three measurements.

Different letters indicate significant differences (P=0.05) between developmental stages of each genotype by the Kruskal–Wallis test with the following non-parametric multiple comparison test
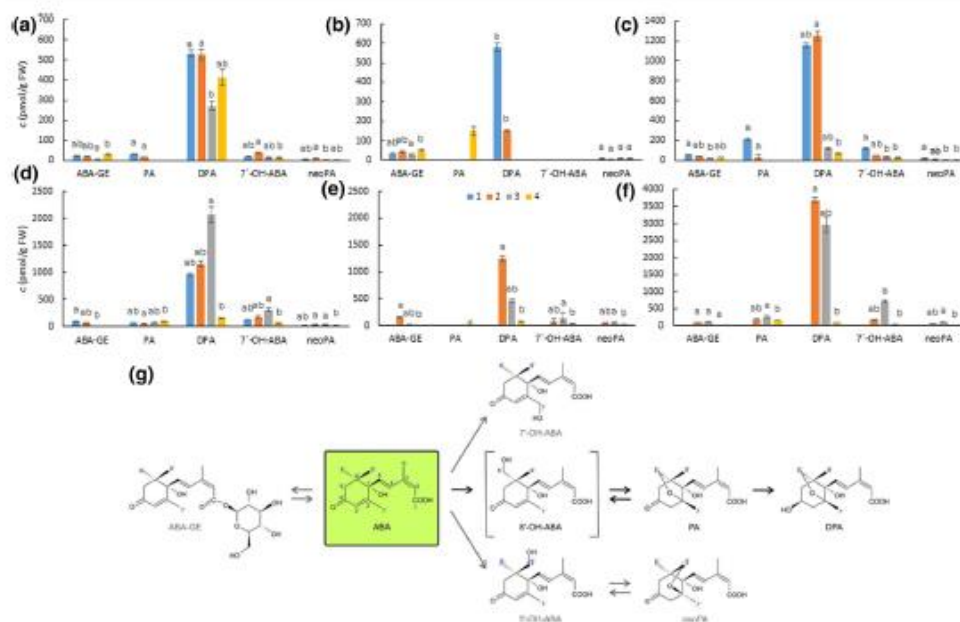
Fig. 2 Quantification of ABA glycosyl ester (ABA-GE), phaseic acid (PA), dihydrophaseic acid (DPA), 7'-hydroxy-ABA (7'-OH-ABA) and neophaseic acid (neoPA) levels in the seed coats (a–c) and embryos (d–f) in the developing seeds of Cameor (a, d), JI92 (b, e) and JI1794 (c, f) peas. Data expressed mean ± SD of three measurements. Differ-ent letters indicate significant differences ($P=0.05$) between develop-mental stages of particular metabolite by Kruskal–Wallis test with the following non-parametric multiple comparison test. The scheme of ABA inactivation (g)

catabolites were determined in developing seed coats and embryos of both cultivated (Cameor, JI92) and wild (JI1794) peas.

In accordance with the literature (Garcia-Martinez et al. 1987; Graebe 1987), GA_1 was found to be the main bioac-tive GA in the developing seeds of all three pea genotypes (Fig. 3). Besides GA_1, also other bioactive GAs such as GA_3 (from the 13-hydroxylation pathway), GA_4 and GA_7 (from the non-13-hydroxylation pathway) were detected (Fig. 3), especially in the seed coat. Interestingly, the seed coat of the pigmented pea genotypes (JI92 and JI1794) contained a more diverse combination of bioactive GAs than the seed coat of the non-pigmented cultivated Cameor seeds. Moreo-ver, the seed coats produced more variable bioactive GAs than the corresponding embryos (Fig. 3).

Unlike ABA, the level of GA_1 was found to be higher in the seed coat than in the embryos of all studied genotypes (except for embryos of JI92 in the 3rd developmental stage). The amount of GA_1 in seed coat of all studied genotypes tended to be highest in the 2nd developmental stage, then it decreased when reaching later developmental stages. On the
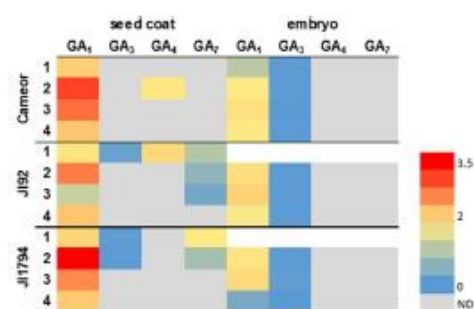
Fig. 3 The bioactive GAs detected in developing seed coat and embryo of cultivated (Cameor, JI92) and wild (JI1794) pea genotypes at four developmental stages (1–4). The heatmap is based on average gibberellin content (pmol/g FW). *ND* not detected

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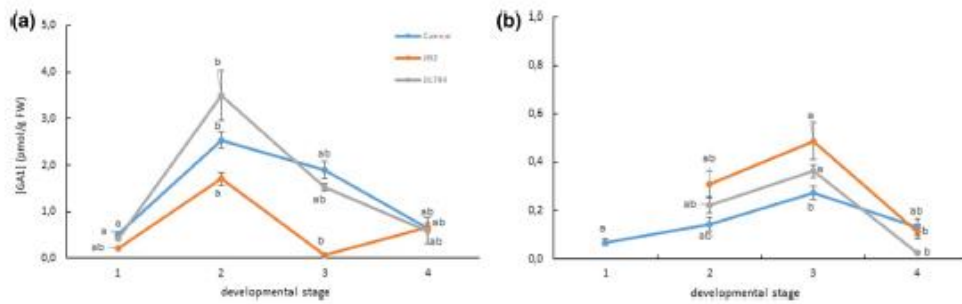
Fig. 4 The level of GA_1 during the development of cultivated (Cameor, JI92) and wild (JI1794) pea seed coat (a) and embryo (b). Data expressed mean $\pm$ SD of three independent measurements. Dif- ferent letters indicate significant differences ($P = 0.05$) among devel- opmental stages of each genotype by Kruskal–Wallis test with the fol- lowing non-parametric multiple comparison test

contrary, the level of GA_1 in the embryos was the greatest in the 3rd developmental stage (Fig. 4).

GAs in both seed coat and embryo were biosynthesized mainly via the 13-hydroxylation pathway (Fig. 5). In our pea samples, GA_1 biosynthetic precursors GA_{53}, GA_{44}, GA_{19},

GA_{20}, as well as GA_{20} degradation product GA_{29} were found (Fig. 6). Notably, GA_{29} was the most abundant gibberellin in the seed coat of all three studied pea genotypes, while GA_{20} was the major gibberellin detected in the embryos of both cultivated and wild peas. In contrast to Nadeau et al. (2011),
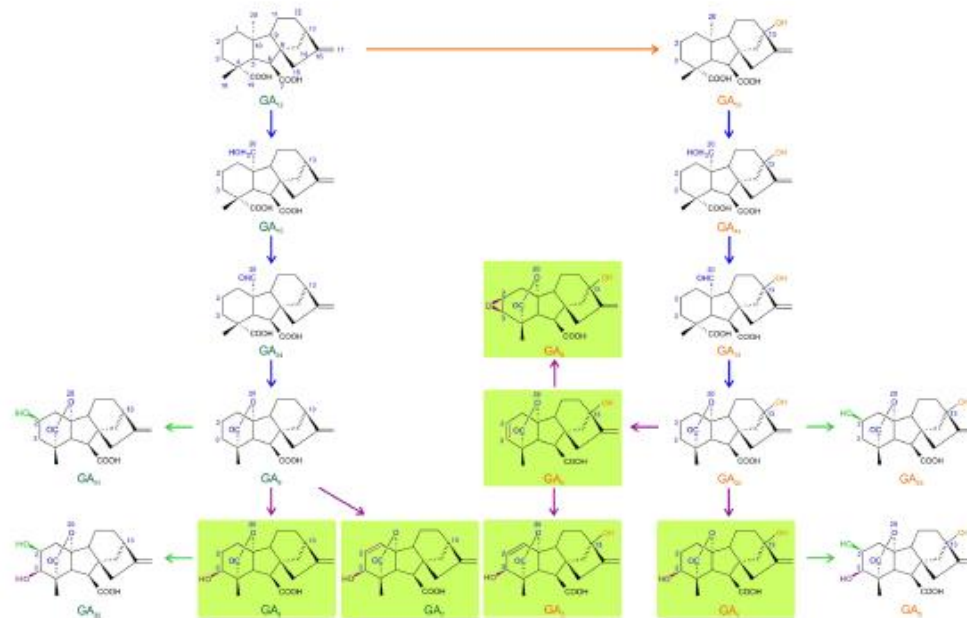
Fig. 5 The simplified scheme of the non-13-hydroxylation (leading to the production of GA_4 and GA_7) and the 13-hydroxylation (leading to the production of GA_1, GA_3, GA_5, GA_6) gibberellin metabolic path- ways. The bioactive GAs are in green rectangles. The arrows indicate enzymes responsible for GA precursor conversion, GA 20-oxidases (the blue arrows) and GA 3-oxidases (the violet arrows), and GA 2-oxidases (the green arrows) ensuring the conversion of bioactive GAs

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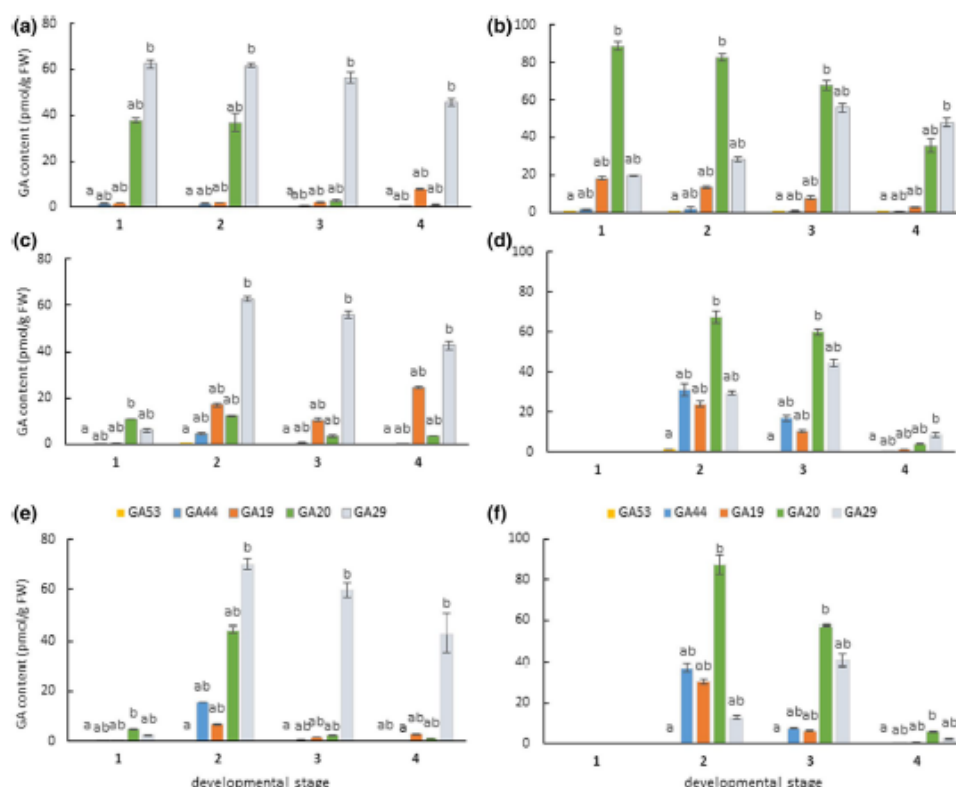
Fig. 6 The level of GAs belonging to the 13-hydroxylation pathway in the developing seed coat (left panel) and embryo (right panel) of Cameor (a, b), JI92 (c, d) and JI1794 (e, f) pea genotypes. Data represent the mean ± SD of three independent measurements. Differ- ent letters indicate significant differences ($P = 0.05$) among various metabolites in each developmental stage by Kruskal–Wallis test with the following non-parametric multiple comparison test

GA_8, the main degradation product of GA_1, was detected only occasionally (its internal standards were recovered in all samples) and at a level close to the limit of detection of the method (data not shown).

The precursors belonging to 13-non-OH pathway (Fig. 5), GA_{15}, GA_9 and its degradation product GA_{51}, were detected in both seed coats and embryos of Cameor and JI1794 (Fig. 7). However, they were not found in samples of JI92. Interestingly, GAs formed in this pathway were more abundant in the embryos compared to the seed coats. Moreover, they were found especially in Cameor genotype. The occurrence of 13-non-OH precursors significantly decreased in both genotypes along the development. Noteworthy, gibberellins GA_{12} and GA_{24} were not found in any tissue of all genotypes tested.
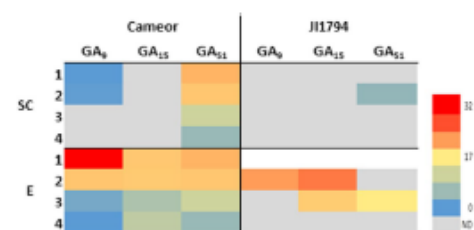
Fig. 7 The precursors formed via the 13-non-hydroxylation pathway and detected in the seed coats (SC) and embryos (E) of cultivated (Cameor) and wild (JI1794) pea seeds at four developmental stages (1–4). The heatmap is based on average gibberellin content (pmol/g FW). The precursors of the 13-non-hydroxylation pathway were not detected in JI92 seed samples. *ND* not detected

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Seed desiccation of domesticated pea is slower than those of landrace and wild pea

The water content of seeds declined with the developmental stage (Fig. 8). At early stage 1, the water content of seeds was around 75–81% of seed FW, with the highest value for JI64. Seeds of wild pea genotype JI64 had the highest water content decline between stages 3 and 4, at around 47%, compared to domesticated and landrace genotypes. The greatest water content decline of landrace JI92 seeds, of around 40%, occurred between the 3rd and 4th developmental stages. This developmental time was associated with the desiccation (maturation) phase when the seed undergoes a fast drying process. In domesticated Cameor, the most rapid water content decrease was observed between the 3rd and 4th stages (about 28%), followed by about 24% between stages 4 and 5. Altogether, the domesticated pea desiccated more slowly and smoothly than primitive landrace JI92 and wild JI64. On the other hand, the desiccation profiles of JI92 and JI64

were characterized by a sudden change between the 3rd and 4th developmental stages.

Besides seed maturation profiles, the water loss rate was measured (Fig. 9). In Cameor (the domesticated pea), the water loss kinetics was similar in the first three developmental stages, then it declined in the 4th and 5th stages, without reaching zero (Fig. 9a, Table 1). Landrace JI92 lost the most significant amount of water in early stage 1 (about 3.5% of FW/hour), whereas in stages 2 and 3, water loss was around 2.3% of FW/h. In JI92, there was no measurable water loss in stages 4 and 5, as the seed water content was already minimal at these stages. (Fig. 9b, Table 1). In wild JI64, the most extensive water loss was detected in the first developmental stage (4.1% FW/h), followed by a decline in the 2nd and 3rd stages (3.0 and 3.4% of FW/h). Similarly to JI92 seeds, developmental stages 4 and 5 of wild JI64 showed no water loss (Fig. 9c, Table 1). Based on water content in these stages, JI92 and JI64 seeds might be considered as
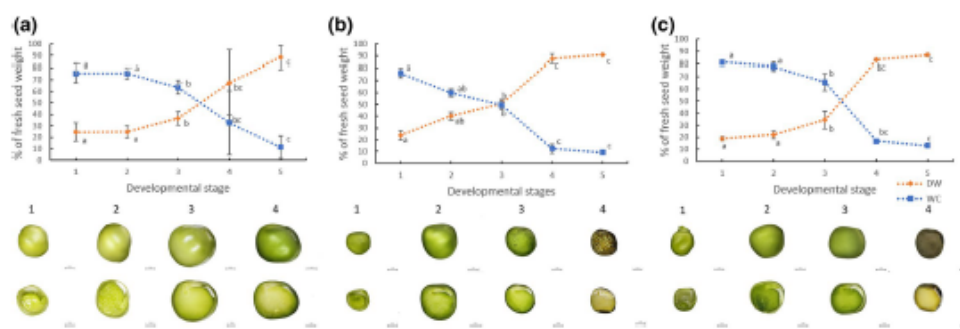
Fig. 8 Maturation profiles of Cameor (**a**), JI92 (**b**), and JI64 (**c**) pea seed. Changes in seed water content (WC) and dry weight (DW) in cultivated (Cameor, JI92) and wild JI64 pea seed during development. The graphs show the percentage of WC and seed DW calcu-lated from 2 to 3 experiments (±SD, $n=20$–40 seeds). Different letters indicate significant differences ($P=0.05$) by Kruskal–Wallis test with the following non-parametric multiple comparison test. Bars = 2 mm
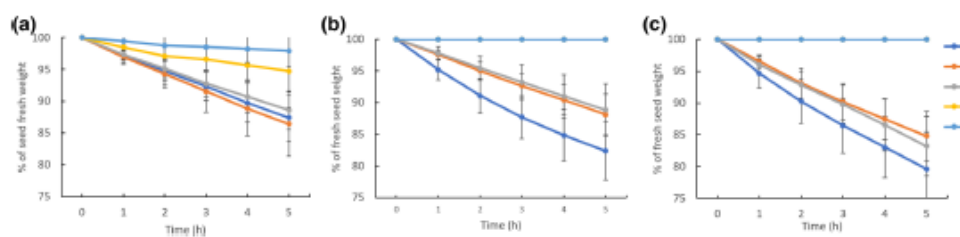
Fig. 9 Water loss during seed development of Cameor (**a**), JI92 (**b**) and JI64 (**c**) pea genotypes. Stage 4 is not visible in **b** and **c** because it is hidden under the line presenting stage 5. Means ± SD calculated from two to three experiments ($n=20$–40 seeds) are presented. The results of the statistical analysis are shown in Suppl. Fig. S3

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Table 1 The water loss rate in pea seeds expressed as the loss of seed mass during the time

Water loss rate (% of mass/hour)

Developmental stage	Domesticated Cameor	Landrace JI92	Wild JI64
1	2.5 ± 0.2	3.5 ± 0.9	4.1 ± 0.8
2	2.7 ± 0.2	2.3 ± 0.1	3.0 ± 0.4
3	2.2 ± 0.2	2.2 ± 0.1	3.4 ± 0.4
4	1.0 ± 0.4	0.0 ± 0.0	0.0 ± 0.0
5	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0

Values were calculated from the slope of the line of fresh seed weight loss (water loss, Fig. 9) and represent the mean of 2–3 measurements (± SD, $n = 20$–40 seeds)

fully matured. Unlike landrace and wild peas, the seeds of domesticated pea still lost some water even at the point of expected seed maturity (Fig. 9a). JI92 water loss rate showed slower desiccation in stages 2 and 3, which could correspond to lower water content of JI92 seed compared to those of JI64 (Fig. 9, Table 1). Interestingly, the water content of Cameor and JI64 was similar in the first three developmental stages (Fig. 9), however, the water loss rate was higher in JI64 seeds (Table 1).

Genes of ABA biosynthesis and GA metabolism are expressed mainly in developing seed coats

To explore the temporal expression of ABA and GA metabolic genes, RNA sequencing data were searched for genes encoding enzymes of ABA and GA biosynthesis and catabolism. Heatmaps of transcription patterns of genes encoding key ABA metabolic enzymes (Fig. 10) showed the differences between tissues but also among genotypes. Generally, these genes were more transcribed in seed coats than in embryos. Genes encoding ABA biosynthetic enzymes tended to be expressed especially later in the seed development in both seed coats and embryos. Interestingly, in the Cameor seed coats *ZEP* and *AO* transcripts prevailed, whereas, in the seed coats of the pigmented genotypes (JI92 and JI1794), the expression of *NCED* (with a peak in the 3rd developmental stage) and *SDR1* (with a peak in the 1st developmental stage) genes were the strongest. *ABA 8′-hydroxylase* genes were expressed particularly at the first two developmental stages in the seed coats, particularly in the two pigmented genotypes, JI92 and JI1794. In the embryos, their expression augmented with the developmental stage.

Heatmaps of transcription patterns of genes encoding main GA metabolic enzymes (Fig. 11) showed that these genes were expressed mostly in the seed coats. In particular, *GA 20-oxidases* and *GA 3-oxidases* were transcribed

Fig. 10 Heatmaps of genes involved in biosynthesis and catabolism of ABA in the seed coats (a) and embryos (b) of cultivated (Cameor, JI92) and wild (JI1794) peas. *ZEP* zeaxanthin epoxidase, *NCED* 9-*cis*-epoxycarotenoid dioxygenase, *SDR1* short-chain dehydrogenase reductase, *AO* abscisic aldehyde oxidase. The heatmap is based on average FPKM (Fragments Per Kilobase Million) values from RNA sequencing

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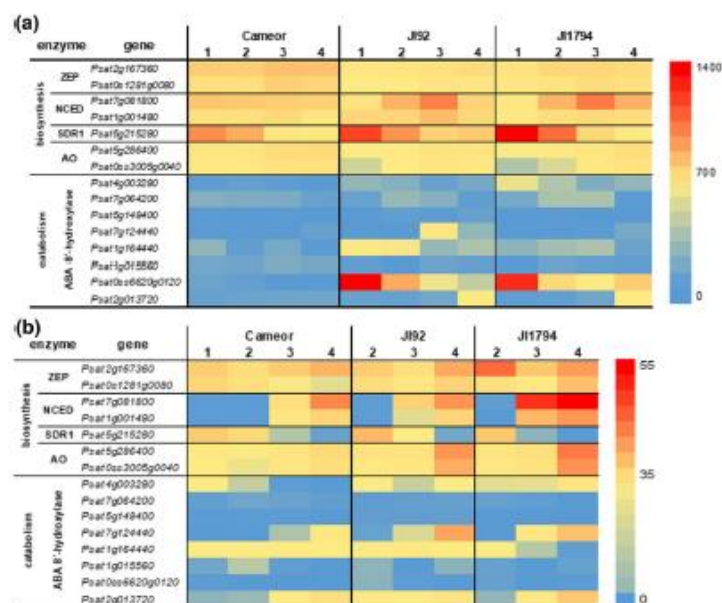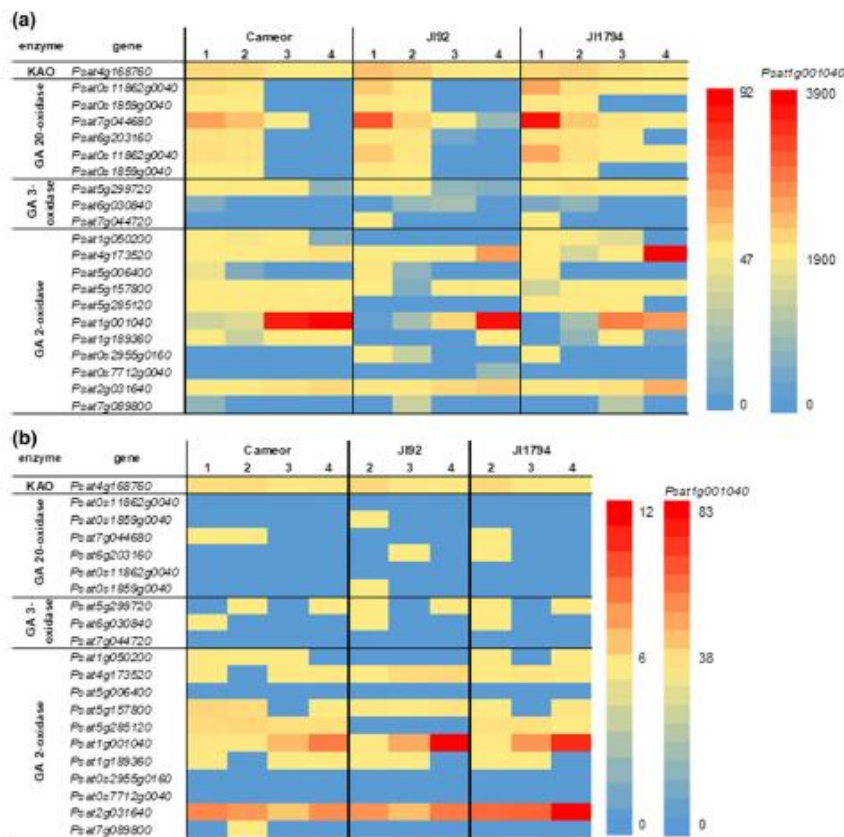
Fig. 11 Heatmap analysis of genes involved in gibberellin metabolism in the seed coats (a) and embryos (b) of cultivated (Cameor, JI92) and wild (JI1794) peas. *KAO* *ent*-kaurenoic acid oxidase. The heatmap is based on average FPKM (Fragments Per Kilobase Million) values from RNA sequencing

mainly in the seed coats of all studied genotypes. In both the seed coats and embryos, the expression of genes encoding *KO*, *GA 20-oxidases* and *GA 3-oxidases*, which are involved in GA precursor formation and production of bioactive GAs, decreased with development. Their expression was higher in the seed coats of pigmented (JI92 and JI1794) genotypes compared to Cameor, while there was no difference among genotypes in the embryo samples. By contrast, the genes encoding enzymes deactivating bioactive GAs (*GA 2-oxidases*) were expressed predominantly later during seed development in both tissues similarly and in all three studied genotypes.

Discussion

Seed germination is one of the key steps in a plant's lifecycle. To germinate successfully, the seed development must be completed appropriately, and seed dormancy must be broken. The wild pea seeds will not germinate unless their seed coat is physically disrupted. The balance of phytohormones has a major influence on numerous morphological traits, including some of the agronomical relevance, such as seed dormancy, branching, tillering, and shoot and root architecture. Since seed dormancy is one of the key

domestication traits (Hradilová et al. 2017; Smýkal et al. 2018), it is not surprising to see the alteration of hormone levels in crops compared to their wild progenitors (Ben-Abu and Itsko 2022). This has been nicely documented in the case of maize domestication (Dong et al. 2019).

Seed, and especially seed coat, structure (Zablatzká et al. 2021) and chemical composition (Janská et al. 2017; Hradilová et al. 2017) were studied in both wild and cultivated peas. However, the hormone levels were extensively monitored only in various pea cultivars (Ribalta et al. 2017, 2019). To the best of our knowledge, this study is the first one focused on ABA and GA changes between cultivated and wild peas during seed development. We focused on the later stages of seed development, from 13 to 28 DAP, in relation to our seed coat study (Balarynová et al. 2022). At the earliest developmental stage (13 DAP), the seed coat structure is already developed, both in wild and cultivated genotypes, and the embryo is embedded in the liquid endosperm (Zablatzká et al. 2021). After gradual endosperm consumption, the growing embryo comes into contact with the seed coat (by 17 DAP, in the second developmental stage). Endosperm depletion is connected with the expansion of parenchyma cells (the innermost layer of the seed coat), which forms nutritional tissue supporting the developing embryo with nutriments unloaded from the phloem (Nadeau et al. 2011). Accordingly, the peak in GA_1 accumulation was found in the seed coat (Fig. 4) of both cultivated and wild peas by 17 DAP, which could be explained by the growth of the parenchyma layer of the seed coat. Subsequently, the developing embryo absorbs nutrients supplied by the seed coat, and the parenchyma is slowly crushed by the expanding cotyledons (Van Dongen et al. 2003). The further growth of the embryo corresponded to an increase in the level of GA_1 (Fig. 4) in the third developmental stage (23 DAP); as the size of seeds increases, the process of desiccation begins, and the seed coat pigmentation (in JI92 and JI1794) starts to develop (Zablatzká et al. 2021).

Seed maturation is associated with reduced water content and changes in ABA content in the embryo. To survive the desiccation occurring during maturation, desiccation tolerance is acquired by an accumulation of antioxidants, various osmoprotectants, late embryogenesis abundant (LEA) proteins, and ABA plays a vital role in this process (Corbineau et al. 2000; Bewley et al. 2013). The level of ABA in the embryos reaches its peak just before the embryos start to desiccate (King 1976; Hsu 1979; Karssen et al. 1983). Afterward, the ABA level is quite low in mature seeds (Karssen et al. 1983). Our data showed that seeds of the semi-domesticated pea, landrace JI92, developed faster. The maturation began earlier than in seeds of wild or domesticated peas (Fig. 8). The peak of ABA level (Fig. 1), indicating the onset of maturation, was detected in the 2nd and 3rd developmental stages of JI92 and JI1794 embryos, respectively. The

level of ABA in Cameor embryos started to increase after the 4th developmental stage (Fig. 1), indicating a slower development and late maturation. A similar trend was shown in experiments on the rate of water loss (Fig. 9, Table 1), which might hence be considered an indicator of seed maturation, the same as changes in ABA content. For some legumes, seed maturity was reported to be reached when water content drops to 55–60% of FW, when maximum dry weight is reached (Ellis et al. 1987). After finishing the seed-filling phase, seeds start desiccating, developing desiccation tolerance (Ellis et al. 1987). In pea, the desiccation tolerance is expected to evolve between 65 and 80% of water content before physiological maturity is acquired (Ellis et al. 1987; Ney et al. 1993). We showed that Cameor seeds typically exhibited a lower water content loss and a slower dry weight increase compared to JI92 (a primitive pea landrace) and wild pea seeds (Figs. 8 and 9, Table 1), which indicated the slower development of Cameor seeds. Moreover, the Cameor seeds showed a slower water loss rate than wild seeds during the first three developmental stages, although their water contents were quite similar. It was unclear why the seeds of domesticated pea lost water more slowly, perhaps this could be related to their lower water potential at the given stages, but this would need to be verified. Matthews (1973) suggested that an initial slow water content decrease helps to develop desiccation tolerance in pea seeds. According to our data, we can assume that maturity was acquired between 2nd and 3rd developmental stages in JI92 seeds, while in JI64 and Cameor seeds it tended to be achieved later, between 3rd and 4th developmental stages.

Comparative analysis of embryos and seed coats of wild and cultivated pumpkin (*Cucurbita maxima*), which has a combination of physical and physiological dormancy, like the model legume *M. truncatula* (Ochatt and Abirached-Darmency 2019), has revealed that while in the embryos, ABA concentrations were similar in both domesticated and wild subspecies, in seed coats, it was threefold higher in the wild subspecies (Martínez et al. 2018). Moreover, in this study with pumpkin, the naked embryos from the wild subspecies were far more responsive to ABA than those from the domesticated subspecies. These results indicate that dormancy in the wild pumpkin is imposed by the seed coat tissues and that this effect is mediated by their high ABA content and the sensitivity of the embryos to ABA (Martínez et al. 2018). Unlike pumpkin, ABA and its metabolites were more present in embryos than in seed coats of all pea genotypes studied (Figs. 1 and 2). Both tissues of the wild pea had the highest amount of ABA and its metabolites (Suppl. File S1) which could be explained by its great resilience and ability to survive in the changing natural localities. The high content of ABA is usually connected with dormancy and resistance to various biotic and abiotic challenges, as shown in pea (Ochatt 2015; Ribalta et al. 2019). It is known

that ABA plays an important role in the initiation of dormancy in developing seeds (Feurtado and Kermode 2007). Although wild pea seed dormancy is primarily determined by the water-impermeable seed coat, and its acquisition in legumes is still poorly understood, the elevated ABA level during seed development might be important to prepare the embryo for survival in the dry dormant seed. Similarly to *Arabidopsis thaliana* seeds, ABA might be involved in the establishment of seed coat impermeability by regulating the synthesis of various compounds such as the hydroxylated fatty acids, phenolics, or pigments (Mendoza et al. 2005), which were shown to be more abundant in the seed coat of wild peas (Cechová et al. 2017; Janská et al. 2019; Krejčí et al. 2022). Pea seed coat showed abundant accumulation of phenolic compounds that, upon oxidation, may impact seed permeability as well as pigmentation, both typical in the seed coat of dormant peas (Balarynová et al. 2022; Krejčí et al. 2022).

Gene expression of ABA and GA metabolism genes has been studied during seed development and imbibition in rice, the species with physiological dormancy (Liu et al. 2014). A comparison of dormant and non-dormant seeds showed differences in the peak of *OsNCED* (ABA biosynthesis) between dormant and non-dormant genotypes. On the contrary, our data rather showed a difference between non-pigmented (Cameor, stable *NCED* gene expression) and pigmented genotypes (JI92 and JI1794, *NCED* gene expression peaked at 3rd developmental stage) instead, with no effect of dormancy (physical dormancy) (Fig. 10). This could indicate the relationship between seed coat pigmentation and ABA. Similarly, *ABA 8'-hydroxylase* genes expression showed similar trends (Fig. 10). On the other hand, *GA 20-oxidases* and *GA 3-oxidases* showed several peaks during the non-dormant rice seed development, whereas their expression in dormant seeds remained stable. This corresponds to a lower accumulation of active GAs in non-dormant genotype (Liu et al. 2014). Conversely, the main difference between studied genotypes in our study was not in the expression patterns of GA metabolism genes but in their levels of expression, which were higher in the pigmented genotypes (Fig. 11). Although the expression of GA and ABA-related genes was analyzed only in cultivated legumes, such as alfalfa (*Medicago sativa*) (Zhao et al. 2022), their expression patterns were in agreement with our data. Finally, we showed that both ABA and GA metabolic genes were expressed predominantly in the seed coat, indicating the crucial role of seed coat in governing ABA and GA hormonal levels in the developing pea seeds.

Based on seed coat pigmentation, the pea genotypes studied here can be divided into pigmented (JI92 and JI1794) and non-pigmented (Cameor) ones (Suppl. Fig. S2). Despite being pigmented, JI92 is a primitive domesticated landrace compared to JI1794, which belongs to wild pea genotypes.

It has been shown that cultivated legume seeds contain fewer carotenoids, the precursors of ABA, than their wild counterparts (Frey et al. 2006; Fernández-Marín et al. 2014). Moreover, ABA is also able to promote or inhibit the biosynthesis of anthocyanin in fruits by cross-talking with other phytohormones including jasmonic acid, GAs, auxin and cytokinin (Xie et al. 2012; Jaakola 2013; An et al. 2018). ABA level and the expression of *NCED* were associated with pigment production (Jia et al. 2011; Karppinen et al. 2018; Li et al. 2019). Correspondingly, the expression of *NCED* was increased in the seed coat of pigmented peas (JI92 and JI1794) (Fig. 10). In peanut (*Arachis hypogaea*), an association not only between ABA signalling and anthocyanins but also proanthocyanidin (condensed tannins) content was suggested (Wan et al. 2016). The fact that proanthocyanidins stimulate ABA synthesis was also observed during the maturation and germination of *Arabidopsis* seeds (Jia et al. 2012).

On the other hand, it has been shown that proanthocyanidins can also act as GA antagonists in pea (Green and Corcoran 1975; Corcoran et al. 1972). In common bean (*Phaseolus vulgaris*) seeds, (+)- catechin (found mainly in seed coat, not in embryo) inhibits the conversion of GA_{12}-aldehyde to GA_{12} (Kwak et al. 1988). In our samples, we detected GAs of the 13-non-hydroxylation pathway, especially in Cameor (Fig. 7), the domesticated genotype with the seed coat low in proanthocyanidins (Hradilová et al. 2017). Besides, GAs were shown to regulate anthocyanin biosynthesis (Loreti et al. 2008). Unlike ABA, the seed coat of the studied pea genotypes contained more bioactive GAs than the embryo (Figs. 3 and 4). Moreover, the seed coat of pigmented genotypes (primitive domesticated landrace JI92 and wild JI1794) contained a more diverse combination of bioactive GAs than Cameor (Fig. 3). The seed coat of JI92 and JI1794 contained GA_3 and GA_7, which were not detected in Cameor. Thus, we might expect that these GAs may play a role in the development of seed coat pigmentation.

Our data showed that GA_{20} and GA_{29} were the most abundant gibberellins detected in the embryos and seed coats (Fig. 6), respectively, of studied pea genotypes. This is in agreement with previous work on immature pea seeds (Sponsel 1983; Zhu et al. 1991). In seeds, GA_{20} is metabolised to GA_{29} in the embryos and then it is transported to the seed coats. In the seed coat, GA_{29} is metabolised to GA_{29}-catabolite (Sponsel 1983). This could explain a decrease in GA_{29} content with development despite very high gene expression of *GA 2-oxidases* in the later stages (Fig. 11).

Alteration of flavonoid pigmentation during crop domestication has been widely reported. Particularly, a loss of pigmentation in the edible parts is one of the domestication symptoms (reviewed in Smýkal et al. 2018; Paauw et al. 2019; Alseekh et al. 2021). The discussion on whether this is the result of direct selection

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or of linkage of other important domestication genes is ongoing. The consumer preference for visual appearance likely drives acting selection. This also acted in the case of grain legumes, such as pea, chickpea, common bean and lentil (Balarynová et al. 2022).

Notably, Wang et al. (2018) identified a gene responsible for seed dormancy that has been subjected to parallel selection in multiple crops. This gene encodes stay-green G gene-affected seed dormancy in soybean through interactions with NCED3 and PSY and in turn, modulated ABA synthesis. The green soybean seed coat is governed by three classical stay-green loci with different mechanisms, among which the G locus specifically dominantly controls the green colour of the seed coat, whereas the other two loci affect other organs as well. Using transgenic and mutant lines, they have shown that in the mutant g lines, less ABA is produced, resulting in the weakening of dormancy and thus facilitating crop management for farmers, which probably led to the parallel selection of g genotypes in various crops. Interestingly, the region around the G locus exhibits selection signatures in soybean domestication. However, if the trait under selection is seed coat colour, it is then perplexing. All wild soybeans with black seeds contain the G allele conferring the green seed coat colour, considering green is invisible against black. It was hypothesized that the trait controlled by G under selection is the reduction of seed dormancy. Indeed, overexpressing the wild-type G allele strengthened seed dormancy. However, G corresponds to a different mechanism from both, and it is a new gene linked to physiological dormancy. Whether this is a case of pea seed dormancy remains to be shown. However, both comparative transcriptomics and genetic mapping did not show any direct involvement of such genes (Hradilová et al. 2017 and unpublished results).

Analysis of seed content of different hormones suggests that the hormonal balance between ABA, GAs, and auxins at crucial time points during this process might underlie seed development differences in these accessions and would thus illustrate the dynamics of pea seed development. Auxin acts upstream of GA during seed coat development (Figueiredo et al. 2016). Despite the fact that the role of hormones in regulating legume seed development is poorly described, it was shown that the embryos of pea seeds do not germinate until physiological maturity (around 18 DAP) is reached (Ribalta et al. 2017), except if cultured in vitro in the presence of exogenous growth regulators (Ribalta et al. 2019). In these studies, it was found that cultivated pea seeds had the highest ABA level after physiological maturity, which can be linked to the biosynthetic pathway for ABA and the positioning of carotenoid biosynthesis in it (Nambara and Marion-Poll 2003; North et al. 2007; Ali et al. 2022).

Conclusion

In this study, we provide the first report of ABA and GAs profiling wild pea seeds during their development and compare them to domesticated peas. Despite the loss of seed dormancy in domesticated legumes, the mechanisms underlying physical dormancy in legumes are still poorly understood. Our data showed that wild pea seed coat and embryo were abundant in ABA and its metabolites, which might be associated with the preparation of its seeds for a period of dormancy and the development of seed coat pigmentation. The seed coats of pigmented seeds differed in the composition of bioactive GAs and were transcriptionally more active in the expression of ABA and GA metabolite genes, highlighting the importance of seed coat during seed development.

Author contribution statement JB, PS conceived, and JB coordinated the research. JB, BK, DT, and VT performed the experiments and JB, BK, DT, VT, and MŠ analysed data. OT contributed to RNA seq data analysis. The first draft of the manuscript was written by JB, SO, PS, all authors commented on previous versions of the manuscript and complemented it. All authors read and approved the final manuscript.

Declarations

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ARTICLE 8

Changes in the concentrations and transcripts for gibberellins and other hormones in a growing leaf and roots of wheat seedlings in response to water restriction

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Contribution: analysis of gibberellins, data evaluation

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Changes in the concentrations and transcripts for gibberellins and other hormones in a growing leaf and roots of wheat seedlings in response to water restriction

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Abstract

Background: Bread wheat (*Triticum aestivum*) is a major source of nutrition globally, but yields can be seriously compromised by water limitation. Redistribution of growth between shoots and roots is a common response to drought, promoting plant survival, but reducing yield. Gibberellins (GAs) are necessary for shoot and root elongation, but roots maintain growth at lower GA concentrations compared with shoots, making GA a suitable hormone for mediating this growth redistribution. In this study, the effect of progressive drought on GA content was determined in the base of the 4th leaf and root tips of wheat seedlings, containing the growing regions, as well as in the remaining leaf and root tissues. In addition, the contents of other selected hormones known to be involved in stress responses were determined. Transcriptome analysis was performed on equivalent tissues and drought-associated differential expression was determined for hormone-related genes.

Results: After 5 days of applying progressive drought to 10-day old seedlings, the length of leaf 4 was reduced by 31% compared with watered seedlings and this was associated with significant decreases in the concentrations of bioactive GA_1 and GA_4 in the leaf base, as well as of their catabolites and precursors. Root length was unaffected by drought, while GA concentrations were slightly, but significantly higher in the tips of droughted roots compared with watered plants. Transcripts for the GA-inactivating gene *TaGA2ox4* were elevated in the droughted leaf, while those for several GA-biosynthesis genes were reduced by drought, but mainly in the non-growing region. In response to drought the concentrations of abscisic acid, *cis*-zeatin and its riboside increased in all tissues, indole-acetic acid was unchanged, while *trans*-zeatin and riboside, jasmonate and salicylic acid concentrations were reduced.

Conclusions: Reduced leaf elongation and maintained root growth in wheat seedlings subjected to progressive drought were associated with attenuated and increased GA content, respectively, in the growing regions. Despite increased *TaGA2ox4* expression, lower GA levels in the leaf base of droughted plants were due to reduced biosynthesis rather than increased catabolism. In contrast to GA, the other hormones analysed responded to drought similarly in the leaf and roots, indicating organ-specific differential regulation of GA metabolism in response to drought.

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Background

Wheat (*Triticum aestivum* L.) is one of the most important staple crops globally feeding a major part of the human population. The worldwide estimated production of wheat in 2019 was 766 million tonnes making it the second most important grain after maize [1]. However, drought is a major constraint to the productivity of wheat and other cereals [2–4] and is anticipated to become an even more serious problem for farmers worldwide as a result of the changing climate [5, 6].

Plants under drought stress adapt their morphology, physiology and biochemistry in an attempt to cope with the water limitation [7–9]. Plant hormones are important components of this stress response, mediating mechanisms to reduce water usage, including restriction of shoot growth, while roots, which first perceive the lack of water, continue to extend [10–12]. Gibberellins (GAs) as growth regulators are prime candidates for involvement in this growth redistribution. Furthermore, it has been shown that plants with reduced GA content, through the application of growth retardants or mutation, are more resistant to abiotic stress, including drought, cold and salt stress [13]. The mechanism of this phenomenon is still not fully understood.

Gibberellins signal through the GID1 receptor, which, in the presence of GAs, interacts with and destabilises DELLA proteins, a family of transcriptional regulators, first identified as repressors of growth [14]. DELLA proteins act in association with transcription factors to modify gene expression, for example by sequestration of the transcription factors or by functioning as a co-activator [15–17]. After GA-dependant binding to GID1, DELLA proteins are polyubiquitinated by an E3 ubiquitin ligase and targeted for degradation by the 26S proteasome [18, 19]. In rice, barley and Arabidopsis DELLA protein loss-of-function mutants have been shown to exhibit a constitutive GA response phenotype [20, 21]. These mutants do not respond to exogenous GA, are taller than the wild-type, flower early and are often sterile. On the other hand, specific mutations in the N-terminal region of DELLA proteins cause their accumulation by preventing their association with the GA-GID1 complex [20–24]. Such gain-of-function mutants are dwarfed; prime examples of which are the semi-dwarfing alleles of the wheat *RHT-1* gene, which were introduced in the Green Revolution and are present in most commercial cultivars [25]. Wheat cultivars containing *RHT-1* dwarfing alleles were found to perform better under water deprivation than those carrying the non-mutant tall allele [26].

During abiotic stress, DELLA proteins accumulate in association with a reduction in GA content [27, 28]. As well as decreasing water requirement through suppressing shoot growth, the accumulation of DELLA was shown to reduce transpiration in tomato by closing stomata [29]. The mechanism involves increased ABA concentration through enhanced expression of an ABA transporter [30], highlighting the action of DELLA as a hub in the cross-talk between the GA signalling pathway and those of other plant hormones [31]. In addition, DELLA was observed to reduce the level of reactive oxygen species [27], which accumulate during abiotic stress and can lead to oxidative damage and cell death when present at high concentrations [32].

Stress-induced DELLA accumulation is enabled primarily by a reduction in GA concentration, although expression of some DELLA paralogues, such as *RGL3* in Arabidopsis, is up-regulated by stress [33, 34]. Biosynthesis of GAs proceeds through the action of plasmid-localised terpene cyclases, *ent*-copalyl diphosphate synthase (CPS) and *ent*-kaurene synthase (KS), and the membrane-associated cytochrome P450 monooxygenases, *ent*-kaurene oxidase (KO) and *ent*-kaurenoic acid oxidase (KAO) to produce the C_{20}-GA GA_{12}, which is 13-hydroxylated by cytochrome P450 monooxygenases (GA13ox) to GA_{53} [35]. GA_{12} and GA_{53} are converted by the soluble 2-oxoglutarate-dependent dioxygenases GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) to the biologically active C_{19}-GA end-products GA_4 and GA_1, respectively. GA turnover occurs primarily through the action of the inactivating GA 2-oxidases (GA2ox), of which there are two families, acting on C_{19}-GAs or C_{20}-GAs, respectively [36–39]. Up-regulation of *GA2ox* genes to reduce GA content and promote DELLA accumulation has been shown to occur in response to abiotic stress, with examples from Arabidopsis for up-regulation of the *AtGA2ox7* paralogue in response to salt [40] and mechanical stimulus [41], and of several *GA2ox* genes in response to cold [33, 36]. Pearce et al. [42] identified 7 C_{19}-*GA2ox* paralogues, excluding homoeologues, and 5 C_{20}-*GA2ox* paralogues in the wheat genome but there is limited information on their involvement in stress responses for this species.

While the growth of shoots and roots is dependent on GA signalling, root growth requires lower concentrations of GAs than does that of shoots [43] and indeed root growth may be inhibited by supraoptimum GA concentrations [44, 45]. Thus, a reduction in GA content in response to drought provides a mechanism

to redistribute growth between shoots and roots, but the role of GA signalling in this response has been little studied. In this report, we describe the effect of water limitation on the growth, transcriptome and hormone content of shoots and roots of wheat seedlings with a focus on GA metabolism and signalling.

Results

The effect of water restriction on growth and physiology

Wheat seedlings were grown in pots under a controlled environment in a field soil with a high sand content until the third leaf was visible. Watering was then withheld from half the plants, while the remainder were continued to be watered to 100% soil capacity. Leaf lengths were monitored throughout the experiment (Fig. 1A). After 5 days, the length of leaf 4 in the droughted plants was reduced by 31% compared with the watered plants, while the lengths of the longest root were not significantly different between the two treatments (Fig. 1B). At this stage, the water content of the droughted soil was 19% full capacity (Fig. 1C), and the relative water content of the 3rd leaf was reduced from 96% for the well-watered plants to 81.5% under drought (Additional file 1: Table S1). The contents of malondialdehyde (MDA), indicative of lipid oxidation, and proline in leaves of the droughted seedlings increased by 2- and 5-fold, respectively, compared with the well-watered seedlings (Additional file 1: Table S1). There was a small, but non-significant ($p = 0.2$)

Fig. 1 Growth response of wheat seedlings to prolonged drought. **A** Mean lengths +/− SD of leaves 1-4 measured from 5 days before and after the cessation of watering. Statistically significant differences between watered and droughted plants are indicated by * ($p < 0.05$) and *** ($p < 0.001$), $n = 20$. **B** Mean length of the longest root +/− SD at 5 days after water cessation, $n = 20$. **C** Soil water content (% full soil water capacity)

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reduction in photosynthetic rate in the droughted seedlings after 5 days of water restriction compared with the well-watered plants, while by 6 days the difference was significant ($p = 0.04$) (Additional file 1: Table S1). Similarly, there were small, non-significant reductions in stomatal conductance and intercellular CO_2 concentrations on day 5 for the droughted plants with greater, significant reductions after 6 days (Additional file 1: Table S1). In order to determine changes in hormone content and gene expression in response to water restriction, the 4th leaf was harvested at day 5 and divided into the bottom 3 cm of the sheath (leaf base), which includes the growing region of the leaf sheath, and the rest of the leaf. Roots were divided into the bottom 3 cm of the primary roots (the root tip) and the remaining root material (Additional file 1: Fig. S1 illustrates the tissue sampling). The four samples from well-watered and droughted seedlings with

replicates were analysed for the abundance of GAs and other hormones by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS-MS) and of transcripts by RNA-sequencing.

Water restriction and gibberellin content

The GAs, including precursors and catabolites, were analysed separately from other hormones due to the requirement for a different protocol in order to achieve adequate sensitivity. Figure 2A and C show the concentrations of 13-hydroxylated (13-OH) and 13-deoxy (13-H) GAs, respectively, in the leaf tissues, while these are presented for the roots in Fig. 2B and D. In the base of the leaf, which contains the extension zone of the sheath [46], the concentrations of most GAs, including the biologically active GA_1 and GA_4 were significantly reduced by water restriction. Notably, the concentration

Fig. 2 Concentrations of gibberellins (GAs) in the 4th leaf and roots of watered and droughted wheat seedlings after 5 days of water cessation. GAs were analysed in the leaf base and remaining leaf (A and C for 13-OH- and 13-H-GAs, respectively) and in the root tip and remaining root (B and D, for 13-OH- and 13-H-GAs, respectively). Values are the means of 3 replicates +/− SD. Statistically significant differences between watered and droughted plants are indicated by * ($p < 0.05$); ** ($p < 0.01$) and *** ($p < 0.001$) determined by the Student t-test

of GA_8, the 2β-hydroxylated catabolite of GA_1, was also strongly reduced by drought. GA_1 and GA_4 contents were unchanged by drought in the remaining leaf, although GA_8 concentration, but not that of GA_{34} (2β-hydroxyGA$_4$), was significantly reduced. The effect of drought on precursor levels in the remaining leaf was variable with only GA_{44} and GA_{20} showing significant decreases. In contrast to the base of the leaf, the root tip showed small, but significant increases in GA_1 and GA_4 concentrations under drought, while the levels of GA_{15}, GA_{20} and GA_{51} (2β-hydroxyGA$_9$) decreased and those of the other GAs remained unchanged. In the remaining root tissue there were small decreases in the levels of several GAs under drought, including GA_1 and GA_4 and the precursor GA_{19}. Notably, the levels of the major catabolites, GA_8 and GA_{34}, were not changed by drought in either root tissue.

Water restriction and the concentration of other hormones
Growth and its response to water limitation are regulated by the combined activity of multiple hormones [47, 48], so we took the opportunity to measure the concentrations of abscisic acid (ABA), indole-3-acetic acid (IAA), cytokinins (CKs), jasmonates (JAs), salicylic acid (SA) and associated metabolites in equivalent samples to those analysed for GAs. Their concentrations are given in Additional file 2, and for selected hormones shown in Fig. 3A and B for the leaf and root samples, respectively. As expected, the concentration of ABA was strongly increased by water limitation in all four tissues, with fold increases of 41 and 94 in the leaf base and remaining leaf, respectively, and of 79 and 219 for the root tip and remaining root tissue, respectively. There were smaller increases under drought in the concentration of the ABA-catabolite phaseic acid in the leaf tissue and lower root (Additional file 2), while it increased more substantially in the remaining root. In contrast to that of ABA, the concentration of IAA was not significantly changed by drought in any of the tissues, although there were higher levels of 2-oxindole-3-acetic acid in all tissues and of IAA-glutamate in the leaf base. The concentrations of JA and its isoleucine conjugate decreased in all four water-stressed tissues, as did that of SA in all tissues except the remaining leaf in which it increased, suggesting a redistribution under drought.

The concentrations of *trans*-zeatin (*tZ*) and its riboside (*tZR*), which were higher in the growing regions of the leaf and root compared with the remaining tissues, were reduced by water limitation in all sampled tissues. The lower *tZ* and *tZR* concentrations were accompanied by increased levels of their *O*-glucosides in the leaf base and root tip, but not in the remaining tissues. In contrast,

the concentrations of *cis*-zeatin (*cZ*) and its riboside *cZR*, which were generally higher than those of the *trans* isomers, were increased by drought in the leaf base and root tip, whereas it was reduced in the droughted remaining leaf tissue and unchanged in the remaining root tissue, in which concentrations were generally low. The concentrations of the *O*-glucosides of *cZ* and *cZR* followed the same trend as their aglycones in the leaf base and root, while there were significant decreases in their levels in the remaining root tissues under drought.

Water restriction and gene expression
Changes in gene expression due to water restriction was determined by RNA-seq from three biological replicates in tissues and treatments equivalent to those used for the hormone determination. The numbers of differentially expressed genes (DEGs) (> 2-fold change) were substantially higher for the leaf tissues (17,298 and 24,343 for the 4th leaf base and remaining leaf, respectively) than for the root (2696 and 8995 for the root tip and remaining root, respectively) (Table 1). The distribution of up- and down-regulated genes is illustrated by the volcano plots in Additional file 1: Fig. S2. As indicated by the dendrogram and principal component analysis plot in Additional file 1: Fig. S3A and Fig. S3B, respectively, there was relatively close sample replication, with the largest discrimination between leaf and roots, followed by leaf tissue types and then by the leaf tissues in response to drought. In contrast to the leaf, there was comparatively little discrimination between the root samples, for tissue type or treatment. The distribution of DEGs between the tissue types is shown in Additional file 1: Fig. S3C. Of the 37,996 DEGs, 89.5% were expressed in the leaf, of which 84.3% (75.5% of the total) were unique to the leaf and 22.4% were expressed in both leaf tissues. The equivalent figures for the root were 9.3% of the total, of which 42.7% (4% of the total) were unique to the roots and 17.1% were expressed in both root tissues. While the number of unique DEGs was 50 and 59% of those expressed in the leaf base and remaining leaf, respectively, it was only 13% for the root tip and 38% for the remaining root. Full lists of genes with mean normalised reads under well-watered and drought conditions and log$_2$-fold change (LFC) are presented in Additional files 3, 4, 5 and 6, in which they are ordered by differential expression. A gene ontology analysis of biological function is shown in Additional file 1: Fig. S4 – Fig. S7 for the leaf base, remaining leaf, root tip and remaining root, showing the 30 most significant down- and up-regulated processes, respectively, for each tissue type. Cellular organisation and metabolism are strongly represented in the down-regulated functions, while responses to abiotic stimuli and related metabolism, and to ABA are major up-regulated processes.

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Fig. 3 Concentration of selected hormones in the 4th leaf and roots of watered and droughted wheat seedlings after 5 days of water cessation. Concentrations were determined in the leaf base and remaining leaf (**A**) and the root tip and remaining root (**B**). Values are the means of 7 replicates +/− SD. Statistically significant differences between watered and droughted plants are indicated by * ($p < 0.05$); ** ($p < 0.01$) and *** ($p < 0.001$) determined by the Student t-test. *tZ, trans*-zeatin; *tZR, trans*-zeatin riboside; *cZ, cis*-zeatin; *cZR, cis*-zeatin riboside; IAA, indole-3-acetic acid; ABA, abscisic acid; JA, jasmonic acid; JA-Ile, jasmonic acid isoleucine conjugate; SA, salicylic acid
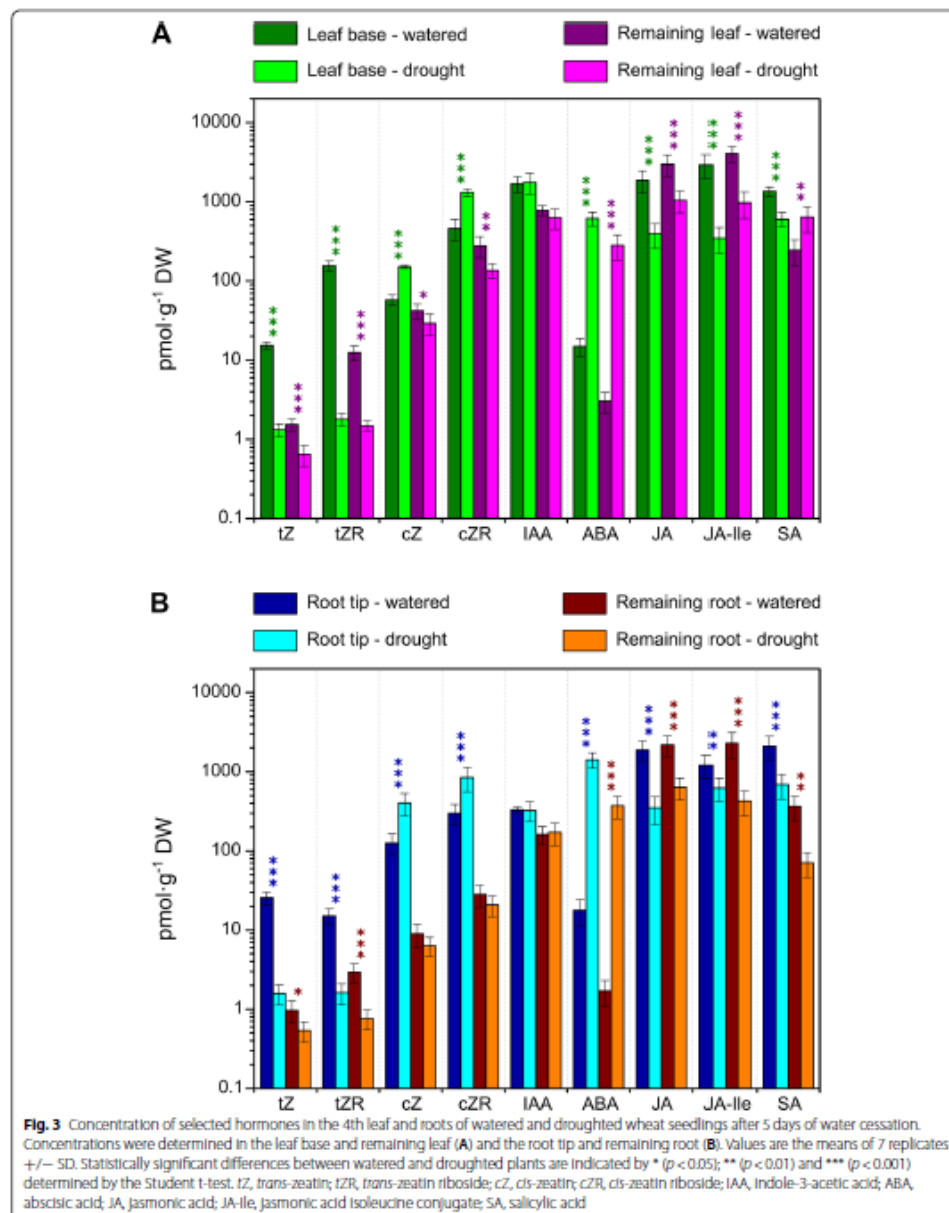
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Table 1 Numbers of differentially expressed genes (log$_2$-fold change between droughted and watered plants > 1 or < 1)

Tissue	Number of differentially expressed genes	
	Up-regulated	Down-regulated
Leaf base	9175	8123
Remaining leaf	9570	14,773
Root tip	1903	793
Remaining root	4103	4892

Annotated genes for hormone metabolism and signalling are listed in Additional file 7, in which their annotation, if not previously published, is based where possible on that of their rice orthologues.

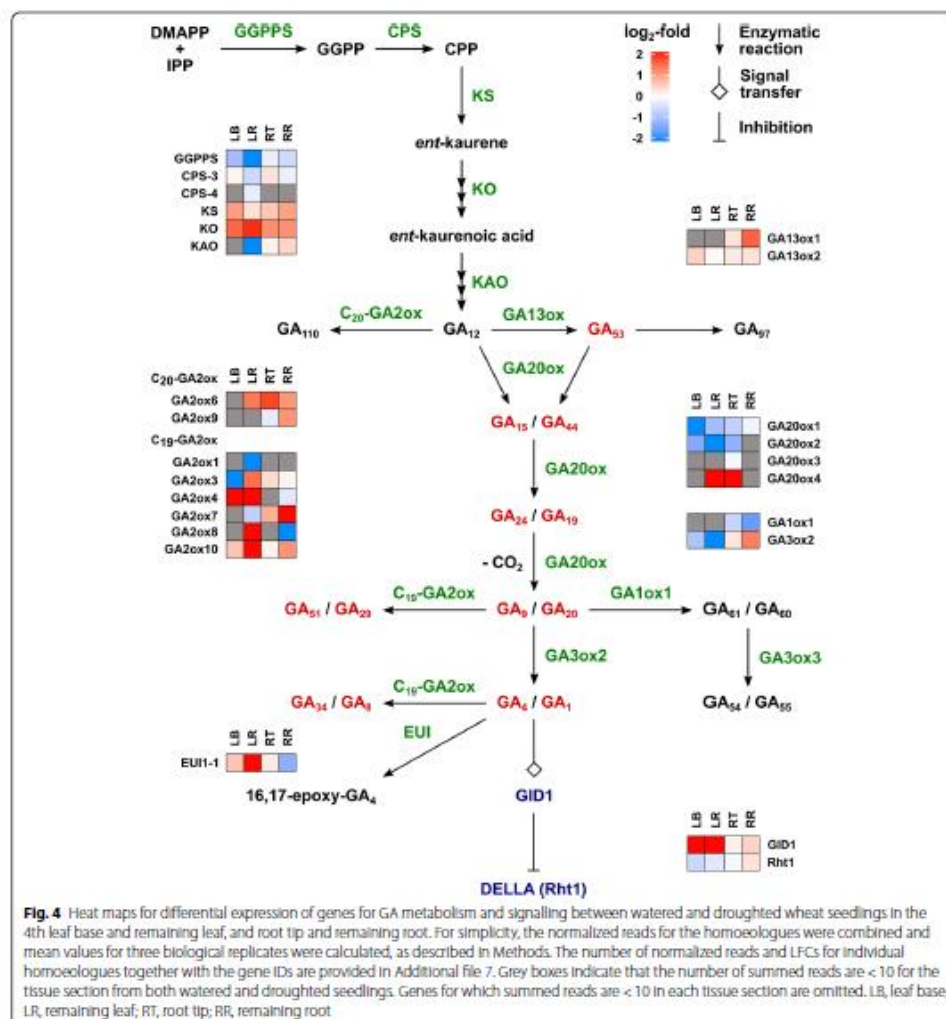### Gibberellin metabolism and signal transduction

The relative expression in response to drought of genes involved in GA metabolism and signalling is shown for each tissue type as heat maps in Fig. 4. In order to simplify the figure, normalised reads for the three homoeologues are summed, while the normalised reads are provided separately for all homoeologues in Additional file 7. The 2-oxoglutarate-dependent dioxygenase genes of GA biosynthesis and inactivation are considered to be major sites of regulation [35]. Transcripts for *TaGA20ox1* and *2* were more abundant in the remaining leaf tissue than in the leaf base and were reduced by drought in both leaf tissues. There was no significant change due to drought in the root tissues. Notably, there was a relatively high expression of *GA20ox4* in the remaining leaf and, for one homoeologue (*TaGA20ox-A4*) only, expression was increased substantially under drought. Roots contained very few reads for this paralogue, except for the A homoeologue in the droughted root tip. In contrast to the *GA20ox* genes, *GA3ox2* transcripts were evenly distributed between the leaf base and remaining leaf and also in the root tissues. Under drought *GA3ox2* expression was reduced in the leaf, but increased in the root, particularly in the remaining root tissue. Both root tissues contain transcripts for *GA1ox1* that encodes a mildly inactivating enzyme and is highly expressed in developing grain [42]. Its expression was reduced under drought.

For the genes encoding enzymes for the early GA-biosynthetic pathway, *CPS* is present as at least four paralogues in wheat, of which *CPS3* and *CPS4* are reported to be involved in GA biosynthesis [49, 50] and are included in Fig. 4 and Additional file 7. Expression of neither gene is affected by drought in any tissue type. The A homoeologue of *KS* was up-regulated in response to drought in all tissues, while *KO* was up-regulated by drought in the leaf tissues but to a less extent in the roots. *KAO* expression in the leaf was mainly in the remaining leaf tissue,

in which it was down-regulated by drought, while it was not differentially regulated by drought in the roots. Transcripts of *trans*-geranylgeranyl diphosphate synthase (*GGPPS*) homoeologues, responsible for diterpene biosynthesis, were reduced by drought in the leaf, particularly in the remaining leaf tissue, but were not affected by drought in the root tissues.

Of the wheat *GA2ox* genes, responsible for GA-turnover by inactivation, the enzymes encoded by *TaGA2ox6* and *TaGA2ox9* act mainly on C$_{20}$-GAs [42], while *TaGA2ox11*, which is orthologous to the rice *GA2ox11*, belongs to the same clade and is assumed also to encode a C$_{20}$-GA2ox [51]. The genes annotated as *TaGA2ox11*, *12* and *13* in Pearce et al. [42] are close homologues of *TaGA2ox6* and are renamed as *TaGA2ox6-2, 6-3* and *6-4*, respectively (Additional file 7). The remaining *TaGA2ox* genes encode enzymes that act mainly on C$_{19}$-GAs [42]. No reads were present for *TaGA2ox2, TaGA2ox6-4* and *TaGA2ox11* in any of the sampled tissues, while expression of *TaGA2ox1, 6-1, 6-2, 6-3, 8* and *9* was low in all tissues. In both sections of the droughted fourth leaf, there was strong up-regulation of *TaGA2ox4*, with *TaGA2ox3* and *TaGA2ox10* also up-regulated in the remaining leaf, but not in the leaf base. Indeed, *TaGA2ox3* transcripts, which were more abundant in the base of the sheath, were present at lower levels in this tissue in response to drought. In contrast, expression of *TaGA2ox4* under well-watered conditions was higher in the remaining leaf, while water restriction caused strong up-regulation in both tissues, particularly in the leaf base. Expression of *TaGA2ox7* was mainly confined to the remaining leaf, in which two of its homoeologues were down-regulated under drought, whereas *TaGA2ox10* was expressed more highly in the leaf base and was the most highly expressed *GA2ox* gene in this tissue under well-watered conditions. Under drought, expression of *TaGA2ox10* increased in the remaining leaf, but only expression of the D homoeologue increased in the leaf base. In the roots, *TaGA2ox3* was by far the most highly expressed *GA2ox* gene and its expression in both tissue types was not affected by drought.

Genes encoding the GA-receptor GID1 and the DELLA protein RHT-1 were highly expressed in all four tissues. Notably, expression of *GID1* was strongly promoted under drought in the leaf tissues, but not in the roots, while *RHT-1* was not differentially expressed under drought in any tissue. Transcripts for genes encoding the F-box protein GID2, a component of the E3-ligase responsible for RHT-1 degradation [52, 53], are not included in the analysis. *GID2* exists in wheat as two paralogues, of which the closest orthologue to the rice gene (TraesCS3A01G055700 and TraesCS3B01G068100) is present in the intron of another gene and its expression

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Fig. 4 Heat maps for differential expression of genes for GA metabolism and signalling between watered and droughted wheat seedlings in the 4th leaf base and remaining leaf, and root tip and remaining root. For simplicity, the normalized reads for the homoeologues were combined and mean values for three biological replicates were calculated, as described in Methods. The number of normalized reads and LFCs for individual homoeologues together with the gene IDs are provided in Additional file 7. Grey boxes indicate that the number of summed reads are < 10 for the tissue section from both watered and droughted seedlings. Genes for which summed reads are < 10 in each tissue section are omitted. LB, leaf base; LR, remaining leaf; RT, root tip; RR, remaining root

could not be determined accurately. The function of its paralogues (*GID2-like*: TraesCS3A01G056000, TraesCS3A01G511800, TraesCS3B01G068800 and TraesCS3D01G056100) is unclear.

In a separate experiment, differential expression in the four tissue types in response to water restriction was determined by qRT-PCR for three *GA2ox* genes (*GA2ox3*, *GA2ox4* and *GA2ox10*), the biosynthetic gene *GA3ox2* as well as *GID1* and the ABA signalling gene *PP2C1*, which is strongly up-regulated by drought (see below). The results from qRT-PCR, for which primers were designed to amplify all three homoeologues, were generally consistent with the differential expression determined by RNA-seq (Additional file 1: Fig. S8).

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Metabolism and signalling for other hormones

Metabolism and signalling genes for the other analysed hormones were curated for wheat and their normalised reads are provided for the four tissue types under well-watered and droughted conditions in Additional file 7, with their differential expression displayed as heat maps in Figs. 5, 6, 7 and 8. Although levels of ethylene,

brassinosteroids and strigolactones were not determined, for completeness expression data for their pathway genes are included in Additional file 7.

Differential expression of genes involved in ABA metabolism and signalling is displayed in Fig. 5. Two of four *NCED* genes encoding 9-*cis*-epoxycarotenoid dioxygenases were up-regulated in all tissues by drought, while

Fig. 5 Heat maps for differential expression of genes for ABA metabolism and signalling between watered and droughted wheat seedlings in the 4th leaf base and remaining leaf, and root tip and remaining root. See the legend for Fig. 4 for details

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Fig. 6 Heat maps for differential expression of genes for cytokinin metabolism and signalling between watered and droughted wheat seedlings in the 4th leaf base and remaining leaf, and root tip and remaining root. See the legend for Fig. 4 for details

NCED-3A was up-regulated in the remaining leaf and remaining root. Of the other ABA-biosynthetic genes, two *AAO3* genes encoding abscisic aldehyde oxidase were up-regulated by drought only in the roots. *ABAox1* and *ABAox2* encoding CYP707A (ABA 8′-hydroxylases) that catalyze ABA catabolism were up-regulated by drought in the leaf, but not significantly in the root. Genes encoding the ABA signalling components A-type 2C protein phosphatases (PP2CAs) were strongly up-regulated by drought in all tissues as were two *TaSAPK* genes encoding stress/ABA-activated protein kinase that acts downstream of PP2CA. Several of the *PYL* (ABA receptor) genes were down-regulated by drought, while

two subfamily I *PYL* genes, *PYL11* and *PYL12*, were up-regulated, particularly in the leaf.

For the other hormones, differential gene expression in response to drought was more evident in the leaf tissues than in the roots, even though the levels of these hormones responded similarly to drought in both organs. The data for CKs are present in Fig. 6. Transcripts for the isopentenyltransferase genes *IPT3 – IPT8*, thought to be responsible for *tZ* biosynthesis [54], were present at very low levels in the leaf, with the most abundant, *IPT8*, down-regulated by drought. Although biosynthesis of *cZ* is not fully understood, there is evidence for the involvement of tRNA-IPTs [55]. These are encoded by *IPT9* and *10*, which were expressed at relatively high levels in

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Fig. 7 Heat maps for differential expression of genes for auxin (**A**) metabolism and salicylic acid (**B**) signalling between watered and droughted wheat seedlings in the 4th leaf base and remaining leaf, and root tip and remaining root. See the legend for Fig. 4 for details
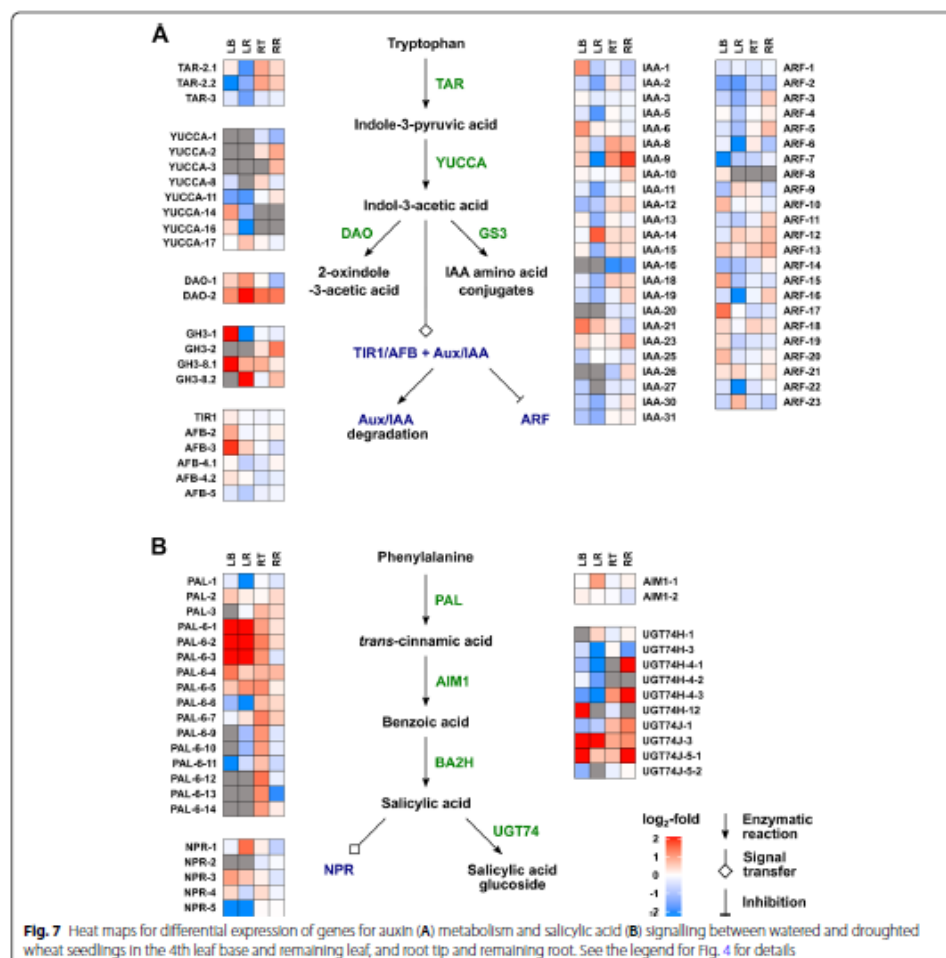
the leaf tissues, with *IPT10* down-regulated by drought. The isopentenyladenine hydroxylase (CYP735A) genes were not expressed in the leaf, while a number of LOGL genes, encoding CK riboside 5′-monophosphate phosphoribohydrolases [56], were expressed in the leaf, with several down-regulated by drought. Expression of CK dehydrogenase genes (*TaCKX*), responsible for CK catabolism, was modified by drought in the leaf tissues, either negatively or positively, but not in the roots. Type A CK response regulator genes were generally

down-regulated by drought in the leaf. With the exception of *TaCOGT-D1.1*, which was strongly up-regulated in the remaining leaf, expression of *O*-glucosyltransferase genes were generally down-regulated by drought in the leaf or unchanged in the root tissues. The IAA-biosynthetic genes *TaTAR2.1* and *TaTAR3.1*, encoding tryptophan aminotransferases and *TaYUCCA11*, encoding a flavin-containing monooxygenase, were slightly down-regulated by drought in the remaining leaf as were AUX/IAA- and ARF-encoding genes involved in auxin-signal

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Fig. 8 Heat maps for differential expression of genes for jasmonate metabolism and signalling between watered and droughted wheat seedlings in the 4th leaf base and remaining leaf, and root tip and remaining root. See the legend for Fig. 4 for details
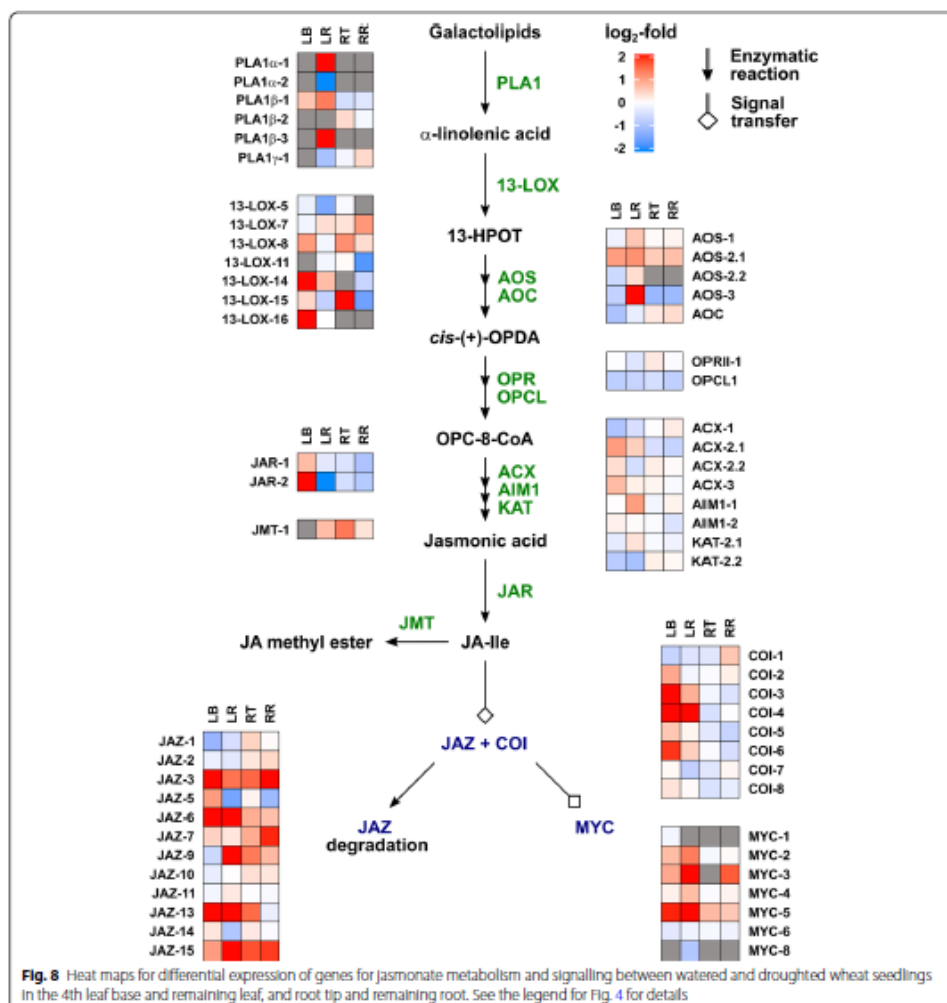
transduction (Fig. 7A), consistent with altered IAA signalling in the droughted leaf. Several *PAL* genes, encoding phenylalanine ammonia-lyase, potentially involved in SA biosynthesis, were differentially regulated under drought in the leaf with smaller changes in the root (Fig. 7B). Notably, a UDP-glucosyltransferase gene *UGT74J3* was strongly up-regulated by drought, while the more highly expressed *UGT74H* genes were down-regulated in the remaining leaf. In rice, these glucosyltransferases have been shown to conjugate SA and regulate its concentration [57, 58]. While there was little uniform change in expression of jasmonate biosynthetic genes in response to drought, several genes encoding COI receptors, the JAZ signalling component and MYC transcription factors were up-regulated by drought in the leaf tissues (Fig. 8).

Discussion

Redistribution of growth between shoots and roots is a common consequence of water restriction [59, 60]; reduced shoot growth moderates water use, while promoted or maintained root elongation maximizes exploitation of the available water. Due to the importance of GAs in regulating growth, this class of hormone is a prime candidate in this growth redistribution, particularly since shoots are much more sensitive to changes in GA concentration than are roots [43]. After five days of progressive water restriction, growth of the elongating third and fourth leaves of the wheat seedlings was significantly reduced, while there was no significant difference in root length (Fig. 1). The growth reduction of the fourth leaf corresponded with substantial decreases in the concentrations of the bioactive GA_1 and GA_4 in the base of the elongating leaf (Fig. 2), while there was no change in the remaining leaf tissue, containing the upper leaf sheath and blade. In the root tip, containing the growing region there were small but significant increases in the concentrations of these GAs consistent with the maintained root growth under water restriction. In order to understand the mechanisms for the drought-induced changes in GA content, RNA-seq was used to determine differential gene expression under drought for the four tissue types. Abiotic stress has been shown to induce increased expression of GA2ox genes [13] with evidence from work with Arabidopsis and tomato that this is at least partly responsible for the stress-induced physiological response [33, 36, 40, 41, 61]. In both sections of the droughted fourth leaf, there was strong up-regulation of TaGA2ox4, with TaGA2ox3 and TaGA2ox10 also up-regulated in the remaining leaf, but not in the lower leaf sheath. Indeed, TaGA2ox3 transcript was present at lower levels in the leaf base in response to drought, potentially in response to the reduced GA content through the homeostasis mechanism, suggesting its expression is not directly regulated by water stress. Potentially through the same mechanism, there was strong drought-induced up-regulation of the GA-receptor gene GID1 in the leaf tissues, although a response to reduced GA signaling is less clear for the remaining leaf in which the GA content was unchanged. More detailed information on the distribution of GA and gene expression along the leaf may be required to understand the relationship between gene expression and GA content. In the root, TaGA2ox3 was the most-highly expressed GA2ox gene, but was not differentially expressed under drought, as was no other GA2ox gene. There are recent reports for maize and rice of up-regulation of GA2ox genes in shoots and down-regulation of these genes in roots in response to osmotic stress, indicating tissue-specific differential regulation of these genes [62, 63].

Despite the up-regulation of GA2ox genes in the leaf, the concentrations of GA catabolites and precursors are not consistent with higher rates of 2-oxidation as the major cause of the reduction in bioactive GA levels in the leaf base under drought. Indeed, the levels of the 2β-hydroxyGAs, GA_8, GA_{34}, GA_{29} and GA_{51} were also reduced. The concentrations of precursor GAs in both the 13-hydroxy and 13-deoxy pathways to GA_1 and GA_4, respectively, were also reduced suggesting a general reduction in GA biosynthesis. Transcripts levels for the biosynthesis genes GGPPS1, KAO and GA3ox2 were reduced by water restriction in the remaining leaf section, but less so in the base of the leaf sheath, in which nevertheless GA levels were reduced. Overall, under well-watered conditions transcripts for most biosynthetic genes were more abundant in the remaining leaf than in the leaf base, despite the latter containing higher concentrations of bioactive GA_1 and GA_4 as is predictable for the elongation zone. The exception is the GA3ox2 transcript, which was equally distributed between the two leaf sections. These results are in agreement with those in [64], in which the distribution of GAs and expression of GA-biosynthesis genes were determined along a growing maize leaf. They showed that bioactive GAs and GA3ox expression peaked at the boundary between the division and cell elongation zones, while precursors and transcripts encoding enzymes for earlier biosynthetic steps were abundant in the more mature part of the leaf. Most strikingly, transcript reads for KAO were very low in the leaf base, as was the case also for the maize leaf [64]. The data would suggest that the early stages of biosynthesis occur predominantly in the remaining leaf, containing the leaf blade, with GAs and/or precursors transported to the base. The leaf blade may experience more stress under drought, although this did not translate into reduced GA content in the remaining leaf. However, this material is heterogeneous and it will be necessary to determine the effect of drought on GA distribution.

Growth and its response to water limitation are regulated by the combined activity of multiple hormones [47, 48], so we took the opportunity to measure the concentrations of ABA, CKs, IAA, JA, SA and associated metabolites (Fig. 3 and Additional file 2) and to determine the effect of drought on expression of genes involved in their metabolism and signaling pathways (Figs. 5, 6, 7 and 8 and Additional file 7). In contrast to GAs, ABA levels were strongly increased by water restrictions in both leaf and root samples, accompanied by up-regulation of genes encoding NCED and ABA8ox metabolism enzymes and the PP2C signaling components, as has been previously reported [65–70]. Several of the PYL (ABA receptor) genes were down-regulated by drought, as has been shown for the subfamily II PYLs (PYL4, PYL5 and PYL6)

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in rice [71]. These genes are also down-regulated by ABA, consistent with their involvement in ABA homeostasis [72]. Two subfamily I *PYL* genes, *PYL11* and *PYL12* were up-regulated, particularly in the leaf, as reported by [71].

The levels of the other analysed hormones responded similarly to drought in the leaf and roots, although expression of the genes monitored for their metabolism and signal transduction changed much more in the leaf tissues than in the roots. The very substantial reduction in the levels of *tZ* and its riboside in the leaf sheath base and root tip in response to water restriction is consistent with reduced leaf growth and maintained root elongation, which is inhibited by CK signalling [73, 74]. The reduced *tZ* and its riboside was accompanied by increased levels of their *O*-glucosides suggesting enhanced conjugation, although, with the exception of *TaCOGT-D1.1*, there was limited indication of increased expression of *O*-glucosyltransferase genes. Of the *IPT* genes thought to be responsible for *tZ* biosynthesis [54], only *IPT8* was expressed in the leaf and was down-regulated by drought. The reduced expression of *CKX* under drought is in agreement with previous reports [75], but was observed only in the leaf. The lack of a clear correlation between changes in CK levels and expression of metabolism genes in rice in response to drought was noted in [76], in which it was suggested that drought affects CK distribution and degradation. In contrast to those of *tZ* and its riboside, *cZ* and *cZR* levels increased in the leaf base and root tip under drought. *cZ* is thought have a role in regulating growth under stress [77, 78], and its increase is consistent with a stress response, although this is not correlated with growth restriction in the root. Unexpectedly, there was no change in IAA content in the leaf or root in response to drought, despite previous reports of reduced IAA under such conditions, for example [76]. However, higher levels of 2-oxindole-3-acetic acid in all tissues and of IAA-glutamate in the leaf base indicate increased IAA turnover. Furthermore, RNA-seq revealed down-regulation of genes encoding Aux/IAA and ARF proteins, consistent with altered IAA signal transduction in the droughted leaf. A role for jasmonate in drought responses is well documented [79–81], with reports of water restriction resulting in increased jasmonate levels [82]. However, in the current experiment, the levels of JA and JA-isoleucine were lower in the droughted organs, although expression of several *JAZ*, *COI* and *MYC* genes encoding components of JA signal transduction was up-regulated in the droughted leaf. SA levels were also reduced by drought in the leaf base and root tip, although they were higher in the remaining leaf, suggesting a redistribution under drought. Munoz-Espinoza et al. [80] reported transient increases in the levels of SA and JA in roots and shoots of tomato plants under water restriction, while ABA levels increased progressively. Their results indicated that ABA reduced the production of SA and JA as the drought progressed, resulting in lower levels of these hormones in prolonged drought, as found in the current study.

Summary and conclusions

Growth restriction in an elongating leaf as a result of progressive drought was associated with a reduction in bioactive GA concentration in the leaf base, which contains the growing zone of the sheath, while small increases in bioactive GAs in the root tip under drought were consistent with maintained root elongation. These changes were not evident in the remaining leaf and root and were thus associated specifically with the growth of these organs. While drought resulted in up-regulation of *GA2ox* gene expression in the leaf, in particular of *TaGA2ox4*, quantitative analysis of GA catabolites and biosynthetic intermediates indicated that reduced biosynthesis rather than increased inactivation explained the reduction in GA content. Genes encoding several GA-biosynthetic enzymes were down-regulated by drought in the leaf, but not in the root. These changes occurred throughout the leaf suggesting that the distribution of GAs and precursors may be an important factor in the response to the stress. ABA and transcripts for *NCED* biosynthesis genes and genes encoding type-A PP2C signalling components were substantially increased in all tissues by drought indicating that the stress was perceived throughout the plant, although overall differentially expressed genes were more abundant in the leaf tissues compared with roots. The concentrations of *tZ* and its riboside were higher in the leaf base and root tip than in the remaining organs and were very highly attenuated under drought, with increased concentrations of their *O*-glucosides. In contrast, *cZ* and *cZR* concentrations increased in the leaf base and root tip under drought, in support of their suggested role in stress responses, whereas the *trans* isomers are associated with growth. The concentration of IAA was unchanged, while those of JAs and SA were reduced under drought for most tissues, potentially due to the prolonged and progressive nature of the stress. For most of the hormones investigated it was not possible to correlate the changes in hormone abundance with expression of metabolism genes, and more detailed knowledge of their distribution and its drought-induced changes will be required.

Methods

Plant growth conditions and sampling

Seeds of *Triticum aestivum* cv. Cadenza were imbibed on wet filter paper at 4°C for 2 d in the dark and then

germinated at room temperature for a further 2 d. Two uniformly germinated seedlings were planted in pots measuring 13x13x12 cm containing 500 g of dried field soil (loamy sand: 88% sand, 5% silt, 7% clay) and 1.5 g of Osmocote® fertilizer (Sierra Chemical), which had been allowed to take up water to full capacity (0.26 g tap water per 1 g of soil). One of the seedlings was removed after 4-5 d. The soil water content was maintained at full capacity for 7-8 days until the 3rd leaf was visible, after which watering of the droughted group was discontinued, while the control group was watered daily as before. The plants were grown in a controlled environment cabinet at 22/18 °C and 70/80% relative humidity (day/night) with a 14-h photoperiod and light flux of 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The length of each visible leaf was recorded daily. After 5 days of drought, the 4th leaf from both sets of plants was harvested after removing the outer leaves and dissected into the bottom 3-cm of the sheath and the remaining leaf (Additional file 1; Fig. S1), which were frozen in liquid N_2. The roots were washed briefly in water to remove soil, dried on a paper towel and the length of the longest root measured. 3-cm of the tips of the major roots (seminal and nodal) were dissected (Additional file 1; Fig. S1) and frozen in liquid N_2, as were the remaining roots.

Evaluation of stress

The relative water content was measured on the 3rd leaf using the protocol in [83]. Free proline content was determined according to [84] with slight modifications. Homogenized leaves (100 mg) were incubated in 3 ml of 3% sulphosalicylic acid at 96 °C for 10 minutes. Samples were clarified by centrifugation and 1 ml of supernatant was mixed with 2 ml of 50% acetic acid, 2 ml of 2.5% acidic ninhydrin solution and boiled for another 30 min. The reaction product was liquid-liquid extracted by 5 ml of toluene and the absorbance of the toluene fraction was measured at 520 nm. The concentration of proline was determined using a standard curve (0-30 µg) and expressed as $\mu g \cdot mg^{-1}$ of protein. Estimation of protein concentration was according to [85] by spectrophotometric measurement of absorbance of PBS (100 mM, pH 7.8) buffered leaf extracts at 260 and 280 nm. The level of lipid peroxidation was determined by measuring the MDA concentration as described in [86]. Gas exchange measurements were made on the 2nd leaf using the LI-COR 6400-XT infrared gas analyzer with attached leaf chamber LI6400-40 (Li-COR, Biosciences). The measurements were performed with a CO_2 reference concentration of 400 $\mu mol \cdot mol^{-1}$, an air flow of 200 $\mu mol \cdot s^{-1}$, block temperature of 20 °C, photosynthetic photon flux density of 1800 $\mu mols \cdot m^{-2} \cdot s^{-1}$ and relative humidity between 55 and 65%.

Hormone quantitative analysis

For analysis of GAs, dissected leaf and root sections from watered and droughted plants (harvested as described above) were combined from 3 plants for each of 3 biological replicates for each tissue type and treatment and then frozen in liquid N_2. The samples were freeze-dried, ground to a powder, from which 10 mg was extracted in the presence of 3 pmol $^2H_2$-labelled GA internal standards (OlchemIm s.r.o., Olomouc, Czech Republic), purified and analysed by UHPLC-MS-MS as described in [87] using a Xevo TQ-XS triple quadrupole mass spectrometer (Waters Milford, MA, USA). For the global hormone analysis, samples were prepared in the same way with 7 biological replicates and 3 mg sample extracted, purified and analysed as described in [88].

Analysis of gene expression by qRT-PCR

Total RNA was extracted from three biological replicates of plant tissues using E.Z.N.A.® Plant RNA kit (Omega Bio-Tek) and treated with RNase-free DNase I (Promega). cDNAs were synthesized by RevertAid H Minus reverse transcriptase using oligo (dT)18 primers (Thermo Fisher Scientific). The subsequent qRT-PCR analysis of each cDNA was performed in three technical replicates on the CFX96 Real-Time System with a C1000 thermal cycler (Bio-Rad). The reaction mixture contained 400 nM of each primer (Additional file 1: Table S2) and Xceed qPCR SYBR Green I mix (Institute of Applied Biotechnologies, CZ; discontinued). PCR efficiencies of primer pairs were determined using a dilution series of cDNA and standard curve method. The wheat α-tubulin gene (*TaTUBa*, GenBank accession number U76558) and a gene with GenBank accession number CJ705892 [89] were used as reference genes. Normalization of relative gene expression was performed with respect to primer amplification efficiency and the internal control genes as described by [90], without taking into account the error propagation from amplification efficiency determination.

RNA sequencing and differential gene expression analysis

Total RNA samples from the four sample groups were isolated using the Monarch Total RNA Miniprep Kit (New England Biolabs Inc., Ipswich, MA, USA) according to the manufacturer's protocol. The RNA quantity and integrity were measured with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples with RNA integrity number (RIN) higher than 6.3 were used in the RNA sequencing (RNA-seq) which was performed at Novogene Co. Ltd. (Beijing, China) on the Illumina NovaSeq 6000 platform with a paired-end 150 bp sequencing strategy. The raw data were further processed, and low-quality bases were removed using Trimmomatic-0.39 [91]. This included Illumina

adapter sequences, bases with Qscore < 13 and reads shorter than 36 bases. Cleaned reads were mapped to the reference genome of *T. aestivum* cv. Chinese Spring, IWGSC RefSeq1.1[1] using HISAT2-2.1.0 [92] with default parameters. Reads count matrix was generated using only uniquely mapped reads with Rsubread-2.0.1 (featureCounts function) [93]. Differential gene expression analysis was performed using DESeq2 software [94]. LFC estimates were shrunken using the ashr method within the lfcShrink function. Only genes with FDR-adjusted *p* values < 0.05 (Benjamin-Hochberg procedure) and LFC of ≥ 1 or ≤ -1 (2-fold change) were considered as differentially expressed. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) was performed after regularized-logarithm transformation (rlog) of count data. For the heat maps in Figs. 4, 5, 6, 7 and 8 the normalized reads for the homoeologues were combined and mean values for three biological replicates were calculated. The LFC was calculated using the formula: $LFC = \log_2(B) - \log_2(A)$, where A and B are the means of summed reads for watered and droughted samples, respectively. The number of normalized reads and LFCs for individual homeologues together with the gene IDs are provided in Additional file 7. The heat maps were generated using pheatmap-1.0.12 [95] R package and the pathways were drawn using Inkscape 1.1. The heat map in Additional file 1 : Fig. S3A was generated using ComplexHeatmap-2.2.0 R package [96]. Volcano plots were generated using the EnhancedVolcano R package [97].

Gene ontology (GO) enrichment analysis

The functional enrichment analysis was performed using g:Profiler and gprofiler2 R package (version e104_eg51_p15_3922dba) [98] with FDR < 0.05 and LFC of ≥ 1 or ≤ -1 for up-regulated and down-regulated genes, respectively.

Abbreviations

ABA: abscisic acid; CK: cytokinin; CKX: cytokinin dehydrogenase; CPS: *ent*-copalyl diphosphate synthase; DEG: differentially expressed gene; GA: gibberellin; GA20ox: GA 20-oxidase; GA2ox: GA 2-oxidase; GA3ox: GA 3-oxidase; GGPPS: *trans*-geranylgeranyl diphosphate synthase; GID: GIBBERELLIN INSENSITIVE DWARF; GO: gene ontology; IAA: indole-3-acetic acid; JA: jasmonate; KAO: *ent*-kaurenoic acid oxidase; KO: *ent*-kaurene oxidase; KS: *ent*-kaurene synthase; LFC: log₂-fold change; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; SA: salicylic acid; *cZ*: *cis*-zeatin; cZR: *cis*-zeatin riboside; *tZ*: *trans*-zeatin; tZR: *trans*-zeatin riboside; UHPLC-MS-MS: ultra-high performance liquid chromatography tandem mass spectrometry.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-022-03667-w.

Additional file 1: Table S1. Effect of water restriction on relative water content, MDA and proline concentration, photosynthetic rate and gas exchange. **Table S2.** Sequences of primers used for qRT-PCR analysis. **Figure S1.** Schematic diagram of a wheat seedling, indicating the tissue sections harvested for analysis. **Figure S2.** Volcano plots of differentially expressed genes in each tissue type. **Figure S3.** Hierarchical clustering heatmap, principal component analysis and tissue distribution of differentially expressed genes. **Figure S4-Figure S7.** Gene ontology analysis for biological function of differentially-regulated genes in the leaf base (Fig. S4), remaining leaf (Fig. S5), root tip (Fig. S6) and remaining root (Fig. S7). **Figure S8.** Comparison of qRT-PCR and RNA-seq for determination of differential expression of selected genes between watered and droughted plants.

Additional file 2. Concentrations of hormones (ABA, CKs, IAA, JAs, SA acid and related metabolites in each tissue type in watered and droughted wheat seedlings.

Additional file 3. Lists of mapped genes from RNA-seq for the 4th leaf base (File 3).

Additional file 4. Lists of mapped genes from RNA-seq for the 4th remaining leaf (File 4).

Additional file 5. Lists of mapped genes from RNA-seq for the 4th root tip (File 5).

Additional file 6. Lists of mapped genes from RNA-seq for and remaining root (File 6) in order of differential expression.

Additional file 7. Normalised reads for wheat hormone metabolism and signalling genes from RNA-seq for each tissue type under watered or droughted conditions.

Authors' contributions
PH, PJ, ALP, KP, MS and SGT designed the experiments. KP performed the drought experiments and prepared samples for RNA-seq. ALP and MS analysed the RNA-seq data. PJ performed qRT-PCR. PJ, ALP and MS assembled the gene lists for display of RNA-seq data. ON, IvaP, IvanP, KP and DT performed the hormone analysis. PH, PJ, ALP, KP, MS, DT and SGT drafted the manuscript and prepared the figures, tables and data files. The authors read and approved the final manuscript.

Availability of data and materials
The datasets supporting the conclusions in this article are included in the article and its additional files.

Declarations

Ethics approval and consent to participate
Not applicable. No approval was required for the use of the plant material. The study complies with all relevant local and national regulations.

Consent for publication
Not applicable.

Competing interests

The authors declare that they have no competing interests.

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ARTICLE 9

The determination of 22 natural brassinosteroids in a minute sample of plant tissue by UHPLC–ESI–MS/MS

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Contribution: design and performance of method development, preparation of figures, literature research, manuscript writing

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ARTICLE 10

Immunoaffinity chromatography combined with tandem mass spectrometry: a new tool for the selective capture and analysis of brassinosteroid plant hormones

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Contribution: performance of method development, preparation of figures, literature research, manuscript writing
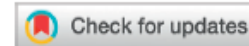
ARTICLE 11

Brassinosteroid biosynthesis is modulated via a transcription factor cascade of COG1, PIF4, and PIF5

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Contribution: analysis of brassinosteroids, manuscript writing

Brassinosteroid Biosynthesis Is Modulated via a Transcription Factor Cascade of COG1, PIF4, and PIF5[1]

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Brassinosteroids (BRs) are essential phytohormones regulating various developmental and physiological processes during normal growth and development. cog1-3D (coguheel1-3D) was identified as an activation-tagged genetic modifier of bri1-5, an intermediate BR receptor mutant in Arabidopsis (Arabidopsis thaliana). COG1 encodes a Dof-type transcription factor found previously to act as a negative regulator of the phytochrome signaling pathway. cog1-3D single mutants show an elongated hypocotyl phenotype under light conditions. A loss-of-function mutant or inducible expression of a dominant negative form of COG1 in the wild type results in an opposite phenotype. A BR profile assay indicated that BR levels are elevated in cog1-3D seedlings. Quantitative reverse transcription-polymerase chain reaction analyses showed that several key BR biosynthetic genes are significantly up-regulated in cog1-3D compared with those of the wild type. Two basic helix-loop-helix transcription factors, PIF4 and PIF5, were found to be transcriptionally up-regulated in cog1-3D. Genetic analysis indicated that PIF4 and PIF5 were required for COG1 to promote BR biosynthesis and hypocotyl elongation. Chromatin immunoprecipitation and electrophoretic mobility shift assays indicated that COG1 binds to the promoter regions of PIF4 and PIF5, and PIF4 and PIF5 bind to the promoter regions of key BR biosynthetic genes, such as DWF4 and BR6ox2, to directly promote their expression. These results demonstrated that COG1 regulates BR biosynthesis via up-regulating the transcription of PIF4 and PIF5.

Brassinosteroids (BRs) are a class of naturally occurring hormone mediating multiple developmental and physiological processes during normal growth and development, such as photomorphogenesis, vascular differentiation, senescence, cell elongation, and cell division and differentiation (Clouse and Sasse, 1998). Within the last four decades, BR biosynthesis, catabolism, and signal transduction pathways have been largely elucidated. It is known that brassinolide (BL), the final product of the BR biosynthetic pathway and the most active BR, can directly interact with the extracellular domain of a Leu-rich repeat receptor-like kinase (LRR-RLK), BRASSINOSTEROID-INSENSITIVE1 (BRI1; Kinoshita et al., 2005; Hothorn et al., 2011; She et al., 2011). BL binding to BRI1 causes the release of BRI1 KINASE INHIBITOR1 from BRI1, allowing BRI1-BL to associate with a second LRR-RLK named BRI1-ASSOCIATED RECEPTOR KINASE1 (BAK1; Li et al., 2002; Nam and Li, 2002; Wang and Chory, 2006; Jaillais et al., 2011). Structure analyses revealed that BL is directly involved in the interaction of the BRI1-BL complex with BAK1, which is consistent with genetic data showing that BAK1 is essential for the early events of the BR signaling pathway (Gou et al., 2012; He et al., 2013; Santiago et al., 2013; Sun et al., 2013). The perception of BL at the cell surface by BRI1/BAK1 initiates a reversible phosphorylation and

dephosphorylation cascade, leading to the dephosphorylation and inactivation of an important negative regulator, BRASSINOSTEROID-INSENSITIVE2 (BIN2), a GSK3-like kinase (Li and Nam, 2002; Mora-García et al., 2004; Kim et al., 2009, 2011).Without early BR signaling, BIN2 phosphorylates BRASSINAZOLE-RESISTANT1 (BZR1) and BRI1-EMS-SUPPRESSOR1 (BES1) to trigger their destruction (Wang et al., 2002b; Yin et al., 2002). Recent studies revealed that BIN2 has additional substrates that modulate downstream BR signaling. One of them is PHYTOCHROME INTERACTING FACTOR4 (PIF4), a basic helix-loop-helix transcription factor mainly regulating cell elongation (Castillon et al., 2007; de Lucas et al., 2008; Oh et al., 2012). BIN2 phosphorylates PIF4, leading this transcriptional regulator to a proteasome-mediated degradation process, which plays a prevalent role in timing hypocotyl elongation to late night (Bernardo-García et al., 2014). When BR signaling is induced, BIN2 is inactivated, and BZR1/BES1 and PIF4 are rapidly dephosphorylated and subsequently move into the nucleus to form a transcription factor complex synergistically activating a common set of BR-regulated genes involved in diverse processes of plant growth and development (He et al., 2002; Wang et al., 2002b; Yin et al., 2002; Sun et al., 2010; Yu et al., 2011; Oh et al., 2012).

The main BR biosynthetic pathway has also largely been elucidated (Zhao and Li, 2012). Campesterol is thought to be the first precursor entering into the specific BR biosynthetic pathway. Campesterol is converted to campestanol through a C-22 oxidation pathway. Campestanol is then converted to castasterone (CS) via an early or a late C-6 oxidation pathway (Suzuki et al., 1994a, 1994b; Fujioka et al., 1995; Choi et al., 1996, 1997). CS is ultimately converted to BL (Yokota et al., 1990; Suzuki et al., 1993). Both CS and BL are active BRs, with CS showing only about 10% of the activity of BL (Kinoshita et al., 2005). Until now, more than 70 BRs have been identified in plants (Fujioka and Yokota, 2003; Bajguz, 2007). Catalytic activities of several BR biosynthetic enzymes, such as DEETIOLATED2 (DET2), CONSTITUTIVE PHOTOMORPHOGENESIS AND DWARFISM (CPD), ROTUNDIFOLIA3 (ROT3), CYP90D1, DWARF4 (DWF4), BRASSINOSTEROID-6-OXIDASE1 (BR6ox1), and BR6ox2, have been elucidated. For example, DET2 catalyzes 5α reduction steps of the early BR biosynthetic pathway (Li et al., 1996; Fujioka et al., 1997, 2002; Noguchi et al., 1999b). CPD is involved in the C-3 oxidation steps of BR biosynthesis (Ohnishi et al., 2012). DWF4 encodes a C-22 hydroxylase that catalyzes the C-22 hydroxylation of multiple BR biosynthesis intermediates (Choe et al., 1998; Fujita et al., 2006). ROT3, also known as CYP90C1, and its homolog CYP90D1 are essential enzymes for the C-23 hydroxylation steps (Ohnishi et al., 2006). BR6ox1 and BR6ox2 catalyze multiple C-6 oxidation reactions connecting the early and late C-6 oxidation pathways. In addition, BR6ox2 participates in a final biosynthetic step, converting CS to BL (Bishop et al., 1999; Shimada et al., 2001; Kim et al., 2005).

Mutants impaired in BR biosynthesis show a severely retarded growth phenotype, whereas application of an excessive amount of BRs also inhibits root growth, suggesting that BR homeostasis is critical for optimal growth and development (Chory et al., 1991; Szekeres et al., 1996; Choe et al., 1998; Clouse and Sasse, 1998). Plants developed several strategies to tightly control endogenous BR levels. For example, the expression of many BR biosynthetic genes is negatively feedback controlled by the BR signaling pathway (Mathur et al., 1998; Bancoş et al., 2002). Activated BZR1 and BES1 can repress the expression of CPD and DWF4 by directly binding to their promoters when BR signaling is strong enough to maintain normal plant growth and development (He et al., 2005; Sun et al., 2010; Yu et al., 2011). Plants also can accelerate the BR biosynthetic rate to quickly produce more BRs when needed for certain developmental stages or environmental conditions. TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR1 (TCP1) and CESTA (CES) can up-regulate the expression of DWF4 and CPD by associating with the conserved motifs in their respective promoters (Guo et al., 2010; Poppenberger et al., 2011; Gao et al., 2015). Auxin or high temperature also can induce DWF4 expression (Chung et al., 2011; Maharjan and Choe, 2011). However, the detailed mechanisms controlling BR biosynthesis by environmental cues are poorly understood.

To identify additional regulators mediating BR biosynthesis, we carried out a large-scale genetic screen in an intermediate mutant of the BR receptor named bri1-5. A number of bri1-5 genetic modifiers have been isolated (Li et al., 2001, 2002; Zhou et al., 2004; Yuan et al., 2007; Guo et al., 2010). One of these mutants is cog1-3D (cogwheel1-3D). COG1 encodes a Dof-type transcription factor that is involved in phytochrome signaling and seed tolerance to deterioration (Park et al., 2003; Bueso et al., 2016). In this report, we provide strong evidence to show that COG1 can directly up-regulate the expression of PIF4 and PIF5 by associating with their promoter regions. PIF4 and PIF5 bind to the promoters of DWF4 and BR6ox2 to directly enhance their expression. Our analyses revealed that PIF4 and PIF5 are key regulators mediating BR biosynthesis, demonstrating that light signaling is critical to BR homeostasis.

RESULTS

cog1-3D Partially Suppresses the Short-Hypocotyl Phenotype of bri1-5

To identify additional components regulating BR signaling or homeostasis, we generated a large-scale activation-tagging pool in a weak bri1 allele, bri1-5, using a pBIB-BASTA-AT2 vector as described previously (Gou and Li, 2012). From about 120,000 transgenic plants, an extragenic suppressor was identified with significantly elongated hypocotyls compared with bri1-5 (Fig. 1, A and B). Scanning electron microscopy analysis indicated that the longer hypocotyls of the

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double mutants are caused mainly by elongated cells (Fig. 1, C and D). Genetic analysis indicated that the mutant phenotype is controlled by a dominant gene that cosegregates with the herbicide-resistant gene from the T-DNA of the activation-tagging construct.

To clone the gene responsible for the elongated hypocotyl phenotype, we backcrossed the double mutant with WS2, and the *bri1-5* mutation was segregated out. The single mutants exhibit significantly longer hypocotyls than those of WS2 (Fig. 2A). Thermal asymmetry interlaced PCR was used to amplify the genomic sequences flanking the T-DNA insertion site (Liu and Whittier, 1995). Sequence analysis indicated that the T-DNA was inserted 1,400 bp upstream of the translation initiation codon of *COG1* (At1g29160; Fig. 2B). The suppressor was named *cog1-3D*, as two dominant alleles, *cog1-D* and *cog1-2D*, were reported previously (Park et al., 2003; Bueso et al., 2016). To further determine whether *COG1* is the gene responsible for the longer hypocotyl phenotype, transgenic plants harboring *35S:FLAG-COG1* were generated. Among 50 total transgenic plants obtained, over 90% of them showed elongated hypocotyl phenotypes, similar to that of the *cog1-3D* single mutant (Fig. 2, A and C). Real-time reverse transcription (RT)-PCR analysis confirmed that *COG1* is indeed overexpressed in both the activation-tagged mutants and the *35S:FLAG-COG1* transgenic lines (Fig. 2D). These results confirmed that elevated expression of *COG1* is the main cause of the longer hypocotyl phenotypes of the activation-tagged line.
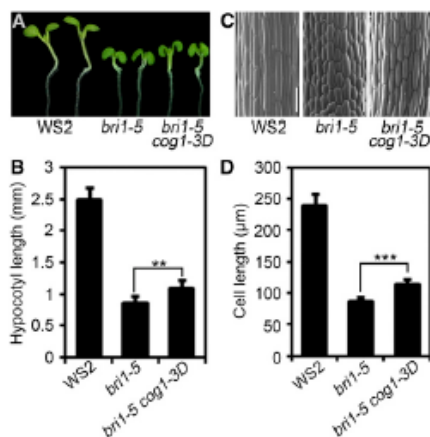

Figure 1. *cog1-3D* partially suppresses the short hypocotyl phenotype of *bri1-5*. A, Phenotypes of WS2, *bri1-5*, and *bri1-5 cog1-3D* seedlings. B, Hypocotyl lengths of the seedlings shown in A. C, Scanning electron micrographs showing that *cog1-3D* partially suppresses the short hypocotyl cell length phenotype of *bri1-5*. Middle parts of the hypocotyls were used for the scanning analyses. Bars = 150 μm. D, Cell lengths of *bri1-5* and *bri1-5 cog1-3D* hypocotyls shown in C.

Loss of Function by Ethyl Methanesulfonate Point Mutation or Inducible Expression of the *COG1-SRDX* Chimeric Gene Results in a Short-Hypocotyl Phenotype

To further determine whether the function of COG1 is required for hypocotyl elongation, loss-of-function mutants of *COG1* were searched from various resources. No T-DNA insertion alleles were identified from available databases. A *cog1-6* mutant was identified as an intragenic suppressor of *cog1-D*, the first activation-tagged allele of *COG1* (Park et al., 2003), from a large-scale ethyl methanesulfonate-mutagenized screen. The *cog1-6* mutant contains a deletion of a single G at position 85, resulting in a premature stop codon (Fig. 2B). The *cog1-6* mutation caused a significant inhibition of the long-hypocotyl phenotype of *cog1-D* and a slightly reduced hypocotyl length compared with that of wild-type seedlings, especially under red light (Fig. 2, E and F; Supplemental Fig. S1).

The subtle developmental phenotypes observed in *cog1-6* plants suggested that homologs of COG1 may play redundant roles with COG1. As a matter of fact, phylogenetic analysis revealed that a number of proteins are closely related to COG1 (Yanagisawa, 2002). Therefore, we employed a dominant negative technology, named chimeric repressor gene silencing, to further examine the roles of COG1 and its homologs. This technology has been used successfully to determine the roles of numerous functionally redundant transcription factors (Hiratsu et al., 2003; Guo et al., 2010; Poppenberger et al., 2011). COG1 was fused with an EAR repressor domain (SRDX), and the chimeric version should effectively repress the expression of the target genes of COG1 and its redundant proteins. Since constitutive expression of *COG1-SRDX* driven by the 35S promoter resulted in a seedling lethality phenotype (Supplemental Fig. S2), we made a dexamethasone (DEX)-inducible *COG1-SRDX* construct (*GVG:COG1-SRDX*) and generated corresponding transgenic plants (Aoyama and Chua, 1997). Homozygous lines for the transgene were selected and examined for the hypocotyl growth phenotype with or without DEX treatment. When 2-d-old seedlings from one-half-strength Murashige and Skoog (MS) medium were transferred to the medium containing DEX and grown for an additional 5 d, the *GVG:COG1-SRDX* transgenic seedlings showed significantly shortened hypocotyls due to reduced cell length (Fig. 2, G and H; Supplemental Fig. S3), which is similar to the *cog1-6* mutant and the *COG1* antisense seedlings (Fig. 2, E and F; Park et al., 2003). Without DEX treatment, the transgenic plants show no defective phenotypes compared with the wild type (Fig. 2, G and H; Supplemental Fig. S3). Nontransgenic plants grown on one-half-strength MS medium with or without DEX treatment did not show any altered phenotypes, confirming that the shortened hypocotyl phenotype was indeed caused by induced expression of *COG1-SRDX*. These results demonstrated that COG1 plays an important role in hypocotyl elongation.
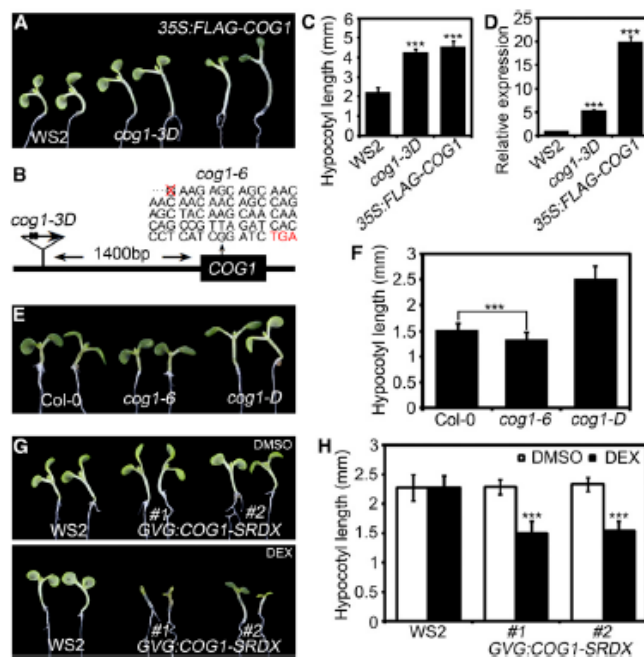
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Figure 2. COG1 plays an important role in regulating hypocotyl elongation. A, Phenotypes of WS2, cog1-3D, and 35S:FLAG-COG1 seedlings. B, In cog1-3D, a T-DNA fragment from the activation-tagging construct pBIB-BASTA-AT2 was inserted at 1,400 bp upstream of the start codon of COG1 (At1g29160). The cog1-6 mutant contains a deletion of a single G at position 85 bp, resulting in a premature stop codon after amino acid 51. C, Hypocotyl measurements of the seedlings shown in A. D, Real-time RT-PCR assay indicated that the expression of COG1 was elevated in both cog1-3D and 35S:FLAG-COG1 seedlings. E, Phenotypes of Columbia-0 (Col-0), cog1-6, and cog1-D seedlings grown under long-day conditions. F, Hypocotyl lengths of the seedlings shown in E. G, Induced expression of a dominant negative chimeric gene, COG1-SRDX, inhibits hypocotyl elongation. Wild-type and GVG:COG1-SRDX transgenic seedlings were germinated on one-half-strength MS medium plates for 2 d and then transferred to one-half-strength MS medium supplemented with mock solution (dimethyl sulfoxide [DMSO]; top) or 10 μM DEX (bottom) for an additional 5 d. H, Hypocotyl lengths of the seedlings shown in G.

The cog1-3D Seedlings Are Insensitive to Exogenously Applied BRs Due to Elevated Endogenous BR Levels

COG1 partially suppressing the short-hypocotyl phenotype of bri1-5 suggests that COG1 is involved in BR-related pathways. To investigate how COG1 regulates BR-related pathways, we first examined the effect of 24-epibrassinolide (24-epiBL) on the hypocotyl growth of cog1-3D. The hypocotyl growth of WS2 is promoted by exogenously applied different concentrations of 24-epiBL ranging from 1 to 1,000 nM (Fig. 3A). The hypocotyl growth of cog1-3D mutants, however, is insensitive to exogenously supplied different concentrations of 24-epiBL (Fig. 3A).

Either blocking the BR signaling pathway or increasing endogenous levels of BRs can result in plant insensitivity to exogenous BRs. The fact that cog1-3D shows an elongated but not a shortened hypocotyl phenotype suggests the BR signaling is at least not blocked in cog1-3D. The phosphorylation status of BES1 was investigated with a specific anti-BES1 antibody in wild-type and cog1-3D seedlings after treatment with or without 1 μM 24-epiBL (Yin et al., 2002). Interestingly, the levels of both phosphorylated and unphosphorylated BES1 in cog1-3D mutants were higher than those of wild-type seedlings without BR treatment (Supplemental Fig. S4A). Upon BR treatment, unphosphorylated BES1 was increased dramatically, and phosphorylated BES1

disappeared in both wild-type and mutant seedlings. The amount of unphosphorylated BES1 in cog1-3D also was greater than that of wild-type seedlings (Supplemental Fig. S4A). Consistently, the expression of a BR signaling marker gene, SAUR-AC1, was increased significantly in cog1-3D plants (Supplemental Fig. S4B). These results confirmed that BR signaling is enhanced in cog1-3D plants.

Next, we investigated whether the endogenous levels of BRs are elevated in cog1-3D. If COG1 promotes BR biosynthesis, the shortened hypocotyl phenotype of COG1-SRDX should result from the reduced BR biosynthesis and the phenotype should be rescued by exogenously applied BRs. Indeed, the shortened hypocotyl phenotype of GVG:COG1-SRDX plants grown on medium containing DEX was rescued by supplying 100 nM 24-epiBL in the medium (Fig. 3B). In addition, blocking BR biosynthesis using a specific inhibitor, brassinazole (BRZ), should shorten the hypocotyls of cog1-3D plants (Asami et al., 2000). Indeed, the cog1-3D seedlings exhibited a hypocotyl length indistinguishable from that of wild-type controls when grown on one-half-strength MS medium agar plates supplied with 1 μM BRZ (Supplemental Fig. S5). These results support the hypothesis that COG1 promotes hypocotyl elongation by increasing endogenous BR levels.

BR profile analysis was performed using WS2 and cog1-3D seedlings. It is apparent that BL, CS, and

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Figure 3. The *cog1-3D* seedlings showed reduced sensitivity to BR due to increased endogenous BR amounts. A, Hypocotyl growth analyses of 7-d-old *cog1-3D* and wild-type seedlings grown on one-half-strength MS agar plates containing different concentrations of 24-epiBL. B, The shortened hypocotyl phenotype of *COG1-SRDX* can be rescued by exogenous 24-epiBL application. WS2 and *GVG:COG1-SRDX* seedlings were grown on one-half-strength MS medium with or without 0.5 μM DEX or with 0.5 μM DEX and 100 nM 24-epiBL for 6 d. C, Endogenous BR levels in *cog1-3D* and wild-type plants. The data shown are averages and sd from three independent replicates. FW, Fresh weight.
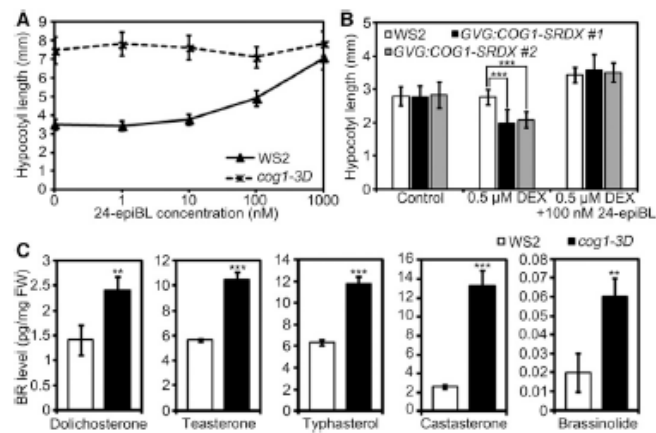
several of their precursors are significantly elevated in *cog1-3D* seedlings (Fig. 3C). For example, dolichosterone, teasterone and typhasterol are increased by approximately 2-fold in *cog1-3D* seedlings compared with the wild type; CS is elevated by about 5-fold in *cog1-3D*; BL is increased by 3-fold in *cog1-3D* seedlings (Fig. 3C). These results suggested that COG1 possibly enhances BR biosynthesis by affecting multiple reactions in the biosynthesis pathway. Alternatively, it is also possible that BR catabolism is decreased in *cog1-3D*.

COG1 Up-Regulates the Expression of BR Biosynthetic Genes

To examine whether BR biosynthesis is enhanced or BR catabolism is decreased in *cog1-3D*, we compared the expression levels of all known key genes involved in both BR biosynthesis and catabolism from *cog1-3D* and wild-type seedlings. The transcript levels of *DET2*, *CYP90D1*, and *BR6ox1* in *cog1-3D* showed no significant differences from those of the wild type (Supplemental Fig. S6), whereas the expression levels of *CPD*, *DWF4*, *ROT3*, and *BR6ox2* are significantly up-regulated in *cog1-3D* and *35S:FLAG-COG1* compared with wild-type seedlings (Fig. 4A). Consistently, inducible expression of *COG1-SRDX* resulted in the down-regulation of these four genes (Fig. 4B). All the catabolism genes are not significantly down-regulated in *cog1-3D* compared with those in wild-type seedlings. Several of the catabolic genes are actually up-regulated in *cog1-3D* (Supplemental Fig. S7). These results suggested that BR accumulation in *cog1-3D* is caused mainly by enhanced BR biosynthesis rather than by decreased BR catabolism.

To further investigate whether COG1 promoting hypocotyl elongation relies on BR biosynthesis, *cog1-3D* plants were crossed with various mutants deficient in BR biosynthesis. The homozygous *det2 cog1-3D*, *cpd*

cog1-3D, *dwf4 cog1-3D*, and *br6ox1 br6ox2 cog1-3D* mutants were obtained and examined for their hypocotyl growth phenotypes. All these double or triple mutants show hypocotyl elongation phenotypes indistinguishable from the corresponding single or double mutants (Fig. 4, C and D). Furthermore, deficiency in BR signal transduction also completely inhibits the hypocotyl elongation of *cog1-3D* (Supplemental Fig. S8). These results clearly indicated that COG1 regulation of hypocotyl elongation depends on BR biosynthesis and signaling.

PIF4 and PIF5 Are Up-Regulated by COG1 and Are Required for COG1-Mediated BR Biosynthesis and Hypocotyl Elongation

Although the expression of *CPD*, *DWF4*, *ROT3*, and *BR6ox2* is transcriptionally regulated by COG1, we failed to detect the direct association of COG1 with their promoters by chromatin immunoprecipitation (ChIP) analyses, indicating that COG1 regulates the expression of these genes possibly via other unknown transcription factors. An RNA sequencing analysis was performed to compare the expression profiles of WS2 and *cog1-3D*. We identified a total of 443 genes displaying statistically significant 2-fold expression changes in *cog1-3D* compared with wild-type seedlings, including 37 transcription factors (Supplemental Table S1). PIF4 and PIF5 are among those 37 transcription factors. PIF4 and PIF5 play a pivotal role in cell elongation by directly elevating the expression of genes involved in cell wall loosening (Huq and Quail, 2002; de Lucas et al., 2008; Oh et al., 2012; Supplemental Fig. S9). To investigate whether PIF4 and PIF5 are involved in COG1-mediated hypocotyl elongation, the expression of *PIF4* and *PIF5* in *cog1-3D* and wild-type plants was confirmed using real-time RT-PCR. Both *PIF4* and *PIF5* are up-regulated in *cog1-3D* as well as *35S:FLAG-COG1*
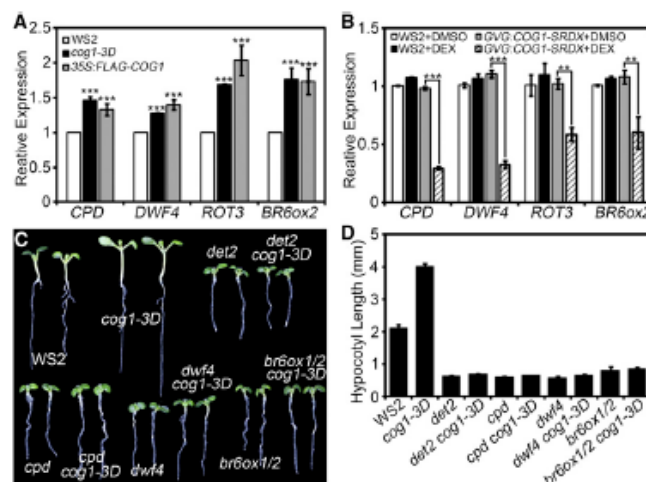
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COG1 Regulates BR Homeostasis

Figure 4. COG1 up-regulates the expression of BR biosynthetic genes, and COG1 regulation of hypocotyl elongation is dependent on BR biosynthesis. A, Expression levels of *CPD, DWF4, ROT3,* and *BR6ox2* in WS2, *cog1-3D,* and *35S: FLAG-COG1* plants were detected by quantitative reverse transcription (qRT)-PCR. B, Expression levels of *CPD, DWF4, ROT3,* and *BR6ox2* in WS2 and *GVG:COG1-SRDX* transgenic plants with or without DEX treatment. WS2 and *GVG:COG1-SRDX* seedlings were treated with mock solution (DMSO) or 10 μM DEX for 24 h before harvest for RNA extraction and qRT-PCR analyses. C, *cog1-3D* showed no or less effect on hypocotyl elongation in various BR biosynthetic mutants. One-week-old seedlings of WS2, *cog1-3D, det2, det2 cog1-3D, cpd, cpd cog1-3D, dwf4, dwf4 cog1-3D, br6ox1 br6ox2,* and *br6ox1 br6ox2 cog1-3D* are shown. D, Hypocotyl lengths of the seedlings shown in C.

seedlings compared with wild-type plants (Fig. 5A). To investigate whether the protein levels of PIF4 and PIF5 also are accumulated, we performed western-blot analysis. Consistently, the protein level of PIF5 is increased significantly in *cog1-3D* and *35S:FLAG-COG1* seedlings (Supplemental Fig. S10). PIF4 levels in *cog1-3D* and *35S:FLAG-COG1* were not examined due to unavailable specific antibody against PIF4. Inducible expression of *COG1-SRDX*, on the other hand, resulted in significantly decreased expression of *PIF4* and *PIF5* (Fig. 5B).

To further determine whether PIF4 and PIF5 are essential for COG1-mediated cell elongation, *cog1-3D* plants were crossed with *pif4* and *pif5* single and *pif4 pif5* double mutants. The *pif4 cog1-3D* and *pif5 cog1-3D* seedlings exhibited partially inhibited hypocotyl elongation compared with the *cog1-3D* single mutant (Fig. 5, C and D), whereas *pif4 pif5 cog1-3D* triple mutant seedlings showed hypocotyl length almost identical to that of the *pif4 pif5* double mutants (Fig. 5, C and D). Plants grown under continuous red light showed similar results (Supplemental Fig. S11). In addition, the expression levels of *CPD, DWF4, ROT3,* and *BR6ox2* in *pif4 pif5 cog1-3D* triple mutants showed no significant differences from *pif4 pif5* double mutants (Fig. 5E). These results suggested that the function of COG1 in regulating BR biosynthesis and hypocotyl elongation is dependent on PIF4 and PIF5.

COG1 Binds Directly to the Promoters of *PIF4* and *PIF5*

Since *PIF4* and *PIF5* are transcriptionally regulated by COG1 in plants and the function of COG1 is dependent on PIF4 and PIF5 (Fig. 5), we hypothesized that COG1 may directly regulate the expression of *PIF4* and

PIF5. To test this hypothesis, COG1 was fused with a hormone-binding domain of a human estrogen receptor (ER) at its C terminus, and transgenic plants harboring the chimeric gene driven by a 35S promoter (*35S: COG1-ER*) were generated. Six-day-old transgenic seedlings were pretreated with 10 μM cycloheximide (CHX) for 2 h to prevent the biosynthesis of new proteins. Estradiol was then added to the liquid culture to induce preexisting COG1 to enter the nucleus. The expression of *PIF4* and *PIF5* was rapidly activated following estradiol induction, demonstrating that COG1 can directly regulate the transcription of *PIF4* and *PIF5* (Fig. 6A). Previous studies demonstrated that Dof-type transcription factors bind to the 5'-A/TAAAG-3' core sequences or their reversibly complementary sequences 5'-CTTTA/T-3' in the promoters of their target genes to regulate gene expression in Arabidopsis (*Arabidopsis thaliana*; Yanagisawa, 2004). Sequence scanning revealed that there are several putative Dof DNA-binding motifs in the promoter regions of *PIF4* and *PIF5* (Fig. 6B). To determine whether COG1 associates with these motifs in the *PIF4* and *PIF5* promoters, *35S:FLAG-COG1* transgenic plants were used for ChIP assays. After immunoprecipitation of protein-DNA complexes from 7-d-old seedlings using an antibody against the FLAG epitope, enriched DNA fragments were amplified by qRT-PCR using primers that annealed near the Dof-binding motifs in the promoters of *PIF4* and *PIF5*. Our results showed that at least one fragment in the *PIF4* or *PIF5* promoter region was indeed enriched in the ChIP assays, suggesting that COG1 associates directly with the promoters of *PIF4* and *PIF5* (Fig. 6C).

To further confirm the direct binding of COG1 to the *PIF4* and *PIF5* promoters, we performed an electrophoretic mobility shift assay (EMSA) using purified GST-COG1 fusion protein from *Escherichia coli*. Our

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Figure 5. PIF4 and PIF5 are essential for COG1 to promote BR biosynthesis and hypocotyl elongation. A, Expression levels of *PIF4* and *PIF5* in WS2, *cog1-3D*, and *35S:FLAG-COG1* seedlings. One-week-old WS2, *cog1-3D*, and *35S:FLAG-COG1* seedlings were harvested for RNA extraction and qRT-PCR analyses. B, Expression levels of *PIF4* and *PIF5* in WS2 and *GVG:COG1-SRDX* transgenic plants with or without DEX treatment. WS2 and *GVG:COG1-SRDX* seedlings were treated with either mock solution (DMSO) or 10 μM DEX for 24 h before harvesting for RNA extraction and qRT-PCR analyses. C and D, Phenotypes and hypocotyl measurements of WS2, *cog1-3D*, *pif4*, *pif4 cog1-3D*, *pif5*, *pif5 cog1-3D*, *pif4 pif5*, and *pif4 pif5 cog1-3D* seedlings. The *pif4 pif5* double mutant was crossed with *cog1-3D* to yield a *pif4 pif5 cog1-3D* triple mutant that was then backcrossed with WS2 for at least five generations. E, qRT-PCR analysis of *CPD*, *DWF4*, *ROT3*, and *BR6ox2* in WS2, *pif4 pif5*, and *pif4 pif5 cog1-3D* seedlings.
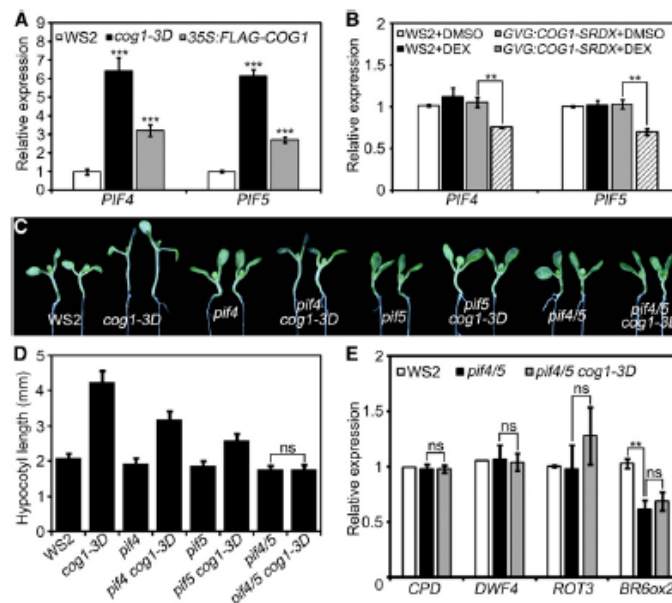
results showed that GST-COG1 bound to the biotin-labeled Dof-binding motif-containing DNA fragments in the promoters of *PIF4* and *PIF5*, as suggested by the ChIP assay (Fig. 6D). Furthermore, the binding could be competed off by adding an excessive amount of unlabeled same DNA fragments. However, the unlabeled DNA probes containing mutated Dof-binding motifs failed to compete off the binding of COG1 to the biotin-labeled wild-type DNA fragments (Fig. 6D). Control experiments indicated that GST protein alone failed to bind to the labeled DNA probes (Fig. 6D). Taken together, we conclude that COG1 can bind directly to the promoter region of *PIF4* and *PIF5* and directly enhance their expression.

PIF4 and PIF5 Directly Regulate the Expression of *DWF4* and *BR6ox2*

Two pieces of evidence suggest that PIF4 and PIF5 might directly regulate the expression of BR biosynthetic genes. First, COG1 regulation of the expression of BR biosynthetic genes is dependent on PIF4 and PIF5 (Fig. 5E). Second, the transcript levels of *BR6ox2* are reduced significantly in *pif4 pif5* double mutants compared with those in the wild type (Fig. 5E). Given the fact that PIFs bind to the G-box motifs in their target gene promoters, we searched for G-box motifs in the promoters of *CPD*, *DWF4*, *ROT3*, and *BR6ox2*. G-box motifs were found in the promoters of *CPD*, *DWF4*, and

BR6ox2 but not in the promoter of *ROT3* (Fig. 7A; Supplemental Fig. S12A). To test whether PIF4 and PIF5 bind to the G-box-containing regions of these three BR biosynthetic genes, we performed ChIP assays using the *35S:PIF4-FLAG* and *35S:PIF5-FLAG* transgenic plants and anti-FLAG antibody. PCR analyses indicated that PIF4-FLAG specifically associates with the G-box-containing promoter regions of *CPD*, *DWF4*, and *BR6ox2* (Fig. 7B; Supplemental Fig. S12B). PIF5-FLAG associates with the G-box-containing promoter region of *DWF4* but not with the promoter regions of *CPD* and *BR6ox2* (Fig. 7B). EMSA experiments using PIF4 and PIF5 proteins expressed in vitro further confirmed that PIF4 directly bound to the G-box-containing DNA fragments in the promoter regions of *DWF4* and *BR6ox2* (Fig. 7, C and D). PIF5 specifically bound to the G-box-containing DNA fragments in the promoter region of *DWF4* (Fig. 7E). The binding could be effectively competed off by the addition of an excessive amount of unlabeled G-box-containing DNA probes (Fig. 7, C–E). As a control, DNA probes containing a mutated G-box motif (CACGGG) failed to compete off the binding of PIF4 or PIF5 to the biotin-labeled DNA fragments (Fig. 7, C–E). Moreover, we examined whether PIF4 and PIF5 regulate the expression of *DWF4* and *BR6ox2* in planta. qRT-PCR analysis showed that the expression of *DWF4* and *BR6ox2* is significantly up-regulated in both *35S:PIF4* and *35S:PIF5* transgenic plants relative to wild-type seedlings (Fig. 7F). PIF4 was taken as an example to further confirm that this regulation is via a

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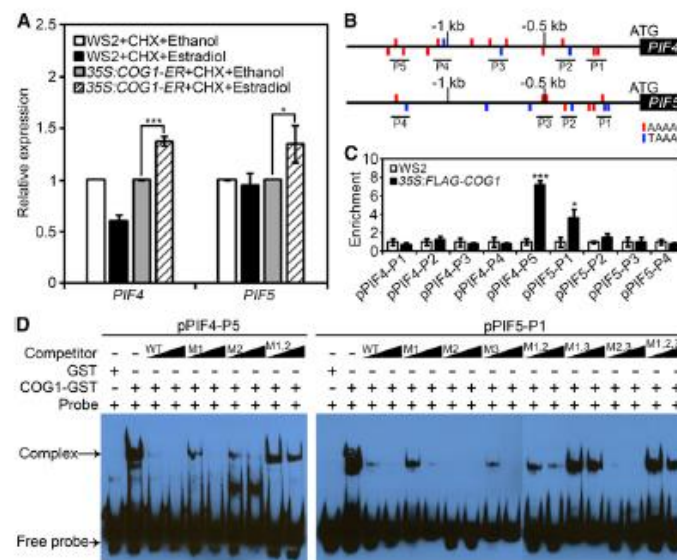
Figure 6. COG1 interacts directly with the promoters of *PIF4* and *PIF5*. A, Expression levels of *PIF4* and *PIF5* in WS2 and *35S:COG1-ER* transgenic plants with or without estradiol treatment in the presence of CHX. WS2 and *35S:COG1-ER* seedlings were pretreated with 10 μM CHX for 2 h, then either mock solution (ethanol) or 10 μM estradiol was added to the liquid solution for another 2 h before harvesting for RNA extraction and qRT-PCR analyses. B, *PIF4* and *PIF5* promoter regions contain multiple Dof motifs (red or blue upright lines). P1 to P5 indicate the DNA fragments amplified in ChIP-PCR assays. C, ChIP-quantitative PCR assays showing that COG1 associates with the promoters of *PIF4* and *PIF5* in vivo. One-week-old WS2 and *35S:FLAG-COG1* seedlings were harvested for ChIP experiments. qRT-PCR was used to quantify enriched DNA fragments in the *PIF4* and *PIF5* promoters. D, EMSA showing that COG1 binds to the Dof motifs in the promoter regions of *PIF4* and *PIF5* in vitro. The enriched DNA fragments in the promoters of *PIF4* and *PIF5* were incubated with GST-COG1 recombinant proteins as indicated. GST proteins were used as negative controls. Competition for COG1 binding was performed with 100× and 200× unlabeled probes containing the enriched DNA fragments (wild type [WT]) or mutated DNA fragments (M).

direct process. Consistently, the expression of *DWF4* and *BR6ox2* can be rapidly up-regulated in *35S:PIF4-ER* transgenic plants upon the treatment with estradiol in the presence of CHX (Fig. 7G). As a result, the final product of the BR biosynthetic pathway, BL, is increased significantly in *35S:PIF4* and *35S:PIF5* transgenic plants (Fig. 7H). Together, these results corroborated that PIF4 and PIF5 redundantly and directly regulate the expression of *DWF4* and *BR6ox2* to promote BR biosynthesis and hypocotyl elongation.

DISCUSSION

Regarding BR related research, the least characterized aspect is probably the mechanisms controlling BR homeostasis. Consistent with other growth-promoting phytohormones, such as auxin, GA, and cytokinin, both deficiency and excessive amounts of BR are harmful to plant growth and development. Unlike other phytohormones, BRs cannot be transported in a long-distance manner. Therefore, controlling BR homeostasis in a

certain tissue or a cell is extremely important to their biological functions. In this study, we identified a dominant genetic suppressor, *cog1-3D*, of a weak BR receptor mutant named *bri1-5*. *cog1-3D* and *cog1-3D bri1-5* showed significantly longer hypocotyl phenotypes relative to their corresponding backgrounds, WS2 and *bri1-5*, respectively (Figs. 1A and 2A). BR profile analyses revealed that multiple BR intermediates, including two biologically active forms, CS and BL, are drastically elevated in *cog1-3D* in comparison with those in wild-type seedlings (Fig. 3C). Transcription assays ruled out the possibility that it was caused by decreasing BR catabolism. Rather, it appeared that the accumulation of BRs is most likely caused by the enhanced BR biosynthesis. Consistently, several key BR biosynthesis genes are up-regulated in *cog1-3D* (Fig. 4A). Detailed analyses further suggested that *cog1-3D* up-regulation of the expression of BR biosynthetic genes is largely dependent on the function of PIF4 and PIF5. In the *pif4 pif5* background, *cog1-3D* cannot promote the expression of BR biosynthetic genes and, as a result, fails to enhance hypocotyl growth (Fig. 5, C–E).

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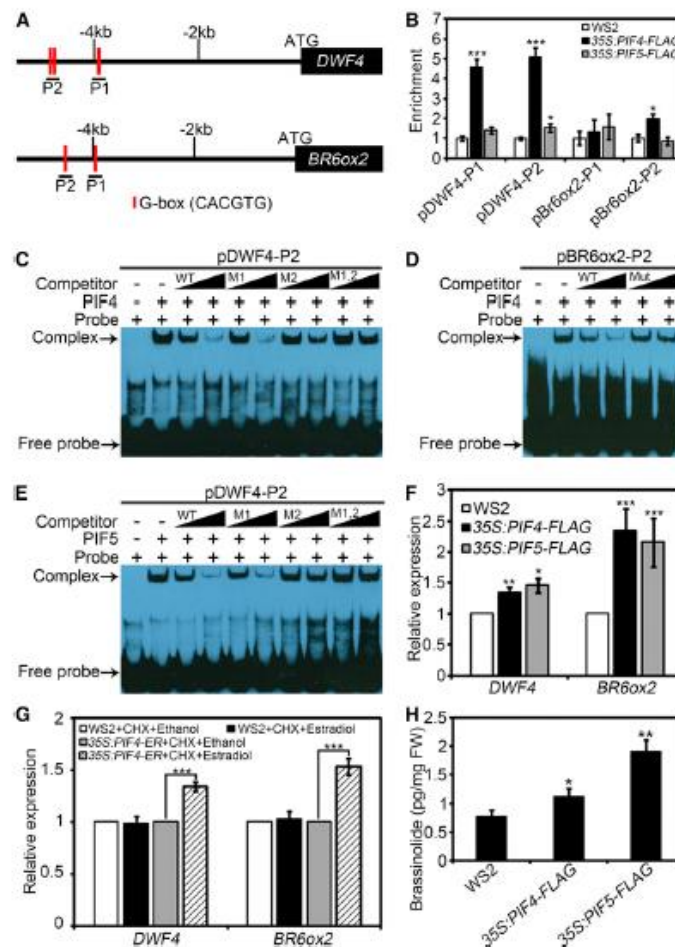
Figure 7. PIF4 and PIF5 bind directly to the promoter regions of BR biosynthetic genes and activate their expression. A, Schematic diagrams of the promoter regions of two BR biosynthetic genes, *DWF4* and *BR6ox2*. Red upright lines represent G-box DNA motifs. P1 and P2 are the DNA fragments for ChIP-PCR amplification. B, ChIP analysis showing that PIF4-FLAG and PIF5-FLAG interact with the G-box-containing regions within the *DWF4* and *BR6ox2* promoters upon precipitation with anti-FLAG antibody. One-week-old WS2, *35S:PIF4-FLAG*, and *35S:PIF5-FLAG* seedlings were harvested for ChIP experiments. qRT-PCR was used to quantify the enriched DNA fragments in the *DWF4* and *BR6ox2* promoters. C and D, EMSA indicates that PIF4 binds to the G-box motifs within the promoter regions of *DWF4* and *BR6ox2*. The biotin-labeled probes containing the G-box elements were incubated with TNT-expressed PIF4 protein, which was used for gel-shift analyses. The probes incubated with the TNT expression system without the PIF4 template were used as negative controls. Nonlabeled probes containing G-box (wild type [WT] or mutated G-box (M) with 10- or 100-fold molar concentrations over the biotin-labeled probe were used as cold competitors. E, EMSA shows that PIF5 binds to the G-box motifs in the promoter of *DWF4*. The fragments containing the G-box motifs present in the promoters of *DWF4* were incubated with TNT-expressed PIF5 protein, which was used subsequently for EMSA analyses. Competition for PIF5 binding was performed using 10- and 100-fold unlabeled probes containing G-box or mutated G-box. F, *DWF4* and *BR6ox2* expression levels in 1-week-old WS2, *35S:PIF4-FLAG*, and *35S:PIF5-FLAG* seedlings. G, Expression levels of *DWF4* and *BR6ox2* in WS2 and *35S:PIF4-ER* transgenic plants with or without estradiol treatment in the presence of CHX. WS2 and *35S:PIF4-ER* seedlings were pretreated with 10 μM CHX for 2 h, then either mock solution (ethanol) or 10 μM estradiol was added to the liquid solution for another 2 h before harvesting for RNA extraction and qRT-PCR analyses. H, Endogenous BL levels in WS2, *35S:PIF4-FLAG*, and *35S:PIF5-FLAG* seedlings. The data shown are averages and sd from three independent replicates. FW, Fresh weight.

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control the transcription levels of *PIFs* (Leivar and Quail, 2011). However, the transcription factors that directly regulate the expression of *PIF4* are largely unknown. A previous study revealed that *COG1* expression could be induced by far-red and red light, which is similar to that of *PIF4* (Huq and Quail, 2002; Park et al., 2003). In this study, we demonstrated that COG1 binds directly to the promoter regions of *PIF4* and *PIF5* to regulate their expression (Fig. 6).

Biosynthetic pathways are frequently feedback regulated by their end products or their signaling pathway either positively or negatively. For example, ethylene can both positively and negatively regulate its biosynthesis by up-regulating the expression of *ACS2* and *ACS4* and down-regulating the expression of *ACS6* (Wang et al., 2002a). Abscisic acid induces the expression of *AtNCED3* to positively regulate its biosynthesis under dehydration stress (Yang and Tan, 2014). BR signaling is utilized to create a feedback inhibitory regulation loop to control the expression of BR biosynthetic genes (He et al., 2005; Sun et al., 2010; Yu et al., 2011). Together with previous studies, our results support a positive feedback regulatory loop of BR biosynthesis by PIF4-mediated signaling (Fig. 8). In the absence of BR, BIN2 phosphorylates PIF4, leading to its degradation process and the inhibition of hypocotyl elongation (Bernardo-García et al., 2014). In the presence of BR, activated BRI1 and BAK1 receptor kinase lead to the inactivation of BIN2. PIF4 then can interact with BZR1 to regulate the expression of their target genes and promote hypocotyl growth (Oh et al., 2012; Bernardo-García et al., 2014). On the other hand, PIF4 activates the expression of *DWF4* and *BR6ox2* to produce more BRs and further promote hypocotyl elongation. Meanwhile, BZR1 can inhibit the expression of BR biosynthetic genes when BRs are excessive to plant growth and development (He et al., 2005). Such positive and negative feedback loops ensure optimal BR effects on plant growth and development. How plants reset the equilibrium of BR in response to developmental and environmental signals is a question to be answered in the near future.

Understanding the mechanisms controlling BR homeostasis can help us to develop strategies to optimize plant growth and development via genetic engineering in the future. BR profile analysis showed that the substrates of DWF4 are always plentiful in plants. However, the products of the DWF4-catalyzed reactions are considerably low or even undetectable, suggesting that DWF4 catalyzes a flux-determining step in BR biosynthesis (Guo et al., 2010). BR6ox2 was identified as a bifunctional enzyme that mediates the conversion of CS to BL, the final and rate-limiting step in BR biosynthesis, as well as BR C-6 oxidation (Kim et al., 2005). Controlling the expression of *DWF4* and/or *BR6ox2* could be an effective approach to alter BR production in response to external and internal cues. TCP1 was identified as a transcription factor promoting the expression of *DWF4* (Guo et al., 2010). Auxin and high temperature also can induce the expression of *DWF4*

via mechanisms yet to be determined (Chung et al., 2011; Maharjan and Choe, 2011). However, transcriptional regulation of *BR6ox2* is not described in the literature. Our data revealed that PIF4 functions as a positive regulator enhancing the expression of *DWF4* and *BR6ox2*, leading to an increase of bioactive levels of BRs in *cog1-3D* (Figs. 3C and 7). PIF5, a homolog of PIF4, also can bind to the promoter of *DWF4* to activate its expression (Fig. 7).

Interestingly, *CPD* also is up-regulated in *COG1*- and *PIF4*-overexpressing plants, and the ChIP assay showed that PIF4 associates with the promoter of *CPD* (Fig. 4A; Supplemental Fig. S12C). However, we failed to detect the direct binding of PIF4 to the *CPD* promoter, indicating the existence of other transcription factors that regulate *CPD* expression together with PIF4. The expression levels of *CPD* and *DWF4* are not altered significantly in *pif4 pif5* double mutants compared with the wild type (Fig. 5E), which should be caused by the functional redundancy of other transcription factors, such as CES and TCP1, that regulate the expression of these two BR biosynthetic genes. And there might be more transcription factors regulating the level of these two BR biosynthetic genes. Teasterone, an earlier BR intermediate converted from cathasterone by ROT3, also is increased in *cog1-3D* (Fig. 3C). However, *ROT3* is not regulated directly by PIF4 and PIF5, suggesting that COG1 may regulate *ROT3* expression through other transcription factors. Typhasterol and CS are both elevated in *cog1-3D*, indicating that COG1 regulates genes essential for C-3 dehydrogenation, C-3 reduction, and C-2 hydroxylation. It is known that OsD2 and OsD11 are responsible for the C-3 dehydrogenation and C-3 reduction reactions in rice (*Oryza sativa*), respectively (Hong et al., 2003; Tanabe et al., 2005). C-2 hydroxylation of BRs is mediated by DDWF1 in pea (*Pisum sativum*; Kang et al., 2001). However, the enzymes catalyzing these reactions in Arabidopsis are not identified. It is possible that homologs of these three cytochrome P450s that are up-regulated in *cog1-3D* are the potential enzymes responsible for the corresponding reactions in Arabidopsis.

MATERIALS AND METHODS

Plant Materials

The Arabidopsis (*Arabidopsis thaliana*) *bri1-5 cog1-3D* double mutant was obtained from a large-scale activation-tagging screen for *bri1-5* genetic modifiers as described previously (Li et al., 2001, 2002; Yuan et al., 2007; Guo et al., 2010). The *cog1-3D* single mutant was generated by backcrossing *bri1-5 cog1-3D* with WS2. Other plant materials, including *bri1-5, bri1-4, det2, cpd, dwf4,* and *cog1-D*, were described previously (Noguchi et al., 1999a; Park et al., 2003; Guo et al., 2010). The *cog1-6* mutant was identified from an ethyl methanesulfonate-mutagenized *cog1-D* seed pool. The *br6ox1* (Salk_148384), *br6ox2* (Salk_148384), *pif4* (Salk_140393), and *pif5* (Salk_087012) T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center. The *bri1-5, bri1-4, det2,* and *dwf4* mutants are in the WS2 background. The *cpd, br6ox1/2, pif4,* and *pif5* mutants are in the Columbia-0 background. The double or triple mutants were generated by genetic crossing using the related single mutants. The *cpd cog1-3D, br6ox1/2 cog1-3D,* and *pif4 pif5 cog1-3D* mutants were then backcrossed with the WS2 ecotype for at least five times.

Identification of the *cog1-3D* Locus

The flanking genomic sequence of the inserted T-DNA of *pBIB-BASTA-AT2* was amplified using thermal asymmetric interlaced PCR as described previously (Liu and Whittier, 1995; Yuan et al., 2007; Guo et al., 2010). The T-DNA insertion site was determined by sequencing the flanking genomic DNA.

Constructs and Plant Transformation

Gateway technology was employed to clone the coding sequences of *COG1*, *PIF4*, and *PIF5* (Gou et al., 2010). The amplified *COG1* coding sequence was cloned into a *pEarleyGate 202* destination vector (Earley et al., 2006). The *PIF4* and *PIF5* coding sequences were cloned into a *pBIB-35S-GWR-FLAG* vector (Gou et al., 2010). For the construction of *35S:COG1-ER* and *35S:PIF4-ER*, the coding sequences of these two genes were introduced in a *pBIB-35S-GWR-ER* vector. For the construction of *35S:COG1-SRDX* and *GVG:COG1-SRDX*, *COG1-SRDX* was PCR amplified and introduced in a *pBIB-35S-GWR* vector and a Gateway-compatible *pTA7002* vector (Aoyama and Chua, 1997; Gou et al., 2010). These expression constructs were then transformed into *Agrobacterium tumefaciens* strain GV3101 for transformation of Arabidopsis plants by the floral dip method (Clough and Bent, 1998). The primers used are listed in Supplemental Table S2.

Hypocotyl Measurements

Seeds were surface sterilized for 15 min in 10% bleach, washed five times with sterilized water, and planted on one-half-strength MS agar plates containing 1% (w/v) Suc, with or without 24-epiBL, or BRZ. Plants were pretreated at 4°C for 3 d and then transferred to a growth chamber set at 22°C with a 16-h-light/8-h-dark photoperiod or in continuous red light for 6 d. The *GVG:COG1-SRDX* seeds were planted on one-half-strength MS agar plates containing 1% (w/v) Suc. Two days after germination, the seedlings were transferred to one-half-strength MS agar plates containing 1% (w/v) Suc and 10 μM DEX, growing for another 5 d. Hypocotyls of the seedlings were then scanned and measured as reported previously (Yuan et al., 2007). All measurements were repeated three times, and at least 20 seedlings were measured for each genotype. Student's *t* test was performed (*, P < 0.05; **, P < 0.01; and ***, P < 0.001).

qRT-PCR Analyses

Long-day-grown seedlings were harvested directly or after treatment with DEX or estradiol and CHX at the same time of day and frozen in liquid nitrogen for RNA extraction. Total RNA was extracted using the RNAprep Pure Plant Kit (Tiangen Biotech). Five micrograms of total RNA was reverse transcribed into first-strand cDNAs using M-MLV reverse transcriptase (Thermo Fisher Scientific) according to the manufacturer's instructions. The cDNA samples diluted 10-fold were used as PCR templates. qRT-PCR was performed using SYBR Premix Ex Taq II (TaKaRa) on a Step One Plus Real-Time PCR machine (Thermo Fisher Scientific). The relative expression level was calculated from three replicates using the ΔΔC_t method after normalization to an *ACT2* control. All the qRT-PCR analyses were performed for three biological replicates, yielding similar results. Data shown are means and se from three biological replicates. Student's *t* test was performed (*, P < 0.05; **, P < 0.01; ***, P < 0.001; and ns, not significant). The primers used are listed in Supplemental Table S2.

BR Profile Assays

Samples were analyzed for BR contents as described previously with a few modifications (Swaczynova et al., 2007). Briefly, 50 mg of fresh Arabidopsis tissue samples was homogenized to a fine consistency using 3-mm zirconium oxide beads and an MM 301 vibration mill at a frequency of 30 Hz for 3 min (Retsch). The samples were then extracted overnight with stirring at 4°C using a benchtop laboratory rotator (Stuart SB3; Bibby Scientific) after the addition of 1 mL of ice-cold 60% acetonitrile and 10 pmol of [^{2}H$_3$]BL, [^{2}H$_3$]CS, [^{2}H$_3$]24-epiBL, [^{2}H$_3$]24-epicastasterone, [^{2}H$_3$]28-norbrassinolide, and [^{2}H$_3$]28-norcastasterone as internal standards (OlChemIm). The samples were further centrifuged, purified on polyamide SPE columns (Supelco), and then analyzed by ultra-high-performance liquid chromatography-tandem mass spectrometry (Micromass). The data were analyzed using Masslynx 4.1 software (Waters), and BR content was finally quantified by the standard isotope dilution method (Rittenberg and Foster, 1940).

ChIP-PCR Assay

Two grams of one-week-old WS2, *35S:FLAG-COG1*, *35S:PIF4-FLAG*, and *35S:PIF5-FLAG* transgenic seedlings was harvested for ChIP experiments. Chromatin was isolated and sonicated to generate DNA fragments with an average size of 500 bp. The solubilized chromatin was immunoprecipitated by an agarose-conjugated anti-FLAG antibody (Sigma-Aldrich). The coimmunoprecipitated DNA was recovered and analyzed by quantitative PCR with SYBR Premix Ex Taq II (TaKaRa). Relative fold enrichment was calculated by normalizing the amount of a target DNA fragment against that of an *ACT2* promoter fragment and then against the respective input DNA samples. Three independent biological repeats were performed, yielding similar results. Shown are representative data from one biological replicate. Student's *t* test was performed (*, P < 0.05; **, P < 0.01; and ***, P < 0.001). The primers used are listed in Supplemental Table S2.

DNA Gel-Shift Assay

The full-length *COG1* coding region was cloned into a *pGEX-4T-3* vector (GE). The resulting constructs were sequenced to confirm the correct open reading frame and sequences and then transformed into the *Escherichia coli* Rosetta strain for recombinant protein expression. Bacteria were grown in 100 mL of Luria-Bertani medium supplemented with 100 μg mL^{-1} ampicillin and 30 μg mL^{-1} chloramphenicol, and the GST-COG1 recombinant proteins were purified with GST-Sefinose Resin (Sangon Biotech). PIF4 and PIF5 were synthesized using the TNT SP6 High-Yield Wheat Germ Protein Expression System (Promega). The probes were synthesized and labeled with biotin using the Biotin 3' End DNA Labeling Kit (Thermo Fisher Scientific). Unlabeled competitor probes were generated from dimerized oligonucleotides. EMSA experiments were performed using the Chemiluminescent Nucleic Acid Detection Module (Thermo Fisher Scientific) according to the manufacturer's instructions. Probe sequences are shown in Supplemental Table S2.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *COG1* (At1g29160), *PIF4* (At2g43010), *PIF5* (At3g59060), *DET2* (At2g38050), *CPD* (At5g05690), *DWF4* (At3g50660), *BR6ox1* (At5g38970), *BR6ox2* (At3g30180), *ROT3* (At4g36380), and *CYP90D1* (At3g13730).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. The *cog1-6* mutant was identified as an intragenic suppressor of *cog1-D*.

Supplemental Figure S2. Phenotypes of *35S:COG1-SRDX* transgenic plants.

Supplemental Figure S3. Induced expression of *COG1-SRDX* leads to reduced cell length.

Supplemental Figure S4. BR signal transduction is elevated in the *cog1-3D* mutant.

Supplemental Figure S5. BRZ inhibits the hypocotyl growth of *cog1-3D* seedlings.

Supplemental Figure S6. Expression of *DET2*, *CYP90D1*, and *BR6ox1* in *cog1-3D*.

Supplemental Figure S7. Expression of BR catabolism genes in *cog1-3D*.

Supplemental Figure S8. The *bri1-4* mutation completely inhibits the long-hypocotyl phenotype of *cog1-3D*.

Supplemental Figure S9. PIF4 and PIF5 promote cell elongation.

Supplemental Figure S10. Protein levels of PIF5 in *cog1-3D* and *35S:FLAG-COG1* seedlings.

Supplemental Figure S11. COG1-mediated hypocotyl elongation is dependent on PIF4 and PIF5.

Supplemental Figure S12. PIF4 regulates the expression of *CPD*.

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Supplemental Table S1. The expression of 37 transcription factors is significantly altered in *cog1-3D* compared with WS2.

Supplemental Table S2. List of primers and probes used in this study.

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ARTICLE 12

Drought-tolerant and drought-sensitive genotypes of maise (*Zea mays* L.) differ in contents of endogenous brassinosteroids and their drought-induced changes

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Contribution: analysis of brassinosteroids, manuscript writing

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Drought-tolerant and drought-sensitive genotypes of maize (*Zea mays* L.) differ in contents of endogenous brassinosteroids and their drought-induced changes

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Abstract

The contents of endogenous brassinosteroids (BRs) together with various aspects of plant morphology, water management, photosynthesis and protection against cell damage were assessed in two maize genotypes that differed in their drought sensitivity. The presence of 28-norbrassinolide in rather high quantities (1–2 pg mg^{-1} fresh mass) in the leaves of monocot plants is reported for the first time. The intraspecific variability in the presence/content of the individual BRs in drought-stressed plants is also described for the first time. The drought-resistant genotype was characterised by a significantly higher content of total endogenous BRs (particularly typhasterol and 28-norbrassinolide) compared with the drought-sensitive genotype. On the other hand, the drought-sensitive genotype showed higher levels of 28-norcastasterone. Both genotypes also differed in the drought-induced reduction/elevation of the levels of 28-norbrassinolide, 28-norcastasterone, 28-homocastasterone and 28-homodolichosterone. The differences observed between both genotypes in the endogenous BR content are probably correlated with their different degrees of drought sensitivity, which was demonstrated at various levels of plant morphology, physiology and biochemistry.

Introduction

The economic losses in crop production due to drought are quite substantial and will undoubtedly further increase with the expected climate changes. To prepare for these changes, various new agricultural technologies are tried and utilised [1, 2]. Some attention has been paid to the application of diverse chemical compounds such as polyethylene glycol (PEG), amino acids, antioxidants, phytohormones, minerals, volatile organic compounds, *etc.* [3]. A group of steroidal phytohormones called brassinosteroids (BRs) has been included among these compounds. Indeed, BRs seem to be destined for agricultural practice due to the fact

they are non-toxic, non-mutagenic and environmentally friendly, as well as their effectivity at low concentrations, ease of application and the possibility of artificially synthesising them on a commercial scale [4–6].

Studies on the impact of BRs on drought-stressed plants began almost 25 years ago [7], and since that time, more than 90 papers on this topic have appeared in various scientific journals or books. BRs are generally regarded as positive regulators of the plant drought response, and the elevation of their contents in plants by exogenous application is often accompanied by an improvement in drought resistance. The studies presenting some data on the response of BR-treated plants or mutants in BR biosynthesis/perception to drought greatly vary regarding the overall design of experiments, plant cultivation conditions, examined species, developmental stage of plants, *etc.* However, these studies usually have one attribute in common: their authors analysed only one genotype/cultivar of the respective plant species. Although some papers deal with more than one genotype, the drought sensitivity/resistance of these genotypes is often unspecified. This fact significantly limits our understanding of the relationship between BRs and drought resistance and could limit the potential practical application of these compounds.

The data on the role of BRs in plant drought response that are currently available from the few studies that have been performed with genotypes of known drought sensitivity are not very conclusive. Logically, any comparison of the impact of exogenously applied BRs on drought-resistant/sensitive genotypes should reveal the BR-induced changes particularly in the sensitive genotypes, because the resistant genotypes should experience less intensive drought effects. This should be similar to the situation observed for the BRs exogenously applied to plants exposed to severe or mild/moderate drought, BRs always have a greater effect on more strongly stressed plants [8–11]. Such response of drought-sensitive and drought-resistant genotypes to BRs has indeed been observed by [12] and [13] in sorghum exposed to PEG. However, a more-or-less similar response of drought-sensitive and drought-resistant genotypes was demonstrated in wheat stressed by the cessation of watering or subjected to PEG or mannitol treatment [14–16], as well as in tomato [17]. There are also some cases where the drought-tolerant genotype showed a more pronounced response to BRs than the drought-sensitive one, this been reported for PEG-stressed maize [18–20] or rice [21]. Thus, the situation is not so simple and probably depends on plant species as well as on a mechanism that is responsible for drought resistance/sensitivity of the respective genotype. In addition, most of the authors of the abovementioned studies simulated drought by the application of PEG. This certainly causes *osmotic* stress, but the application of PEG induces a specific stress level very rapidly and very strongly, thus evading the natural course of drought response with its gradual changes. Therefore, the results obtained from such studies might not mimic drought situations occurring in nature.

Moreover, although diverse aspects of plant biology have been examined during research on the role of BRs in plant drought response, most scientists have focused only on the possible alleviation of stress symptoms by exogenously applied BRs. Almost no one has pursued the possibility that drought *per se* could induce changes in the content of *endogenous* BRs. Indeed, such analysis has rarely been performed for any type of stress and only three studies give some information on this topic. Jäger *et al.* [22] assessed the content of one bioactive BR (castasterone, CS) in the drought-stressed pea. They found that the exposure of their experimental plants to adverse environment increased the content of this BR, but only non-significantly. Gruszka *et al.* [23], who examined barley plants subjected to water deficiency, reported that drought induced the levels of endogenous CS and 24-*epi*brassinolide (*epi*BL) but did not change the amounts of brassinolide (BL), 24-*epi*castasterone (*epi*CS) or 28-homocastasterone (homoCS). Finally, Pociecha *et al.* [24] assessed the CS content in two cultivars of cold-acclimated winter rye. The winter-resistant cultivar showed the same elevation of CS (approximately 2-fold) after 3 or 6 weeks of cultivation in cold, while the less winter-resistant cultivar

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was characterised by a 2-fold increase after 3 weeks and an additional increase after 6 weeks of cold acclimation.

We thus decided to compare the contents of various endogenous BRs in drought-resistant and drought-sensitive maize genotypes to examine their changes under drought stress. To our knowledge, such analysis has not been previously performed. Additionally, to determine the possible relationship between these changes and various processes that occur during plant drought response, we also examined several aspects of plant morphology and physiology. Because BRs are generally considered to play a positive role in plant stress resistance, we hypothesised that the resistant genotype could be characterised by higher levels of endogenous BRs than the sensitive genotype already under non-stress conditions. However, because the resistant genotype would not experience stress to such a degree as the other genotype, we also expected that the resistant genotype would not need to further elevate the BR contents when subjected to drought.

Material and methods

Plant material, experimental design and BR treatment

Two maize (*Zea mays* L.) inbred lines, drought-sensitive 2023 and drought-resistant CE704, were used for our experiments. The degree and mechanisms of their drought-sensitivity are described in [25, 26]. Kernels of both genotypes were obtained from the *CEZEA Maize Breeding Station* (Czech Republic) and were sown in pots (diameter 12 cm, height 12 cm, one plant per pot) filled with a mixture (15:1, v:v) of Garden Compost (*Agro CS*, Czech Republic) and Hawita Baltisches Uni 20 Tonsubstrat 1 (*Hawita*, Germany). The pots were placed in a naturally lit greenhouse located on the Faculty of Science campus at Charles University, Prague, Czech Republic (54°04'N, 14°25'E) under semi-controlled conditions (mean air temperature 24/16°C, mean relative air humidity 71/86% day/night, natural irradiance). All plants were sufficiently watered until 30 days after the date of sowing. At this time, all the plants had three fully developed leaves and the fourth leaf was visible from at least one half of its final length. They were then divided into four groups (experimental variants) according to genotype (2023 or CE704) and watering treatment (control, *i.e.*, the 20% soil water content, or drought stress, *i.e.*, the cessation of watering for 14 days resulting in the 3% soil water content at the end of the drought period). Each group consisted of 60 plants organised in a randomised plot design. This enabled us to use different plants for the determination of various parameters (*i.e.*, we had separate groups of plants allocated for the assessments of i) the contents of BRs, ii) plant morphology, iii) gas exchange and the osmotic potential, iv) chlorophyll fluorescence and the contents of chlorophylls and carotenoids, and v) index of cell membrane injury (CMI), the contents of malondialdehyde (MDA), H_2O_2, proline, and the activities of ascorbate peroxidase (APX) and catalase (CAT). The fourth leaf (counting from the base) was utilised for all the measurements and samplings. The evaluation of plant morphology, gas exchange analysis, chlorophyll fluorescence measurements and determination of the photosynthetic pigment content and CMI were conducted immediately after the end of the experiments. For other parameters, the respective leaves were sampled at that time and kept at -80°C (in case of the leaf osmotic potential in -18°C) until their analyses. The exact number of biological replicates (3 to 12) for the individual parameters is always indicated in the legends to the respective figures/tables.

Content of brassinosteroids

Samples were analysed for the BR contents according to the method described in [27]. Briefly, 50 mg of fresh maize tissue samples were homogenised to a fine consistency using 3-mm

zirconium oxide beads and an MM 301 vibration mill at a frequency of 30 Hz for 3 min (*Retsch GmbH & Co. KG*, Haan, Germany). The samples were then extracted overnight with stirring at 4°C using a benchtop laboratory rotator Stuart SB3 (Bibby Scientific Ltd., Staffordshire, UK) after adding 1 mL ice-cold 60% acetonitrile and 10 pmol of [^{2}H$_3$]BL, [^{2}H$_3$]CS, [^{2}H$_3$]24-*epi*BL, [^{2}H$_3$]24-*epi*CS, [^{2}H$_3$]28-norBL, [^{2}H$_3$]28-norCS and [^{2}H$_3$]typhasterol as internal standards (OlChemIm Ltd., Olomouc, Czechia). The samples were further centrifuged, purified on polyamide SPE columns (Supelco, Bellefonte, PA, USA) and then analysed by UHPLC-MS/MS (Micromass, Manchester, UK). The data were analysed using Masslynx 4.1 software (Waters, Milford, MA, USA) and the BR contents were finally quantified by the standard isotope-dilution method [28]. Our study focused on the content of fifteen BRs: teasterone (TE), 28-norteasterone (norTE), typhasterol (TY), CS, *epi*CS, homoCS, 28-norcastasterone (norCS), BL, *epi*BL, 28-homobrassinolide (homoBL), 28-norbrassinolide (norBL), dolicholide (DL), 28-homodolicholide (homoDL), dolichosterone (DS) and 28-homodolichosterone (homoDS). Each experimental variant was represented by three biological replicates, where the level of each of these replicates was calculated as an arithmetic mean of two independent technical replicates. One biological replicate represents an individual leaf sampled at the end of the drought period and kept at -80°C until UHPLC-MS/MS analysis.

The assessment of plant morphology and the measurements of the leaf osmotic potential

The height of plants was measured from the base of the shoot to the tip of the youngest leaf visible at the top whorl of leaves. The number of visible leaves was also counted. The area of individual leaves was calculated from their lengths and widths using a previously determined coefficients [26]. The total area of the photosynthetically active leaves was calculated as the sum of the area of all the leaves that were at least 50% green. Dry masses of individual leaves and the rest of shoot (their sum constitutes the total dry mass of shoot) and the total dry mass of roots were assessed after drying the respective parts of the plants for seven days at 80°C and weighing on analytical balances. The specific mass of the 4th leaf was determined from ten leaf discs (diameter 6 mm) cut from the middle portion of the leaf blade, oven-dried at 80°C for two days and weighed on analytical balances with 0.1 mg precision.

For the measurements of the leaf osmotic potential, the samples of plant leaves were kept gently compressed in syringes sealed with Parafilm M till the time of measurements when the samples were thawed at 2°C. Approximately 0.05 mL of leaf sap was pressed out from each sample and put into the chamber of the potentiometer WP4C (*Decagon Devices*, Pullman, WA, the U.S.A.) to measure this parameter.

Gas exchange measurements, chlorophyll fluorescence analysis and determination of the content of photosynthetic pigments

The net photosynthetic rate, transpiration rate and stomatal conductance were determined *in situ* using an LCi Portable Photosynthesis System (*ADC BioScientific*, Hoddesdon, the United Kingdom) with the following conditions in the measurement chamber: air temperature at 25°C, ambient CO$_2$ concentration at 550±50 μL L^{-1}, air flow rate at 205±30 μmol s^{-1}, and irradiance at 300 μmol m^{-2} s^{-1} [25].

The polyphasic rise of the chlorophyll fluorescence transient (OJIP) was measured at the upper surface of the dark-adapted (20 min) leaves *in situ* with the portable fluorometer *FluorPen FP100max* (*Photon System Instruments*, Brno, Czech Republic) as described in [26]. The parameters of the JIP test (see S1 Table) were calculated according to the theory of energy flow in the photosynthetic electron-transport chain [29, 30]. The relative variable fluorescences

W_{OI}, W_{OJ}, W_{OK} and W_{IP} (*i.e.*, normalizations of the whole fluorescence transients) and the difference kinetics ΔW_{OJ} and ΔW_{OK} (as the differences between the drought-stressed and control plants) were also calculated according to [31] and their graphical representation was utilised to obtain further information on the primary photosynthetic processes.

The chlorophyll *a* and *b* contents and total carotenoids were determined spectrophotometrically [32] in the *N*,*N*-dimethylformamide extracts prepared as described in [33].

The determination of various indicators of cell damage and protective mechanisms

Except for the CMI determination, all parameters were evaluated spectrophotometrically using *Anthelie Advanced 2* (*Secomam*, Alès, France) or *Evolution 201* (*Thermo Fisher Scientific*, Waltham, MA, the U.S.A.). The CMI was determined from the measurements of electrical conductivity of the samples consisting from 15 leaf discs with the *GRYF 158* conductometer (*Gryf HB*, Havlíčkův Brod, Czech Republic). The CMI was calculated as $(100 \times C_1/C_2)$, where C_1 is the electrical conductivity of a sample after 24 h incubation and C_2 is the conductivity of the same sample after its denaturation. The method described in [34] was utilised for the determination of the content of MDA, resp. thiobarbituric acid reactive substances. This procedure serves as a method for the determination of a degree of membrane lipid peroxidation. The content of H_2O_2 was determined using the potassium iodide method described in [35]. The activities of APX and CAT in the leaves were assessed according to [36] and [37], for further details, see [26]. The protein content was determined by the Bradford method [38] using bovine serum albumin as a standard. The proline content was determined using the acid-ninhydrin method according to [39].

Statistical analysis

The original data that constituted the basis for the statistical analysis are available in the S1 File. The datasets containing parameters that had to be measured on the same plants (see above) were subjected first to two-way analyses of variance (genotype, treatment (control/stress) and the interaction between these two factors were included as the sources of possible variability). To correct for false discovery rates (FDR), the Benjamini and Hochberg [40] correction was applied within the respective datasets with FDR set as 0.05. Tukey's HSD tests were then performed for each parameter separately. Additionally, to determine the relevant differences between the control and drought-stressed variants of the respective genotype, or the differences between both examined genotypes in plants either stressed or non-stressed by drought, one-way analyses of variance followed by Tukey's HSD tests were also performed. The statistical evaluation was conducted with CoStat (Version 6.204) statistical software (*CoHort Software*, Moterey, CA, the U.S.A.).

Results

The contents and composition of BRs distinctly differed between both genotypes and in some cases also depended on water supply. CE704 displayed significantly higher levels of total BRs in its leaves compared to 2023 (Table in S2 File, Fig 1A). The most abundant BR detected in CE704 was TY followed by norBL. On the other hand, 2023 contained only small amounts of these two BRs per leaf fresh mass (FM) and the differences between both genotypes were statistically significant (Table in S2 File, Fig 1B and 1F). The CE704 stressed plants showed a significantly lower homoCS content in their leaves compared to 2023 (Table in S2 File, Fig 1G). The homoCS and homoDS contents were reduced by drought, particularly in 2023 (in case of the homoDS content non-significantly; Table in S2 File, Fig 1G and 1H). On the other hand, 2023

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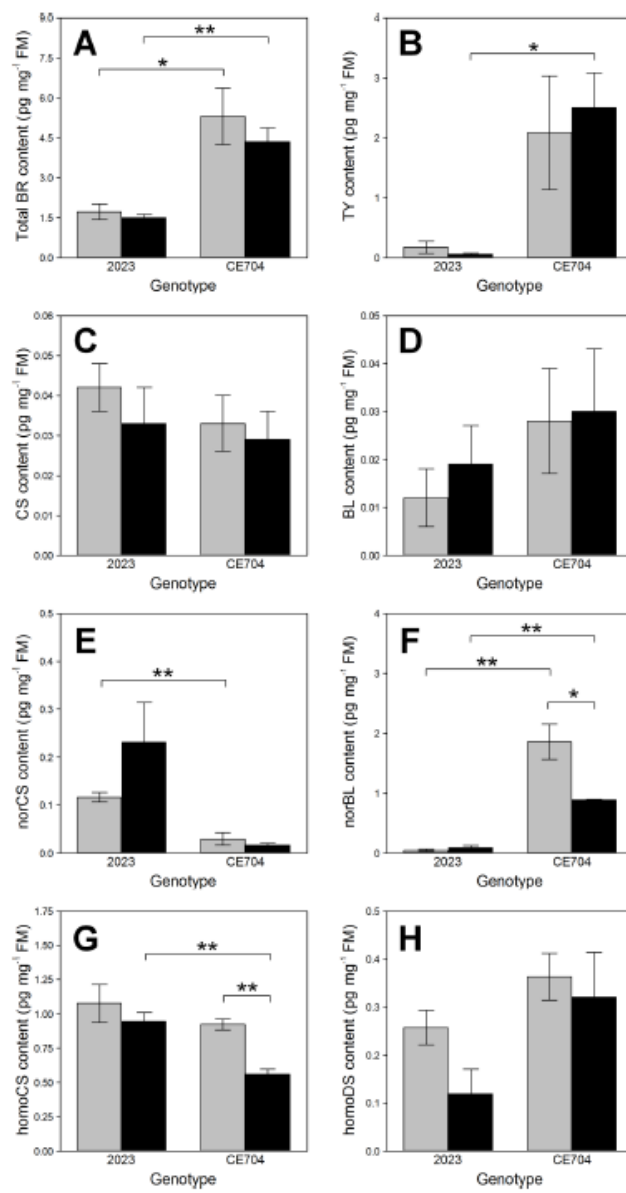
Fig 1. Contents of total and individual brassinosteroids (BRs) in two maize genotypes (2023 and CE704). Plants were either subjected to normal watering (control; grey columns) or to 14 days of withholding water (stress; black columns). Mean values ± SEM are shown (n = 3). Asterisks indicate significant ($p \leq 0.05$; *) or highly significant ($p \leq 0.01$; **) differences between mean values according to Tukey's tests made separately for each genotype (in case of the differences between control and stress treatment) or for each treatment (in case of the differences between both genotypes). BL ... brassinolide, CS ... castasterone, DS ... dolichosterone, FM ... leaf fresh mass, TY ... typhasterol.

showed a distinct increase in the norCS content after drought, contrary to CE704. CE704 was generally characterised by a lower content of this BR (Table in S2 File, Fig 1E). Regarding two other BRs that were detected, i.e., CS and BL, no significant differences were observed between genotypes or between watering treatments (Table in S2 File, Fig 1C and 1D). All other analysed BRs (TE, norTE, epiBL, homoBL, epiCS, DL, homoDL, DS) were below a limit of a detection method.

CE704 showed significantly higher values of plant height compared to 2023 but only under stress conditions (Fig 2A). This genotype also had higher number of visible leaves compared to 2023 (Table in S2 File, Fig 2E). While the older leaves of 2023 plants subjected to drought displayed strong symptoms of senescence (the first two leaves were completely dry or yellow, the third leaf also started to senesce), this did not apply to CE704, which developed almost normally (only slightly slowing down) even under stress conditions (S1 File, S1 Fig). Drought significantly reduced the total leaf area as well as the shoot dry mass in 2023 but not in CE704, resulting in genotypic differences under stress conditions (Table in S2 File, Fig 2B and 2C). The stressed plants of CE704 also had a greater root biomass compared to 2023; however, this did not apply to the control plants (Table in S2 File, Fig 2D). The SLM of the 4th leaf did not significantly differ between both genotypes or between watering treatments (Table in S2 File, Fig 2F).

The leaf osmotic potential values were lower in the drought-stressed plants of both genotypes compared to their respective controls (Table in S2 File, Fig 3A). However, the differences between the genotypes were statistically significant only for the control plants, not for the stressed ones (Fig 3A). The transpiration rate was reduced after 14 days without watering. This was less pronounced in CE704 than in 2023 (Fig 3B). Both the net photosynthetic rate and stomatal conductance were also negatively affected by drought and the changes in the values of these parameters were again more evident in 2023 than in CE704 (Table in S2 File, Fig 3C and 3D). This was reflected in the presence of significant differences between both genotypes in the stomatal conductance, which were found under stress conditions (Fig 3D).

The drought-stressed plants of CE704 contained more chlorophyll and carotenoids in its leaves compared to 2023 (Table in S2 File, Fig 3E and 3F). This was caused by the fact that the content of these chlorophylls actually increased in CE704 after 14 days without watering, contrary to 2023.

Drought also reduced the efficiency of the primary photosynthetic processes. There were apparent differences between the control and drought-stressed plants, particularly for the parameters describing electron transport within the photosystem (PS) II reaction centre, such as φ_{P0}, φ_{E0} and ψ_{E0} as well as the performance index PI_{ABS}, and these differences were more pronounced in the 2023 genotype (S1 File). However, no true statistically significant differences were found between the genotypes for the numerical parameters of the JIP test (Table in S2 File). The dissipation of excess excitation energy significantly increased with drought stress (parameters φ_{E0} and DI_0/RC). The oxygen-evolving complex of PSII did not seem to be negatively affected in the drought-stressed plants of either genotype, as seen from the location of the K-band around zero on the graph of the ΔW_{OJ} difference kinetics (Fig 4A). The excitonic connectivity between the individual PSII units (inferred from the L-band positions above zero on the graph of the ΔW_{OK} difference kinetics) was slightly negatively affected by drought, but

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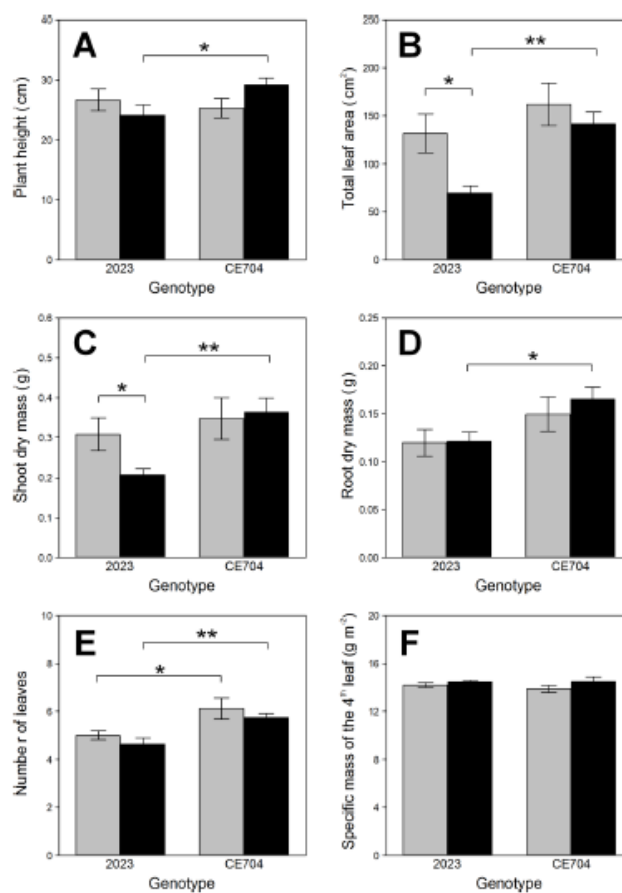
Fig 2. Selected parameters of plant morphology in two maize genotypes (2023 and CE704). Plants were either subjected to normal watering (control; grey columns) or to 14 days of withholding water (stress; black columns). Mean values ± SEM are shown (n = 8). Asterisks indicate significant ($p \leq 0.05$; *) or highly significant ($p \leq 0.01$; **) differences between mean values according to Tukey's tests made separately for each genotype (in case of the differences between control and stress treatment) or for each treatment (in case of the differences between both genotypes).

both genotypes did not differ in this respect (Fig 4B). Regarding the electron transport beyond PSII, our experimental plants were not affected much by drought (S1 File, Table in S2 File, parameters δ_{RE01}, φ_{RE01} and ψ_{RE01}). However, based on the greater observed difference in the positions of the respective W_{OI} curves, 2023 showed a slightly more pronounced decline in the reduction rate of electron acceptors at the end of the electron-transport chain due to drought than CE704 (Fig 4C). The size of the pool of these acceptors was similar in all the experimental variants (based on the fact that the positions of the W_{IP} curves did not differ much; Fig 4D).
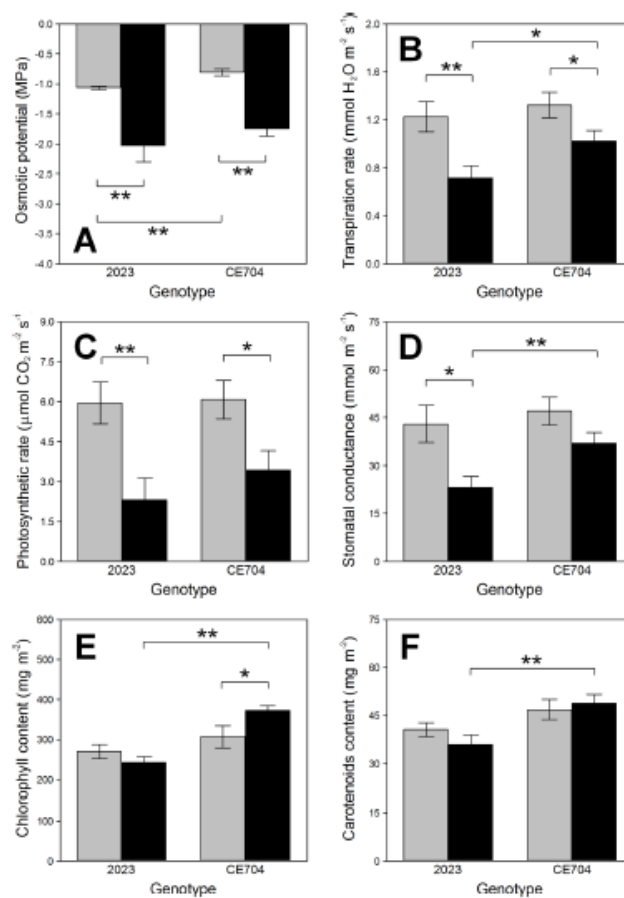
Fig 3. Selected parameters of gas exchange, the osmotic potential and the contents of chlorophylls and carotenoids in leaves of two maize genotypes (2023 and CE704). Plants were either subjected to normal watering (control; grey columns) or to 14 days of withholding water (stress; black columns). Mean values ± SEM are shown (n = 8 for gas exchange and the contents of photosynthetic pigments, n = 12 for osmotic potential). Asterisks indicate significant ($p \leq 0.05$; *) or highly significant ($p \leq 0.01$; **) differences between mean values according to Tukey's tests made separately for each genotype (in case of the differences between control and stress treatment) or for each treatment (in case of the differences between both genotypes).

CMI had higher values in the stressed plants than in the control ones. This increase was not as prominent in CE704 as in 2023 (Fig 5A). The 2023 genotype generally also showed slightly higher peroxidation of membrane lipids based on the MDA content compared to CE704, although lipid peroxidation seemingly increased more in CE704 than in 2023 after the exposure of plants to drought (Fig 5B). CE704 was also characterised by a greater proline content in

Genotypic differences in endogenous brassinosteroid contents in drought-stressed maize
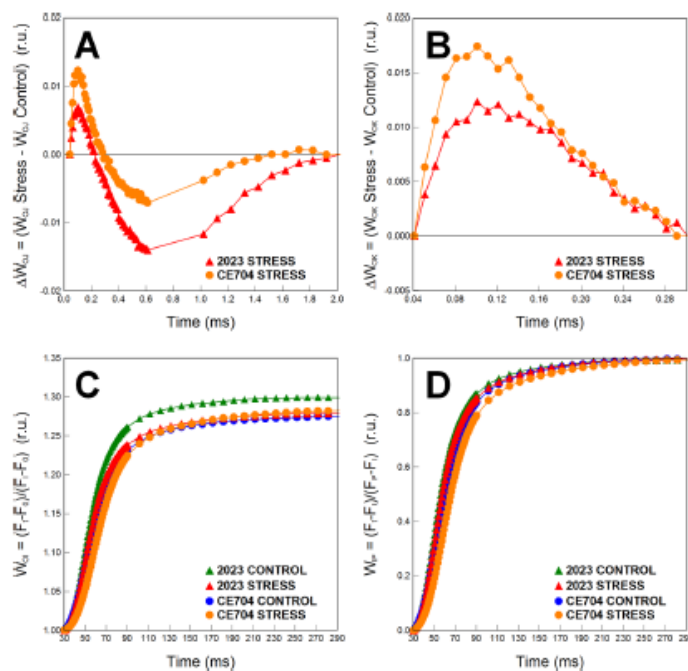
Fig 4. The difference kinetics and the relative variable fluorescences calculated from OJIP analysis of two maize genotypes (2023 and CE704). The difference kinetics ΔW_{OJ} (A) reveals the K-band; ΔW_{OK} (B) reveals the L-band. Only the part between the I and P points of the OJIP curve is shown for the relative variable fluorescence W_{OI} (C). The normalization of OJIP curve between the I and P points with the maximum amplitude fixed as 1 is shown as the relative variable fluorescence W_{IP} (D). Plants were subjected either to normal watering (control) or to 14 days of withholding water (stress). ΔW_{OJ} and ΔW_{OK} were calculated from the comparisons of the stressed and control plants; the latter are represented by the zero point of the respective y axes in graphs A and B. Mean values (n = 8) are shown. r. u. . . . relative units.

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its leaves compared to 2023 and drought induced a further elevation of this osmoprotectant content in CE704 (Fig 5D). While the APX activity in the leaves of 2023 increased after 14 days without watering, the reverse was true for CE704 and a similar trend was observed for the CAT activity (Fig 5E and 5F). However, almost none of the described differences in the parameters characterising cell damage were actually statistically significant due to high variability in the samples (S1 File, Table in S2 File). No differences between genotypes or between control and stressed plants were found for the H_2O_2 content (Fig 5C).

Discussion

Endogenous BRs are important regulators of various processes that occur in plants. Several studies have revealed that the contents of endogenous phytosterols (including campesterol and cholesterol which are the main precursors of BR biosynthesis) in plant leaves can significantly differ between genotypes [41, 42]. However, whether these differences also affect the levels of
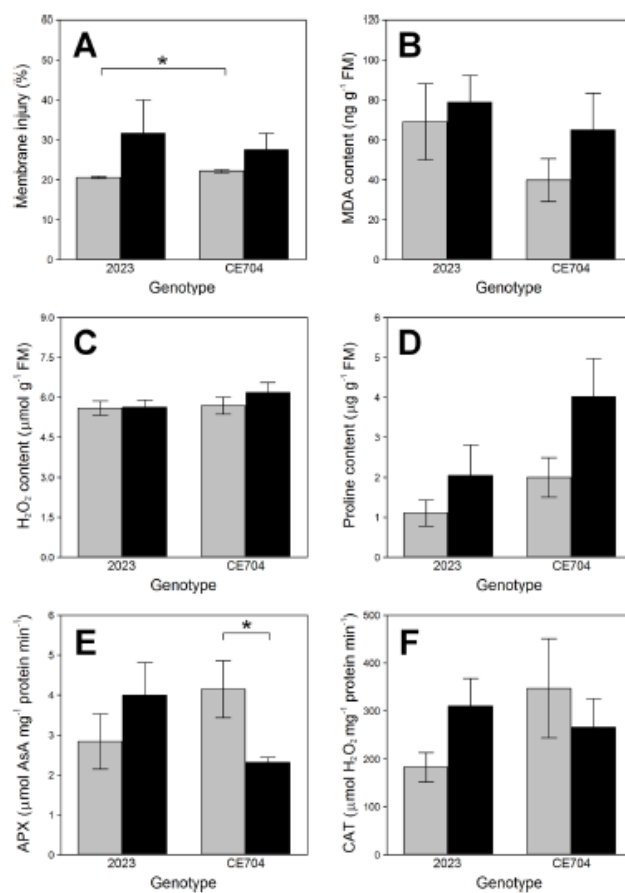
Fig 5. The contents of proline, H_2O_2, malondialdehyde (MDA), the antioxidant activities and the index of membrane injury in two maize genotypes (2023 and CE704). Plants were either subjected to normal watering (control; grey columns) or to 14 days of withholding water (stress; black columns). Mean values ± SEM are shown (n = 6 for the contents of MDA, n = 4 for the other parameters). Asterisks indicate significant ($p \leq 0.05$; *) or highly significant ($p \leq 0.01$; **) differences between mean values according to Tukey's tests made separately for each genotype (in case of the differences between control and stress treatment) or for each treatment (in case of the differences between both genotypes). AsA ... ascorbate, APX ... ascorbate peroxidase, CAT ... catalase, FM ... leaf fresh mass.

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endogenous BRs has not been previously examined. To date, only Pociecha *et al.* reported the existence of genotypic differences in the response of CS levels in the leaves of two winter rye cultivars to cold acclimation [24].

The determination of various endogenous BRs in the leaves of our two maize genotypes with different drought sensitivities provided several interesting results. We found that the presence/content of the individual natural BRs in the leaves may depend on the genotype. The

most abundant BR present in the leaves of our plants was TY (~2–3 pg mg^{-1} FM), followed by norBL (~1–2 pg mg^{-1} FM) and homoCS (~1 pg mg^{-1} FM). The drought-resistant CE704 genotype had particularly higher levels of both TY and norBL compared with the drought-sensitive genotype 2023. The presence of TY was reported previously for maize pollen [43] and the observed levels of TY in the leaves of our plants are mostly in agreement with those reported for the leaves or shoots of several other plant species [44]. However, the norBL content in the CE704 leaves reached 1–2 pg mg^{-1} FM, which might seem to be a rather high level for any BR detected in vegetative tissue, such as leaves [44], since these tissues generally contain significantly lower amounts of phytohormones compared with the reproductive tissues, such as pollen or seeds [45]. Indeed, the norBL level in our drought-resistant CE704 genotype is approximately 15 times higher compared to the norBL level reported for maize leaves by Oklešťková et al. [46]. This seems to be an interesting feature of this particular maize genotype. We are not aware of any previous study that reported the presence of this type of BR in such high quantity in this plant species or even its presence in any other monocot plant.

In addition to TY, norBL and homoCS, we also detected other endogenous BRs substances in smaller quantities, namely, homoDS, norCS, CS and BL. Although the biosynthetic pathways and mutual conversions of the C_{27}, C_{28} and C_{29} BRs are not entirely clear at the present time, there is some evidence that the C_{27} BRs are formed from C_{27} sterol cholesterol while the C_{29} plant steroidal biosynthetic precursor for the production of C_{29} BRs is sitosterol [47]. In the C_{28} BRs group, TY is a precursor of CS which is converted into the biosynthetic end product BL. These reactions are probably unidirectional [48]. The conversion of CS to BL is catalysed by a cytochrome P-450 monooxygenase encoded by the *CYP85A2/3 (BR6ox2)* gene in the dicot plants *Arabidopsis* and tomato [49, 50]. BL plays an important role in the development of reproductive organs in these species, whereas CS probably serves as a regulator of plant vegetative growth [51]. Although BL has already been reported in rice shoots [52], other authors showed that the step of BR biosynthesis involving the conversion of CS to BL does not seem to exist in rice [53, 54]. The same situation seems to apply for other monocot grasses [55, 56]. However, BL was positively detected in leaves of graminaceous plants such as barley and wheat [57–59]. Therefore, the presence of BL and norBL in the leaves of our maize plants is perhaps not as exceptional if we also consider the lower detection limits of the method we used for their determination, compared with those used earlier by other authors. Clearly, monocot plants have either some means to perform the Baeyer-Villiger oxidation of CS to yield BL, or they can produce BL (and convert it to its various metabolites) by completely different reaction(s), although no details of this have been revealed.

Details of BR biosynthetic pathways were identified mostly in *Arabidopsis* and *Catharanthus roseus* [60]. Similar situation applies for BR signaling; an overwhelming majority of our knowledge of this topic comes from the model plant *Arabidopsis* [61]. Information on monocot plants is rather limited; although many aspects of BR biosynthesis and signaling seem to be conserved between dicots and monocots, some differences also exist [62]. Maize has not yet been much investigated in order to identify genes coding for proteins that catalyse individual steps of BR synthesis or participate in BR signaling pathways. To this date, only six such studies exist. The first discovery of a BR biosynthetic gene in maize was made by Tao et al. [63], who found a functional homolog of the *Arabidopsis DWF1* gene (*ZmDWF1*). Its product catalyses the reduction of 24-methylenecholesterol to campesterol at the beginning of the BR biosynthetic pathway and the gene was shown to be expressed particularly in roots. This agreed well with the subsequent studies of Kim et al. [64, 65], who demonstrated that BR biosynthesis in maize indeed occurs in roots. However, another study indicated that the *ZmDWF1* gene is expressed in all maize tissues (at least at the transcriptional level) with the highest degree of expression in the leaf collars and lower expression in older tissues [66]. Contrary to [63], the

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authors of this study also found not only one but two paralogs of *ZmDWF1* gene in the maize genome.

Another BR biosynthetic gene that was established in maize was *ZmDWF4* which codes for C-22 hydroxylase [67]. This rate-limiting enzyme catalyses various hydroxylation events at the beginning of both early and late C-6 oxidation pathways as well as the early C-22 oxidation pathway [60]. A higher level of its transcripts was found in shoots of young maize seedlings than in their roots. The third gene experimentally confirmed to participate in BR biosynthesis in this plant species was the maize homolog of *DET2*, expressed particularly in anthers [68]. Its product is 5α-steroid reductase, functioning mainly in the early C-22 oxidation and the late C-6 oxidation pathways [60]. The final BR biosynthetic gene documented to function in maize was revealed by [55]: *ZmBRD1*, an ortholog of the *BR6ox1* gene. The enzyme produced from this gene catalyses the final steps of early and late C-6 oxidation pathways, *i.e.* the synthesis of CS. Again, the highest expression of this gene was recorded in anthers but its transcripts were at least at some level present in all tissues. In addition to the four known maize BR biosynthetic genes, the gene coding for the BR receptor (*ZmBRI1*) was reported and experimentally confirmed by [69]. Although various additional orthologs of *Arabidopsis* genes known to participate in BR biosynthesis or signaling are present in the maize genome, their role in these processes has yet to be verified and it seems that at least some of them actually participate in other biological processes in this species (Tables in S3 File).

The organ-specific or developmental information on the expression of maize genes known/predicted to be involved in BR biosynthesis or signaling pathways can be easily found in the MaizeGDB (https://www.maizegdb.org/); it is based on the transcription data obtained by [70] and subsequently further updated [71]. Unfortunately, this does not give us any information on the possible changes of the expression of these genes that could be caused by unfavourable environmental conditions such as drought. Indeed, the relationship between BRs and drought at the molecular level is rather ill-defined. Recently, one group of scientists showed that some transcription factors from WRKY and NAC families, which are involved in plant stress responses, either directly interact with or influence the levels of some downstream components of the BR signaling pathway (particularly the transcription factor BES1) in *Arabidopsis* [72–75]. Based on their results, it seems that drought and BR pathways antagonize each other: BR signaling inhibits drought-induced gene expression pathways and *vice versa*. Some authors who worked with BR mutants, transgenic plants or plants treated with exogenous BRs and subjected to drought conditions, assessed the levels of a few specific transcripts presumed to be correlated to plant drought response. These studies were made with *Arabidopsis* [76], rapeseed [76, 77], cucumber [78], tobacco [79], potato [80], *Brachypodium* [81] or barley [82]. However, with the exception of Zhou *et al.*, who found gradual increase in the levels of *CPD* transcripts during the first 24 hours of PEG-induced osmotic stress in potato (and subsequent drop to the original level after additional 24 hours) [80], the expression of BR biosynthetic or signaling genes in drought-stressed plants had not yet been purposefully analysed. The CPD protein catalyses some steps of the early C-22 oxidation and the late C-6 oxidation pathways [60].

We examined various differential gene expression studies available for drought-stressed maize (identified by searching the public databases NCBI GEO DataSets and GEO Profiles (https://www.ncbi.nlm.nih.gov/gds, https://www.ncbi.nlm.nih.gov/geoprofiles/) and the EMBL-EBI Expression Atlas (https://www.ebi.ac.uk/gxa/home) based on the Array Express database (https://www.ebi.ac.uk/arrayexpress/) and found some cases where the expression of maize genes known/predicted to be involved in BR biosynthesis and/or signaling (or maize orthologs of *Arabidopsis* BR genes) changed after the simulated drought (Table in S3 File). Generally, it seems that drought usually reduces the expression of BR biosynthetic genes at the transcriptional level; the maize orthologs of *Arabidopsis* BR biosynthetic genes, which showed

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elevated amounts of transcripts in drought-stressed plants, are probably involved more in the abscisic acid (ABA) or other metabolic pathways then directly in the BR biosynthesis (Tables in S3 File). However, the observed changes evidently depend on the analysed organ (*e.g.*, [83–85]), its developmental stage [84, 85], the intensity/duration of stress [86–87] or a particular maize cultivar [86, 88]. The changes in the expression of genes predicted to be involved in BR signaling are even more variable (Tables in S3 File). Thus, no obvious conclusions on the relationship between the transcription of these genes and drought can be drawn, particularly as true participation of the majority of these genes in BR-signaling pathways of maize has yet to be confirmed.

Moreover, although transcriptomic analyses are rather popular, they can give only a limited picture of gene expression changes. Drought-induced changes of plant cell transcriptome do not have to be reflected in the changes of cell proteome (due to many steps and factors influencing gene expression between RNA and protein levels). Indeed, among studies dealing with drought-induced changes in plant proteome, that also simultaneously analysed the changes of transcriptome [89–96], all (with the exception of [93]) reported that no true correlation between these two sets of gene expression data existed. Our previous analyses of drought-induced changes of leaf proteome performed in the same two maize genotypes we used in our present study did not reveal any significant change in the amounts of any protein known or predicted to be involved in BR biosynthesis or signaling [25, 26]. Nolan *et al.* [97], who examined the regulation of *BES1* expression in *Arabidopsis* plants exposed to drought conditions, found that although no change in *BES1* transcript levels was observed in stressed plants, the levels of the BES1 protein were reduced after drought exposure. Thus, the expression of this component of BR-signaling pathway was regulated at the posttranscriptional level and probably even at the posttranslational level (the stability of the BES1 protein). Lei *et al.* [98] performed a ribosome profiling assay together with a RNAseq-based transcriptome analysis in maize seedlings grown in normal and drought conditions. Several genes involved in BR biosynthesis (*e.g.*, *ZmDWF1* and an ortholog of *Arabidopsis DWF7*) or signaling (*e.g.*, *ZmBRI1* and orthologs of *Arabidopsis BES1, BRH1, BEH3/BEH4*) showed reduced translational efficiency but no changes at the level of their mRNAs [98]. Clearly, the relationships between the transcriptional, translational and post-translational levels of expression of BR-associated genes in plants exposed to drought and their influence on the contents of endogenous BRs and general plant response to this stress factor can be very complex and deserve further examination.

Our experiments with drought-stressed maize plants revealed several changes in the content of endogenous BRs induced by this stress factor. The most conspicuous change was the significant reduction of the homoCS and norBL levels in the drought-resistant CE704 genotype. This differs from the results of Gruszka *et al.* [23], who did not observe any particular drought-induced reduction in the homoCS content in the leaves of their barley plants. On the other hand, they reported that drought specifically induces *epi*BL formation. Interestingly, this drought-specific elevation of the *epi*BL content did not depend on functional BR biosynthesis or signalling, because it was observed in BR mutants as well as in *wild type* plants. They also found rather high CS content that increased with drought, which also does not agree with our observations, as the levels of CS in the leaves of our plants did not change with water deficiency. The difference between our results regarding the changes in the CS content and its derivatives and the results of Gruszka *et al.* [23] could perhaps arise from the evolutionary divergence of the *BR6ox* gene between barley and maize species. Maize has only one homologue of the *BR6ox* gene [55], whereas the barley genome contains two homologs of this gene (*HvDWARF* and *HvBRD*), which are probably partly redundant at least in CS biosynthesis [99]. Whether one of these proteins could still have another function under specific (drought) conditions remains to be seen.

Another possible explanation for the absence of any significant changes of the CS content in our experimental plants could lie in the mutual conversions of the C_{27}, C_{28} and C_{29} BRs. CS can be converted to norCS and, likewise, norBL can be produced from BL. Both these norBRs belong to the C_{27} group and their biosynthesis in *Arabidopsis* and tomato originates from cholesterol [100–103]. However, norCS can also be methylated to yield CS, with DS acting as an intermediate. Reportedly, this conversion occurs also in monocot plants [47, 65]. Furthermore, CS can also be produced from C_{29} BRs such as homoCS or homoDS in rice [47]. Both homoBRs mentioned were also detected at a certain quantity in our maize samples; thus, we dare to say that this pathway operates in maize as another representative of monocot plants.

As the leaves of our drought-sensitive 2023 inbred line showed an elevation of the norCS content whereas the homoDS content was reduced, it is possible that the conversions between these BRs masked an actual inducement of CS biosynthesis by drought in this genotype. Jäger *et al.* [22], who described an increase in the CS content after pea plant exposure to a water deficit, proposed that the induction of CS levels could be correlated with a large enhancement of the ABA content, which occurs under conditions of *severe* drought stress. This applied for their experiment, as well as to the abovementioned experiments of Gruszka *et al.* [23], who also observed a substantial increase in the levels of ABA in their drought-exposed barley. The drought-sensitive 2023 clearly suffered from drought more than CE704, as evident, from the more pronounced reduction of its biomass and photosynthetic efficiency, and suggested by some other parameters correlated to cell damage and antioxidative protection. It could be thus experiencing symptoms of more severe drought stress, contrary to CE704. The different degree of drought sensitivity of both genotypes could also explain why we did not find similar changes in the norCS and homoDS contents in the CE704 genotype, because this genotype was much less stressed by water deficiency.

Our CE704 genotype has one interesting trait: it can keep the stomata opened (at least to some degree) when watering ceases. We have previously shown [25, 26] that this ability enables it to maintain a sufficiently high photosynthetic rate during the *early* stage of drought response, which results in the unimpeded production of the energetically rich compounds necessary for proteosynthesis. This early acclimation mechanism, together with some other aspects of CE704 physiology and biochemistry (regarding, *e.g.*, the higher proline content), enables it to better counteract the negative consequences of drought stress during the later, more severe phases [25, 26]. BRs are known to indirectly regulate stomatal function in a concentration- and species-dependent manner [104, 105]. While low concentrations of exogenously applied BRs do not affect stomatal movements, higher BR concentrations can operate in two opposite ways. They either induce stomatal closure [105, 106] or, conversely, facilitate the opening of the stomata whose closure was induced by ABA [107] or impaired by BR deficiency [108]. It is thus possible that the observed differences in the endogenous BR levels in the leaves of our two maize genotypes could perhaps be somehow connected to the behaviour of their stomata. A further analysis of this phenomenon is certainly needed.

Conclusions

This is the first study investigating the endogenous BR content in maize genotypes differing in their drought sensitivity. We found that TY together with norBL and homoCS are the main representatives of this group of phytohormones in maize leaves. Importantly, this is also the first report of presence of a rather high quantity of norBL in a monocot plant. We also revealed for the first time that drought-resistant and drought-sensitive maize genotypes differ in the presence/contents of individual, naturally-occurring BRs. The observed differences between both genotypes in the endogenous BR content are probably correlated with their different degrees of drought sensitivity, which was demonstrated at the levels of plant morphology,

physiology and biochemistry. We confirmed our original hypothesis that the drought-resistant genotype displays higher levels of endogenous BRs compared with the sensitive genotype already under non-stress conditions. Indeed, this could be one of the reasons for its higher drought resistance. Our second hypothesis suggested that the resistant genotype should not need to elevate its endogenous BR contents when subjected to water deficiency, because it would not experience stress to such a degree as the drought-sensitive genotype, which was also supported by our data.

Supporting information

S1 Table. Selected photosynthetic parameters of the JIP test derived from the measurements of the polyphasic rise of chlorophyll *a* fluorescence. F_0—the initial fluorescence intensity (at 50 μs), F_K—the fluorescence intensity at the K-step (300 μs), F_J—the fluorescence intensity at the J-step (at 2 ms), F_I—the fluorescence intensity at the I-step (at 30 ms), $F_M \approx F_P$—the maximum fluorescence intensity, Area—area between the fluorescence curve and F_M, PSI—photosystem I, PSII—photosystem II, RC—reaction centre.
(DOCX)

S1 File. The original data on the brassinosteroid contents, plant morphology and various physiological and biochemical characteristics measured in two maize genotypes (2023 and CE704). Plants were either subjected to normal watering (control) or to 14 days of withholding water (stress).
(XLSX)

S2 File. Tables with the results of the two-way ANOVA and Tukey's tests applied to various physiological and biochemical parameters measured in two maize genotypes (2023 and CE704). Plants were either subjected to normal watering (control) or to 14 days of withholding water (stress).
(DOCX)

S3 File. Tables with the main results and experimental aspects of the transcriptome studies performed with drought-stressed maize, which showed differential expression of genes involved in brassinosteroid biosynthesis, catabolism/homeostasis or signaling.
(DOCX)

S1 Fig. Phenotypic representation of two maize genotypes, 2023 (A, B) and CE704 (C, D). Plants were subjected either to normal watering (control; **A, C**) or to 14 days of withholding water (stress; **B, D**).
(TIF)

Acknowledgments

Author Contributions

Conceptualization: Lenka Tůmová, Marie Kočová, Dana Holá.

Data curation: Lenka Tůmová, Dana Holá.

Formal analysis: Lenka Tůmová, Dana Holá.

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ARTICLE 13

Contents of endogenous brassinosteroids and the response to drought and/or exogenously applied 24-*epi*brassinolide in two different maize leaves

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Contribution: analysis of brassinosteroids, manuscript writing

Contents of endogenous brassinosteroids and the response to drought and/or exogenously applied 24-epibrassinolide in two different maize leaves

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Exogenously applied brassinosteroids (BRs) improve plant response to drought. However, many important aspects of this process, such as the potential differences caused by different developmental stages of analyzed organs at the beginning of drought, or by BR application before or during drought, remain still unexplored. The same applies for the response of different endogenous BRs belonging to the C_{27}, C_{28}- and C_{29}- structural groups to drought and/or exogenous BRs. This study examines the physiological response of two different leaves (younger and older) of maize plants exposed to drought and treated with 24-epibrassinolide (epiBL), together with the contents of several C_{27}, C_{28}- and C_{29}-BRs. Two timepoints of epiBL application (prior to and during drought) were utilized to ascertain how this could affect plant drought response and the contents of endogenous BRs. Marked differences in the contents of individual BRs between younger and older maize leaves were found: the younger leaves diverted their BR biosynthesis from C_{28}-BRs to C_{29}-BRs, probably at the very early biosynthetic steps, as the levels of C_{28}-BR precursors were very low in these leaves, whereas C_{29}-BR levels vere extremely high. Drought also apparently negatively affected contents of C_{28}-BRs (particularly in the older leaves) and C_{29}-BRs (particularly in the younger leaves) but not C_{27}-BRs. The response of these two types of leaves to the combination of drought exposure and the application of exogenous epiBL differed in some aspects. The older leaves showed accelerated senescence under such conditions reflected in their reduced chlorophyll content and diminished efficiency of the primary photosynthetic processes. In contrast, the younger leaves of well-watered plants showed at first a reduction of proline levels in response to epiBL treatment, whereas in drought-stressed, epiBL pre-treated plants they were subsequently characterized by elevated amounts of proline. The contents of C_{29}- and C_{27}-BRs in plants treated with exogenous

*epi*BL depended on the length of time between this treatment and the BR analysis regardless of plant water supply; they were more pronounced in plants subjected to the later *epi*BL treatment. The application of *epi*BL before or during drought did not result in any differences of plant response to this stressor.

1 Introduction

Drought is one of the most significant stressors affecting agricultural production worldwide; thus, it is crucial to alleviate its negative effect on plants. Various approaches to achieve this are possible. Aside from classical breeding and genetic engineering, the treatment of plants with chemical compounds such as antioxidants, phytohormones, polyethylene glycol, *etc.*, has also been reportedly tried. One group of these compounds are brassinosteroids (BRs): steroid phytohormones showing a wide range of functions in the regulation of plant growth and development and significantly participating in plant defense against diverse environmental stressors (Ahammed et al., 2022).

More than 170 studies focused on BRs and drought stress have been published during the past three decades. Most of these studies were performed with plants treated with exogenous BRs, although mutants in genes associated with BR synthesis or signaling have also been utilized (for review see, *e.g.*, Holá, 2019; Sidhu and Bali, 2022). However, the results of these studies are so variable that it is still impossible to draw a clear conclusion about the role of BRs in plant response to drought. To demonstrate this, let us randomly choose just two parameters strongly associated with plant drought response: *i)* catalase activity (CAT; an important enzymatic antioxidant) and *ii)* the proline content (important osmoprotectant as well as a non-enzymatic antioxidant). Chandrasekaran et al. (2017) and Sivakumar et al. (2017) reported a reduced CAT activity in drought-stressed BR-treated tomato plants compared to non-treated ones. Completely opposite results were published in some previous analyses performed with the same species (Behnamnia et al., 2009; Yuan et al., 2010). Similarly, in several studies made with various drought-stressed plant species, reduced levels of proline were observed after BR treatment (Li et al., 2012; Gursude et al., 2014; Ahmed et al., 2017; Hemmati et al., 2018). Other studies on this topic, where proline was also analyzed, reported its increased levels in drought-stressed BR-treated plants (*e.g.*, Zhu et al., 2014; Shahana et al., 2015; Younesian et al., 2017; Lv et al., 2020).

The above-mentioned variability of the data on the relationship between BRs and plant drought response results from many factors such as the inter- or intra-species differences, the developmental stage of analyzed plants/organs, duration of the drought period, the method utilized for the drought simulation, the BR type and concentration, the method of plant treatment with BRs, timepoints of BR application, *etc.* (for more details see Holá, 2019). Unfortunately, studies that would purposely focus on the effects these possible sources of variability can have on plant responses to BRs are very rare. Thus, we decided to focus on two following aspects in order to ascertain how they could affect plant responses to BRs: *i)* to analyze the response of two different leaves (already developed and still developing ones at the beginning of drought period) of plants exposed to drought and treated with BRs, and *ii)* to compare two timepoints of BR application (prior to drought and during drought).

There is extremely little knowledge on the possible different response of leaves already developed before drought compared to leaves developing during this abiotic stress to the treatment with exogenous BRs. So far, only Gomes et al. (2013) examined some photosynthetic parameters in three different leaves of papaya stressed by drought and treated with BRs. They reported that BR application led to slightly more pronounced drought-induced degradation of chlorophyll (Chl) in the older leaves compared to the younger ones. On the other hand, Wang et al. (2015) compared the stomatal density, width and length in BR-treated drought-stressed young and mature leaves of grapevine plants and found that BR treatment significantly affected these parameters only in the young leaves. This seems to indicate that fully developed leaves could indeed respond to the combination of drought and BR treatment in a different manner than leaves that are still developing. However, these differences could depend on both plant species and/or the respective evaluated parameter.

Similarly, there is very little knowledge on the potentially different impact of BR treatment before or during drought. If we compare the results of studies dealing with BR application only before the stress period with studies where BRs were applied only during the stress period, we can perhaps discern slightly different responses of plants to these treatments. For example, Behnamnia et al. (2009) sprayed tomato plants before the onset of drought and found mostly increased activities of several antioxidant enzymes including CAT in their drought-stressed BR-treated plants. On the other hand, Sivakumar et al. (2017) treated plants of the same species with BRs during stress and in this case, the CAT activity decreased. However, due to the high variability of many different aspects of experimental setups in BR/drought studies, it is very

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complicated to ascertain whether there truly could be some significant differences between these two main potential timepoints of BR application. So far, only two groups of authors applied BRs at these timepoints in the same study in order to purposely compare the respective effects. Unfortunately, in both cases, a different type of BR application for each timepoint was used. Hashemi et al. (2015) compared the effect of seed priming before drought and leaf spraying during the stress period. They found almost no difference between these two approaches/timepoints, but each method required a different BR concentration for the maximum effect. In an earlier experiment by Farooq et al. (2009), no difference between the effect of seed soaking prior to drought and leaf spraying during the drought period was observed.

In addition to the two above-mentioned aims of our study, we further wanted to examine the effect of drought per se and in combination with the exogenous application of BRs on their endogenous levels in leaves (and roots). Our focus was again on the possible differences caused by the leaf age/development before or during the drought period. Several studies revealed that plant exposure to drought can cause changes in BR levels in pea (Jager et al., 2008), soybean (Janeczko et al., 2011), rice (Ding et al., 2014; Zhang et al., 2020; Zhang et al., 2022b), barley (Gruszka et al., 2016; Malaga et al., 2020), tobacco (Duan et al., 2017), foxtail millet (Tang et al., 2017), Chinese cabbage, white cabbage and kale (Pavlović et al., 2018), maize (Tůmová et al., 2018), Kentucky bluegrass (Chen et al., 2019) and tomato (Nie et al., 2019). However, different plant species showed a rather varied response in this respect and the majority of these studies analyzed either only the contents of total BRs or the contents of the two most biologically active BRs, i.e., castasterone (CS) and/or brassinolide (BL). No other member of big BR family was studied. Regarding the effects of the exogenous BR application on the endogenous BR levels, all studies dealing with the such topic have been made under non-stress conditions (with the exception of Efimova et al., 2014, who studied salinity stress) and focused again mostly on the total BR content (Mao et al., 2017; Fu et al., 2019; Nie et al., 2019; Setsungnern et al., 2019; Chen et al., 2021; Zhang et al., 2021; Liu et al., 2022). Beside CS and BL, some authors also determined the contents of some C_{27}- and C_{29}-BRs (Janeczko and Swaczynová, 2010; Janeczko et al., 2010; Janeczko et al., 2011; Filek et al., 2019; Tarkowská et al., 2020) and more detailed analyses including some BR biosynthetic precursors were performed by Bajguz et al. (2019) and Chmur and Bajguz (2021). However, all these studies were made with plants under non-stress conditions. To our knowledge, no data on the possible changes in the contents of various individual endogenous BRs or their precursors, induced by the treatment of plants with exogenously applied BRs under drought conditions, are available at this time.

Thus, the purpose of our study can be summarized into several main objectives. Firstly, we wanted to examine potential differences between the response of younger and older leaves to exogenous BR application, both in drought conditions and conditions of sufficient water supply (we hypothesized that the older leaves, as well as the leaves of stressed plants, should probably show a more pronounced response, because BRs generally seem to function particularly under suboptimal conditions). The second objective was comparison of the effect of BR

application before or during drought period (our hypothesis here was that the application during drought could affect plants more strongly, because they were already stressed and BRs could thus immediately show their anti-stress effects). The third objective consisted in the evaluation of the general effect that plant treatment with 24-epiBL has on the portfolio/contents of various BRs belonging to three different structural groups, and the potential differences in this respects caused by the length of time between exogenous BR application and endogenous BR determination (we hypothesized that the effects of exogenous BRs will probably diminish with time and that plants treated with 24-epiBL, a representative of C_{28}-BRs, could probably divert their BR biosynthesis into C_{29}- or C_{27}-BR biosynthetic pathways). Finally, we also wanted to examine whether the contents of individual BRs differ between younger and older leaves (and roots) and whether these contents change due to drought treatment (we expected a positive answer but could not predict the nature of these differences and/or changes).

2 Materials and methods

2.1 Plant material and cultivation conditions

The drought-sensitive maize (Zea mays L.) inbred line 2023 (Benešová et al., 2012) from the CEZEA Maize Breeding Station (Čejč, Czech Republic) was used for the experiments. Plants were grown in plastic pots (15 cm diameter, 23 cm height; 1 plant per pot) filled with the mix (10:1) of garden soil (Garden Compost, Agro CS, Czech Republic) and sand (Spielsand Sahara sand, WECO, Germany) at the greenhouse facility of the Faculty of Science, Charles Univerzity, Prague, the Czech Republic, 50°04' N, 14°25' E, 238 m above the sea level) under semi-controlled conditions during the spring season (April, May). The conditions in the greenhouse were: the average temperature of 25/20°C, the average relative air humidity of 60/80% day/night, natural irradiance, and watering of plants with tap water as necessary. Moderate drought stress was simulated by the cessation of watering starting at the day 35 after the date of sowing and maintained for two weeks.

The first measurements/samplings (Timepoint 1) were made from the 32 d-old plants (at this time all plants had three fully developed leaves). The second measurement/sampling point (Timepoint 2) was executed after additional 3 d when the drought simulation started. Timepoint 3 occurred after 7 d of drought and the last samplings/measurements (Timepoint 4) were at the end of the drought period. All morphological, physiological, and biochemical parameters were assessed during these four timepoints (with the exception of the determination of endogenous BR contents, which was done only at Timepoint 4). The volumetric soil water content was 24.1% at Timepoint 1, 22.6% at Timepoint 2, 22.5% for the normally-watered plants and 13.8% for stressed plants at Timepoint 3, and 21.8% for normally-watered plants and 9.3% for stressed plants at Timepoint 4.

Separate plants were used for i) plant morphology assessment (16 biologic replicates), ii) determination of the relative water content (RWC), photosynthetic pigments contents and Chl fluorescence measurements (8 biologic replicates); iii) the

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membrane damage index (MDI) (6 biologic replicates); *iv*) gas exchange measurements (6 biologic replicates); *v*) determination of the malondialdehyde (MDA) content (8-9 biologic replicates); *vi*) determination of the proline content (9 biologic replicates); and *vii*) determination of the BR contents (3-4 biologic replicates). In all cases, the 3rd (*i.e.*, fully developed at the beginning of drought) or the 4th (*i.e.*, developing during drought) leaves were used for the physiological/biochemical analyses. In addition, the BR contents were also determined in the roots.

2.2 BR treatments

The 10^{-6} M aqueous solution of 24-*epi*brassinolide (*epi*BL; (22R,23R,24R)-2α,3α,22,23-tetrahydroxy-24-methyl-7-oxa-7-homo-5α-cholestan-6-one; *Sigma-Aldrich-Merck*) used for treatments was prepared from the 10^{-4} M stock solution containing distilled water:96% ethanol (10:1; ethanol was used for the dissolution of *epi*BL and water was then gradually added with continuous stirring till the stock solution of fully dissolved *epi*BL was achieved) and contained also 0.05% of nonionic detergent Tween® 20. The corresponding control solution (C) had the same composition except *epi*BL. The concentration of *epi*BL was chosen based on our previous experiments (unpublished data). One group of plants was sprayed with the *epi*BL solution at Timepoint 1 (the BR1 variants, application prior to drought), another group of plants was sprayed at Timepoint 3 (the BR2 variants, application during drought). The respective samplings/measurements at these timepoints were always done before the *epi*BL treatment. In all cases, the whole aboveground part of plants was always sprayed (the amount of solution applied per plant was approximately 10 ml). Thus, at the end of the experiments (Timepoint 4), six experimental variants were available: three originated from plants that were well-watered during the whole experiment, and another three were from plants exposed to drought simulation.

2.3 Plant development and morphology

Plant development was monitored by counting the number of fully developed leaves in all plants of the respective variants throughout the whole experiment. The dry masses of the shoot (DMS) and roots (DMR) together with the plant height (measured from the surface of the soil in the pots to the youngest fully developed leaf node) were also assessed.

2.4 RWC and gas exchange

The RWC was evaluated by a standard method described in Čatský (1960). A small piece (approx. 3-4 cm^2) was cut from the respective leaf and immediately weighed (FW). Then it was put into distilled water and left saturating for 5 hours. At that moment the saturated weight (SW) was obtained and the leaf piece was left to dry completely at 80°C and again weighed (DW). The RWC was calculated as (FW-DW)/(SW-DW).

The net transpiration rate (E), the stomatal conductance (g_S) and the net photosynthetic rate (P_N) were determined by gasometric measurements using the portable LCpro+ device (*ADC BioScientific, Hoddesdon, UK*) with the following conditions in the measuring chamber: the temperature 25°C, the ambient CO_2 concentration 550 ± 50 μL L^{-1}, the airflow rate 205 ± 30 μmol s^{-1}, irradiance 650 μmol m^{-2} s^{-1} of photosynthetically active radiation. These measurements were performed between 9:00 and 12:00 AM, Central European Time, at the middle part of the respective leaves.

2.5 Photosynthetic pigments contents and Chl fluorescence (OJIP) analysis

Six small discs (0.6 cm^2) were cut from the middle part of the leaf blade and incubated for 7 days in 5 mL of *N,N*-dimethylformamide at 4°C in the dark (the extracts were stirred several times during this period). After extraction, the absorbances at 480, 664, 647 and 710 nm were measured spectrophotometrically. The contents of Chl *a*, *b* and total carotenoids (Car) were evaluated using the formulae of Wellburn (1994).

Chl fluorescence was measured on the top side of dark-adapted (20 min) leaves with the portable fluorometer FluorPen FP100max (*Photon System Instruments, Brno, Czech Republic*) between 8:30 and 9:00 AM, Central European Time. The measurements were started with a saturating pulse (blue light, 455 nm, 3000 μmol m^{-2} s^{-1}). After that the Chl fluorescence transient was recorded at a time scale from 10 μs to 2 ms, representing the so-called OJIP curve. Fluorescence values F_0 (the initial fluorescence intensity recorded at 40 μs), F_K (the fluorescence intensity at the K-step of the OJIP curve, 300 μs), F_J (the fluorescence intensity at the J-step of the OJIP curve, 2 ms), F_I (the fluorescence intensity at the I-step of the OJIP curve, 30 ms), and $F_M = F_P$ (the maximum fluorescence intensity) were used for the calculations of various parameters of the JIP test according to Strasser et al. (2000) and Stirbet and Govindjee (2011). These parameters can be utilized for the description of the performance of various steps of the photosynthetic electron transport chain (PETC; see Supplementary Table 1 for their definitions and formulae).

To obtain further information on the primary photosynthetic processes in various *epi*BL-treated/control or stressed/well-watered experimental variants, the normalizations of chlorophyll fluorescence transients leading to calculations of relative variable fluorescences W_{OI}, W_{OJ} and W_{OK} were performed according to Yusuf et al. (2010). The positions/amplitudes of the W_{OI} curves after the I-step can inform about the size of the pool of end electron acceptors in the PETC after Photosystem (PS) I (the lower positions reflect the lower size of this pool). W_{OJ} and W_{OK} serve for further calculations of so-called difference kinetics (ΔW), which are always based on comparisons of some treatment *versus* the respective control. In our case they were based either on the comparison of *epi*BL-treated (BR1, BR2) and control (C) plants subjected to the same watering conditions, or on the comparison of non-watered and well-watered plants subjected to the same type of treatment. The difference kinetics ΔW_{OJ} and ΔW_{OK} enabled us to visualize K- and L-bands of OJIP curves, respectively. If the respective K-band

showed a negative amplitude, the state of the oxygen-evolving complex (OEC) of PSII was more active in the BR1/BR2 variants than in C plants (or, alternatively, in the non-watered plants than in the well-watered ones), if it showed a positive amplitude, the reverse was true. The position/amplitude of the L-band which yields information on the energetic connectivity among individual PSII units in the PETC of compared variants, can be interpreted in a similar way (Yusuf et al., 2010).

2.6 Membrane damage and proline content

The MDI was determined according to Sullivan (1972). Twelve small discs (0.6 cm^2) were cut from the middle part of the leaf blade and incubated in distilled water for 24 h at 4°C. After adjustment to the room temperature, the electrical conductances of the samples were measured using the Gryf 158 conductometer (*Gryf HB spol. s.r.o., Czech Republic*). The samples were then boiled in a water bath for 15 min, again adjusted to room temperature and the conductances were measured again. The MDI was calculated as the ratio of the conductance values before and after boiling.

To determine the MDA content, a modified method by Hodges et al. (1999) was utilized. 0.2 g of leaf tissue were ground in liquid nitrogen and then homogenized in 80% ethanol and centrifuged at $14000 \times g$ and 4°C for 20 min. The supernatant was added separately to the thiobarbituric acid (TBE) reaction mixture (TBA+; 0.65% TBA, 20% trichloroacetic acid and 0.01% butylated hydroxytoluene) and the TBA- reaction mixture (20% trichloroacetic acid and 0.01% butylated hydroxytoluene). In the next step, the samples with TBA+/- reaction mixtures were tempered at 95°C for 30 min. After cooling to room temperature, the samples were centrifuged again ($14000 \times g$, 4°C, 20 min). The absorbance of the supernatant was measured at 440 nm, 532 nm and 600 nm. The MDA content was calculated according to Hodges et al. (1999).

The proline content was determined spectrophotometrically according to Bates et al. (1973). The frozen samples were homogenized in 3% aqueous sulfosalicylic acid and treated with acid-ninhydrin and acetic acid. This reaction mixture was boiled in a water bath for 30 min and then cooled on ice. 3 mL of toluene were added to the cooled samples. After phase stabilization (approximately 20 min at room temperature), the absorbance of the toluene phase was measured at 520 nm and the proline content was calculated based on its calibration curve.

2.7 BR contents

Contents of various BRs were determined after their extraction and purification using ultra-high performance liquid chromatography (UHPLC) followed by mass spectrometry ((+) ESI-MS/MS) analysis according to Tarkowská et al. (2016).

Frozen maize tissue samples of 50 mg FW were homogenized to a fine consistency using 2.8-mm zirconium oxide beads (*Retsch GmbH & Co. KG, Haan, Germany*) and a MM 400 vibration mill (*Retsch GmbH & Co. KG, Haan, Germany*) at a frequency of 27 Hz

for 3 min. The samples were then extracted overnight with stirring at 4°C using a benchtop laboratory rotator Stuart SB3 (*Bibby Scientific Ltd., Staffordshire, UK*) after adding 1 mL ice-cold 60% acetonitrile and a mixture of stable isotope internal standards (*OlChemIm Ltd., Olomouc, Czech Republic*) including [^{2}H$_3$]BL, [^{2}H$_3$]CS, [^{2}H$_3$]24-*epi*BL, [^{2}H$_3$]24-*epi*CS, [^{2}H$_3$]28-norBL, [^{2}H$_3$]28-norCS, [^{2}H$_3$]TY, [^{2}H$_3$]campestanol, [^{2}H$_3$]campesterol, [^{2}H$_3$]6-deoxocathasterone, [^{2}H$_3$]6-deoxotyphasterol and [^{2}H$_3$]6-oxocampestanol. The samples were further centrifuged, purified on polyamide SPE columns (*Supelco, Bellefonte, PA, USA*) and then analyzed by UHPLC-MS/MS (*Micromass, Manchester, UK*). The data were analyzed using Masslynx 4.2 software (*Waters, Milford, MA, USA*) and the BR contents were finally quantified by the standard isotope-dilution method (Rittenberg and Foster, 1940).

2.8 Statistical analysis

The original data are shown in Supplementary File 1. Mean values and standard deviations (SD) were calculated for all parameters. The data were first subjected to Welch's ANOVA. Where appropriate, the pairwise differences between experimental variants were analyzed using Welch's *t*-tests, for multiple comparisons, Games-Howell *post hoc* tests were applied. The data from the 3rd and 4th leaves were statistically analyzed separately because it was not technically possible to determine BR contents (or some of the evaluated biochemical parameters) in all sample variants (3rd and 4th leaves, roots) together in one run and we did not want to introduce artificial differences to our data that could be actually caused by the different analytical runs.

3 Results

3.1 Timepoint 1

The data from Timepoint 1 are shown in Supplementary File 1; they were obtained only in order to characterize basal levels of plant performance prior to any *epi*BL treatment or stress induction. Thus, there is no point in their presentation here or in the subsequent discussion.

3.2 Timepoint 2 (no drought, early epiBL treatment)

The BR1 treatment significantly reduced levels of proline in the 4th leaves whereas in the 3rd leaves this treatment had no effect (Supplementary Table 2). Slight OEC inactivation and reduction of the energetic connectivity among PSII complexes (inferred from the positions of the ΔW_{OJ} or ΔW_{OK} curves above zero), as well as moderate reduction in the pool size of end electron acceptors in the PETC (inferred from the relative positions of the respective W_{OI} curves) in the 3rd leaves of the *epi*BL-treated plants (but not in the 4th leaves), was suggested by the graphical analysis of Chl fluorescence curves (Figure 1, Supplementary Figure 1). Aside from this, plants of

the BR1 and C variants did not significantly differ in any other parameter at this timepoint (Supplementary Table 2).

3.3 Timepoint 3 (drought, early epiBL treatment)

One week of drought stress significantly reduced the plant height in comparison to the well-watered plants, as well as the DMS of the *epi*BL-treated plants (Table 1). RWC, E, P_N and g_s values also significantly decreased due to drought (Table 1; for RWC, this decrease was statistically significant only in the 3rd leaves of plants treated with the control solution, for the gas exchange parameters, it was significant only in the 4th leaves of both *epi*BL-treated and control plants and in the 3rd leaves of the *epi*BL-treated plants). Similar drought-induced reduction was observed for the contents of photosynthetic pigments in the 3rd leaves; this was more pronounced in the control plants compared to the *epi*BL-treated ones (Table 1). One week of drought led also to an increase of the MDI values in the leaves of the control plants and the proline content in the leaves of both control and *epi*BL-treated plants, but

not to any significant changes in the MDA content (Table 1). The efficiency of the PETC did not seem to be particularly affected by drought at this timepoint (Figure 2, Supplementary Figure 2); the exceptions being mostly the parameters related to the electron transport after PSII (φ_{RE01}, ψ_{RE01}, δ_{RE01}, RE_{01}/RC, PI_{TOTAL}) in the 4th leaves of the *epi*BL-treated plants (Table 1).

The early *epi*BL treatment had no statistically significant effect on the values of most parameters evaluated in plants subjected to either well-watered or drought conditions, with the exception of the DMS which was greater in the well-watered BR1 plants compared to the respective C variant. Additionally, their 4th leaves were again characterized by significantly lower contents of proline. They also showed higher values of the JIP test parameters related to the electron transport after PSII (Table 1) and had a greater size of the pool of end electron acceptors in the PETC (Figure 2); however, this did not apply to the 3rd leaves of these plants or to the drought-stressed *epi*BL-treated plants (Figure 2, Supplementary Figure 2). The inactivation of the OEC in the 4th leaves of the *epi*BL-treated well-watered plants appeared at this timepoint as well, although to a less extent than in the 3rd leaves at the Timepoint 2. However, in the drought-stressed plants, the *epi*BL treatment positively affected the

FIGURE 1
Graphical analysis of the OJIP chlorophyll fluorescence data measured in maize 3rd leaves at Timepoint 2. Plants were well-watered and either treated with 24-epibrassinolide (the BR1 variant) or with water (the C variant). The OJIP curve (A), the relative variable fluorescence W_{OJ} (B) and the difference kinetics ΔW_{OJ} (C) and ΔW_{OK} (D) are shown. Only the part between the J and P points of the OJIP curve is shown for the W_{OJ}. ΔW_{OJ} reveals the K-band; ΔW_{OK} reveals the L-band and were calculated from the comparison of BR1 and C variants; the latter are represented by the zero point of the respective y axes in graphs in panels (C, D). Mean values (n = 8) are shown. a.u., alternative units.

TABLE 1 Selected morphological, physiological and biochemical parameters measured at Timepoint 3 in maize leaves.

| | 3rd leaf (or the whole plant *) | | | | 4th leaf | | | |
| | Well-watered plants | | Drought-stressed plants | | Well-watered plants | | Drought-stressed plants | |
	C	BR1	C	BR1	C	BR1	C	BR1
Plant height (mm) *	190.86 ± 11.16	205.88 ± 29.79	177.20 ± 14.62	166.63 ± 21.45				
Number of leaves *	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00				
DMS (g) *	2.05 ± 0.30	2.56 ± 0.64	1.87 ± 0.50	2.00 ± 0.46				
DMR (g) *	0.66 ± 0.15	0.70 ± 0.15	0.63 ± 0.10	0.64 ± 0.15				
RWC (%)	98.42 ± 0.79	98.39 ± 1.08	96.47 ± 1.41	97.37 ± 0.95	97.84 ± 0.62	97.19 ± 1.26	92.53 ± 10.79	94.22 ± 3.06
E (mmol H_2O m^{-2} s^{-1})	2.09 ± 0.77	1.87 ± 0.43	1.57 ± 0.21	1.53 ± 0.36	2.02 ± 0.52	2.00 ± 0.71	1.33 ± 0.17	1.17 ± 0.60
g_s (mol m^{-2} s^{-1})	0.13 ± 0.02	0.14 ± 0.02	0.10 ± 0.04	0.09 ± 0.03	0.10 ± 0.03	0.10 ± 0.03	0.05 ± 0.02	0.05 ± 0.03
P_N (μmol CO_2 m^{-2} s^{-1})	21.29 ± 1.23	20.66 ± 1.37	16.85 ± 4.85	16.04 ± 4.25	20.20 ± 3.26	21.69 ± 2.39	12.29 ± 5.37	12.37 ± 7.13
Chl a content (g kg^{-1})	17.86 ± 0.97	17.71 ± 0.90	15.97 ± 1.08	16.03 ± 0.72	15.89 ± 0.76	15.26 ± 1.28	15.16 ± 1.14	14.54 ± 1.14
Chl b content (g kg^{-1})	4.91 ± 0.26	4.77 ± 0.49	4.41 ± 0.33	4.12 ± 1.05	4.57 ± 0.30	4.34 ± 0.31	4.27 ± 0.34	4.10 ± 0.14
Car content (g kg^{-1})	3.29 ± 0.20	3.12 ± 0.21	2.92 ± 0.14	2.93 ± 0.15	2.78 ± 0.13	2.75 ± 0.10	2.68 ± 0.18	2.63 ± 0.18
MDI (%)	30.46 ± 2.60	30.76 ± 1.82	28.70 ± 1.95	29.12 ± 0.83	31.21 ± 2.08	31.45 ± 1.06	34.31 ± 2.14	32.46 ± 1.66
MDA content (nmol g^{-1})	19.59 ± 12.79	12.38 ± 10.35	16.84 ± 13.95	13.40 ± 5.37	23.34 ± 6.22	30.82 ± 11.42	32.32 ± 17.46	26.69 ± 7.25
Proline content (mg g^{-1})	25.59 ± 4.10	26.46 ± 4.46	58.57 ± 33.12	49.81 ± 16.21	58.81 ± 12.88	47.25 ± 9.49	448.30 ± 129.32	547.49 ± 191.93
φ_{Po}	0.78 ± 0.02	0.78 ± 0.03	0.77 ± 0.02	0.77 ± 0.01	0.79 ± 0.01	0.77 ± 0.04	0.77 ± 0.02	0.78 ± 0.01
φ_{Eo}	1.41 ± 0.03	1.41 ± 0.05	1.40 ± 0.06	1.41 ± 0.01	1.39 ± 0.03	1.38 ± 0.04	1.38 ± 0.03	1.38 ± 0.03
φ_{RE01}	0.22 ± 0.02	0.22 ± 0.03	0.21 ± 0.03	0.21 ± 0.02	0.25 ± 0.01	0.27 ± 0.03	0.25 ± 0.03	0.24 ± 0.02
φ_{Do}	0.22 ± 0.02	0.22 ± 0.02	0.23 ± 0.02	0.23 ± 0.01	0.21 ± 0.01	0.23 ± 0.04	0.23 ± 0.02	0.22 ± 0.01
ψ_{Eo}	0.55 ± 0.01	0.55 ± 0.01	0.55 ± 0.01	0.55 ± 0.01	0.57 ± 0.01	0.55 ± 0.03	0.56 ± 0.02	0.56 ± 0.01
ψ_{RE01}	0.29 ± 0.02	0.28 ± 0.04	0.27 ± 0.04	0.27 ± 0.02	0.31 ± 0.01	0.36 ± 0.05	0.33 ± 0.04	0.31 ± 0.02
δ_{RE01}	0.52 ± 0.04	0.50 ± 0.07	0.49 ± 0.07	0.49 ± 0.04	0.55 ± 0.02	0.64 ± 0.11	0.60 ± 0.09	0.56 ± 0.04
$\gamma RC2$	0.56 ± 0.01	0.56 ± 0.02	0.56 ± 0.01	0.56 ± 0.01	0.58 ± 0.01	0.57 ± 0.02	0.57 ± 0.02	0.58 ± 0.01
ABS/RC	0.80 ± 0.04	0.77 ± 0.05	0.79 ± 0.03	0.79 ± 0.04	0.72 ± 0.03	0.76 ± 0.08	0.75 ± 0.05	0.74 ± 0.02
TP_0/RC	0.61 ± 0.03	0.60 ± 0.02	0.61 ± 0.01	0.61 ± 0.03	0.57 ± 0.02	0.58 ± 0.03	0.58 ± 0.03	0.57 ± 0.01
ET_0/RC	0.34 ± 0.01	0.33 ± 0.01	0.34 ± 0.01	0.34 ± 0.01	0.32 ± 0.02	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01
RE_{01}/RC	2.16 ± 0.23	2.20 ± 0.37	2.29 ± 0.41	2.28 ± 0.25	1.82 ± 0.09	1.65 ± 0.17	1.76 ± 0.19	1.84 ± 0.14
DI_0/RC	0.18 ± 0.02	0.17 ± 0.03	0.18 ± 0.01	0.18 ± 0.02	0.16 ± 0.01	0.18 ± 0.05	0.18 ± 0.03	0.16 ± 0.01
PI_{ABS}	5.52 ± 0.88	5.78 ± 1.32	5.39 ± 0.59	5.06 ± 0.52	6.60 ± 0.66	5.73 ± 1.96	5.62 ± 1.33	6.17 ± 0.68
PI_{TOTAL}	6.31 ± 1.37	5.97 ± 1.95	5.27 ± 1.55	4.77 ± 0.79	8.24 ± 1.20	10.41 ± 2.25	8.60 ± 2.57	7.80 ± 1.45

Plants were either treated with 24-epibrassinolide (the BR1 variant) or with water (the C variant) and subjected to normal watering (well-watered plants) or 7 d of withholding water (drought-stressed plants). Statistically significant differences (p ≤ 0.05) according to Welch's t-tests between the respective C and BR1 variants subjected to the same cultivation conditions are shown in bold, statistically significant differences between the respective well-watered and drought-stressed variants treated with the same solution are shown in italics. For the explanation of abbreviations see the Material and Methods section of the article, for the biological meaning of the JIP test parameters see Supplementary Table 1.

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FIGURE 2

Graphical analysis of the OJIP chlorophyll fluorescence data measured in maize 4th leaves at Timepoint 3. Plants were either treated with 24-epibrassinolide (the BR1 variant) or with water (the C variant) and subjected to normal watering (non-stressed plants) or 7 d of withholding water (stressed plants). The OJIP curve (A), the relative variable fluorescence W_{OI} (B) and the difference kinetics ΔW_{OJ} (C, E) and ΔW_{OK} (D, F) are shown. ΔW_{OJ} and ΔW_{OK} were calculated either from the comparison of the respective BR1 and C variants subjected to the same watering regime (C, D) or from the comparison of the respective non-stressed and stressed plants subjected to the same type of treatment (E, F). For other information see legend to Figure 1.

OEC function (Figure 2). No particular effect of the early *epi*BL treatment on the energetic connectivity between PS II units was found at this timepoint (Figure 2).

3.4 Timepoint 4 (drought, early and late epiBL treatments)

Further drought-induced reduction of the values of all morphological parameters in comparison to the well-watered plants was observed after an additional week of drought simulation with the only exception for the DMR of the C variant

(Figure 3). The same applied to the RWC and all gas exchange parameters; the reduction of the values of these parameters was more pronounced in the 3rd leaves than in the 4th leaves (Figures 4, 5). The contents of photosynthetic pigments and the efficiency of the PETC also significantly decreased due to drought. In this case, both leaves responded more-or-less similarly, although the most pronounced changes for the 3rd leaves were usually observed in the BR2 variants, whereas for the 4th leaves in the C variants (Figures 4–7, Supplementary Table 3). On the other hand, the exposure of plants to drought caused a significant increase in the dissipation of the excess excitation energy in the PETC (Supplementary Table 3) and the MDI values (similar in both leaves, Figures 4, 5). The same

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FIGURE 3

Plant height (A), the number of fully developed leaves (B), the dry mass of shoot (C) and the dry mass of roots (D) measured in maize at Timepoint 4. Plants were subjected to normal watering (non-stressed plants) or 14 d of withholding water (stressed plants) and treated with 24-epibrassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2). Mean values ± SD are shown (n = 16). Asterisks indicate significant (p ≤ 0.05; *) or highly significant (p ≤ 0.01; **) differences between mean values according to Welch´s t-tests, resp. Games-Howell tests, made separately for each treatment (in case of the differences between non-stressed and stressed plants), resp. for each watering regime (in case of the differences between C, BR1 and BR2 variants).

applied to the content of proline; the change of this parameter was particularly pronounced in the 4th leaves (Figures 4, 5). We found almost no statistically significant differences in the MDA content between drought-stressed and well-watered plants, probably due to the high biologic variability of the samples (Figures 4, 5).

The BR1 group of well-watered plants showed significantly higher DMR values in comparison to the C group and the number of fully developed leaves was also significantly higher in both epiBL-treated groups under these cultivation conditions (Figure 3). Regarding the DMS and the plant height, although we could find a slight trend of an epiBL-caused increase of the values of these parameters in the well-watered plants, due to the high biologic variability these differences were not statistically significant. Under drought conditions, neither BR1 nor BR2 variants significantly differed from their respective control in either morphological parameter (Figure 3).

There were almost no statistically significant differences in the RWC or the gas exchange parameters between our epiBL-treated and non-treated plants, although a trend of a slight increase of values of E, g_s and P_N from the C to the BR2 group could be seen in the 3rd leaves of the well-watered plants (Figure 4). Neither there were any significant differences between the epiBL-treated and non-treated variants in the contents of photosynthetic pigments. The exception from this was the Chl a content in the 3rd leaves of the drought-stressed plants, which was significantly lower in both BR1 and BR2 variants (Figure 4). The JIP test parameters describing the efficiency of the PETC also did not show any effect of the epiBL treatment either in the well-watered or in the drought-stressed plants (Supplementary Table 3). However, the late epiBL treatment showed a negative effect on the function of the OEC, as well as on the energetic connectivity among PSII complexes in the 3rd leaves of

our drought-stressed plants (Figure 6); whereas in the 4th leaves or in the well-watered plants these effects were negligible (Figure 7). The size of the pool of end electron acceptors in the PETC was also slightly reduced after both epiBL treatments in the 3rd leaves of the well-watered plants, whereas for the drought-stressed plants, the BR1 variant showed a greater size of this pool compared to the C or BR2 variants (Figure 6). The differences between individual variants in this respect were less obvious in the 4th leaves (Figure 7).

Although the BR1 and BR2 variants exposed to drought showed a reduction of the MDA content in their 3rd leaves in comparison with the respective control, these differences were statistically non-significant due to the high biologic variability of samples (Figure 4). No significant differences were found between our epiBL-treated and non-treated plants in the MDI (the only exception being the difference between the BR2 and C variants in the 4th leaves of the well-watered plants; Figure 5). The proline content was significantly higher in the 4th leaves of the drought-stressed BR1 variant in comparison with the drought-stressed C variant (Figure 5).

Drought caused significant increase of the levels of BR biosynthetic precursors CR and CN in the 3rd leaves of the control plants while this increase was insignificant in root tissue (Figures 8, 9). In the 4th leaves, the levels of these compounds were much lower and no changes due to plant exposure to drought were observed (Figure 10). The levels of TY, a direct precursor of bioactive CS, were reduced at the end of the drought simulation period in leaves of the C and BR2 variants (and insignificantly also in roots; Figures 8–10). The levels of CS also showed a certain reduction, but statistically significant difference was found only for the 4th leaves of the C variant and roots of the BR2 variant, mostly due to the otherwise high variability of samples taken from the well-watered plants (Figures 8–10). A similar situation was observed for

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FIGURE 4

The relative water content (A), the membrane damage index (B), the transpiration rate (C), the malondialdehyde content (D), the stomatal conductance (E), the proline content (F), the net photosynthetic rate (G), the contents of chlorophylls a and b (H, J) and total carotenoids (I) measured in maize 3rd leaves at Timepoint 4. Plants were subjected to normal watering (non-stressed plants) or 14 d of withholding water (stressed plants) and treated with 24-epi/brassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2). Mean values $\pm$ SD are shown (n = 6 for gas exchange parameters, 8-9 for other parameters). For other information see legend to Figure 3. DM, dry matter. Asterisks indicate significant ($p \leq 0.05$; *) or highly significant ($p \leq 0.01$; **) differences between mean values according to Welch´s t-tests, resp.

the content of a C_{29}-analogue of CS, homoCS, with the exception that a significant difference from the C variant was found in the 3rd leaves (Figures 8–10). No statistically significant drought-induced changes in the norCS (C_{27}-BR) levels were found, although there was a visible trend of the increase of the contents of this compound in the drought-stressed plants of C and BR2 variants compared to the well-watered ones (Figures 8–10). There were also no statistically significant differences in the BL content between the

well-watered and the drought-stressed plants either in the 3rd leaves or in roots (Figures 8, 9). However, drought led to a significant reduction of the content of the most biologically active BR brassinolide in the 4th leaves of plants not treated with epiBL., i.e., BL (Figure 10). Maize was found to be capable for synthesizing 28-homodolichosterone (homoDS) whose levels showed also a reduction as a direct result of drought in both leaves and roots, resp. (Figures 8–10). The presence of epiBL and norBL was detected

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FIGURE 5

The relative water content (A), the membrane damage index (B), the transpiration rate (C), the malondialdehyde content (D), the stomatal conductance (E), the proline content (F), the net photosynthetic rate (G), the contents of chlorophylls a and b (H, J) and total carotenoids (I) measured in maize 4th leaves at Timepoint 4. Plants were subjected to normal watering (non-stressed plants) or 14 d of withholding water (stressed plants) and treated with 24-epibrassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2). Mean values ± SD are shown (n = 6 for gas exchange parameters, 8-9 for other parameters). For other information see legend to Figure 3. DM, dry matter. Asterisks indicate significant ($p \leq 0.05$; *) or highly significant ($p \leq 0.01$; **) differences between mean values according to Welch's t-tests, resp.

only in the 3rd leaves and (in much lower amounts) in roots (Figures 8, 9). The levels of these BRs did not significantly differ between well-watered and stressed plants.

The exogenous *epi*BL treatment did not significantly affect the levels of endogenous BRs detected in leaves or roots of our experimental plants (Figures 8–10). The exception from this were

BL levels in the 4th leaves of the BR2 variants, which significantly differed from the C variants regardless of the cultivation conditions (although with opposite trends; Figure 10). The 4th leaves of the well-watered BR2 plants had also higher levels of norCS (and, non-significantly, also of homoCS) compared to their controls (Figure 10). A statistically significant increase in the *epi*BL levels

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FIGURE 6

Graphical analysis of the OJIP chlorophyll fluorescence data measured in maize 3rd leaves at Timepoint 4. Plants were either treated with 24-epibrassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2), and subjected to normal watering (non-stressed plants) or 14 d of withholding water (stressed plants). The OJIP curve (A), the relative variable fluorescence W_{OJ} (B) and the difference kinetics ΔW_{OJ} (C, E) and ΔW_{OK} (D, F) are shown. ΔW_{OJ} and ΔW_{OK} were calculated either from the comparison of the respective BR1/BR2 and C variants subjected to the same watering regime (C, D) or from the comparison of the respective non-stressed and stressed plants subjected to the same type of treatment (E, F). For other information see legend to Figure 1.

4 Discussion

was observed in the 3rd leaves of the well-watered BR2 variant. There was also an increase in the stressed BR1 and BR2 variants. However, in this case, it was not statistically significant (Figure 8).

BRs are generally considered to play many beneficial roles in plant development, physiology and biochemistry, particularly in plants subjected to various sub-optimum conditions. Their exogenous application was shown to improve plant response to both abiotic and biotic stress factors, such as high or low temperature, water deficit or water excess, high or low irradiance, salinity, nutrient deficiency or excess, exposure to heavy metals, herbicides, pesticides, viral or bacterial pathogens, fungi, etc. The number of studies dealing with this topic reaches many hundreds and as such, exceeds the possibilities of this paper to be thoroughly discussed. However, although many studies are currently available, various aspects of BR relationship to plant stress response still remain very poorly examined or not explored at all. Our

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FIGURE 7

Graphical analysis of the OJIP chlorophyll fluorescence data measured in maize 4th leaves at Timepoint 4. Plants were either treated with 24-epibrassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2), and subjected to normal watering (non-stressed plants) or 14 d of withholding water (stressed plants). The OJIP curve (A), the relative variable fluorescence W_{OJ} (B) and the difference kinetics ΔW_{OJ} (C, E) and ΔW_{OK} (D, F) are shown. ΔW_{OJ} and ΔW_{OK} were calculated either from the comparison of the respective BR1/BR2 and C variants subjected to the same watering regime (C, D) or from the comparison of the respective non-stressed and stressed plants subjected to the same type of treatment (E, F). For other information see legend to Figure 1.

experiments presented in this paper tried to add to our knowledge on some of these poorly understood topics. Thus, in the following paragraphs we will focus only on the five main aspects of our study, as stated in the list of our objectives at the end of the Introduction section. The reader interested in the general topic of BRs in relation to a particular type of plant stress, is referred to recent reviews and book chapters, e.g., (Ahammed et al. 2020; Ahammed et al., 2022), Ramirez and Poppenberger (2020); Basit et al. (2021); Li et al. (2021); Rehman et al. (2022); Sidhu and Bali (2022) and many others.

4.1 Effects of exogenous BR application on leaves of different age

One of the aims of our study was to analyze whether the exogenous application of BRs might differently mitigate the negative impact of drought on plants in two different leaves: those that were already fully developed at the beginning of stress and those that were still developing during stress period. Several authors (Mostajeran and Rahimi-Eichi, 2009; Gilgen and Feller, 2014; Budič et al., 2016) found that older leaves are more affected by

FIGURE 8

The contents of campesterol (A), campestanol (B), typhasterol (C), castasterone (D), brassinolide (E), 24-epibrassinolide (F), 28-norcastasterone (G), 28-norbrassinolide (H), 28-homocastasterone (I) and 28-homodolichosterone (J) measured in maize 3rd leaves at Timepoint 4. Plants were subjected to normal watering (non-stressed plants) or 14 d of withholding water (stressed plants) and treated with 24-epibrassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2). Mean values ± SD are shown (n = 4). For other information see legend to Figure 3. FM, fresh matter. Asterisks indicate significant ($p \leq 0.05$; *) or highly significant ($p \leq 0.01$; **) differences between mean values according to Welch´s t-tests, resp.

drought than younger leaves. However, in relation to the BRs role in plant drought response, this aspect has not been previously examined. We exposed 5-weeks old maize plants to gradually induced moderate drought stress, which significantly reduced the values of the majority of the observed parameters associated with plant morphology and physiology. A trend of decreasing values after the exogenous epiBL application was found in the older, 3rd leaves, especially for the RWC and parameters characterizing photosynthesis. On the other hand, no such trend was observed for the younger 4th leaves for which we even registered a few signs of

BR-induced improvement at the end of our experiments (efficiency of some parts of the PETC and the proline content).

The difference in observed responses of analyzed leaves to BRs application could be connected to BR-induced acceleration of leaf senescence. Several authors (e.g., Sağlam-Çağ, 2007; Barış and Sağlam-Çağ, 2016; Fedina et al., 2017; Kim et al., 2019) showed that BRs promote leaf senescence (although the exact mechanism is still unknown and might be probably an indirect one; Jan et al., 2019). This is usually accompanied by an increase in membrane damage, reduced Chl contents and decreased photosynthetic

FIGURE 9

The contents of campesterol (A), campestanol (B), typhasterol (C), castasterone (D), brassinolide (E), 24-epibrassinolide (F), 28-norcastasterone (G), 28-norbrassinolide (H), 28-homocastasterone (I) and 28-homodolichosterone (J) measured in maize roots at Timepoint 4. Plants were subjected to normal watering (non-stressed plants) or 14 d of withholding water (stressed plants) and treated with 24-epibrassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2). Mean values ± SD are shown (n = 4). For other information see legend to Figure 3. FM, fresh matter. Asterisks indicate significant (p ≤ 0.05; *) or highly significant (p ≤ 0.01; **) differences between mean values according to Welch's t-tests, resp.

efficiency. Thus, the older (3rd) leaves of our drought-stressed plants treated with epiBL could, particularly at Timepoint 4, already enter the beginning stage of the senescence (reflected in the more pronounced reduction of their Chl content and the PETC efficiency compared to the non-treated stressed plants). Even before the beginning of the drought simulation, the 3rd leaves of plants treated with epiBL already showed a worse efficiency of their PETC than the non-treated plants. On the other hand, the 4th leaves were younger so they have not entered the senescence phase even at Timepoint 4 and the BR treatment thus did not affect them

negatively. Gomes et al. (2013) also found lower chlorophyll content as a sign of the beginning of senescence in the older drought-stressed papaya leaves after BR treatment but not in the younger ones (this did not apply to the non-stressed plants, similarly to our results). Interestingly, while the later study of these authors made with the same species (Gomes et al., 2018) confirmed that the development of younger leaves does not seem to be particularly accelerated by BRs, leaves that were already mature at the time of BR application showed slightly delayed senescence in BR-treated plants in this case. However, the respective plants were

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FIGURE 10

The contents of campesterol (A), campestanol (B), typhasterol (C), castasterone (D), brassinolide (E), 28-norcastasterone (F), 28-homocastasterone (G), and 28-homodolichosterone (H) measured in maize 4th leaves at Timepoint 4. Plants were subjected to normal watering (non-stressed plants) or 14 d of withholding water (stressed plants) and treated with 24-epibrassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2). Mean values ± SD are shown (n = 4). For other information see legend to Figure 3. FM, fresh matter.

not subjected to any stressor so perhaps it might be the combination of stress and the BR treatment that particularly accelerates leaf senescence.

The expected mitigating effect of BRs on our drought-stressed plants was thus found only in their 4th leaves, but it was very mild and usually statistically non-significant. The reason for this could be that the 4th leaves of our plants did not particularly suffer from drought stress (their RWC values were still above 90% even after 2 weeks of drought simulation). The overwhelming majority of the authors that analyzed the BR/drought relationship by measuring various physiological and biochemical leaf parameters did not state the water status of leaves of their experimental plants at all and those that did usually worked with severely stressed leaves (RWC much below 70% or leaf water potential below -1.5 MPa) (Holà, 2019). It is thus highly probable that there was no opportunity for the epiBL treatment to show its potential as a drought-mitigating

agens because the 4th leaves of our experimental plants simply did not need it.

The observed change in proline levels in the response to epiBL treatment was another interesting phenomenon found only in the 4th leaves of our plants. We found that the epiBL application prior to any drought simulation results in significantly reduced proline contents in these leaves, but only in non-stressed plants. Furthemore, this effect did not persist with time: it was observed only during Timepoints 2 and 3, while at Timepoint 4 it faded away and was replaced by a positive effect of epiBL pre-treatment on proline levels in the drought-stressed plants. Proline in drought-stressed plants generally functions as an osmoprotectant and its increased accumulation also results in plant protection against oxidative stress (Ghosh et al., 2022). The majority of authors who analyzed the proline content in leaves of drought-stressed plants treated with exogenous BRs (or plants overproducing BRs due to

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transgenic modification of some BR biosynthetic gene) reported a positive effect of such treatment on this parameter. However, such effects were usually observed only in stressed plants, not in well-watered (control) ones, where BR treatment either did not have any significant effect or even resulted in reduced proline levels (e.g., Zhang et al., 2008; Anjum et al., 2011; Li and Feng, 2011; Talaat et al., 2015; Ahmed et al., 2017; Duan et al., 2017; Khamsuk et al., 2018; Kaya et al., 2019). Although Fu et al. (2019) reported that exogenous epiBL pre-treatment significantly elevates the expression of two key proline biosynthetic genes, P5CS and P5CR (as well as the activities of the respective enzymes and the proline content) in Elymus nutans plants, this applied only for plants subjected to cold stress but not for their non-stressed plants. On the other hand, Ábrahám et al. (2003) showed that treatment of non-stressed Arabidopsis plants with exogenous epiBL inhibits the expression of a P5CS gene and thus actually leads to reduced proline levels. It is possible that something similar occurred in our experimental plants. Exogenous BR treatment per se certainly does not seem to specifically induce proline biosynthesis (it can even act antagonistically) and it is only in combination with drought (or some other stress factor) that BR application can result in elevated proline levels and thus better osmoprotective and antioxidant parameters of BR-treated plants.

The reason that the effects of epiBL application took place only in the younger leaves of our plants is currently unknown. Whether it could be somehow associated with the general differences between younger and more mature (or senescing) leaves in proline levels (which can be different in various plant species, e.g., Cechin et al., 2006; Xue et al., 2008; Sperdouli and Moustakas, 2015), and whether it could be somehow associated with differences in the contents of specific brassinosteroids between these two types of leaves (see below) remains to be seen. The detailed examination of the expression of various genes associated with proline metabolism and/or transport together with a thorough analysis of the activities of the respective proteins in BR-treated, drought-stressed (or non-stressed) plants at different timepoints after the onset of drought and/or BR application, and in leaves in different developmental stage would be certainly worthwhile and while such analysis was outside the scope of our experiments presented in this paper, it is something we would like to focus on in the future.

4.2 Effects of exogenous BR application before or during drought

The second aspect we examined in our work was the role of a time when BRs were applied to our experimental plants. We used the epiBL application before (BR1 variants) and during (BR2 variants) drought simulation but we found almost no difference in the BR effects on plant physiology and morphology between these two timepoints. The earlier application slightly improved the plant morphology of our non-stressed plants (perhaps because epiBL had more time to act on plant growth and development), whereas the later application resulted in slightly higher gas exchange (particularly in their 3rd leaves). However, these differences were mostly negligible and certainly did not apply to the drought-

stressed plants. There is very little knowledge on the difference between BR applications before drought and during drought. In two studies available on this topic, the authors used a different type of application and BR concentration for each timepoint, which makes a subsequent comparison inappropriate (Farooq et al., 2009; Hashemi et al., 2015). However, even in these cases, the authors did not find any difference between the effect of BR pre-treatment and post-treatment on their drought-stressed plants. The only marked difference between our drought-stressed BR1 and BR2 variants was that there was a clearly evident more negative type of effect of epiBL applied during the stress period on the performance of the OEC and the excitonic connectivity among individual PSII complexes observed in their 3rd leaves but not in the 4th ones. The primary photosynthetic processes are always more affected by BR treatment in more intensively stressed leaves and the OEC seems to be particularly sensitive to any change in BR homeostasis (Holá, 2022). However, all things considered, it certainly does not seem that the time of exogenous BR application is a particulary important factor that influences the response of drought-stressed plants to these phytohormones.

4.3 Effects of exogenous BR application on endogenous C_{27}, C_{28}-and C_{29}- BRs

Our selection of these two separate timepoints for the BR application also meant that the endogenous BR contents were determined in the leaves and roots of our plants at either 17 d (BR1 variants, i.e., plants treated at Timepoint 1) or 7 d (BR2 variants, treated at Timepoint 3) after this application, respectively. Although in most cases the differences in the contents of individual BRs and/or their precursors between BR1/BR2 variants and the non-treated plants were not statistically significant, there was a clearly pronounced trend of a higher increase of the amounts of C_{29}-BRs (homoCS, homoDS) in the 4th leaves and roots of the non-stressed BR2 variant. For norCS, which is a representative of C_{27}-BRs, this increase in the 4th leaves was even statistically significant, whereas the content of BL (a representative of C_{28}-BRs) significantly increased under stress conditions (particularly after the later BR application) but showed an opposite trend in our well-watered plants. These observed trends suggest that the changes in the levels of endogenous BRs caused by the exogenous BR application probably diminish with time (e.g. due to metabolization – see Schneider et al., 1994; Kolbe et al., 1995) and that the shorter period between the BR treatment and the analysis of endogenous BR contents is better for them to be detectable. This is supported also by our observation that the contents of epiBL in the 3rd leaves of our plants significantly increased in the order of C < BR1 < BR2, following the decreasing length of the period between epiBL spraying and BR content determination. We think that the detected epiBL levels in our BR1 and BR2 variants could be residues after the respective epiBL treatments, which persisted in a tissue of interest to the last sampling point. A similar situation was described for another monocot (wheat) plant by Janeczko and Swaczynová (2010). The absence of epiBL in the 4th leaves could be explained either by the fact that these leaves were yet rather small during the spraying and thus offered less area for BR entry (the BR1 variant), or by the possibility that these leaves could use a

completely different mechanism how to deal with exogenously applied BRs. For instance, Nishikawa et al. (1995) showed that while hypocotyls and roots of cucumber are not much able to metabolize epiBL, petioles and leaves do have this ability. A similar situation was observed by the same research team in intact wheat seedlings. Thus, there might be some differences in the metabolization of particular exogenous substance in various plant parts/organs in this respect.

The effect of the exogenous BR application on the endogenous BR contents depends not only on *when* but also on *how* BRs are applied, as well as on plant species and BR concentration. We utilized foliage spraying as it is the most frequently employed method in BR/drought studies. However, the majority of information available on the possible changes in endogenous BR contents caused by the exogenous BR application comes from studies where BRs were either added directly into the growth medium and plants received them through roots, or by soaking of seeds in BR solutions (Supplementary File 2). Only Janeczko and Swaczynová (2010) sprayed wheat plants in the V2 stage with epiBL solutions and they did not observe any changes in the CS or BL contents in the 3rd leaves of their plants 11 d after spraying. Janeczko et al. (2011) also used direct injection of epiBL into the 1st and 2nd leaves of barley. Using this approach, they did not found any significant effect on endogenous CS or BL contents in the 7th leaf after 20 d. Notably, both CS and BL belong to the C_{28}-BR group and none of these authors analyzed C_{27}- or C_{29}-BRs in their plants at that time. In later studies, some authors that applied BRs directly into the growth medium or by seed soaking also observed an increase in the levels of C_{27}- or C_{29}-BRs but not C_{28}-BRs (Filek et al., 2019; Tarkowská et al., 2020) while others found that such type of application can elevate the contents of C_{28}-BRs as well (depending on the concentration and/or plant species and analyzed plant organ) (Bajguz et al., 2019; Chmur and Bajguz, 2021). It seems that while the application of BRs into growth medium, particularly in hydroponic cultures, can evidently affect their contents in leaves/shoots (either by enabling their direct transport through a plant as suggested by Janeczko and Swaczynová, 2010, or, more probably, by an indirect effect on the regulation of BR biosynthesis), the direct treatment of leaves/shoots (as in our case) seems to have a more negligible effect on the endogenous BR levels. This is probably due to the fact that BRs applied in this manner are not transported over long distances (Symons and Reid, 2004; Symons et al., 2008) and their effect is only temporary and diminishes with time.

4.4 Effects of analyzed organ and leaf age on endogenous C_{27}, C_{28}- and C_{29}-BRs

The biosynthetic precursors of C_{29}-BRs (as well as the respective end products in monocots) are frequently found to be present in much higher amounts compared to C_{28}-BRs in various plant species that have been examined to this date (Supplementary File 2). This is in good agreement with our findings: both the 3rd and 4th leaves and roots of our plants contained more homoCS and homoDL than BRs belonging to the C_{28} group. When the levels of BRs and/or their precursors in leaves and roots of our experimental plants were compared, we also found a good agreement between our data and the majority of data

published previously by authors who simultaneously analyzed endogenous BR levels in shoots/leaves and roots of various plant species (Supplementary File 2). The contents of CS and BL are consistently reported to be higher in the aboveground vegetative parts of plants than in roots and our results supports this observation as well. The contents of TY were found by some authors to be also higher in leaves than in roots (Jiang et al., 2012, in rice, Pavlović et al., 2019, in Chinese cabbage, Lu et al., 2021 in sunflower), which also corresponds to our observations and to the results of earlier reports describing the lowest levels of BRs in root tissue (Bancos et al., 2002; Shimada et al., 2003; Symons and Reid, 2004). In contrast, there are some other authors who reported either similar levels of TY in shoots and roots or even TY elevation in roots (Supplementary File 2). Altogether it seems that the differences in the TY content between shoots/leaves and roots might depend on plant species or some other experimental aspects. A similar discrepancy of data can be observed also for CR and CN: while we found comparable amounts of these C_{28}-BR precursors in the 3rd leaves and roots of our maize plants (much lower amounts were detected in the 4th leaves) and Nakamura et al. (2006) reported higher levels of CR and CN in rice shoots than in roots, the opposite situation was found by Shimada et al. (2003) and Kim et al. (2006) for *Arabidopsis*. Regarding C_{27}- and C_{29}-BRs, our data were again in good agreement with the findings of Yokota et al. (2001) and Luo et al. (2018), who reported lower levels of the end products of the respective biosynthetic pathways (or even the complete absence of these BRs) in roots of tomato and rice as compared to shoots.

The situation with the BR contents in the 3rd and the 4th leaf is more interesting than the simple comparison of BR quantity in leaves and roots. Actually, we think that this is probably our most interesting finding presented in this study. The aspect of the comparison of a profile of BRs belonging to various structural groups in younger and more mature leaves has not been much dealt with. When searching the available literature, we found only two studies discussing the content and portfolio of C_{27}- to C_{29}-BRs in the leaves of different ages. Zhang et al. (2022a) reported a slightly higher content of CS (a C_{28}-BR) for older leaves compared with younger leaves of their tea (*Camellia sinensis*) plants. This is in agreement with our own findings, although in our case, the difference between leaves was of a much higher order of magnitude. On the other hand, the amount of norCS (a C_{27}-BR, which is synthesized mostly from 6-oxocholestanol with cholesterol and cholestanol as earlier precursors (Bajguz et al., 2020) was lower in the 4th (younger) leaves of our plants, whereas Zhang et al. (2022a) did not report any differences. The second study published on this topic is that of Parada et al. (2022) using a grapevine (*Vitis vinifera*) as a model plant. In their case, CS and homoDS (C_{28}- and C_{29}-BRs, resp.) were detected in neither young nor mature leaves, epiCS was present in the young leaves only and the level of norTE and homoCS (C_{27}- and C_{29}-BRs, resp.) was higher in the mature leaves compared to younger ones. This is inconsistent with our results where the levels of homoCS are about an order of magnitude higher in our younger leaves (4th) than in the older leaves (3rd).

Our above-mentioned data lead strongly to the suggestion that the younger, 4th leaves of maize probably for some reason divert their BR biosynthesis from C_{28}-BRs (TY, CS, BL, epiBL) to C_{29}-BRs like homoCS. The biosynthesis of C_{27}-, C_{28}- and C_{29}-BRs is

interconnected at various levels. CS can be synthesized from many C_{27}- to C_{29}-BRs precursors including norCS, homoCS and homoDS (Bajguz et al., 2020). However, we think that the divergence of BR biosynthesis in our younger leaves probably occurs at the very early biosynthetic steps, prior to the beginning of the late C-22 oxidation pathway, because the 4th leaves appeared to be a very poor source of CR and CN. Thus, the BR biosynthesis in the younger maize leaves (at least in this inbred line) is probably strongly redirected from the CR (and/or cholesterol) branches to the parallel sitosterol branch of the BR biosynthetic pathway (Bajguz et al., 2020; Wei and Li, 2020). This might explain the highly elevated level of homoCS then. With respect to the levels of the homoDS, one can only speculate that *within* the sitosterol branch the biosynthesis is not further diverted *via* 22S-hydroxyisofucosterol, which is proposed to be a precursor of homoDS (Bajguz et al., 2020).

4.5 Effects of drought on endogenous C_{27}, C_{28}-and C_{29}-BRs

The final part of our experiment to be discussed is the effect of drought *per se* on the levels of individual BRs and their precursors. We found that drought slightly enhanced amounts of CR and CN and reduced amounts of TY, homoCS and homoDS in the leaves (and to a less extent in roots) of our experimental plants. The reduction of homoCS levels was particularly pronounced in the 4th leaves. Drought also led to reduced levels of CS in the 3rd leaves (in the 4th leaves there were mostly no changes or even an increase), while for BL the situation was usually the opposite. Regarding C_{27}-BRs, exposure of plants to drought was associated with generally higher amounts of norCS but mostly no changes of the norBL content. The observed changes in the levels of individual BRs are generally consistent with the results of our previous study made with the same inbred line of maize (Tůmová et al., 2018). Similarly to that study, we also did not detect the presence of either DS, DL, homoBL, homoDL or norTE. The unchanged levels of CS in our 4th leaves are also in agreement with previous studies on drought-stressed young pea leaves (Jager et al., 2008), tobacco leaves (Duan et al., 2017) or rice shoots (Ding et al., 2014). Additionally, in the last study, BL was detected in well-watered plants but not in the drought-stressed ones.

As stated above, CR and CN are precursors in the late C-22 oxidation pathway leading to the biosynthesis of the C_{28}-BRs (Bajguz et al., 2020). Their slightly increased accumulation in our drought-stressed plants together with the reduced levels of the products of the subsequent early C-6 oxidation pathway (TY, CS) could indicate at least partial inhibition of this BR biosynthetic pathway after plant exposure to drought. However, CS can be also synthesized from homoCS and homoDS (Bajguz et al., 2020) and the reduced levels of these two BRs in our plants suggest the association of drought exposure not only with the inhibition of the C_{28}-BRs biosynthetic pathway but also with the reduced biosynthesis of C_{29}-BRs. Alternatively, CS can be also synthesized from norCS (Bajguz et al., 2020), and here the observed reduction of CS amounts in our 3rd leaves together with the accumulation of norCS could perhaps mean that the C_{27}-biosynthesis *per se* is not

inhibited by drought in maize but that the conversion of norCS to CS is. Thus, we suggest that drought inhibits C_{28}-BRs and C_{29}-BRs biosynthetic pathways whereas C_{27}-BRs biosynthesis is not particularly affected. Further, the inhibition of the biosynthesis of C_{28}-BRs applies particularly for the older leaves whereas the inhibition of C_{29}-BR biosynthesis is more pronounced in the younger ones (which can be associated with the above-mentioned preferences for one or the other biosynthetic pathway in these two types of leaves).

It is worth emphasizing that this endogenous BR response to drought in maize might not be a universal response and does not have to apply to other plant species (or even cultivars of the same species). Even closely related species can show very different responses of their endogenous BR contents to drought as demonstrated by Pavlović et al. (2018) who analyzed three species of *Brassica* under control and drought conditions. They found a reduction of TY amounts, an elevation of CS amounts, and an even more significant elevation of BL amounts in their stressed Chinese cabbage plants whereas there were no changes in the other two species except a mild reduction of BL levels in kale and a mild increase of TY levels in white cabbage (Pavlović et al., 2018). Unfortunately, scientists who studied endogenous BR contents in plants under drought conditions usually analyzed only several (or only one) C_{28}-BRs and no C_{27}- or C_{29}-BRs (Supplementary File 2). Only Gruszka et al. (2016) found increased CS levels and mostly unchanged homoBL levels in barley exposed to the insufficient water supply as compared to the well-watered plants. On the other hand, Malaga et al. (2020) reported mostly no changes for CS but a significant increase of homoCS amounts in leaves of some drought-sensitive barley cultivars but not in cultivars with moderate or high drought tolerance. Our previous study with maize showed intraspecific differences in drought-induced changes of endogenous BR levels as well: the drought-tolerant inbred line had been analyzed together with the drought-sensitive one and differed in the contents of some individual BRs (particularly TY, norCS, norBL) both under normal and drought conditions (Tůmová et al., 2018). Thus, to better understand the relationship between drought and biosynthesis/metabolism of various BRs, more detailed analyses of more plant species, more cultivars of the same species, made under mild, moderate, or severe drought conditions, and including plants/leaves of different developmental stages are clearly very much needed.

5 Conclusions

The results of our experiments presented in this paper offered several interesting answers to the five main objectives of our study. Firstly, when determining the response of these two types of leaves to the combination of drought exposure and the application of exogenous epiBL, we found that the response of leaves of different ages differs: the older leaves can show accelerated senescence under such conditions reflected in their reduced chlorophyll content and diminished efficiency of the primary photosynthetic processes, whereas the younger leaves are characterized by interesting changes of proline levels in response to epiBL treatment (this deserves a further, more detailed exploration).

Secondly, the exogenous application of *epiBL* before or during drought did not result in a different response of plants to this stress factor (which disproved our original hypothesis on this topic). Thirdly, we showed that the contents of C_{27}- and C_{29}-BRs in plants treated with exogenous *epiBL* depended on the length of time between this treatment and the BR analysis (which confirmed our original hypothesis) Finally, we demonstrated marked differences in the contents of individual BRs between younger and older maize leaves, which is, in our opinion, the most interesting result of our study. We propose that the younger maize leaves divert their BR biosynthesis from C_{28}-BRs to C_{29}-BRs, probably at the very early biosynthetic steps (prior to the beginning of the late C-22 oxidation pathway). Drought also apparently negatively affected biosynthetic pathways of C_{28}-BRs (particularly in the older leaves) and C_{29}-BRs (particularly in the younger leaves) but not C_{27}-BRs in our maize plants.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

HM and DH conceived the project, DT performed endogenous brassinosteroids measurements, HM and PČ carried out other biochemical analyses, MK and OR performed *in situ* measurements and samplings. HM, DH and DT drafted the manuscript and all authors reviewed and approved the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2023.1139162/full#supplementary-material

SUPPLEMENTARY FILE 1

The original data on the brassinosteroid contents, plant morphology and various physiological and biochemical characteristics measured at four different timepoints in the 3rd and 4th leaves (or roots) of maize subjected to normal watering or withholding water and treated with 24-epibrassinolide (the BR1 and BR2 variants) or with water (the C variant) either before the start of drought period (C, BR1) or during the drought period (BR2).

SUPPLEMENTARY FILE 2

Heat maps showing the approximate contents of various brassinosteroids (BRs) and their precursors in the non-reproductive parts/organs of various plant species as reported from previously published studies. Only studies dealing with *i)* the effect of exogenous BR application on their endogenous contents; *ii)* the analysis of at least some BRs from both C_{28}-, C_{29}- and/or C_{27}-groups at the same time; *iii)* the analysis of BR contents in both shoots/leaves and roots at the same time, or *iv)* the analysis of BR contents in plants subjected to drought are included here.

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ARTICLE 14

Plant ecdysteroids: plant sterols with intriguing distributions, biological effects and relations to plant hormones

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Contribution: review outline, literature research, manuscript writing

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ARTICLE 15

Plant triterpenoid crosstalk: the interaction of brassinosteroids and phytoecdysteroids in *Lepidium sativum*

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Contribution: design of the study, analysis of brassinosteroids and ecdysteroids, literature research, manuscript writing

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Brief Report

Plant Triterpenoid Crosstalk: The Interaction of Brassinosteroids and Phytoecdysteroids in *Lepidium sativum*

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Abstract: Plant steroid alcohols, plant sterols, are essential components of cell membranes that perform many functions. Their most prominent function is maintaining membrane semipermeability and regulating its fluidity through their specific interaction with phospholipids and membrane proteins. This work is focused on the study of the interaction of two groups of plant sterols, brassinosteroids (BRs) and phytoecdysteroids (PE). Steroid substances belonging to both groups are important signaling molecules essential for plant growth and development, but while the first group has all the known attributes of plant hormones, the second lacks hormonal function in plants. The aim of this preliminary study was to determine at what concentration level and to what extent substances of this type are able to interact with each other, and thus influence the early growth and development of a plant. It was found that exogenously applied PE 20-hydroxyecdysone (20E) significantly reduced the level of endogenous BRs in four-day-old garden cress (*Lepidium sativum*) seedlings. On the other hand, exogenously applied BRs, 24-*epi*brassinolide (*epi*BL), caused the opposite effect. Endogenous 20E was further detected at the picogram level in garden cress seedlings. Thus, this is the first report indicating that this plant species is PE-positive. The level of endogenous 20E in garden cress seedlings can be decreased by exogenous *epi*BL, but only at a relatively high concentration of $1 \cdot 10^{-6}$ M in a culture medium. The image analysis of garden cress seedlings revealed that the length of shoot is affected neither by exogenous BRs nor PE, whereas the root length varies depending on the type and concentration of steroid applied.

Keywords: triterpenoids; brassinosteroids; phytoecdysteroids; *Lepidium sativum*; plant growth

1. Introduction

Plants, lacking motility, have never developed a nervous system, but they have evolved an intricate network of signaling molecules—chemical messengers—that play an indispensable role in the regulation of their responses to environmental stimuli, as well as their growth and development [1]. Brassinosteroids (BRs) and phytoecdysteroids (PEs) belong to a family of plant sterols that are a part of this network, and are thus active players in cell–cell communication in planta [2,3]. Chemically, both of these tetracyclic triterpenoid classes comprise of C_{27} to C_{29} polyhydroxylated steroid structures with an oxygenated B-ring (Figure 1, marked in teal).

However, there are a few structural differences between these two groups of phytosterols that ultimately have a direct effect on the dissimilarity of their biological activity, namely: (1) the B-ring in BRs bears only a carbonyl group at C-6, while PEs have a characteristic 14α-hydroxy-7-en-6-one chromophoric moiety (Figure 1, marked in teal); (2) the orientation of hydroxyl groups at C-2, C-3,

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and C-22 is mirrored; and lastly, (3) the junction of the A- and B-rings is in a cis-orientation in the skeleton of PEs, while BRs have a trans-configuration. Importantly, six-membered B-ring in BRs may be expanded during their biosynthesis to form a lactone, which occurs in the case of brassinolide (BL) and its epimer 24-*epi*brassinolide (*epi*BL, Figure 2). These 7-oxalactone BRs show greater biological activity than 6-oxo types (e.g., castasterone, Figure 2) [4]. However, the structure of the side chain in PEs and BRs could be more important for their biological activity than that of their steroidal core skeleton, as demonstrated by Watanabe [5]. He found that hydroxylation at C-20 is detrimental to the plant's hormonal activity of BRs, whereas the stereochemistry of the hydroxy group at C-22 is of pivotal importance for the molting hormonal activity.
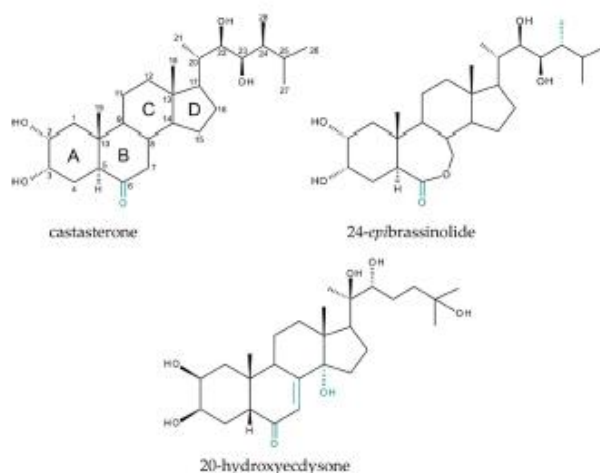

Figure 1. Chemical structure of prominent representatives of brassinosteroids (castasterone, 24-*epi*brassinolide) and phytoecdysteroids (20-hydroxyecdysone, 20E).

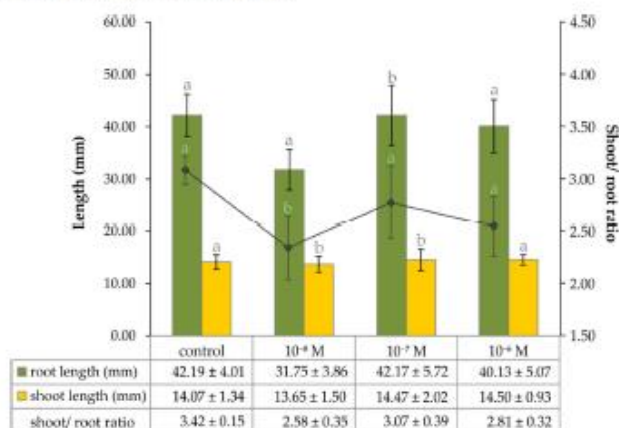|	control	10^{-8} M	10^{-7} M	10^{-6} M
root length (mm)	42.19 ± 4.01	31.75 ± 3.86	42.17 ± 5.72	40.13 ± 5.07
shoot length (mm)	14.07 ± 1.34	13.65 ± 1.50	14.47 ± 2.02	14.50 ± 0.93
shoot/ root ratio	3.42 ± 0.15	2.58 ± 0.35	3.07 ± 0.39	2.81 ± 0.32

Figure 2. The graph of dependence of the shoot and root growth of four-day-old *Lepidium sativum* seedlings on the concentration of exogenous 20E. The data/error bars represent the mean/standard deviation of six independent measurements.

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BRs are generally accepted as plant hormones with known receptor [6] and well-described biological functions [7], while PEs are most often regarded as signaling molecules providing protection against non-adapted insects and/or soil nematodes [8,9] without hormonal properties. Whereas BR receptors show a high specificity, PEs exhibit weak or no activity in bioassays of BRs [10,11] and vice versa. Nevertheless, there are a range of physiological processes that are affected by both BRs and PEs, although the mechanism of their mutual interaction is not known yet. For instance, selected representatives of BRs (*epi*BL) and PEs (20E) have been shown to increase the yield of photosynthesis [12], and exogenously applied *epi*BL induces changes in PE content as well as PE profiles in the plant tissue [13].

The aim of this work was to show that the presence of exogenous PEs can also cause changes in the levels of endogenous steroid hormones, BRs. For this purpose, an earlier well-established sensitive analytical technique based on ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) was used [14].

2. Results and Discussion

2.1. The Effect of Exogenous Phytoecdysteroid on Growth of Lepidium Seedlings and the Level of Endogenous Brassinosteroids

The data obtained by the image analysis of four-day-old garden cress seedlings showed that the length of their shoots was not affected by the presence of 20E at all, and reached an average of 14.2 mm in all of the experimental setups (Figure 2). The roots of the same seedlings were found to be about three times longer compared to corresponding shoots. A significant change occurred only when the growing media was treated with 20E at a concentration of $1 \cdot 10^{-8}$ M, when the length of seedlings' roots decreased by 23.5 %. Therefore, the trend of shoot-to-root ratio reflects the trend observed for the root length (Figure 2).

By determining the levels of naturally occurring BRs in garden cress seedlings grown on media with 20E at the indicated concentrations, it was found that this PE generally reduced the levels of endogenous BRs by an average of 38% compared with the control plants (Figure 3A). The greatest influence for 20E was at a concentration of $1 \cdot 10^{-7}$ M, where there was a reduction in the total level of BRs by 44% compared with the control, i.e., garden cress seedlings grown on a medium without 20E. This observation might be explained by the ability of PEs to affect BRs biosynthesis by so far unknown mechanisms. By analyzing the profile of BRs arising at the end of the early C-6 oxidation biosynthetic pathway (typhasterol (TY) → castasterone (CS) → brassinolide (BL)), we found that 20E had the greatest effect on the level of the most biologically active BRs, BL (Figure 3B), which is the end product of the biosynthetic pathway of all BRs [7]. Its analogue, lacking the CH_3 group at carbon 28, 28-norbrassinolide (norBL), is the second most abundant BR of all endogenous BRs detected in the garden cress (accounting for 31.7 % of all BRs; Figure 3C) and the trend in its levels exactly imitated the situation described for the sum of all BRs in the studied tissue (Figure 3A). It also coincided with the trend in BL levels (Figure 3C). However, the highest level was determined for 28-norcastasterone (norCS; accounting for 43.5 % of all BRs), decreasing with increasing concentration of 20E in the culture medium, and reaching 68.8 % to 48.8 % of the norCS content in the control plants untreated with 20E (Figure 3D).

Reciprocally, the BRs levels were also quantified in garden cress seedlings cultured on one of the selected BRs (*epi*BL) at the same concentrations as in the case of 20E, i.e., at $1 \cdot 10^{-6}$ M, $1 \cdot 10^{-7}$ M, and $1 \cdot 10^{-8}$ M in the medium. It was found that *epi*BL acts on the levels of endogenous BRs exactly the opposite way of the effect of 20E. *Epi*BL increased the levels of endogenous BRs by an average of 32 % (Figure 4A). It had the greatest influence on the levels of BRs at a concentration of $1 \cdot 10^{-7}$ M, when the total amount of BRs in the garden cress tissue increased by up to 69.9 % compared with the control. This increase was 19.8% and 4.6% for $1 \cdot 10^{-8}$ M and $1 \cdot 10^{-6}$ M of *epi*BL in the medium, respectively. An analysis of the BR profiles revealed that among all of the detected BRs, the highest levels observed were again of norCS (Figure 4B), followed by norBL (Figure 4C). This is in agreement with the data

obtained in the experiment with the cultivation of garden cress seedlings on a medium with 20E (Figure 3). The trend of norCS levels across exogenous concentrations of *epi*BL applied in garden cress culture media corresponded with the trend for the sum of all BRs (Figure 4B vs. Figure 4A). It follows from the above that the PEs and BRs in garden cress tissue have an antagonistic effect, and exogenously applied BRs have a much greater influence on the biosynthesis of BRs than PE at the same concentration (see below). This might be related to the presence of the BR receptors in plant cells, which transmitted an exogenous BR signal much more sensitively than that of PE under the same experimental conditions. The reports about antagonism between PEs and BRs are relatively limited. However, Lehmann et al. showed that BRs bind competitively to ecdysteroid (EC) receptors partially purified from larvae of the blowfly *Calliphora vicina* and inhibit biological responses to 20E [15]. Furthermore, using *Drosophila melanogaster* B_{II} cell bioassay, a series of synthetic hybrid BR/EC structures have been assessed for their EC agonist/antagonist activities where only three compounds weakly antagonized the action of 20E at $5 \cdot 10^{-8}$ M [16]. Nevertheless, none of the ECs showed activity in the rice lamina inclination test, a well-established BR bioassay, demonstrating the high specificities of the insect EC receptor and the plant BR receptor.
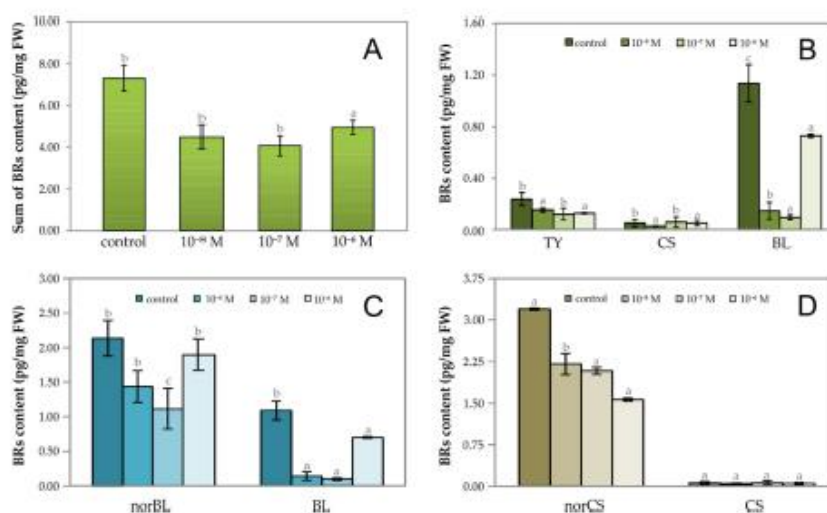

Figure 3. The effect of 20E at different concentrations on the (**A**) total content of endogenous brassinosteroids (BRs); (**B**) the content of typhasterol (TY), castasterone (CS), and brassinolide (BL); (**C**) the level of norBL (28-norbrassinolide) vs. BL; and (**D**) the level of norCS (28-norcastasterone) vs. CS in four-day-old seedlings of *Lepidium sativum* grown in vitro. Data/error bars represent the mean/standard deviation of three independent determinations.

2.2. The Effect of Exogenous Brassinosteroid on Growth of Lepidium Seedlings and the Level of Endogenous Phytoecdysteroid

Based on the image analysis of four-day-old *Lepidium* seedlings grown on an agar MS (Murashige Skoog) medium containing either $1 \cdot 10^{-8}$ M, $1 \cdot 10^{-7}$ M, or $1 \cdot 10^{-8}$ M *epi*BL, it was found that the length of their roots decreased in a concentration-dependent manner, while the length of the shoots seemed to increase only moderately (Figure 5). The root length of the control plants grown on media without *epi*BL was comparable with those grown on media containing $1 \cdot 10^{-8}$ M of *epi*BL, which then slowly decreased with the increased concentration of these BRs in culture media, and reached about 77 % of the control root length at $1 \cdot 10^{-6}$ M of *epi*BL. The length of the seedlings' shoots was not affected

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for those grown in the presence of *epi*BL at concentrations of $1 \cdot 10^{-8}$ M and $1 \cdot 10^{-7}$ M, whereas the concentration of $1 \cdot 10^{-6}$ M of this plant growth regulator in the culture medium caused an increase in this parameter by 21%. The shoot-to-root ratio therefore decreased by 49%, from 3.36 (control plants) to 1.66 (plants grown in the presence of $1 \cdot 10^{-6}$ M *epi*BL; Figure 5).
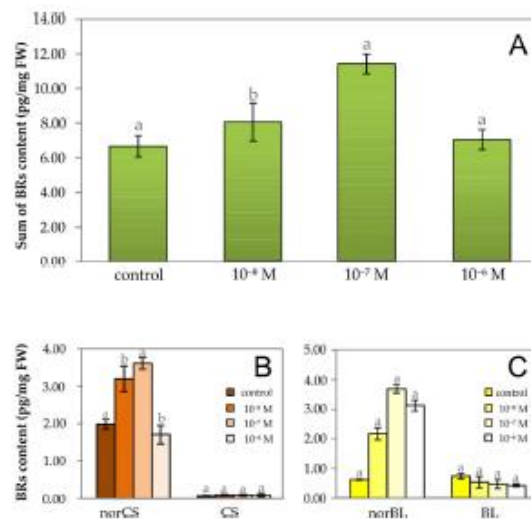

Figure 4. The effect of exogenous *epi*BL (24-*epi*brassinolide) at different concentrations on the **(A)** total content of endogenous BRs, **(B)** the level of norCS vs. CS, and **(C)** the level of norBL vs. BL in four-day-old seedlings of *Lepidium sativum* grown in vitro. Data/error bars represent mean/standard deviation of three independent determinations.
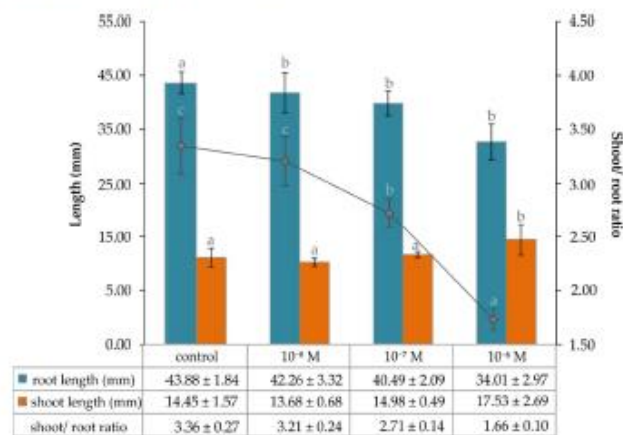

	root length (mm)	shoot length (mm)	shoot/ root ratio
control	43.88 ± 1.84	14.45 ± 1.57	3.36 ± 0.27
10^{-8} M	42.26 ± 3.32	13.68 ± 0.68	3.21 ± 0.24
10^{-7} M	40.49 ± 2.09	14.98 ± 0.49	2.71 ± 0.14
10^{-6} M	34.01 ± 2.97	17.53 ± 2.69	1.66 ± 0.10

Figure 5. The graph of dependence of shoot and root growth of four-day-old *Lepidium sativum* seedlings on the concentration of exogenous *epi*BL. The data/error bars represent the mean/standard deviation of six independent measurements.

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The quantitative analysis of 20E in extracts of four-day-old seedlings of garden cress by UHPLC-MS/MS confirmed the earlier published finding [12] that exogenous *epi*BL is able to affect the endogenous level of PEs. As can be seen from Figure 6, the amount of 20E was about 1.9 pg/mg of fresh weight (FW) in the control *Lepidium* seedlings (no *epi*BL in growth medium), and remained almost unchanged even in the presence of $1 \cdot 10^{-7}$ M and $1 \cdot 10^{-8}$ M of *epi*BL. This substance, at a concentration of $1 \cdot 10^{-6}$ M, caused the level of endogenous 20E to drop to 1.3 pg/mg FW, which is about 32%. This is in agreement with data of Kamlar et al. [13], who used *Spinacia oleracea* as a model plant, examining PEs for their experiment. In the case of garden cress, this is the first report of the presence of 20E in this plant species to our knowledge.
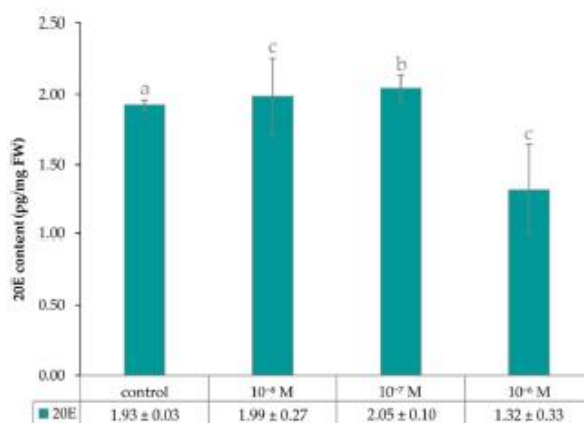

Figure 6. Graph of 20-hydroxyecdysone (20E) content in four-day-old seedlings of *Lepidium sativum* grown in vitro on media containing different concentrations of *epi*BL. Data/error bars represent the mean/standard deviation of three independent determinations.

3. Material and Methods

3.1. Plant Material, Growing, and Cultivation Conditions

The seeds of *Lepidium sativum* L. (Moravoseed Plc., Czech Republic) were surface-sterilized using a 70% ethanol solution containing 0.1% of Tween® 20, and were cultivated in vitro on vertical plates ($120 \times 120 \times 17$ mm; P-LAB, Czech Republic; 10 seeds in two separate lines/plate) containing 70 mL of Murashige Skoog (MS) basal growth medium including vitamins (4.4 g of MS salt/L; 3% sucrose; pH 5.6; agar 5.5 g/L—all purchased from Dutchefa Biochemie, the Netherlands). The cultivation was performed for four days under long day conditions of 18 h light, 23 °C/8 h dark, 18 °C, and light intensity 300 μmol·m^{-2}·s^{-1} (Photon Systems Instruments Ltd., Czech Republic). After four days of cultivation, a picture of the seedlings was taken for image analysis (see below), and the seedlings were then collected, frozen in liquid nitrogen, and stored at −80 °C in a freezer until extraction and analysis.

In the experiment related to the study of BRs' effect on PEs (20E), vertical plates containing 0 mol/L (control), $1 \cdot 10^{-6}$ mol/L, $1 \cdot 10^{-7}$ mol/L, and $1 \cdot 10^{-8}$ mol/L of *epi*BL (OlChemIm Ltd., Olomouc, Czech Republic) in an agar MS medium were prepared, with three identical plates for each sample. Similarly, when studying the effect of 20E on the levels of BRs, vertical plates containing 0 mol/L, $1 \cdot 10^{-6}$ mol/L, $1 \cdot 10^{-7}$ mol/L, and $1 \cdot 10^{-8}$ mol/L of 20E (Merck, Darmstadt, Germany) in an agar MS medium were each replicated three times.

The Java-based open source image-processing program ImageJ (the National Institutes of Health and the Laboratory for Optical and Computational Instrumentation, University of Wisconsin, USA)

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was used for image analysis of the garden cress seedlings. Deionized (Milli-Q) water obtained from a Simplicity® UV Water Purification System (Darmstadt, Germany) was used to prepare all of the aqueous solutions. All of the other chemicals (analytical grade or higher purity) were from Biosolve Chimie (Dieuze, France).

3.2. Sample Preparation and Analysis of Brassinosteroids by Ultra-High-Performance Liquid Chromatography–Electrospray Tandem Mass Spectrometry (UHPLC–ESI–MS/MS)

The extraction and analysis of the BRs was performed in three technical replicates according to Tarkowska et al. [14], with some modifications. Briefly, fresh plant tissue samples of 10 mg fresh weight (FW) were homogenized to a fine consistency, with 1 mL of 60% acetonitrile (ACN) as the extraction solution. The samples were then extracted overnight after adding BRs internal standards (OlChemIm Ltd. Olomouc, Czech Republic). The crude extracts were centrifuged (36,670× g, 10 min, and 4 °C; Beckman Avanti™ 30), and the corresponding supernatants were further purified using polyamine solid phase extraction columns Discovery DPA®-6S (50 mg, Supelco®, Bellefonte, PA, USA). Finally, the samples were analyzed by UHPLC-MS/MS (Micromass, Manchester, UK). The quantitation of BRs was performed using the isotope dilution method [17] after processing the MS data with MassLynx software (version 4.2, Waters, Manchester, UK).

3.3. Sample Preparation and Analysis of Phytoecdysteroids by Ultra-High-Performance Liquid Chromatography–Electrospray Tandem Mass Spectrometry (UHPLC–ESI–MS/MS)

The extraction and analysis of PEs was performed according to Kamlar et al. [13], with some modifications. Fresh plant tissue samples of 10 mg FW were homogenized to a fine consistency with 1 mL of 80 % methanol (MeOH) as the extraction solution. The samples were then extracted overnight, followed by centrifugation of the crude extracts (36,670× g, 10 min, and 4 °C; Beckman Avanti™ 30) and purification of corresponding supernatants using polyamine solid phase extraction columns Discovery DPA®-6S (50 mg, Supelco®, Bellefonte, PA, USA). Finally, the samples were analyzed by UHPLC-MS/MS (Micromass, Manchester, UK).

3.4. Statistical Analysis

All data were subjected to a standard analysis of variance. The means were compared using Tukey's HSD test, with the significance threshold set at 0.05. All calculations were performed using the Sigma Plot software v12.3.

4. Conclusions

The experiments performed in this preliminary study show that the presence of 20E, the most widely distributed PE in nature, reduces the levels of endogenous BRs in four-day-old seedlings of *Lepidium sativum* belonging to the Brassicaceae family. The largest decrease in the sum of endogenous BRs was observed on the medium with 20E at a concentration of $1 \cdot 10^{-7}$ M, while the greatest effect had the presence of exogenous 20E on the level of brassinolide, the most biologically active BR. The reciprocal treatment of culture media with exogenous *epi*BL caused the reduction in 20E endogenous level only at the highest concentration of $1 \cdot 10^{-6}$ M used. The presence of endogenous PEs in this plant species has not yet been published, so this is the first report about *Lepidium sativum* being PE-positive.

Author Contributions: D.T. designed the outline of the article, and composed the manuscript and figures. E.K. performed the experiments and analyzed the data. M.S. provided scientific feedback and critical comments. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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ARTICLE 16

A fast and reliable UHPLC-MS/MS-based method for screening selected pharmacologically significant natural plant indole alkaloids

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Contribution: design and performance of the study, literature research, manuscript writing

* corresponding author

Article

A Fast and Reliable UHPLC–MS/MS-Based Method for Screening Selected Pharmacologically Significant Natural Plant Indole Alkaloids

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Abstract: Many substances of secondary plant metabolism have often attracted the attention of scientists and the public because they have certain beneficial effects on human health, although the reason for their biosynthesis in the plant remains unclear. This is also the case for alkaloids. More than 200 years have passed since the discovery of the first alkaloid (morphine), and several thousand substances of this character have been isolated since then. Most often, alkaloid-rich plants are part of folk medicine with centuries-old traditions. What is particularly important to monitor for these herbal products is the spectrum and concentrations of the present active substances, which decide whether the product has a beneficial or toxic effect on human health. In this work, we present a fast, reliable, and robust method for the extraction, preconcentration, and determination of four selected alkaloids with an indole skeleton, i.e., harmine, harmaline, yohimbine, and ajmalicine, by ultra-high performance liquid chromatography coupled with tandem mass spectrometry. The applicability of the method was demonstrated for tobacco and *Tribulus terrestris* plant tissue, the seeds of *Peganum harmala,* and extract from the bark of the African tree *Pausinystalia johimbe.*

1. Introduction

Alkaloids are a well-known and important group of natural substances. The name "alkaloid" is derived from the alkaline nature of most of these substances, thanks to which it forms salts with acids. Most alkaloids occur in flowering plants as products of secondary metabolism, mainly in the form of salts with organic acids (acetic, oxalic, citric, lactic, tartaric), rarely in free form [1]. Alkaloids occur most often in the higher dicotyledon plants, and, to a lesser extent, in monocotyledons, e.g., the Liliaceae family. Even some seedless plants contain alkaloids, e.g., the yew tree (*Taxus*), joint pine (*Ephedra*), ground pine (*Lycopodium*), or horsetail (*Equisetum*). However, alkaloids are also present in some fungi, such as *Claviceps purpurea* or *Psilocybe*. The structure of alkaloids is very diverse: they can be aliphatic, cyclic, and aromatic, and they can also often have a steroidal structure. Biogenetically, alkaloids are mostly derived from amino acids, namely, ornithine, lysine, phenylalanine, tyrosine, tryptophan, and histidine. The subject of this article is indole alkaloids (IAs) in particular. This group of alkaloids have a common structural feature, the indole skeleton, which is biosynthetically derived from amino acid tryptophan (Trp; Figure 1). Most IAs also contain isoprenoid building blocks being formed via both known isoprenoid pathways—the mevalonate pathway (MVA) and the nonmevalonate pathway, also called the 2-C-methyl-D-erythritol 4-phosphate pathway (MEP) [2]. According to the structure of

the terpene unit, we distinguish simple IAs (including one C_5 unit—ergot alkaloids) and terpene IAs (including two C_5 units and containing 9–10 carbons: *Catharanthus*, *Rauvolfia*, and *Strychnos* alkaloids). Simple IAs are derivatives of tryptamine (Figure 1), which is a decarboxylation product of Trp.
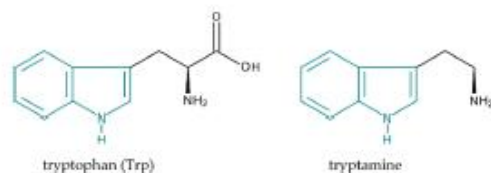

tryptophan (Trp)   tryptamine

Figure 1. Structure of tryptophan (Trp) and tryptamine with indole ring marked in teal.

Well-known members of this group are psylocin and psilocybin, which are alkaloids isolated from species of the *Psilocybe* genus. The subjects of interest of this study were harmine, harmaline, yohimbine, and ajmalicine (Figure 2), which are compounds belonging to a group of terpene IAs commonly called monoterpenoid indole alkaloids (MIAs); they are formed from tryptamine and the C_{10} terpene unit. It is the largest family of alkaloids in the plant kingdom, consisting of over 2000 compounds of the 10,000 alkaloids identified so far [3]. Many of these natural secondary metabolites are physiologically active in mammals, and they are isolated from various medicinal plants for their specific pharmacological effects.
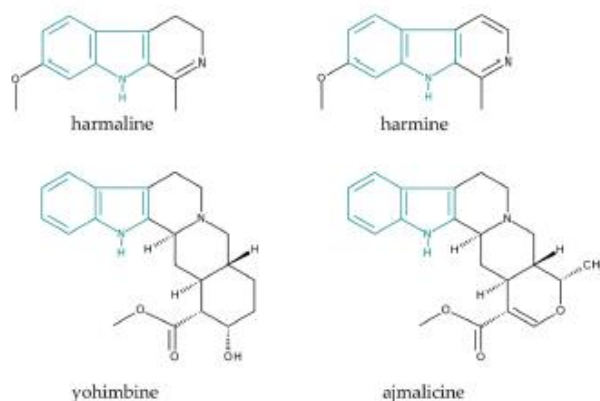

harmaline   harmine

yohimbine   ajmalicine

Figure 2. Structure of monoterpenoid indole alkaloids selected for this study (indole ring marked in teal).

Among them, harmala alkaloids (isolated for the first time from *Peganum harmala* [4] with harmine, harmaline, harmalol, harmol, and tetrahydroharmine as the most important representatives) are potent inhibitors of monoamine oxidase (MAO, EC 1.4.3.4), i.e., they can decrease the rate of degradation of monoamine neurotransmitters; therefore, they can be used as antidepressants [5]. The other two MIAs included in this study, yohimbine and ajmalicine, are present in many plant species, especially in those of the *Rauvolfia* and *Catharanthus* genera (both of the Apocynaceae family). They grow in tropical and subtropical forests of Asia, Africa, and Latin America, and have been commonly used in the Indian and Western systems of medicine, and in traditional Chinese medicine since antiquity [6–8]. The main therapeutic effects of ajmalicine, yohimbine, and other IAs of this type (e.g., reserpine) include antihypertensive and antiarrhythmic properties [9,10]. However, there are also studies where an antitumor effect was demonstrated [11,12].

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Although the occurrence of MIAs is relatively well-known, approaches for microscale extraction and sample pretreatment prior to their determination by sensitive analytical method are limited. Similarly, the relative distribution and content of alkaloids in plants are often inconsistent or not well-established [13–15]. Consumers in countries where plants rich in MIAs do not grow may receive these plants in the form of dried herbs, extracts, and other preparations that are usually sold as food supplements. In many countries, products of this type are not thoroughly controlled for active and other substances. When we remember the statement of Paracelsus (1493–1541; Swiss alchemist, astrologer, and physician) that 'the dose makes the poison' (Sola dosis facit venenum in Latin), i.e., a substance can cause the harmful effect associated with its toxic properties when it reaches a biological system in a certain concentration, we come to the conclusion that these natural products may be dangerous to some individuals. Therefore, the aim of the present research was to establish a fast, reliable, and high-throughput method for the determination of four pharmacologically significant substances from the MIA family for the rapid testing of their content in food supplements of plant origin. The method was validated and subsequently applied for quantitative analysis of these compounds in different, previously unpublished, plant materials. Additionally, part of the study was focused on profiling the selected MIAs in different parts of the tobacco plant to concurrently demonstrate the dependence of alkaloid level on the type of plant tissue (alkaloid distribution across the plant).

2. Results

2.1. Extraction and Purification Procedure

The extraction and purification procedure was developed with respect to both the relatively hydrophobic (indole ring) and the alkaline character of all studied compounds, which was demonstrated by their dissociation constant pK_a ranging from 6.31 (ajmalicine) to 9.55 (harmaline) [16]. Substances of this character were extracted from plant material using 60% MeOH (solvent for relatively hydrophobic substances) containing 0.25% of NH_4OH (alkaline additive). For the purification of crude extracts containing MIAs, a mixed-mode solid phase extraction (SPE) sorbent Bond Elut Plexa PCX having cation-exchange properties was used to effectively retain MIAs on the sorbent, while substances with acidic properties, and neutral substances were removed from the sample matrix. The ion-exchange group of the used cation-exchange SPE sorbent was bound to a polymer matrix, ensuring its stability in a wide pH range (1–14) and was intended for substances with a dissociation constant pK_a of 6–10, which met the range of dissociation constants of the substances of interest. Because the selected extraction solvent contained a base (ammonia), it was necessary to remove it from the sample by evaporation to dryness before applying the sample to the cation-exchange SPE, as the base retention occurs on the cation-exchange resin in an acidic environment where sorbent functional groups are negatively charged, and analytes are positively charged. To induce an acidic environment after evaporation to dryness, a solution containing 60% MeOH with 2% formic acid was selected for the loading of MIA-containing samples. After retention of the basic substances, the hydrophobic interferents were removed from the sorbent by washing with 100% MeOH, while the acidic and neutral substances passed through the column without retention. For the elution of basic substances, including the studied alkaloids, an elution solution containing 60% MeOH with the addition of 5% NH_4OH was chosen. Using this extraction and purification procedure, the following recovery values for the four standards of monitored alkaloids from the PCX sorbent were found: harmine, 87.86%; harmaline, 51.13%; yohimbine, 80.55%; and ajmalicine, 68.38%.

2.2. MIA Analysis by Liquid Chromatography–Mass Spectrometry

For the LC separation of the four MIAs of interest, two reversed-phase columns were tested: a ultra-high performance liquid chromatography (UHPLC) column with a sorbent containing a phenyl-hexyl group that selectively retained polyaromatic compounds through π–π interactions (Acquity UPLC® CSH™ Phenyl–Hexyl, 2.1 × 50 mm, 1.7 μm; Waters, Dublin, Ireland), and a UHPLC

column with C_{18} particles made by technology offering a superior peak shape for basic compounds under weak ionic strength mobile-phase conditions (Acquity UPLC® CSH™, 2.1×50 mm, 1.7 μm; Waters, Dublin, Ireland). Ionization efficiency and peak shape were found to be satisfactory when acetonitrile (ACN) (solvent A) and 10 mM ammonium acetate solution were used as components of the mobile phase. Under these conditions, retention times were acceptable and ranged from 2.15 (harmaline) to 5.38 min (ajmalicine) on the CSH column, and from 2.24 to 4.97 min on the phenyl-hexyl CSH column (Figure 3).
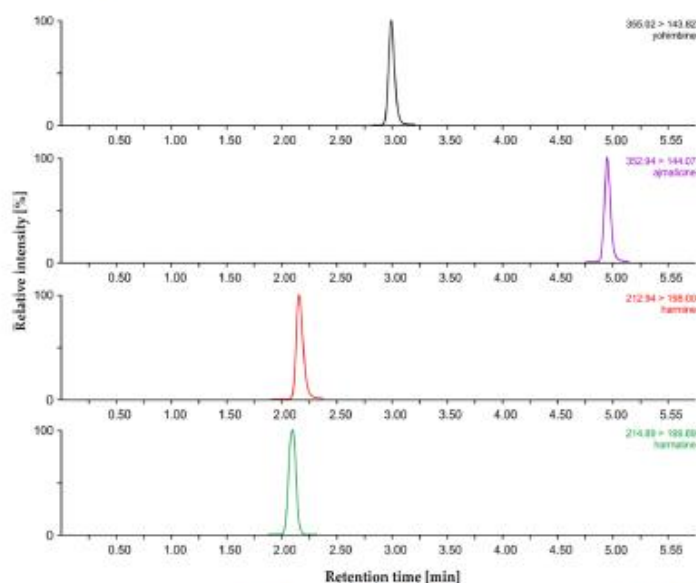

Figure 3. Ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) chromatogram of four-indole-alkaloid standard mixture on Acquity UPLC® CSH™ Phenyl–Hexyl column using acetonitrile and 10 mM ammonium acetate; pH 10.0.

However, two important chromatographic characteristics deteriorated when separated on a CSH vs. phenyl-hexyl CSH column. The resolution of the harmine and harmaline critical pair significantly decreased from 90% to 70%, and the average width of all peaks increased by 27% on the CSH column compared to the phenyl-hexyl counterpart (the largest difference in peak width was found for harmaline when it reached 36%). For these reasons, the phenyl-hexyl column was chosen for the further optimization of MIA separation. When optimizing the acidity of the mobile phase, 10 mM ammonium acetate solution adjusted to different pH levels was prepared. At a pH below 8.0 when all analytes were positively charged, significant widening of peak width was observed. Specifically, the width of the harmaline peak was 25% higher when using 10 mM ammonium acetate of pH 7.0 than that of the same mobile phase adjusted to pH 8.6. Moreover, large peak tailing was observed at pH 7.0 to 8.6 for this compound. The symmetry of this peak was achieved by increasing the pH of the mobile phase to a value of 10.0. This behavior might have been related to the dissociation constant of harmaline, which was the highest of the dissociation constants of all monitored substances and reached the value of 9.55 (see above). Therefore, in order to successfully separate such basic substances on this type of stationary phase, it was necessary to adjust the pH of the mobile phase so that the separated substances were in neutral form.

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2.3. Method Validation

The following performance studies were carried out to prove the suitability of the newly developed method for routine analysis of selected MIAs in biological samples of plant origin: calibration and linearity, limit of detection and limit of quantitation, analytical accuracy, precision and matrix effect.

2.3.1. Method Calibration and Linearity

Calibration curves were created by preparing solutions containing varying amounts of each unlabeled MIA and a known, fixed amount of the methyl-^{13}C, D$_3$-yohimbine as internal standard (IS) across concentration ranges of 5 to 100 pmol/2 μL of injection. Four separate injections were used to give the resulting calibration curves, which were linear in the selected concentration range for all four MIA compounds investigated (correlation coefficient, R^2, values obtained were in the range of 0.9910 to 0.9985; see Table 1). The linear range for all calibration curves was shown to cover 2 orders of magnitude.

Table 1. Validation data for UHPLC–MS/MS analysis of four indole alkaloids.

Compound	Retention Time (min)	Calibration Equation	Correlation Coefficient R^2	LOD (pg)	LOQ (pg)	Dynamic Range (pmol)
Harmaline	2.13 ± 0.01	log y = 0.9707 (log x) − 0.9819	0.9958	0.31	1.04	5–100
Harmine	2.16 ± 0.01	log y = 0.7374 (log x) − 0.2451	0.9935	0.18	0.60	5–100
Yohimbine	2.98 ± 0.01	log y = 1.0147 (log x) − 0.3091	0.9910	0.01	0.05	5–100
Ajmalicine	4.94 ± 0.01	log y = 0.8678 (log x) − 0.2006	0.9985	0.11	0.36	5–100

LOD, limit of detection; LOQ, limit of quantitation.

2.3.2. Limit of Detection and Quantitation

The limit of detection (LOD) was evaluated using an approach based on the standard deviation, s_b, of the calibration curve and the slope, and k, of a regression curve (LOD = $3 \times s_b/k$). The limit of quantitation (LOQ) was evaluated using a standard deviation/slope ratio approach (LOQ = $10 \times s_b/k$). The LODs and LOQs for MIAs are summarized in Table 1. The lowest LOD/LOQ was found for yohimbine (0.01/0.05 pg), while the most basic harmaline showed the highest values (0.31/1.04 pg).

2.3.3. Analytical Accuracy and Precision

The analytical accuracy of the newly developed UHPLC–(+)electrospray (ESI)–MS/MS method for the determination of selected MIAs was evaluated by the "standard addition method" using two sets of samples: purified extracts of *Nicotiana tabacum* tissue (5 mg fresh weight (FW) of leaf tissue—9th leaf—in 1 mL of extraction solution, six replicates) with the addition of 125 and 250 pmol of authentic MIA standards prior to one step sample purification by SPE. The concentration of each analyte was calculated using the standard isotope dilution method for each plant extract spiked before extraction and compared with the concentration of appropriate standard solution. The analytical accuracy values ranged between 97.7 and 102.0% of the true amount (Table 2). The analytical precision was determined to be in the range of 1.5 to 3.5% (Table 2).

Table 2. Analytical accuracy and precision for determination of monoterpenoid indole alkaloids (MIAs) by the UHPLC–(+) electrospray (ESI)–MS/MS method.

Compound	Determined Spiked Content [A] (pmol)	Analytical Precision [A] (%)	Analytical Accuracy [A] (%)	Determined spiked Content [B] (pmol)	Analytical Precision [B] (%)	Analytical Accuracy [B] (%)
Harmaline	125.5 ± 0.2	3.5	100.4	252.5 ± 1.4	2.5	101.0
Harmine	123.8 ± 0.9	1.5	99.0	245.5 ± 0.2	2.2	98.2
Yohimbine	125.0 ± 0.3	2.1	100.3	255.1 ± 0.9	2.9	102.0
Ajmalicine	122.5 ± 1.9	2.4	98.0	244.2 ± 1.2	2.3	97.7

[A] Extracts of 5 mg fresh weight (FW) *Nicotiana tabacum* tissue spiked with 125 pmol or 250 pmol [B] of authentic MIA standards, purified by the one solid phase extraction (SPE) step approach and analyzed by UHPLC–MS/MS. The values represent the mean ± standard deviation obtained for six technical replicates prepared and analyzed separately.

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2.3.4. Matrix Effect

The matrix effect (ME) is a known factor in the MS analysis of samples of biological origin, which causes changes in the ionization efficiency of the analyte in the ion source due to the presence of interfering substances originating from the biological matrix of the sample [17]. ME is manifested by the detection of less analytes than is actually the case, which significantly affects the results of quantitative analysis. For this reason, the study of ME was included in this work. ME is of great importance, especially in the determination of substances occurring in trace amounts, but it is also observed for more abundant substances that belong to the group of secondary metabolites of plants, including substances of alkaloid character. The aim of the experiment was to determine the optimal weight of biological material (plant tissue) for the determination of these substances, i.e., to find out what the optimal amount of tissue is leading to the maximal response of individual MIAs in LC–MS analysis that would be affected by interfering substances from the tissue (matrix) to a minimal extent. The comparison of alkaloid and chemical background levels, an experiment with different weights of plant sample, was performed on a commercially available extract of *Pausinystalia yohimbe*, for which the presence of MIAs, especially yohimbine and ajmalicine, is well-described in the literature [18]. Samples were extracted and purified using the one-step SPE-based procedure described in Section 4.3. The presence of yohimbine and ajmalicine was then confirmed by analysis using our newly developed UHPLC–MS/MS method (Section 4.4.), while harmine was also detected; harmaline was not detected in this plant tissue. The highest concentration of all monitored substances was reached by yohimbine, whose levels were found at the level of $\mu g \cdot mg^{-1}$ dry weight (DW), while the two other alkaloids detected were present at $pg \cdot mg^{-1}$ DW level, i.e., about a thousand times less abundant (Table 3).

Table 3. Levels of monitored indole alkaloids in yohimbe bark extract determined by UHPLC–MS/MS.

Sample Weight (mg)	Harmaline ($pg \cdot mg^{-1}$)	Harmine ($pg \cdot mg^{-1}$)	Yohimbine ($\mu g \cdot mg^{-1}$)	Ajmalicine ($pg \cdot mg^{-1}$)
5	ND	32.34 ± 3.69	5.29 ± 0.63	3.46 ± 0.92
10	ND	25.93 ± 2.79	4.01 ± 0.34	7.46 ± 1.24
15	ND	13.59 ± 3.11	3.31 ± 0.37	10.51 ± 1.28

ND, not detected; values represent mean ± standard deviation of six independent measurements.

The effect of the matrix on alkaloid level was observed for harmine and yohimbine, where their amount decreased with increasing sample weight. However, for ajmalicine, the found trend was the opposite (Table 1). The recovery for stable isotopically labelled yohimbine ranged from 67.62% for the 5 mg DW of the biological sample to 50.53% for the 15 mg DW, and clearly represented the ME phenomenon (Figure 4).
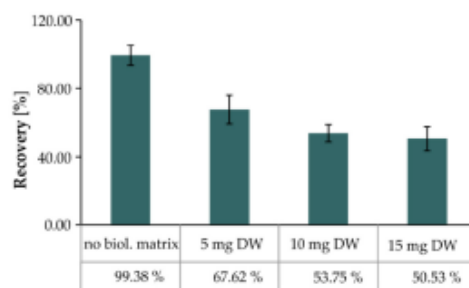

	no biol. matrix	5 mg DW	10 mg DW	15 mg DW
	99.38 %	67.62 %	53.75 %	50.53 %

Figure 4. Demonstration of matrix effect—dependence of recovery of methyl-^{13}C, D_3-yohimbine on biological tissue (yohimbe bark) amount. Data/error bars represent mean/standard deviation of three independent determinations.

Therefore, these experiments showed that the optimal amount of the sample for MIA analysis in yohimbe bark was 1–5 mg DW. As shown in Table 1, using 5 mg sample weight, the content of yohimbine was determined to be 5.29 ± 0.63 µg·mg^{-1} DW ($0.53\% \pm 0.06\%$) in this commercially available sample of yohimbine bark. However, this was not in agreement with the information of the supplier of this product that declared yohimbine content to be 20% (see Section 4.1). In fact, yohimbine content was actually 40 times lower than that stated by the supplier of this food supplement, which might qualify as fraudulent misrepresentation.

The matrix effect can be further presented in the form of a matrix factor (MF), which is a quantity calculated as the ratio of peak area response for each analyte in the presence of the plant matrix ions and peak area response in the absence of matrix multiplied by 100. To study this effect typical for the samples of biological origin, the standard mixture of MIAs (125 pmol each) was added to pure extraction solvent (60% aqueous MeOH, *v/v* containing 0.25% of NH_4OH) and to the extracts of a fresh tobacco tissue (9th leaf) weighing 5 mg, 10 mg and 15 mg FW after SPE process. Having established a new method for MIA analysis, we tested the extent to which the plant matrix from our samples suppressed the MS signals of interest. The data are summarized in Table 4 and confirm that the strongest matrix effect can be observed for the 15 mg FW (MF mean $\sim 61.1\%$), while about one-third lower ME was found for 5 mg FW samples (MF $\sim 81.1\%$),

Table 4. The effect of plant matrix on ionization efficiency of indole alkaloids in fresh tissue of tobacco.

Sample Weight (mg)	Matrix Factor (%)			
	Harmaline	Harmine	Yohimbine	Ajmalicine
5	76.57 ± 0.50	85.55 ± 0.16	94.05 ± 1.34	96.27 ± 1.12
10	69.20 ± 0.33	82.70 ± 0.30	74.74 ± 0.37	69.27 ± 0.50
15	58.68 ± 0.46	70.51 ± 0.21	65.48 ± 1.06	49.75 ± 0.48

The values represent mean ± standard deviation of six independent measurements.

2.4. MIA Determination in Tobbaco

To accurately determine the distribution of individual substances of interest in different types of tobacco tissue, the seeds of *Nicotiana tabacum*, ecotype SR1 (wild type), were sown and grown for 30 days; after that, the plant was divided into 10 parts according to the scheme described in Section 4.1. Analysis of individual tissue types revealed that the highest content of all four monitored substances was of yohimbine, which represented 89.6% of the total amount of all detected indole alkaloids (Figure 5).
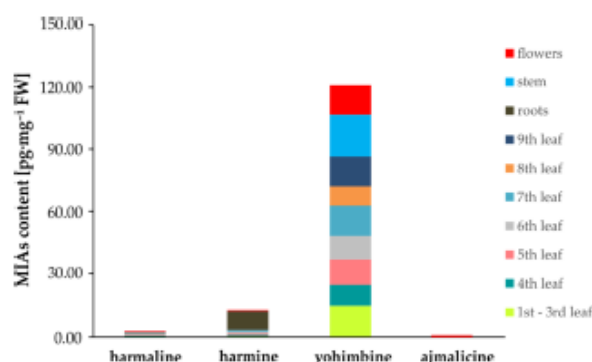

Figure 5. Distribution of individual indole alkaloids in 30-day-old *Nicotiana tabacum* plant. Data/error bars represent mean/standard deviation of three independent determinations.

The richest source of yohimbine was found to be a stem of tobacco plant, where its level reached 20.28 pg·mg^{-1} FW (Table 5). Conversely, the poorest sources of this substance were found to be the youngest leaves of the plant (7.76 pg·mg^{-1} FW; Table 5).

Table 5. Levels of indole alkaloids in 30-day-old *Nicotiana tabacum* plant.

Tissue	Alkaloid Content (pg·mg^{-1})			
	Harmaline	Harmine	Yohimbine	Ajmalicine
Flowers	0.45 ± 0.04	0.53 ± 0.11	14.40 ± 1.68	0.0062 ± 0.0011
1st–3rd leaf	0.38 ± 0.05	0.44 ± 0.15	7.76 ± 1.11	0.0111 ± 0.0010
4th leaf	0.25 ± 0.09	0.59 ± 0.02	10.02 ± 1.36	0.0047 ± 0.0008
5th leaf	0.41 ± 0.11	0.76 ± 0.09	12.38 ± 1.33	0.0135 ± 0.0002
6th leaf	0.36 ± 0.05	0.70 ± 0.10	11.30 ± 1.61	0.0065 ± 0.0015
7th leaf	0.19 ± 0.02	0.89 ± 0.04	14.65 ± 1.09	0.0080 ± 0.0016
8th leaf	0.23 ± 0.06	0.07 ± 0.02	9.53 ± 0.72	0.0067 ± 0.0007
9th leaf	0.23 ± 0.07	0.09 ± 0.01	14.12 ± 1.71	0.0048 ± 0.0011
Stem	0.23 ± 0.07	0.07 ± 0.01	20.28 ± 1.22	0.0090 ± 0.0022
Roots	ND	0.07 ± 0.02	8.04 ± 1.07	0.0056 ± 0.0021

ND, not detected; values represent mean ± standard deviation of three independent measurements.

When we focused on the two structural analogs harmine and harmaline, their total content in this plant was, on average, 1 pg·mg^{-1} FW from flowers to the 7th leaf; then, their total content decreased to 0.32 pg·mg^{-1} FW in the oldest leaves, stems, and roots of the plant, respectively (Figure 6). The lowest levels were found for ajmalicine, which made up only about 1% of the total content of harmine and harmaline. While the content of harmine increased with the age of the plant's leaves (from the apical part of the plant to the 7th leaf), the trend for harmaline was the opposite, while none was detected in the roots. The ratio of harmine to harmaline basipetally increased from 1.1 in the apical part of the plant to the 7th leaf, where their ratio reached 4.7. In the oldest leaves (8th and 9th) and plant stem, it dropped rapidly to 0.4 (Figure 6).
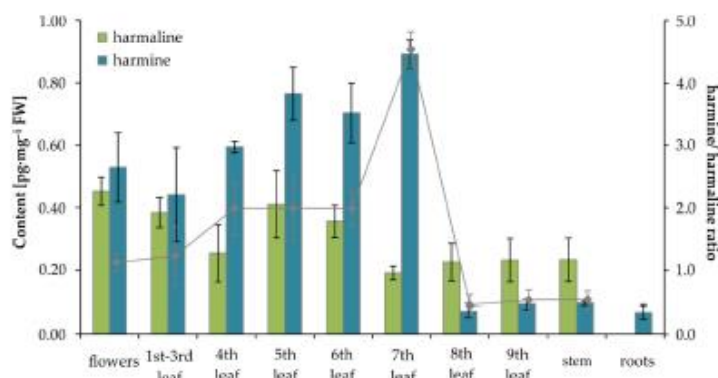

Figure 6. Graph of harmaline and harmine content in different tissue types of 30-day-old *Nicotiana tabacum*. Data/error bars represent mean/standard deviation of three independent determinations.

2.5. Determination of MIAs in Tribulus terrestris

Tribulus terrestris is a plant in traditional Chinese medicine that is well known for its myriad effects on human health. According to the available literature, it also contains a number of alkaloids, including harmine, harmaline [19], harmane, and norharmane [20]. The content of the two other alkaloids studied in this work was not found in the available literature sources. For quantitation of

MIAs using the new UHPLC–MS/MS method, samples of *T. terrestris* were used originating from different countries, and in different compositions of seeds, stems, and other parts of the plant, supplied by Conneco Co., as described in detail in Section 4.1. Analysis showed that the content of harmine and harmaline, when compared to harmane, was at a very low level in the *Tribulus* tissue (Table 6), while harmine was not detected in the *Tribulus* sample originating from Bulgaria and in the sample from China containing only burrs. Furthermore, the *Tribulus* samples were found to contain relatively high levels of yohimbine, and, regardless of the country of origin and composition of the sample, its average content was about 50 $pg \cdot mg^{-1}$ DW, which was the highest level of all indole alkaloids monitored in this type of observed sample. No ajmalicine was detected by this method in *Tribulus* samples.

Table 6. Indole alkaloid levels in *Tribulus terrestris*.

Tissue	Alkaloid Content ($pg \cdot mg^{-1}$)			
	Harmaline	Harmine	Yohimbine	Ajmalicine
Burrs + stems (Bulgaria)	0.012 ± 0.005	ND	36.14 ± 2.35	ND
Whole plant (China)	0.004 ± 0.002	1.76 ± 0.18	62.74 ± 5.02	ND
Burrs only (China)	0.004 ± 0.001	ND	43.21 ± 0.77	ND
Flower, burrs (India)	0.005 ± 0.001	0.21 ± 0.05	57.59 ± 3.25	ND

ND, not detected; values represent mean ± standard deviation of three independent measurements.

2.6. MIA Determination in Peganum harmala Seeds

As is described in the literature, *Peganum harmala*, similarly to *Tribulus terrestris*, is a very well-known plant in traditional East Asian medicine (eastern Iran, western India). For the purposes of this study, seed samples were commercially obtained (see Section 4.1). Companies that are allowed to supply them to the European Union (EU) market must declare that they are only intended for agricultural purposes, as they cannot be imported as food supplements according to European Union regulations. Reportedly, *P. harmala* seeds contain 2.5% to 4% of a mixture of harmala alkaloids [21], which are characterized by many pharmacological effects, including hallucinogenic effects [22], but also antitumor [23], antimicrobial, or vasorelaxant properties [24]. Analysis using the newly developed UHPLC–MS/MS method revealed that harmala seeds do contain all studied MIAs, with the highest content for harmine (1097 $ng \cdot mg^{-1}$ DW), followed by harmaline (20 $ng \cdot mg^{-1}$ DW), and the lowest for yohimbine and ajmalicine (Table 7).

Table 7. Indole alkaloid levels in *Peganum harmala* seeds.

Alkaloid content ($pg \cdot mg^{-1}$)			
Harmaline	Harmine	Yohimbine	Ajmalicine
19.76 ± 0.44	1096.67 ± 14.77	7.25 ± 0.83	0.24 ± 0.03

3. Discussion

Alkaloids are the constituents of plant (and, less often, animal or fungal) cells. With regard to their efficient extraction and subsequent precise analysis, it is important to have sound knowledge of the chemical and analytical principles underlying these processes. First, homogenization of plant tissue prior to extraction is necessary for breaking cell walls in tissue [25]. This action allows for the substances present inside the cell to migrate to an appropriate extraction solvent. It is most often completed by grinding or by ultrasonic devices [5,26]. In our method, milligram amounts of plant material were homogenized in plastic safe-locked microtubes with a selected extraction solvent and chemically/mechanically resistant zirconium oxide beads for an appropriate time at a selected frequency. This approach was also successfully applied for sample preparation in ultra-trace analysis of other substances of plant origin, and gives highly repeatable and reproducible results [27,28]. Analyte losses that usually occur during the sample purification procedure can be accounted for by adding internal standards (usually labelled with stable isotopes) to the plant extracts. In addition, this procedure allows

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a measure of percentage recovery of the target metabolites throughout the purification procedure. Although recovery markers should ideally be included for every analyte that is being quantified, only one internal standard (methyl-^{13}C, D$_3$-yohimbine) was used for the determination of the four substances in this study. This procedure was chosen because there was a minimal risk of errors in the determination of the three other compounds due to the dissimilarity of their chemical nature. Conditions for the optimal extraction of MIAs were provided by the choice of a solvent with a chemical character similar to the chemical nature of the analyzed substances. Although pure organic solvents such as methanol and ethanol are often commonly used [14,26], the extraction efficiency for compounds with a basic character could be significantly increased by the addition of a basic component to an extraction solution [29]. For this purpose, we added NH$_4$OH that met compatibility requirements with MS detection, i.e., it is volatile, does not reduce the signal of the analytes, and does not contaminate the inner part of the quadrupole-based MS analyzer. In order to reduce levels of interfering compounds in highly complex plant extracts, liquid–liquid and/or SPE is a first choice. To ensure high sample throughput and high reproducibility of the method, as well as to reduce the consumption of chemical agents, we used the SPE approach with a sufficient bed size of the mixed-mode sorbent, giving satisfactory recovery and a relatively low volume of cartridge. Moreover, mixed-mode SPE columns packed with a mixture of two types of sorbent allowed us to reduce the number of required purification steps due to more than one separation mechanism that could be exploited using a single column while maintaining high sample-clean-up efficiency. With regard to advanced analytical methods applied for MIA detection and quantitation in samples of plant origin, micellar electrokinetic chromatography (MEKC) [19], high performance liquid chromatography (HPLC) [5,8,15,30], high performance thin layer chromatography (HPTLC) [31], direct analysis in real-time mass spectrometry (DART–MS) [7], and LC–MS/MS [18,26] have been reported to date. Among many available sophisticated instruments, LC–MS/MS offers many advantages in the analysis of these types of compounds mainly due to its separation efficiency and sensitivity, and it was also employed in this study. A comparison of newly developed method parameters with those that were earlier published in regard mainly to the limit of detection and amount of tissue needed for analysis is summarized in Table 8. Unlike commonly used C$_8$ [26] or C$_{18}$ [5] stationary phase columns, an LC sorbent modified with phenyl-hexyl groups was shown to be highly efficient for the separation of MIAs as polycyclic compounds.

Table 8. The comparison of different methods used for the determination of indole alkaloids.

Instrumental Method Used	Amount of Tissue	Type of Tissue	Limit of Detection	Reference	Capacity of the Method Used
UHPLC–(+)ESI–MS/MS	5 mg	whole plant	0.01–0.31 pg	method presented	4 IAs
HPLC–UV	n.a.	whole plant	0.77–56 mg·g^{-1}	[5]	2 IAs
HPLC–UV	0.1 g	roots	6–8 µg·mL^{-1}	[8]	3 IAs
HPLTLC	n.a.	seeds		[13]	4 IAs
HPLC–UV	1 g	seeds	0.01–0.05 µg·mL^{-1}	[14]	2 IAs
HPLC–UV	n.a.	cell culture	n.a.	[15]	22 IAs
UPLC/IM–QTOF–MS *	0.1 g	yohimbe bark	n.a.	[18]	55 IAs
MEKC–UV/LIF	n.a.	none	n.a.	[19]	6 IAs
HPLC–(+)ESI–QTOF–MS/MS	50 g	roots	n.a.	[26]	47 IAs
HPLC–UV–MS	0.5 g	aphrodisiac products	3–60 ng·mL^{-1}	[29]	yohimbine
HPLC–UV	2 g	roots	0.05–0.39 µg·mL^{-1}	[30]	5 IAs
HPLC–UV	5 g	roots	n.a.	[31]	6 IAs

* ultra-performance liquid chromatography/ion mobility quadrupole time-of-flight mass spectrometry; LIF, laser-induced fluorescence; IAs, indole alkaloids; n.a., not available.

4. Materials and Methods

4.1. Plant Materials

The following plant materials were used for this study: (1) Yohimbe bark extract (*Pausinystalia johimbe*) with declared yohimbine content of 20% (country of origin: not specified, Africa), purchased from Herbal Store, Czech Republic (www.herbal-store.cz). (2) Fresh tobacco tissue (*Nicotiana tabacum* cv. Petit Havana SR1, wild type), with plants grown in the soil of a campus greenhouse of the Centre

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of the Region Haná for Biotechnological and Agricultural Research, harvested after 30 days of growing under natural conditions, and divided into 10 individual samples, as shown in Figure 7. Samples were immediately frozen in liquid nitrogen and stored in a freezer at −80 °C until extraction and analysis. (3) *Tribulus terrestris*: dry tissue of different types and different country of origin (Figure 8)—burrs (Figure 9) and stems (Bulgaria); stems, whole plants and burrs (China); flowers and burrs (India)—all provided by Conneco Chemicals Ltd. (4) Seeds of *Peganum harmala* (country of origin: Mexico), purchased from Botanico Ltd., Czech Republic (www.botanico.cz).
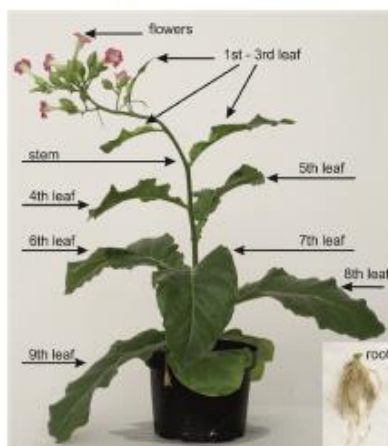
Figure 7. Thirty-day-old tobacco plant. Scheme of plant division into individual parts according to type and age of tissue before extraction and analysis by LC–MS/MS.
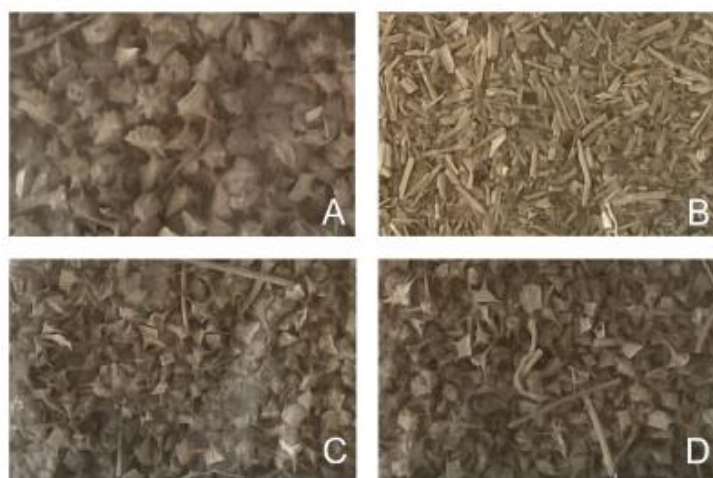
Figure 8. Photo of dry *Tribulus terrestris* tissue provided by Conneco Chemicals Ltd. and used in this study. (**A**) *T. terrestris* burrs including flowers; origin: India. (**B**) Dried whole plant of *T. terrestris* excluding burrs; origin: China. (**C**) *T. terrestris* burrs including stems; origin: Bulgaria. (**D**) Dried whole plant of *T. terrestris* including burrs; origin: China.
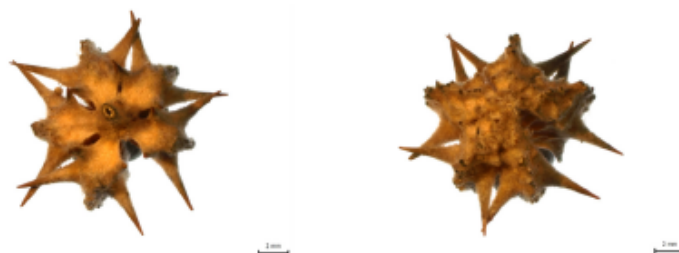
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Figure 9. Detailed photo of *Tribulus terrestris* burrs used in this study.

4.2. Chemicals

Indole alkaloids (harmine, CAS 442-51-3; harmaline, CAS 304-21-2; yohimbine, CAS 146-48-5; and ajmalicine, CAS 483-04-5) were obtained from Sigma Aldrich (Steinheim, Germany) at $\geq$ 95% or higher purity. Stabile isotope-labelled standard of methyl-^{13}C, D_3-yohimbine (CAS 1261254-59-4) was purchased for the purpose of quantitation from Sigma Aldrich, Germany. Formic acid (FA), aqueous ammonia solution (25%, v/v), ammonium bicarbonate, ammonium acetate, methanol (MeOH, HPLC grade), and acetonitrile (ACN, HPLC grade) were purchased from Merck (Darmstadt, Germany). Deionized (Milli-Q) water obtained from a Simplicity® UV Water Purification System (Darmstadt, Germany) was used to prepare all aqueous solutions. All other chemicals (analytical grade or higher purity) were from Biosolve Chimie (Dieuze, France).

4.3. Extraction and Isolation Procedure

Aliquots of 5 to 15 mg fresh or dry weight (FW, DW) of plant tissue were weighed into 2 mL Eppendorf tubes and 1 mL of 60% MeOH containing 0.25% NH_4OH as an extraction solution, and 2 mm ceria stabilized zirconium oxide beads (Next Advance Inc., Averill Park, NY, USA) was added for further homogenization using an MM 400 vibration mill at a frequency of 27 Hz for 3 min (Retsch GmbH & Co. KG, Haan, Germany). For quantitative analysis, an internal standard solution containing 125 pmol of methyl-^{13}C, D_3-yohimbine was also added to the samples. Samples were extracted for 20 min in a DT 510 ultrasonic bath (Bandelin GmbH & Co. KG, Berlin, Germany). Homogenates were then centrifuged (36, 670 g, 10 min, 4 °C; Beckman Avanti™ 30); supernatants were placed into a clean borosilicate glass test tube and evaporated to dryness at 37 °C using nitrogen evaporator Turbovap® (Biotage, Sweden). After that, the dried sample residues were reconstructed in 60% MeOH containing 2% FA, and subsequently purified using a Visiprep™ solid phase extraction (SPE) vacuum manifold (Supelco®, Bellefonte, PA, USA) and mixed-mode polymer-based cation-exchange cartridges (Bond Elut Plexa PCX, 60 mg/3 mL, Agilent Technologies, Santa Clara, CA, USA), activated with 100% MeOH and equilibrated with 60% MeOH + 2% FA before sample loading. After washing the cartridge with 100% MeOH, MIA elution was performed with a solution consisting of 60% MeOH with 5% of NH_4OH. The elution fraction was evaporated to dryness in vacuo (CentriVap® acid-resistant benchtop concentrator, Labconco Corp., Kansas City, MO, USA).

4.4. Indole Alkaloid Analysis by Ultra-High Performance Liquid Chromatography–Tandem Mass Spectrometry (UHPLC–MS/MS)

An Acquity UPLC™ system (Waters, Milford, MA, USA) consisting of binary solvent manager and sample manager modules coupled to a Xevo® TQ MS triple-stage quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an electrospray (ESI) interface and collision cell, Scan Wave, was utilized for MIA quantitation. The entire LC–MS/MS system was controlled by Masslynx™ software (version 4.1, Waters, Manchester, UK). After purification by PCX SPE cartridges, dried samples were reconstituted in 50 µL of 100% MeOH; then, 2 µL of each sample was injected

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onto a reversed-phase UHPLC column (Acquity CSH™ Phenyl–Hexyl, 2.1 × 50 mm, 1.7 μm, 130 Å; Waters) coupled to the ESI–MS/MS system. MIAs were analyzed in positive ion mode as [M+H]$^+$. MIAs of interest were separated (Figure 3) by a linear gradient of ACN (A) and 10 mM ammonium acetate, pH 10.0 adjusted by aqueous ammonia solution (B) at a flow rate of 0.3 mL·min^{-1}, 0–1 min, 30:70 (A:B, *v/v*)—15 min, 100:0 (A:B)—15–16 min, 100:0 (A:B). Under these conditions, the substances of interest were eluted within 5 min. Lastly, the column was washed with 100% ACN for 1.5 min and re-equilibrated to initial conditions (30:70 A:B, *v/v*) for 1.5 min. See Table 9 for the retention times of the studied MIAs. The thermostat of the column was programmed to 40 °C, and the temperature inside the autosampler was maintained at 4 °C. Capillary voltage, cone voltage, collision cell energy, and ion source temperatures were optimized for each individual compound using the same setup. Mass spectrometer settings were as follows: capillary voltage, 0.5 kV; cone voltage, 40 V; source temperature, 100 °C; desolvation gas temperature, 350 °C; cone gas flow, 70 L·h^{-1}, desolvation gas flow (nitrogen), 500 L·h^{-1}; and collision gas flow (argon), 0.15 L h^{-1}. The dwell time of each multiple-reaction-monitoring (MRM) channel was calculated to provide 16 scan points per peak with an inter channel delay of 0.1. MS data were recorded in MRM mode (Table 9). All data were processed using MassLynx™ software (ver. 4.1, Waters).

Table 9. Optimized MS conditions for detection of each studied indole alkaloid.

Compound	RT (min)	MRM (Q)	Cone Voltage (V)	Collision Energy (V)	MRM (C)	Cone Voltage (V)	Collision Energy (V)
Harmaline	2.13	214.89 > 199.89	40	25	214.89 > 172.11	40	30
Harmine	2.16	212.94 > 198.00	40	25	212.94 > 169.84	40	30
Yohimbine	2.98	355.02 > 143.82	40	30	355.02 > 211.93	40	25
Methyl-^{13}C, D$_3$-Yohimbine	2.96	359.05 > 143.94	40	30	359.05 > 216.02	40	25
Ajmalicine	4.94	352.94 > 144.07	40	25	352.94 > 177.91	40	25

RT, retention time; MRM (Q), multiple reaction monitoring transition for compound quantitation; MRM (C), multiple-reaction-monitoring transition for confirmation of compound identity.

5. Conclusions

This work focused on the development of a new microscale method for the extraction, preconcentration, and determination of harmine, harmaline, ajmalicine, and yohimbine, which are indole alkaloids with sympatholytic activity derived from the tryptophan amino acid in plant tissue. We chose 60% MeOH containing 0.25% NH$_4$OH as a suitable solvent for the extraction of these substances from plant tissue. Solid phase extraction based on a mixed-mode sorbent with strong cation-exchange properties proved to be suitable for the preconcentration of natural substances exhibiting basic and relatively hydrophobic character. Furthermore, we developed a new and reliable UHPLC–MS/MS-based high-throughput method for profiling the selected indole alkaloids. The applicability of the method was demonstrated for tobacco and *Tribulus terrestris* plant tissue, seeds of *Peganum harmala,* and extract from the bark of *Pausinystalia johimbe.* Experimentally obtained data on the occurrence of individual alkaloids in the given tissue were in agreement with the available literature, except for the presence of ajmalicine and yohimbine in *Tribulus terrestris,* which was detected in this plant species for the first time.

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Sample Availability: Samples of the compounds are available from the authors.