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RESEARCH PAPER

Distribution, biological activities, metabolism, and the conceivable function of *cis*-zeatin-type cytokinins in plants

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Abstract

Cytokinins (CKs) are plant hormones affecting numerous developmental processes. Zeatin and its derivatives are the most important group of isoprenoid CKs. Zeatin occurs as two isomers: while *trans*-zeatin (*trans*Z) was found to be a bioactive substance, *cis*-zeatin (*cis*Z) was reported to have a weak biological impact. Even though *cis*Z derivatives are abundant in various plant materials their biological role is still unknown. The comprehensive screen of land plants presented here suggests that *cis*Z-type CKs occur ubiquitously in the plant kingdom but their abundance might correlate with a strategy of life rather than with evolutionary complexity. Changing levels of *trans*Z and *cis*Z during *Arabidopsis* ontogenesis show that levels of the two zeatin isomers can differ significantly during the life span of the plant, with *cis*Z-type CKs prevalent in the developmental stages associated with limited growth. A survey of the bioassays employed illustrates mild activity of *cis*Z and its derivatives. No *cis*↔*trans* isomerization, which would account for the effects of *cis*Z, was observed in tobacco cells and oat leaves. Differences in uptake between the two isomers resulting in distinct bioactivity have not been detected. In contrast, *cis*Z and *trans*Z have a different metabolic fate in oat and tobacco. Analysis of a CK-degrading enzyme, cytokinin oxidase/dehydrogenase (CKX), reveals that *Arabidopsis* possesses two isoforms, AtCKX1 expressed in stages of active growth, and AtCKX7, both of which have the highest affinity for the *cis*Z isomer. Based on the present results, the conceivable function of *cis*Z-type CKs as delicate regulators of CK responses in plants under growth-limiting conditions is hypothesized.

Key words: *Arabidopsis thaliana*, *cis*-zeatin, cytokinin, cytokinin oxidase/dehydrogenase, growth-limiting conditions, oat, tobacco BY-2 cells, *trans*-zeatin.

Introduction

Cytokinins (CKs) represent a large group of plant hormones which affect various vital processes throughout plant growth and development. Natural CKs are derivatives of adenine containing an isoprenoid or aromatic moiety at the N^6 position. Typical representatives of isoprenoid CKs are N^6-(2-isopentenyl)adenine (iP) and its hydroxylated forms

zeatin (Z) and dihydrozeatin (DHZ). Zeatin occurs in two isomers, *cis*Z and *trans*Z, referring to the position of the terminal hydroxyl group on the isoprenoid side chain.

Compelling evidence from several studies has shown that CK signals in plants are able to delay leaf senescence (Gan and Amasino, 1995; Kim *et al.*, 2006, and references therein), promote shoot branching (Thimann and Sachs, 1964; Tanaka *et al.*, 2006), and specifically affect plant shoot and root growth (Werner *et al.*, 2001, 2003). In addition, CKs mediate the signalling of the availability of inorganic nitrogen to the roots (Miyawaki *et al.*, 2004), seed gemination, and responses to pathogens (Mok, 1994).

Most of the physiological activity of zeatin as a free hormone has been attributed for years to *trans*Z, while typically *cis*Z has been considered as an inactive or weakly active form of CK. These conclusions were based on data from various bioassays (Schmitz *et al.*, 1972; Kaminek *et al.*, 1987). *cis*Z has been reported to be an important component of certain tRNAs localized as a modified adenosine residue immediately adjacent to the 3' end of the anticodon of tRNAs that recognize the UUN codon (Skoog and Armstrong, 1970). A strong decrease of free *cis*Z was monitored after knocking down two genes; *AtIPT2* and *AtIPT9*, coding for the CK biosynthesis enzyme isopentenyltransferase (IPT; Miyawaki *et al.*, 2006). They are the only tRNA-specific IPT genes which share substantial similarities with the prokaryotic *miaA* gene catalysing transfer of dimethylallyl diphosphate (DMAPP) to tRNA (Persson and Bjork, 1993; Leung *et al.*, 1997).

Despite the presumed inactivity of *cis*Z as a free hormone, the presence of free *cis*Z-type CKs in plant tissues has been reported repeatedly. Some plant species contain detectable levels of diverse *cis*Z-type CKs, and even occasionally as the predominant group of total CKs. Different *cis*Z-type CKs were identified in a large number of seed plants including both monocots (e.g. Parker *et al.*, 1988; Veach *et al.*, 2003) and dicots (e.g. Nicander *et al.*, 1995; Emery *et al.*, 1998, 2000; Dobrev *et al.*, 2002; Ananieva *et al.*, 2004; Gaudinová *et al.*, 2005; Malkawi *et al.*, 2007; Stirk *et al.*, 2008; Van Staden *et al.*, 2010), as well as in algae (Stirk *et al.*, 2003; Ördög *et al.*, 2004) and mosses (Von Schwartzenberg *et al.*, 2007). Moreover, enzymes catalysing specifically *cis*Z metabolic reactions were reported, namely glucosyltransferases (*cis*Z*O*G1 and *cis*Z*O*G2) in maize (Martin *et al.*, 2001; Veach *et al.*, 2003) and *cis–trans*-isomerase in beans (Bassil *et al.*, 1993). Likewise signal perception by particular histidine kinases can be mediated by *cis*Z and/or its riboside *cis*ZR with similar efficiency to *trans*Z (Spíchal *et al.*, 2004; Yonekura-Sakakibara *et al.*, 2004; Romanov *et al.*, 2006). These findings overall have indicated that *cis*Z derivatives are more prevalent and more relevant to CK biology than previously thought, and probably have unique functions in plant tissues. Moreover, it is speculated that they are synthesized in plant cells in a distinct way(s) compared with their corresponding *trans* isomers (Kasahara *et al.*, 2004). However, a precise role for *cis*Z-type CKs in plants still remains to be elucidated.

The aim of this study was to unravel the distribution of the *cis*Z-type CKs in the plant kingdom, their activity in bioassays, as well as their uptake and metabolism including possible *cis–trans* isomerization in plants. The validity of analytical techniques, excluding spontaneous *cis*Z↔*trans*Z isomerization during the extraction and/or purification procedure, has been evaluated here. These aspects have been surveyed using both monocot and dicot plant models (oat, *Arabidopsis*, and tobacco). Based on the results it is hypothesized that the role of *cis*Z might reside in a delicate regulation of CK response(s) and maintenance of minimal CK activity under growth-limiting conditions.

Materials and methods

Chemicals

All CKs were procured from Olchemim Ltd (Olomouc, Czech Republic); other chemicals were purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Radiolabelled CKs [2-^{3}H]iP (specific activity 1200 TBq mol^{-1}), [2-^{3}H]*trans*Z (850 TBq mol^{-1}), and [2-^{3}H] *cis*Z (780 TBq mol^{-1}) were synthesized at the Isotope Laboratory, Institute of Experimental Botany AS CR (Prague, Czech Republic) according to Hanus *et al.* (2000). [$^{13}C_5$]*cis*-zeatin CKs were prepared according to an unpublished method which will be described elsewhere. Other deuterium-labelled CK standards were purchased from Olchemim Ltd.

Plant material

Seeds of plant species chosen for CK screening were obtained from SELGEN Ltd (Pernarec, Czech Republic) and SEMO Ltd (Smržice, Czech Republic). The tissues (in most cases vegetative shoots, otherwise leaves) for analyses of CK content were collected from plants cultivated in optimal greenhouse conditions or from plants growing in the open air and sampled during June and July 2008 in the north-eastern region of the Czech Republic. (A list of the species analysed in this study can be found in Supplementary Table S1 available at *JXB* online.)

Cytokinin analysis

The procedure for CK purification was based on a modification of the method described by Faiss *et al.* (1997). Deuterium-labelled CK internal standards (Olchemim Ltd) were added, each at 1 pmol per sample to check the recovery during purification and to validate the determination (Novák *et al.*, 2008). The samples were purified using a combined cation (SCX-cartridge) and anion (DEAE-Sephadex-C18-cartridge) exchanger and immunoaffinity chromatography (IAC) based on a wide range of specific monoclonal antibodies against CKs (Novák *et al.*, 2003). The metabolic eluates from the IAC columns were evaporated to dryness and dissolved in 20 µl of the mobile phase used for quantitative analysis. The samples were analysed by ultra-performance liquid chromatography (UPLC) (Acquity UPLC™; Waters, Milford, MA, USA) coupled with a Quatro *micro*™ API (Waters, Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray interface. The purified samples were injected onto a C18 reversed-phase column (BEH C18; 1.7 µm; 2.1×50 mm; Waters). The column was eluted with a linear gradient (0 min, 10% B; 0–8 min, 50% B; flow rate of 0.25 ml min^{-1}; column temperature of 40 °C) of 15 mM ammonium formate (pH 4.0, A) and methanol (B). Quantification was obtained by multiple reaction monitoring (MRM) of [M+H]$^{+}$ and the appropriate product ion. For selective MRM experiments, optimal conditions, dwell time, cone voltage, and collision energy in the collision cell corresponding to an exact diagnostic transition were optimized for each CK

(Novák *et al.*, 2008). Quantification was performed by Masslynx software using a standard isotope dilution method. The ratio of endogenous CK to an appropriate labelled standard was determined and then used to quantify the level of endogenous compounds in the original extract, according to the known quantity of an added internal standard (Novák *et al.*, 2003).

An analysis of profiles and concentrations of individual CKs in plant species with a prevalence of *cis*Z(s) was performed by HPLC-MS/MS. CKs were extracted from plants by methanol/formic acid/water (15/1/4, v/v/v), homogenized in liquid nitrogen, and purified using a dual-mode solid phase extraction method (Dobrev and Kaminek, 2002). CK ribotides were determined as the corresponding ribosides following their dephosphorylation by alkaline phosphatase. The HPLC-MS analysis was performed as described by Dobrev *et al.* (2002) using a Rheos 2000 HPLC gradient pump (Flux Instruments AG, Reinach, BL, Switzerland) and HTS PAL autosamples (CTC Analytics AG, Zwingen, Switzerland) coupled with a Finnigan MAT LCQ-MS^n ion trap mass spectrometer (Finnigan, San Jose, CA, USA) equipped with an electrospray interface. Detection and quantification were carried out using a Finnigan LCQ operated in the positive ion full-scan MS/MS mode using a multilevel calibration graph with ^{2}H-labelled CKs as internal standards. Detection limits were calculated for each compound as 3.3 σ/S (σ is the standard deviation of the response and S is the slope of the calibration curve) and ranged between 0.5 and 1.0 pmol per sample. The results indicate averages of analyses of three independent samples and two HPLC-MS/MS injections for each sample.

Chlorophyll retention bioassay

Oat (*Avena sativa* L. cv. Abel) seeds were soaked for 24 h in aerated distilled water (26 °C, 16 h light /8 h dark) and sown into saturated perlite with a 2-fold concentrated Knop's nutrient solution. Plants were cultivated in a growth chamber (SANYO MLR 350H; Sanyo, Osaka, Japan) with an 18 h light/6 h dark photoperiod (photon flux of 19 995 lx), 20 °C/18 °C, and ~80% relative humidity. The first fully developed leaves were excised from 10-day-old plants when the second leaf just started to develop. The leaf apexes were cut into 7 cm long segments and incubated in test tubes containing 1 ml of CK solutions for 4 d at 26 °C in darkness. Four replicates were prepared from each variant. Chlorophyll was extracted with 80% ethanol according to Kaminek *et al.* (1987); the optical absorbance was measured at 665 nm on a Unicam 5625 spectrometer. CK activities were compared in accordance with EC_{50} values, defined as the concentration at which 50% of the maximum response was recorded. For microscopic studies, the leaves (~2 mm wide) were cut off 1 cm from their apical tips, put into a drop of water on a microscopic slide, covered with a coverslip, and immediately observed under a confocal laser scanning microscope (Leica TCS NT, Heidelberg, Germany) (Schwarzerová *et al.*, 2006). Tested compounds were applied at concentrations of 3.2×10^{-8}, 1.6×10^{-7}, 8×10^{-7}, 4×10^{-6}, 2×10^{-5}, 1×10^{-4}, and 5×10^{-4} M.

Tobacco callus bioassay

CK-dependent tobacco (*Nicotiana tabacum* cv. Wisconsin 38) callus derived from a 4-week-old culture was cultivated on solid MS medium (Murashige and Skoog, 1962) supplemented with sucrose (30 g l^{-1}), naphthaleneacetic acid (NAA; 1 mg l^{-1}), and the CK to be tested (in a concentration ranging from 0.640 nM to 10 μM). CK activities were expressed as average fresh weight (FW) of tissue per flask after 7 weeks of cultivation in darkness at 23 °C and compared in accordance with the EC_{50} values (see above). Three independent experiments were set up for each CK. The control stock calli were grown on the same medium containing benzyladenine (0.2 mg l^{-1}). The tested compounds were applied in concentrations of 6.4×10^{-10}, 3.2×10^{-9}, 1.6×10^{-8}, 8×10^{-8}, 4×10^{-7}, 2×10^{-6}, and 1×10^{-5} M.

Amaranthus *bioassay*

A standard bioassay based on the dark induction of betacyanin synthesis in *Amaranthus* cotyledons was carried out as previously described (Holub *et al.*, 1998). The final concentration of solvent [dimethylsulphoxide (DMSO)] in the medium did not exceed 0.2% (v/v). Five replicates were prepared for each CK concentration, and the complete tests were repeated at least three times, the results being compared in accordance with EC_{50} values (see above).

Arabidopsis ARR5:GUS *reporter gene assay*

This assay was carried out as described in Romanov *et al.* (2002), with slight modifications. For quantitative assays, *ARR5:GUS* seedlings were grown for 2–3 d (22 °C, 16 h light/8 h dark) in six-well plates (Techno Plastic Products Ltd, Zurich, Switzerland) and then *cis*Z, *trans*Z, or control solvent [DMSO, final concentration 0.1% (v/v)] was added to the desired final concentration. The seedlings were then incubated in the dark for 17 h at 22 °C. The results were compared in accordance with EC_{50} values (see above).

*Uptake and metabolism of [^{3}H]*cis*Z and [^{3}H]*trans*Z in tobacco* BY-2 *cell culture and in detached oat leaves*

Radiolabelled [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z (2×10^{-9} mol l^{-1} each) uptake and accumulation were measured in CK-autonomus tobacco [*N. tabacum* L. cv. Bright Yellow 2 (BY-2) (Nagata *et al.*, 1992)] cell suspension (0.5 ml aliquots) according to Delbarre *et al.* (1996) as described in Petrášek *et al.* (2003). BY-2 cells were harvested immediately after the addition of CK to the liquid medium (time 0 min) and at 5 min intervals during incubation. The uptake and metabolism of [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z in detached oat leaves were investigated in 8 cm long oat leaf segments incubated in 2.5 ml of water containing 42–47 kBq of radiolabelled *cis*Z and *trans*Z, respectively. The incubation proceeded under continuous light (photon flux of 19 995 lux) at 20 °C for 2, 5, 8, 24, 48, and 96 h. CKs were extracted and purified from 14–16 primary leaf segments (~1 g FW) per sample; for each time interval two independently incubated samples were analysed. Radiolabelled CK metabolites were analysed by HPLC coupled to an online radioactivity detector as described by Gaudinová *et al.* (2005) and identified by comparing their retention times with those of authentic standards.

Computational studies of potential cis–trans *isomerization of zeatin*

Calculations of potential *cis–trans* isomerization of zeatin were performed by a GAUSSIAN 03 program (Frisch *et al.*, 2004) using the DFT Kohn–Sham method with B3LYP exchange and correlation functionals (Becke, 1993) and the 6-31+G(d,p) basis set. Twisted transition state (TS) geometries were obtained using a TS search. The energy differences between the ground state and TS electronic energies corresponded to the activation energy of the isomerization reaction.

Determination of cytokinin oxidase/dehydrogenase activity and substrate specificity

The cytokinin oxidase/dehydrogenase (CKX) from oat and tobacco leaves and BY-2 cells was extracted and partially purified according to Motyka *et al.* (2003) and its activity and substrate specificity were determined by *in vitro* radioisotope assays based on the conversion of 2-^{3}H-labelled CKs ([2-^{3}H]iP, [2-^{3}H]*trans*Z, and [2-^{3}H]*cis*Z) to [^{3}H]adenine. The assay mixture (50 μl final volume) included a 100 mM TAPS-NaOH buffer containing 75 μM 2,6-dichloroindophenol (pH 8.5), 2 μM [2-^{3}H]CK (7.4 TBq mol^{-1} each), and enzyme preparation equivalent to 500 mg tissue FW (corresponding to 0.225 and 0.07 mg protein g^{-1} FW for oat and tobacco leaves, respectively) or 20 mg of tissue FW (corresponding to 0.02 mg protein g^{-1} FW for BY-2 cells). After incubation (1 h and 4 h for oat

and tobacco, respectively) at 37 °C the reaction was terminated and the substrate was separated from the product of the enzyme reaction by HPLC, as described elsewhere (Gaudinová *et al.*, 2005). Protein concentrations were determined according to Bradford (1976) using bovine serum albumin as a standard.

For analysis of the substrate specificity of recombinant CKX enzymes of *Arabidopsis* (AtCKX1, AtCKX2, AtCKX3, AtCKX4, AtCKX5, AtCKX6, and AtCKX7) the CKX genes were cloned into expression vectors pPICZ-A, pPICZ-Aα, and pGAPZα (Invitrogen) with a subcloned N-terminal His-tag. Constitutive expression from pGAPZα clones in the *Pichia pastoris* strain X33 was carried out for 3–5 d in YNB medium supplemented by 2% (w/v) glucose and buffered to pH 6.7 by potassium phosphate. The expression of a pPICZ-A construct (AtCKX7) was maintained in a BMMY medium under the same conditions as above and induced by 1% (v/v) methanol each day. Yeast medium or, for pPICZ-A::AtCKX7, cell extract containing recombinant CKX was concentrated and underwent low-pressure chromatography onto Bio-gel hydroxyapatite (Bio-Rad), octyl-Sepharose CL-4B, and Ni Sepharose HP (Pharmacia) columns as recommended by the supplier [a detailed description of this procedure is given in Morris *et al.* (1999) and Pertry *et al.* (2009)]. The enzyme assay was based on the decolorization of an appropriate electron acceptor—100 μM 2,6-dichlorophenolindophenol (DCIP; Laskey *et al.*, 2003) or 500 μM ferricyanide (FC; Pertry *et al.*, 2009) followed at 420 nm and 600 nm, respectively, within 15 min. The reaction mixture further contained 100 mM McIlvaine buffer, pH 6.5 (DCIP) or pH 6.0 (FC) and 50 μM substrate. The apparent K_m values of CKXs were determined with FC in the same assay mixture as described above and the substrate CKs used in the concentration range of 12.5–100 μM.

Results

Abundance of cis*Z-type cytokinins in the plant kingdom*

In order to define the distribution between *cis*- and *trans*Z-type CKs in land plants, the ratio of these two isomers (as sums of their free bases, ribosides, ribotides, *O*-glucosides, and *N*-glucosides) in fully developed leaves or shoots of >150 representative species of all the main land plant groups in the vegetative stage, including species of bryophytes, lycophytes, and ferns, gymnosperms, and angiosperms, was determined (Fig. 1A; for a detailed list and a phylogenetic tree of all analysed plant species see Supplementary Fig. S1 and Table S1 at *JXB* online). As shown in Fig.1, plants containing *cis*Z derivatives in very high concentrations considerably exceeding those of their *trans* counterparts can be found across the whole evolutionary tree. Representatives of liverworts (*Conocephalum conicum*) and mosses (*Plagiomnium undulatum*), both non-vascular, spore-producing land plants belonging to the bryophytes, were found to contain the *cis*Z type almost exclusively (Supplementary Table S1). The same was true for some of the spore-bearing vascular plants such as ferns, where orders Equisetales, Schizaeales, Salviniales, and mainly Polypodiales *sensu lato* (*s.l.*) were found to include families with strong preferences for *cis*Z forms (Fig. 1B and Supplementary Table S1). Among seed plants, *cis*Zs were dominant in many analysed taxa of monocots (e.g. Liliales, Zingiberales, and Poales; Fig. 1E) but also in dicot plants (e.g. Malphigiales, Fagales, Cornales, Solanales, and Asterales; Fig. 1F), as described in more detail below and in Supplementary Table S1. On the other hand, gymnosperms and ancestral angiosperms, such as Nymphaeales or Magnolliids, seem instead to prefer *trans*Z isoforms (Figs. 1C, D, and Supplementary Table S1).

Next, to depict the contribution of *cis*Z to the whole CK content, the CK spectra were divided into two groups of interest including (i) *cis*Z-type CKs and (ii) non-*cis*Z-type CKs (represented by *trans*Z, iP, and DHZ with their derivatives). In agreement with the structures and physiological activities, both *cis*Z- and non-*cis*Z-type CKs were classified into four functionally different groups, comprised as described in Fig. 2. The total amount of CKs among all tested species varied between ~0.7 pmol g^{-1} FW and 1378 pmol g^{-1} FW. Monocot plants belonging to the families Zingiberaceae (*Elettaria cardamomum*), Musaceae (*Musa acuminatea*), and Liliaceae (*Lilium* cv. *Elodie*, *Lilium martagon*) were found to contain *cis*Z derivatives in concentrations representing >50% of the whole CK content (Fig. 2, Supplementary Table S1). In particular, in leaves of plants of the Poaceae family (*Zea mays*, *Avena sativa*, *Triticum aestivum*, *Dactylis glomerata*, *Agropyron repens*, and *Phragmites australis*) *cis*Z derivatives represented the major CKs (Fig. 2, Supplementary Table S1). The most abundant CK metabolites detected in these genera were *cis*Z-*O*-glucoside (*cis*Z*O*G) and its riboside (*cis*ZR*O*G), representing altogether >90% of the total CKs. In dicot plants, for instance, a similar CK profile with a clear predominance of *cis*Z*O*G and *cis*ZR*O*G was found in *Manihot* sp. (Euphorbiaceae) leaves (Fig. 2). Relatively high levels of *cis*Z-type CKs (exceeding 65% of the total CKs) were also detected in leaves of *N. tabacum*, however, with *cis*Z-N^7-glucoside (*cis*Z7G) as a prevailing CK compound. All CK-type levels of bioactive CK bases and ribosides as well as CK ribotides were rather low in all tested plants. Irrespective of the plant material the prevailing CK forms were *N*- or *O*-glucosides. It can be concluded that *cis*Z-type CKs were detected in all tested plants, but a parallel with evolutionary complexity was not confirmed and they are more likely to be connected by means of a reproductive strategy.

Levels of cis- *and* trans*-zeatins differ during ontogenesis of* Arabidopsis

Further attention was focused on the distribution of the metabolites of the *cis*- and *trans*Z isoforms (free bases, ribosides, ribotides, *O*-glucosides, and *N*-glucosides) throughout plant ontogenesis using *Arabidopsis* as a model plant. It was found that levels of *trans*- and *cis*Zs change dramatically throughout the *Arabidopsis* life span. The ratio between *cis*- and *trans*Z-type CKs is shown in Fig. 3A. Dry seed *cis*Zs were the major CKs, comprising ~70% of the total CK content, with *cis*ZR and *cis*ZRMP as dominant metabolites. No significant changes were observed from the initial observation after the first 24 h of imbibition (Fig. 3A). The situation changed noticeably in the first days of seedling development. On the sixth day after germination (DAG), the level of *cis*Zs decreased dramatically (13%) and

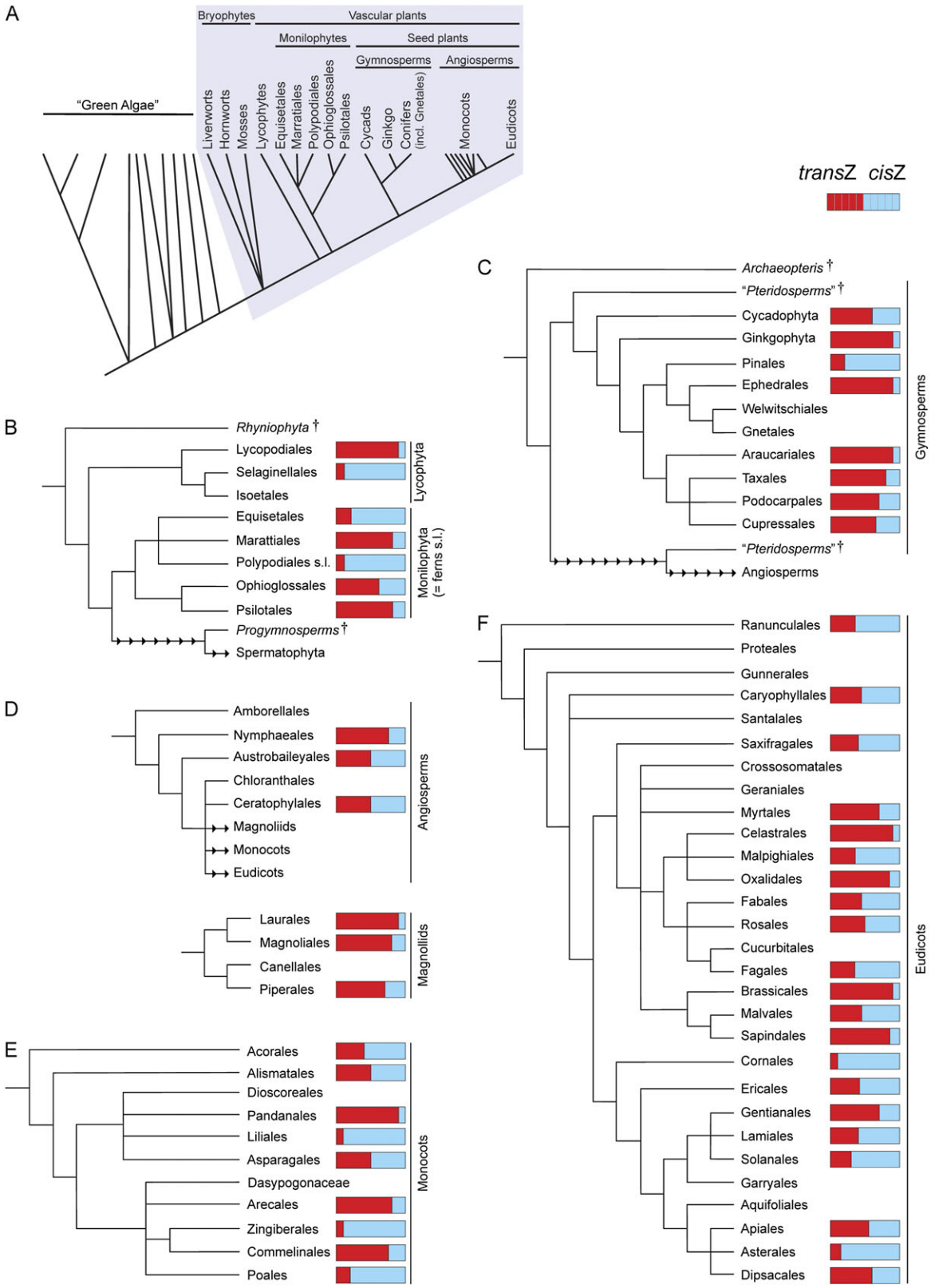

Fig. 1 Distribution between *trans*- and *cis*Z-type CKs throughout the evolution of the plant kingdom. (A) A simplified evolutionary tree of green plants with highlighted main extant plant groups analysed for the ratio of *trans*Z- and *cis*Z-type cytokinins (as the sums of their free bases, ribosides, ribotides, *O*-glucosides, and *N*-glucosides) in this work. (B–F) Phylogenetic relationships and ratios of *trans*Z- and *cis*Z-type cytokinins in the main groups of vascular plants, i.e. Lycophyta and Ferns *s.l.* (B), Gymnosperms (C), Angiosperms and Magnolliids (D), Monocots (E), and Eudicots (F). For a detailed list and phylogenetic tree of all analysed plant species see Supplementary Fig. S1 and Table S1 at *JXB* online. Phylogenetic trees were compiled and modified according to Simpson (2006). Taxa marked with † and written in italics are extinct.

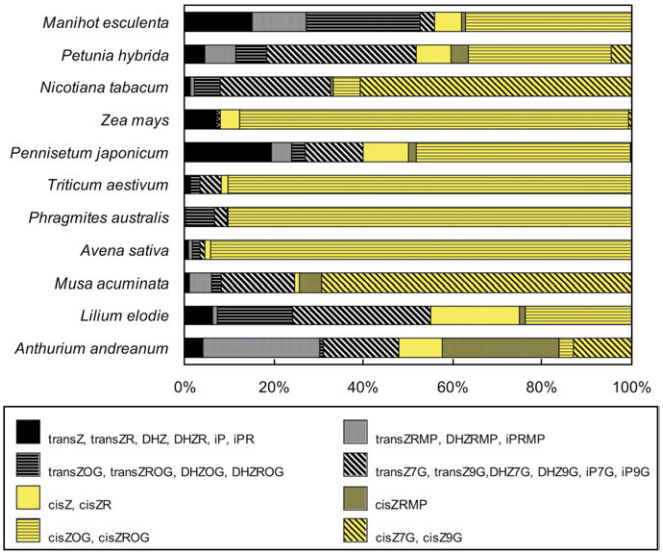

Fig. 2 Proportions of CK groups in leaves of various seed plants and contribution of *cis*Z to the whole CK content. Plant species are arranged from the oldest to the youngest in evolutionary terms (from the bottom to the top); the CK derivatives are divided into eight groups of interest according to structure and activity. Non-*cis*Z-type CKs are shown by black and white bars and *cis*Z-type CKs are shown by black and yellow bars.

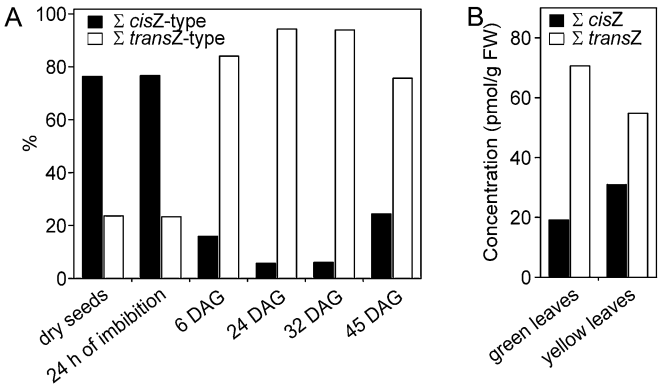

Fig. 3 The ratio between *trans*Z- and *cis*Z-type cytokinins in aerial parts throughout the ontogenesis of *Arabidopsis*. (A) Changes in the *trans*Z- and *cis*Z-type cytokinins ratio during the *Arabidopsis* lifespan from seed to late senescence. (B) Comparison of *trans*Z- and *cis*Z-type cytokinins levels in the youngest green and oldest senescent rosette leaves of 65-day-old *Arabidopsis*.

the major CKs became *trans*Zs (84%; Fig. 3A). The dominance of *trans*Z-type CKs was even more pronounced during the vegetative stage (94%; 24 DAG, 32 DAG), whereas *cis*Z-type CKs were represented by only 5% of the whole CK content (Fig. 3A). During early senescence (45 DAG) the balance shifted towards *cis*Zs (21%; Fig. 3A). The partial rearrangement of the *cis*- and *trans*Z-type CK levels during senescence was also confirmed by analysis of CK content in the youngest green and oldest senescent rosette leaves of 65-day-old *Arabidopsis* plants. In comparison with the youngest green leaves, the abundance of *cis*Zs in the old leaves was 1.6 times higher (Fig. 3B), with the highest increase being in the concentration of *cis*ZR (6.5-fold). The level of *trans*Zs decreased 1.3-fold (Fig. 3B). In the senescent leaves, *cis*Zs represented almost 30% of the total CK content. It seems that the proportion of *cis*Z-type CKs changes during *Arabidopsis* ontogenesis and increases in organs which have completed growth.

Natural cis–trans*Z isomerization without a catalyst is not possible*

In order to exclude the occurrence of *cis*Z in plant samples as an artefact formed during extraction and purification procedures, a computational analysis excluding such a possibility was performed as a part of this study. Alkene *cis*↔*trans* isomerization is generally a photochemical reaction involving excitation of the electron from π to π* orbital. This excitation requires energy of ~5–6 eV, corresponding to the UV region (~230 nm; Foo *et al.*, 1974). An alternative thermal pathway of isomerization has been studied, particularly for the so-called push–pull alkenes (Matus *et al.*, 2003). Such alkenes possess an electron donor substituent on one side of the double bond and an electron acceptor on the other, which results in weakening of the double bond. For extreme cases, the barrier for rotation around the double bond was shown to be as low as 7 kcal mol^{-1}. For the present computational studies, the zeatin molecule was considered as a substituted alkene lacking a purine moiety. The model system (*2E*)-4-amino-2-methyl-but-2-en-1-ol) is depicted in Supplementary Fig. S3 at *JXB* online. The activation energy for thermal isomerization was found to be 58 kcal mol^{-1}, which is comparable with an unsubstituted double bond and corresponds to a reaction rate order of 10^{-28} mol s^{-1} for a monomolecular reaction. As neither a photochemical nor a thermal pathway is accessible under standard laboratory conditions, *cis*Z↔*trans*Z isomerization during the isolation procedure can therefore be excluded without the presence of a catalyst. Conversion catalysed by intensive cool white fluorescent light in an aqueous solution was observed to prefer a *cis* to a *trans* course of isomerization (Bassil *et al.*, 1993) with the ratio balance at 13%. However, the influence of such conditions on the extraction or purification process can be excluded since both zeatin standards, which are exposed to the same light and solvent conditions as samples, are usually checked for stability before the HPLC/MS analysis.

Since zeatins yield identical parent $[M+H]^+$ ions and basic fragment peaks under electrospray mass spectrometry conditions, it was essential to ensure definite separation of the two isomers in order to obtain accurate estimates of their endogenous levels. The analyses without and/or with imperfect separation and internal standardization used sometimes to increase the sample throughput (Prinsen *et al.* 1995) would inevitably cause an overestimation of endogenous concentrations. For this reason, the ring-labelled $[^{13}C_5]$*cis*Z, having a different fragmentation pathway (*cis*Z: 225→141) from a classical side chain-labelled $[^{2}H_5]$*trans*Z (*trans*Z: 225→136), was synthesized. The eluting ions were

measured by MRM of $[M+H]^+$ in a positive ion mode and of the appropriate product ion. The addition of the labelled zeatins to different extracts further facilitated their quantification, also giving better resolution in natural samples as well as a measure of possible *cis*↔*trans* interconversion. In no samples analysed in this study was *cis*↔*trans* Z isomerization observed.

Biological activity of cis- *and* trans-*zeatin in different cytokinin biotests*

Most of the physiological activity of zeatin has been attributed for years to *trans*Z, while *cis*Z has been considered mainly as a weakly active CK. For this reason the biological activity of *cis*Z and *trans*Z metabolites (for free bases, see Fig. 4; the results for ribosides and *O*-glucosides are given in the Supplementary data at *JXB* online) were compared in various CK bioassays.

In the oat leaf senescence assay all tested *trans*Z-type CKs suppressed chlorophyll degradation efficiently (Fig. 4A, Supplementary Fig. S2A, B). All *cis*Z-type CKs were also effective in delaying dark-induced senescence but with lower efficiency (between 5- and 50-fold when compared in accordance with EC_{50} values) than their corresponding *trans* counterparts (Fig. 4A, Supplementary Fig. S2A, B). The most pronounced chlorophyll retention was observed after *cis*ZR application at a concentration 500 μM, when ~92% of the maximum responses induced by corresponding *trans* isomers were reached. Corresponding results demonstrating the activities of *cis*Z-type CKs and their *trans* counterparts (free bases, ribosides, and *O*-glucosides) in delaying dark-induced chlorophyll degradation were also obtained with excised wheat leaves in the same assay and with maize leaf segments in a drop bioassay (data not shown). To support the chlorophyll retention data for inhibition of plastid disintegration in oat leaves treated with *cis*- or *trans*Z, leaf specimens were monitored with a laser scanning microscope and the images are depicted in Fig. 4B.

Biological activities of *cis*Z- and *trans*Z-type CKs in promoting tobacco callus growth are demonstrated in Fig. 4C, and Supplementary Fig. S2C and D at *JXB* online. All tested *trans* isomers considerably enhanced tobacco cell division. The lowest effective concentrations were *trans*Z < *trans*ZR < *trans*Z*O*G, with the maximum stimulatory responses at concentrations ~25 times higher. All tested *cis*Z-type CKs were remarkably effective in promoting tobacco callus growth, displaying activities in the order *cis*ZR > *cis*Z > *cis*Z*O*G (based on EC_{50} values; Fig. 4C, Supplementary Fig. S2C, D). The most pronounced biological response on callus growth was recorded for *cis*ZR at 400 nM, which represented >90% of the highest activity observed for its *trans* counterpart. In general, the *trans*-Z:*cis*Z activity ratio of assayed CK derivatives varied between ~3 and 27 (in accordance with EC_{50} values; Fig. 4C). These data suggest a possible interchangeability of *trans*Z-type CKs by high concentrations of *cis*Z-type CKs at least in terms of maintaining cell division.

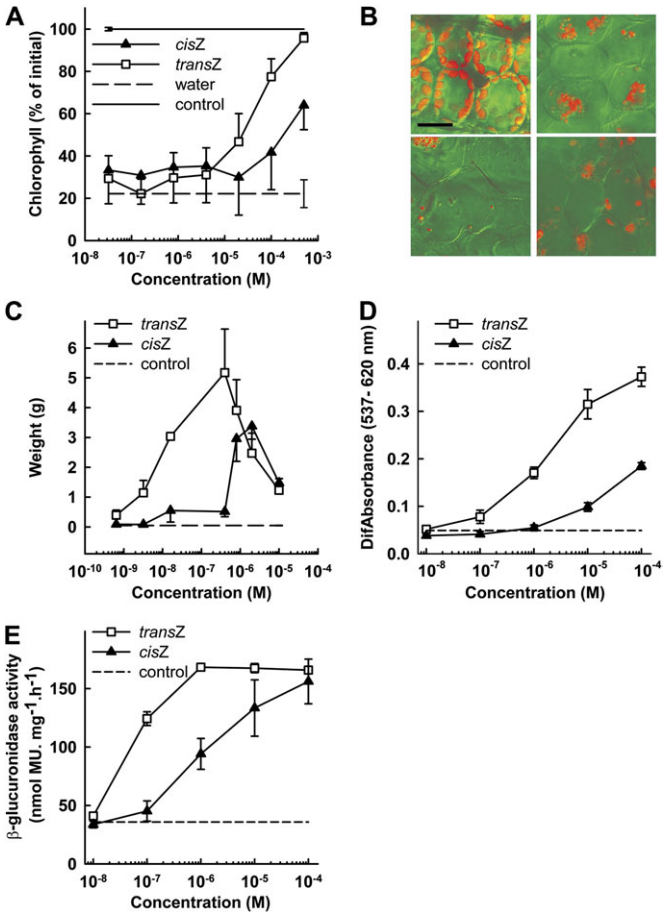

Fig. 4 Comparison of the activity of *trans*Z and *cis*Z in different CK bioassays and their induction of CK signalling. (A) Effect on retention of chlorophyll in senescing oat leaves. Values are expressed as a percentage of the initial chlorophyll content of fresh leaves before incubation. (B) Images of oat leaf mesophyll cells and their chloroplasts (original red autofluorescence). From the upper left corner to the right: control (fresh oat leaves before the experiment), and leaves treated with *cis*Z, water, or *trans*Z after 10 d of cultivation in the dark. The bar represents 20 μm. (C) Effect on fresh weight yield of tobacco cytokinin-dependent W-38 callus tissue. (D) Effect on dark induction of betacyanin synthesis in *Amaranthus* cotyledon–hypocotyl explants. (E) Induction of expression of the *ARR5:GUS* cytokinin reporter in transgenic *Arabidopsis* seedlings. Error bars show the SD (n=4 for A, C, n=5 for D, and n=2 for E); entire tests were repeated at least twice. Dashed lines indicate control treatment without added cytokinin.

In the *Amaranthus* bioassay, *trans*Z was again the more active isomer in dark induction of betacyanin synthesis and reached an EC_{50} value of 1.8 μM, whereas the EC_{50} value for *cis*-zeatin was >100 μM (Fig. 4D). At this concentration *cis*Z reached only 44% of *trans*Z maximal activity.

The competency of *cis*Z to activate CK signalling components subsequent to CK receptor interaction was investigated by means of the *ARR5:GUS* reporter gene response, which is known to be rapidly up-regulated by CK (D'Agostino *et al.*, 2000; Romanov *et al.*, 2002). Data presented in Fig. 4E show that both *trans*- and *cis*Z were able to induce the level of ARR5:GUS in a dose-dependent

manner, although with different effectivity. While *trans*Z was a potent inducer of ARR5:GUS expression, reaching a submicromolar EC_{50} value of 0.07 μM, *cis*Z reached an EC_{50} value of 1.2 μM, indicating the ability of *cis*Z to induce a CK response effectively at the molecular level. Apparently a high abundance of *cis*Z is needed for biological activity in all CK bioassays.

Uptake and accumulation of [^{3}H]cisZ and [^{3}H]transZ by tobacco BY-2 cells

To acquire information about the uptake and accumulation of *cis*- and *trans*Z-type CKs by plant cells, radiolabelled [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z were added to a liquid medium of the tobacco BY-2 suspension culture. The results showed progressive accumulation of both *cis*Z and *trans*Z in cultured tobacco cells with no preference for either of the isomers (Fig. 5). Most of the radioactivity of *cis*Z as well as *trans*Z (up to 80–90% of the total) was taken up early, within 15 min. It would seem that both zeatin isomers entered cells relatively rapidly and in the same or a similar way. Transport does not therefore seem to be a regulatory point in *cis*Z activity.

Short-term metabolism of [^{3}H]cisZ and [^{3}H]transZ in tobacco BY-2 suspension culture

The fate of radiolabelled zeatin metabolites in tobacco BY-2 cells was determined at four time points during the first 15 min of the [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z accumulation experiment. Radiolabelled *cis*Z was metabolized in BY-2 cells, with only a few metabolites detected. A gradual decrease of [^{3}H]*cis*Z (from 72% to 31% of total radioactivity within 15 min incubation) was accompanied by a corresponding increase in radioactivity associated mostly with *cis*ZRMP and to a lesser extent with *cis*ZR. Accumulation of adenine indicated degradation of *cis*Z by CKX (Fig. 6A). Adenosine was detected only in minute amounts. Compared wiht *cis*Z, the application of [^{3}H]*trans*Z to BY-2 cells led to the detection of more CK forms including *N*- and *O*-glucosides. Similarly to *cis*Z, the prevailing metabolites of [^{3}H]*trans*Z were its corresponding ribotide (*trans*ZRMP), riboside (*trans*ZR), and adenine (Fig. 6B). In addition *trans*Z-N^7-glucosides and *trans*Z-*O*-glucosides were found in cells immediately after the addition of [^{3}H]*trans*Z to the media; however, their contribution to the total pool of metabolites remained low. The metabolic fate of *cis*Z differs from that of *trans*Z in terms of slower metabolic changes generating different spectra of metabolites. Interestingly, no *cis*Z↔*trans*Z isomerization was observed during the feeding experiments in BY-2 cells.

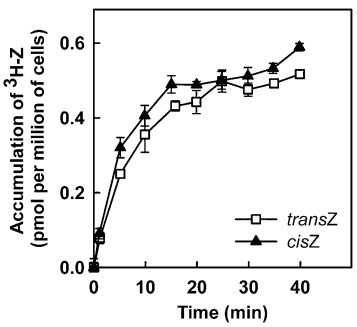

Fig. 5 Uptake of [^{3}H]*cis*Z and [^{3}H]*trans*Z by tobacco BY-2 suspension-cultured cells. Error bars represent the SD.

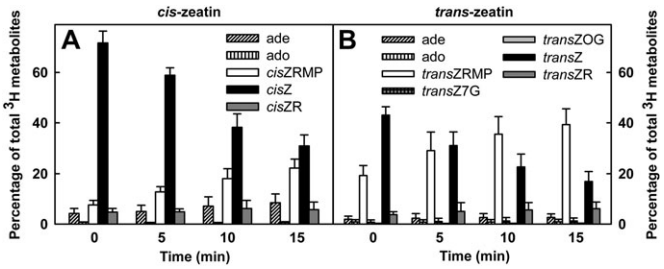

Fig. 6 Metabolism of [^{3}H]*cis*Z and [^{3}H]*trans*Z in tobacco BY-2 suspension-cultured cells. The bars represent the distribution of radioactivity associated with individual CK metabolites 5, 10, and 15 min after application of [^{3}H]*cis*Z (A) or [^{3}H]*trans*Z (B); the values are expressed as a percentage of the total extracted radioactivity taken up by cells. Error bars represent the SD (Ade, adenine; Ado, adenosine; *cis*Z, *cis*-zeatin; *cis*ZR, *cis*-zeatin riboside; *cis*ZRMP, *cis*-zeatin riboside-5′-monophosphate; *trans*Z, *trans*-zeatin; *trans*ZR, *trans*-zeatin riboside; transZRMP, *trans*-zeatin riboside-5′-monophosphate; *trans*Z7G, *trans*-zeatin 7-glucoside; *trans*-ZOG, *trans*-zeatin *O*-glucoside).

Metabolism of [^{3}H]cisZ and [^{3}H]transZ in detached oat leaves

Having in view obtaining information about long-term interconversions of *cis*- and *trans*Z-type CKs in plants, the uptake and metabolic fate of [2-^{3}H]*cis*Z and [2-^{3}H]*trans*Z were investigated in detached oat leaves. Most of [^{3}H]*cis*Z was metabolized to adenine (up to 50% within 8 h) and to a lesser extent but progressively to adenosine (~8% after 96 h; Fig. 7A) indicating degradation of *cis*Z by CKX. In accordance with the prominent occurrence of endogenous *cis*Z*O*G in oat leaves (Fig. 2), radiolabelled *cis*Z*O*G was detected as an abundant [^{3}H]*cis*Z metabolite (Fig. 7A). During the whole incubation period, a significant proportion (12% of total metabolites) of [^{3}H]*cis*Z was retained in an unmetabolized form. The degradation products adenine and adenosine were also found as prominent metabolites of [2-^{3}H]*trans*Z in oat leaves (Fig. 7B). In contrast to *cis*Z, the application of [^{3}H]*trans*Z to oat leaves led to the detection of corresponding N^7- and N^9-glucosides (Fig. 7B). Neither [^{3}H]*cis*Z nor [^{3}H]*trans*Z was found to be a subject of *cis*Z↔*trans*Z isomerization. Similarly, a variation in metabolism between [^{3}H]*cis*Z and [^{3}H]*trans*Z, with more metabolites detected after [^{3}H]*trans*Z treatment, was observed in detached tobacco cv. Samsun leaves (data not shown). Also these plant materials confirmed a distinct pattern for *cis*Z and *trans*Z metabolism and slower accumulation of *cis*Z metabolites.

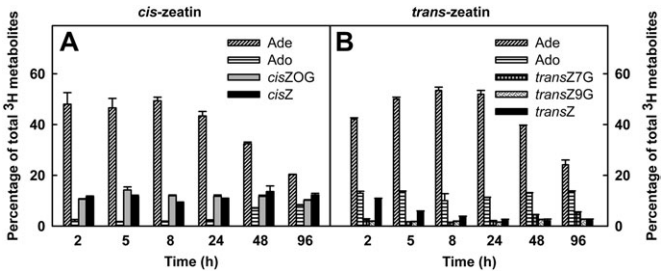

Fig. 7 Metabolism of [^{3}H]*cis*Z and [^{3}H]*trans*Z in oat leaf segments. The bars represent the distribution of radioactivity associated with individual CK metabolites 2, 5, 8, 24, 48, and 96 h after application of [^{3}H]*cis*Z (A) or [^{3}H]*trans*Z (B); the values are expressed as a percentage of the total radioactivity taken up by excised leaves. (Ade, adenine; Ado, adenosine; *cis*Z, *cis*-zeatin; *cis*ZOG, *cis*-zeatin *O*-glucoside; *trans*Z, *trans*-zeatin; *trans*Z7G, *trans*-zeatin 7-glucoside; *trans*Z9G, *trans*-zeatin 9-glucoside).

CKX activity and substrate specificity toward zeatin isoforms

With respect to the intense *in vivo* formation of adenine and adenosine as products of *cis*Z and *trans*Z metabolism in tobacco BY-2 cells and oat leaves (Figs 6, 7), degradation of [2-^{3}H]*cis*Z, [2-^{3}H]*trans*Z, and [2-^{3}H]iP by CKX from crude extracts of two plant materials was determined. The *in vitro* enzymatic studies revealed the order of preference of potential CKX substrates iP > *cis*Z > *trans*Z for tobacco BY-2 cell and cv. Samsun leaf, and iP > *cis*Z = *trans*Z for oat leaf enzymes (Table 1), which demonstrate their higher or identical affinities for the *cis* isomer compared with the *trans* isomer.

From this point on, the influence of the cellular localization and expression pattern of CKX isoforms on their capability to degrade *cis*Z and *transZ* was studied using purified recombinant CKXs of *Arabidopsis*. The activity of CKX enzymes toward iP, *trans*Z, *cis*Z, and their ribosides was estimated as the initial rates of decolorization of the electron acceptors DCIP or FC (Table 2). Whereas reaction rates of iP and *trans*Z were not significantly varied for any of the characterized enzymes, the capability to degrade *cis*Z and its riboside differed with respect to an individual isoform. Two major apoplastic CKXs of *Arabidopsis*, AtCKX2 and AtCKX4, with relevant expression in different tissues throughout plant ontogenesis, showed only limited degradation of *cis*Z (Table 2, Supplementary Table S2 at *JXB* online). *cis*Z was almost resistant to vacuolar-targeted AtCKX3, showing considerable expression during flowering (Table 2, Supplementary Table S2). Interestingly, the second *Arabidopsis* isoform targeted to vacuoles, AtCKX1, deactivated *cis*Z very effectively (Table 2). As both vacuolar CKXs showed different patterns of expression, selective accumulation of *cis*Z derivatives in vacuoles might have a temporal or tissue-specific character. The CKX isoform AtCKX7 with cytosolic localization was found to degrade *cis*Z as a preferred substrate in comparison with other isoprenoid CKs (Table 2).

Table 1. *Substrate specificity of crude extracts of CKX enzymes toward iP,* trans*Z, and* cis*Z*

The CKX activity was determined using [2-^{3}H]N^6-(2-isopentenyl)adenine, [2-^{3}H]*trans*-zeatin, and [2-^{3}H]*cis*-zeatin, 2 μM each, as substrates. Enzyme activity was measured in 100 mM TAPS-NaOH buffer containing 75 μM 2,6-dichloroindophenol at pH 8.5 and is expressed as relative rates toward iP degradation (100%).

	iP	*transZ*	*cisZ*
Oat leaves	100	19	21
Tobacco leaves	100	8	37
BY-2 cells	100	16	62

Discussion

*cis*Z has usually been thought of as being a CK with low activity. However, a number of reports previously demonstrated a high abundance of *cis*Z and/or its derivatives in algae, mosses, and various seed plants. The present comprehensive screen throughout the evolution of land plants endorses the hypothesis that *cis*Z-type CKs occur in the plant kingdom ubiquitously. Surprisingly *cis*Z and *trans*Z isomer ratios do not seem to have a direct connection to evolutionary relationships among species (Figs 1, 2, Supplementary Table S1 at *JXB* online). It is known that wheat and other cereals have adopted a strategy of switching from vegetative to generative reproductive growth after pollination to ensure seed filling, and resulting in regulated shoot withering (Sykorova *et al.*, 2008). It is supposed that the prevalence of *cis*Z derivatives especially in Poaceae might be related to this phenomenon and emanate from ontogeny.

It is shown here that the proportions of isomers can differ significantly during the life span of *Arabidopsis*, with particular stages where *cis*Zs represent the major CKs (Fig. 3A, B). Seeds, imbibed seeds, and senescent leaves containing high levels of *cis*Z CKs are characterized by growth cessation but preserved capability for physiological processes. It is assumed that CKs with low activity such as *cis*Zs might be responsible for the maintenance of basal CK activity necessary for plant survival and subsequent recovery. High levels of *cis*Z-type CKs were also found in *Mercurialis* associated with the induction of male sterility (Louis *et al.*, 1990). Recently, rapid accumulation of *cis*ZRMP has been found in maize roots exposed to salinity stress, while *trans*Z levels remained nearly unchanged (Vyroubalová *et al.*, 2009). Remarkable increases in *cis*Z derivatives were observed in plants exposed to severe drought (Havlová *et al.*, 2008), heat (Dobrá *et al.* 2010), or biotic stress (Pertry *et al.*, 2009), and after administration of inhibitors limiting growth (Blagoeva *et al.*, 2003, 2004). Lower seed dormancy of annual rye grass was accompanied by higher levels of *cis*Z (Goggin *et al.*, 2010). Also a reduction of *cis*ZR levels in buds of chickpea was recorded after decapitation (Mader *et al.*, 2003) and probably facilitates release of the bud from dormancy. Obviously all these states are associated with growth-limiting conditions resulting from internal or external cues, and *cis*Z CKs may have a role on these occasions.

Table 2. *Substrate specificity of CKX enzymes toward iP,* trans*Z,* cis*Z and its ribosides, and the spatial and temporal pattern of expression of CKX genes*

Enzyme activity was determined as the initial rate of 2,6-dichlorophenolindophenol decolorization at pH 6.5 and is given as relative rates toward iP degradation (100%). Each number represents the mean value of three replicates where the standard error does not exceed 10%. Specificity of expression was derived from Genevestigator (Zimmermann *et al.*, 2004).

	iP	iPR	*trans*Z	*trans*ZR	*cis*Z	*cis*ZR	Significant expression during development	Organs with the highest expression
Apoplastic								
AtCKX2	100	15	78	7	2.5	0.2	Senescence	Endosperm
AtCKX4	100	13	89	11	3.7	1.0	Rosette development, onset of flowering	Root cap, leaf
AtCKX5	100	72	68	61	68	46	Germination, flowering, senescence	Testa, old leaf
AtCKX6	100	98	13	11	37	22	Bolting	Hypocotyl, shoot apex
Vacuolar								
AtCKX1	100	96	72	58	116	72	Seedlings, bolting	Endosperm, hypocotyl
AtCKX3[a]	100	128	20	32	0.1	0.5	Late flowering	Sepals
Cytosolic								
AtCKX7	100	45	30	5	120	33	No data available[b]	No data available[b]

[a] Ferricyanide decolorization at pH 6.0 was used instead of DCIP which is not an efficient electron acceptor for this enzyme.
[b] Not present on the ATH1 22K Arabidopsis Affymetrix GeneChip.

The occurrence of *cis*Z as an artefact formed during the extraction procedure was proposed in a few studies (Tay *et al.*, 1986) and posed a challenging question concerning *cis*Z stability (Bassil *et al.*, 1993). The present computational analysis showed clearly that the energy necessary for *cis–trans* isomerization between *cis*Z and *trans*Z and vice versa is too high to allow spontaneous transition. The addition of [$^{13}C_5$]*cis*Z and [$^{2}H_5$]*trans*Z, which have a different fragmentation pathway, to the analysed extracts proved the absence of *cis*Z↔*trans*Z isomerization. Therefore, uncontrollable *cis*Z–*trans*Z isomerization during the sample extraction and purification by the procedure used in this study can reliably be excluded.

Part of the problem concerning diverging experimental data relating to the biological activity of *cis*Z could arise from the synthesis and purity of distinct *cis*Z preparations, namely from their possible contamination with *cis*-isozeatin (Leonard *et al.*, 1971). It was found here that preparations of *cis*Z obtained from different sources often exhibited very different biological activities (results not shown).

The data further imply that there is no difference in uptake and accumulation of *cis*Z and *trans*Z by tobacco BY-2 cells that could lead to the differential activity of isomers. Both zeatin isomers are taken up by cells rapidly and in the same or a similar way (Fig. 5). It is, however, questionable whether they enter cells by simple diffusion or via specific transporters. The possible involvement of purine permeases (PUPs) AtPUP1 and AtPUP2 (Bürkle *et al.*, 2003) suggests a plausible function for PUPs in the transport of CK bases including *cis*Z. Transport of CK ribosides can be mediated by a group of equilibrative nucleoside transporters (ENTs) (Hirose *et al.*, 2005), but elucidating the role of other transporters in active transport of zeatin-type CKs requires further investigation. However, transport seems not to be crucial for the difference in zeatin bioactivity. It should be stressed that the absence of a difference in uptake and accumulation of the two zeatin isomers in cultured tobacco cells does not provide appropriate information about the up- and downloading as well as translocation of different CKs including the *cis*- and *trans*Zs in plants. Significant differences in levels and dynamics of the two forms of zeatins in the xylem and phloem channels during development of white lupine seeds indicate their functioning in CK supply and control of development of these organs (Emery *et al.*, 2000). However, speciation of the involvement of xylem and phloem sap CKs in control of CK levels in plant organs is complicated by expression of IPTs in cells associated with both xylem and phloem channels (Miyawaki *et al.*, 2004).

The most important conclusion stemming from metabolic studies consists of unobserved conversion of the labelled *cis* into the *trans* isomer and vice versa regardless of whether short or long feeding experiments were studied (Figs 6, 7). It briefly questions the role of *cis–trans* isomerase (Bassil *et al.*, 1993), but it needs to be fully understood that the enzyme may be activated only under certain circumstances and thus the conditions or type of material used herein might be insufficient to provoke its activity. In general, the metabolic fate of both isomers differs. The two isomers can be *N*- or *O*-glucosylated in a species-specific manner (Figs 2, 6, 7) and degraded by specific CKX enzymes (Table 2). It can be concluded that *cis*Z plays a role in metabolism separately from *trans*Z. *cis*Z interaction with an AHK3 receptor (Spíchal *et al.*, 2004; Romanov *et al.*, 2006) and expression of ARR5 in *Arabidopsis* confirms a relevant CK signalling cascade evoked by *cis*Z. Recent data indicate that *cis*Z might influence—probably by means of competition with other more active CKs on the AtAHK3 receptor—the transport of auxin (Saleem *et al.* 2010) and thus could elicit responses resulting in the preservation of only essential physiological processes.

Biological activity of *cis*Z and its derivatives was demonstrated in tobacco callus, *Amaranthus*, and oat chlorophyll retention bioassays (Fig. 4). CK activity of *cis*Zs is generally lower than that of the corresponding *trans* isomers. The exception is *cis*ZR as the most effective tested CK among

all tested *cis*Z derivatives and only slightly less active than its *trans* counterpart (Supplementary Fig. S2 at *JXB* online). Data demonstrating high bioactivities of CK ribosides compared with other CK forms were also reported in other studies (Kamínek *et al.*, 1979, 1987; Tarkowská *et al.*, 2003; Doležal *et al.*, 2007). High *cis*ZR activity in bioassays might be due to the more efficient transport of CK ribosides and/or in lower degradation by CKX (Sakakibara, 2006). It also hints at a preference for *cis*ZR rather than *cis*Z in the mediation of the extracellular signal transduction to influence physiological effect(s). It is known that potato hinders vegetative growth of its aerial part during tuberization (Fischer *et al.*, 2008). Therefore, the *cis*ZR increase documented in above-ground potato tissue preceding the onset of tuberization (Mauk and Langille, 1978) might serve in preservation of vital but not growing green tissue, ensuring allocation of more nutrients for tuber formation to stolons. Also *cis*ZR accumulation at the end of embryogenesis in developing pea embryos (Quesnelle and Emery, 2007) might be associated with cessation of growth of the embryo. Additionally *cis*ZR has a high resistance against degradation by AtCKX2, the CKX isoform that is expressed during senescence.

It is suggested that the *cis*Z function might consist of maintaining a minimal level of CK response accompanied by restriction of shoot growth to retain plant fitness while another more vital process requiring energy would be preserved or completed. Therefore, the *cis*Z isomer and/or its derivatives may be relevant under growth-limiting conditions associated with a developmental programme or external signals leading to plant optimal survival via reduction but not complete cessation of CK signalling. This hypothesis does not, however, exclude (a) potential role(s) of *cis*Zs in regulation of other physiological processes in plants.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Phylogenetic relationships of vascular plants (compiled and modified according to Simpson, 2006). Taxa marked with † and in italics are extinct. Taxa in red were analysed in this study.

Figure S2. Comparison of activity of ZR and Z*O*G isomers in different CK bioassays. Effect on retention of chlorophyll in senescing oat leaves of ZR (A) and Z*O*G (B). Values are expressed as a percentage of the initial chlorophyll content of fresh leaves before incubation. Effect of ZR (C) and Z*O*G (D) on fresh weight yield of tobacco cytokinin-dependent W-38 callus tissue.

Figure S3. A molecule of *cis*-zeatin (A) and a simplified model of *cis*-zeatin used for the computational study (B).

Table S1. Distribution of *cis*- and *trans*Z-type CKs throughout the evolution of the plant kingdom. Complete list of plants analysed for the ratio of *trans*Z- and *cis*Z-type of cytokinins (as sums of their free bases, ribosides, ribotides, *O*-glucosides, and *N*-glucosides). Means of two independent measurements are shown. The standard deviation did not exceeded 10%.

Table S2. Apparent Michaelis constants for *Arabidopsis* CKX2 and CKX3. All values represent the mean concentration of at least three replications.

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