S2012

Applying Mendel's Laws of Genetics to Plant Biology

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**Lecture and
Practical book**

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University Campus, Brno

Faculty of Science

Applying Mendel's laws of genetics to plant biology in the lab S2012

Lecture and Practical book

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Program

Mon.	5/10	9.00	A26, 2.22	**Theory part 1** – Role of auxin for plant development – Auxin signalling and auxin transport – *pin1* and *monopteros* mutants **Preparative lecture Practical 1** – Experiment flow	
Fri.	9/10	9.00	A26	**Practical part 1** **–** Preparation of seeds and plates	
Mon.	12/10	9.00	A26, 2.22	**Theory part 2** – Mendel's law of genetics – Molecular genetics in Arabidopsis thaliana **Practical 1** – Transfer plates to growth chambers	
Mon.	19/10	9.00	A26	**Practical part 1** – Phenotyping – sample collecting – DNA extraction	
Mon.	26/10	9.00	A26	**Practical part 1** – PCR reactions (genotyping)	
Mon.	2/11	9.00	A26, 2.22	**Theory part 3** - Root development – Effects of auxin for root growth **Preparative lecture Practical 2** – Experiment flow **Practical part 1** – Electrophoresis and evaluation	
Mon.	9/11	9.00	A26	**Practical part 2** – Preparation of the plates – Sowing seeds	
Thur.	12/11	-	A26	**Practical part 2** – Scan the plates –Transfer plates to growth chambers	
Mon.	16/11	9.00	A26	**Practical part 2**	

				– Scanning plates at T=4 DAG
Wed.	18/11	9.00	A26	**Practical part 2** – Scanning plates at T=6 DAG
Mon.	23/11	9.00	A26	**Practical part 2** – Scanning plates at T=11 DAG – GUS staining
Mon.	30/11	9.00	A26	**Practical part 2** – Expression analysis
Mon.	7/12	9.00	A26, 2.22	**Practical part 2** – Results analysis
Mon.	11/1		A26	Report handling

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1. Auxin and its role in plants

1.1. Plant hormones

Hormones are chemical messengers that coordinate the cellular functions of multicellular organisms. Animals produce many chemical hormones, each of which usually targets a small number of cells and triggers a specific response. By contrast, plant hormones (phytohormones) are fewer in number, usually affect most if not all cells, and trigger diverse responses. Furthermore, the accumulation and effects of each phytohormone are modulated by environmental and developmental influences as well as the activities of other phytohormones. Unravelling the complex networks of hormonal action and signaling pathways in plants is ongoing; several hormone receptors have only recently been identified, and many signaling components are still unidentified.

In their 1937 book *Phytohormones*, Frits Went and Kenneth Thimann define a hormone as "a substance which, being produced in any one part of the organism, is transferred to another part and there influences a specific physiological process." They emphasize the functional aspect of hormones, stating that "these hormones are characterized by the property of serving as chemical messengers, by which the activity of certain organs is coordinated with that of others."

The plant hormones (auxin, cytokinin, gibberellins, brassinosteroids, ethylene, abscisic acid, jasmonates, and salicylates) are active during the plant's life, from seed-to-seed. The five classical hormones are auxin (isolated in 1926 by F. Went), cytokinins (1950s, F. Skoog), ethylene (1901, D. Neljubow), gibberellins (1926, E. Kurosawa), and abscisic acid (ABA; 1950s, T. Bennett-Clark and N. Kefford). Within the past 50 years or so, several other compounds have been identified that meet the criteria of hormones. Four of the more recently identified types of hormones are brassinosteroids (BRs), jasmonates, salicylates, and strigolactones.

The functions of plant hormones are diverse, but all have profound effects on growth and development. Hormones affect all phases of the plant lifecycle from seed to seed, and their responses to environmental stresses, both biotic (from a living organism) and abiotic (from the physical environment). Because of their pleiotropic effects, unravelling the functions of plant hormones has been challenging and continues to be one of the most active areas of plant biology research. Because of their fundamental roles as integrators and regulators, the study of plant hormones and the genes that control their synthesis, transport, and downstream effects has identified many new tools for agricultural improvements.

1.2. Auxin

1.2.1. Early studies on auxin

Auxin is a remarkable small molecule that plays a role in nearly every aspect of plant growth and development. No mutants have been identified that can growth without auxin. It appears to be absolutely required for plant survival. Auxin is universally present in all plants and is found in green algae as well as the more distantly related red and brown algae, although its function in these organisms is not well characterized. In angiosperms, auxin synthesis or signaling mutants are frequently small, underscoring auxin's role as a growth promoter. However, auxin's role is much more that a growth promoter; it is also necessary for the specification and maintenance of the root apical

meristem, the initiation of lateral roots and leaves, and the formation of developmental patterns.

Auxin was the first plant hormone isolated, and it is probably the most thoroughly studied of all plant growth regulators. Early botanists carefully described plant growth, development, and movement and even proposed the existence of mobile signals to coordinate these activities. In the late 19th and early 20th centuries, a series of elegant experiments into the nature of shoot phototropism (moving toward light) led directly to the identification of auxin as a mobile signal regulating cell elongation. Most famously, Charles Darwin and his son Francis studied phototropism in coleoptiles, a tissue in monocots that protects young leaves during germination. In 1880, they determined that light given from one side is perceived at the coleoptile tip but that "some influence is transmitted from the upper to the lower part, causing the latter to bend." In 1913, Peter Boysen-Jensen furthered these studies, observing and that the "influence" can move through an agar block but not a solid substance (Figure 1). Subsequently, Arpad Paal (1919) showed that removing the tip of a dark-grown coleoptile and replacing the tip asymmetrically onto the coleoptile base could induce curvature in the absence of a light stimulus.
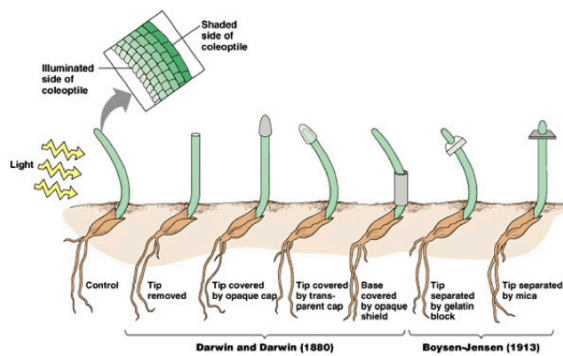

Figure 1.1 - A mobile molecule for the bending response

Building upon these studies, Frits Went placed coleoptile tips onto agar blocks and showed that these treated blocks were capable of promoting growth; they had captured the growth-promoting substance (Figure 1.2). Went's experiments led to the purification and identification of the auxin indole-3-acetic acid (IAA). Auxins in fact are a family of related compounds, some of which are entirely synthetic but mimic auxin effects, whereas others are low-abundance compounds or found in only some plant families. In most discussions, auxin is used synonymously with IAA, which is the most abundant naturally occurring auxin.

Once it was available in purified form, auxin's contributions to root initiation, fruit development, cell elongation, and the suppression of lateral buds by the shoot apex (apical dominance) were recognized, as were some of the fundamental properties that contribute to auxin action. In the 1930s, Kenneth Thimann observed that different tissues differ in their sensitivity to auxin (Figure 3).
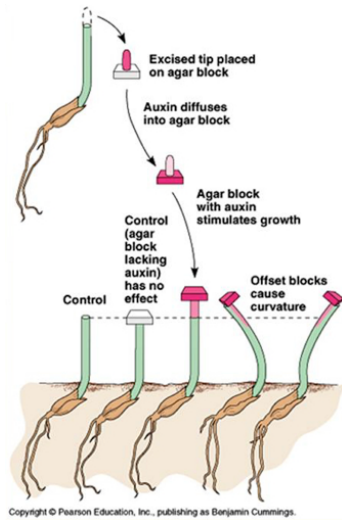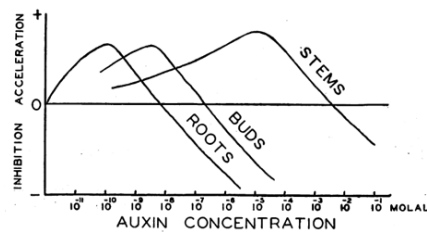
Figure 1.2 - Isolation of the moving substance

Figure 1.3 - Tissue-sensitivity to auxin

1.2.2. Polar auxin transport

Many hormones can be translocated through the plant by way of the xylem or phloem, but the directional movement of auxin between cells and tissues is particularly well described. Polar auxin transport is fundamental to many of its functions in pattern formation, organogenesis, and directional growth responses. The **Cholodny-Went theory** proposed in the 1930s as the chemi-osmotic model postulated that the asymmetries in growth rate in light- or gravity-responding organs are caused by an auxin gradient. After many years, this theory is now widely accepted, largely because of our ability to detect the proposed auxin gradient, and the identification of the chemical and cellular basis by which the auxin gradients are established and maintained.

Because IAA is a weak acid, it exists in a charged anionic form (IAA$^-$) in the neutral pH of the cytoplasm (pH ~7). In the more acidic cell wall environment (pH ~5.5), ~15% of the molecules are in the protonated form (IAAH), which can transit through the plasma membrane. The pH differential between the cytoplasm and wall means that auxin can move into (as IAAH) but not out of plant cells. Plants employ specific transport proteins to move auxin precisely (Figure 4).

Many auxin transporter proteins were identified through **genetic screens** for abnormal auxin responses, including **agravitropism**. The extremely agravitropic *aux1* mutant is deficient in polar auxin transport. AUX1 encodes an auxin influx carrier that augments auxin's chemiosmotic influx into cells. AUX1 and its related LIKE-AUX1 genes seem to be particularly important for auxin influx in conditions when auxin efflux rates are high.

The ATP Binding Cassette subgroup B (ABCB) transporters belong to a family of 21 proteins that contributes to auxin transport in diverse ways; some function in auxin influx and some in auxin efflux. Unlike PIN proteins, their cellular position seems to be relatively stable and they may interact with and

stabilize PIN proteins at specific microdomains of the membrane. These transporters may link auxin responses and stress responses.

The PIN genes (named for the *pin-formed* mutant) encode auxin efflux carriers with asymmetric, polar distributions on cell membranes. Through their polarity, PIN proteins contribute to the highly directional, polar transport of auxin that underlies developmental patterning and differential growth responses. In Arabidopsis, there are eight PIN genes with different tissue-specific expression patterns. Furthermore, the individual PIN proteins themselves can have different cellular distributions within cells. To some extent, these different family members are specialized for specific functions. For example, PIN1 is expressed in the xylem parenchyma throughout the plant and has a major role in the polar transport of auxin from shoot tip to root tip. PIN2 plays a key role in root gravitropism; loss-of-function mutants have a strongly agravitropic phenotype. Localization of the PIN3 protein changes upon a change in light or gravity orientation and is important for establishing the auxin gradients that mediate tropic growth responses, and PIN5 and PIN8 are localized to the endoplasmic reticulum and thought to be involved in intracellular active auxin transport.
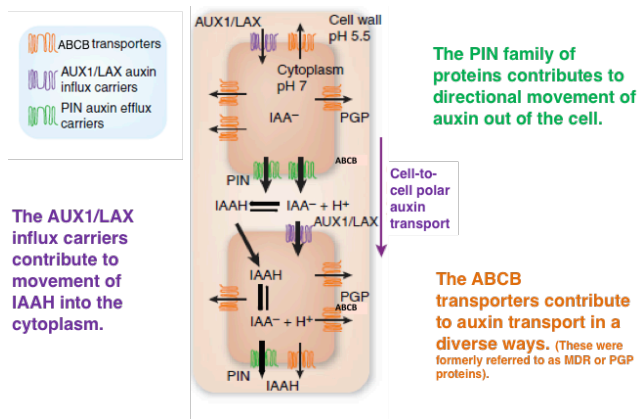
Figure 1.4 - Polar auxin transport and its transporters

PIN protein redistribution is critical for the movement of auxin that regulates pattern formation and organogenesis at the shoot apical meristem and during embryogenesis. It is thought that the localization of PIN proteins at the plasma membranes are indicative of the direction of the auxin flow. Auxin maxima are required for and precede the initiation of lateral roots, leaves, and flowers at the shoot apical meristem and the embryonic formation of the radicle (embryonic root) meristem and cotyledons (Figure 5).
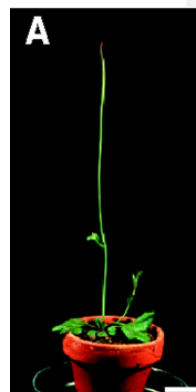
Figure 1.5 - The pin1 *mutant*

1.2.3. Auxin signalling pathway

In 2005, the protein TRANSPORT INHIBITOR RESPONSE1 (TIR1) was identified as an auxin receptor, connecting auxin with the regulated proteolysis (= protein degradation) of auxin response repressors. TIR1 is an F-box protein, a component of an SCF (SKP1, CUL1, and F-box protein) ubiquitin ligase complex. Ubiquitin is a small protein that is conjugated to other proteins by ubiquitin ligase complexes, including SCF^{TIR1}. Because the F-box protein confers specificity to this complex by binding to the target proteins, SCF complexes are identified by their specific F-box protein component as indicated. Ubiquitinated proteins are proteolyzed by the 26S proteasome, which selectively degrades proteins.

When bound to auxin, TIR1 also specifically binds to Aux/IAA repressor proteins with the auxin holding the proteins together like a molecular glue, targeting them for proteolysis. Genes encoding Aux/IAA proteins were identified in the 1980s and were among the first auxin-induced genes to be identified through the newly developed tools of molecular biology. Aux/IAA proteins are short-lived, nuclear-localized proteins, whose rate of degradation is enhanced by auxin. Aux/IAA proteins have four conserved domains. A short amino acid sequence in domain II was identified as the "degron" and is necessary for auxin-induced instability. In the early 1990s, several research groups identified dominant, gain-of-function mutants in *Aux/IAA* genes; these mutations were mapped to amino acid changes in the degron that interfere with auxin-induced protein degradation. Auxin signaling is dependent on the degradation of the Aux/IAA repressors and that stabilized mutant proteins confer an auxin-resistant phenotype because they are resistant to degradation.

Analysis of the promoters of several auxin-induced genes led to the identification of the auxin response element and a family of proteins that specifically bind to the auxin response element called auxin response factors (ARFs). Arabidopsis has 23 ARF encoding genes. All ARFs have DNA binding domains; some have a transcriptional activation domain and function as transcriptional activators, whereas others function as transcriptional repressors.
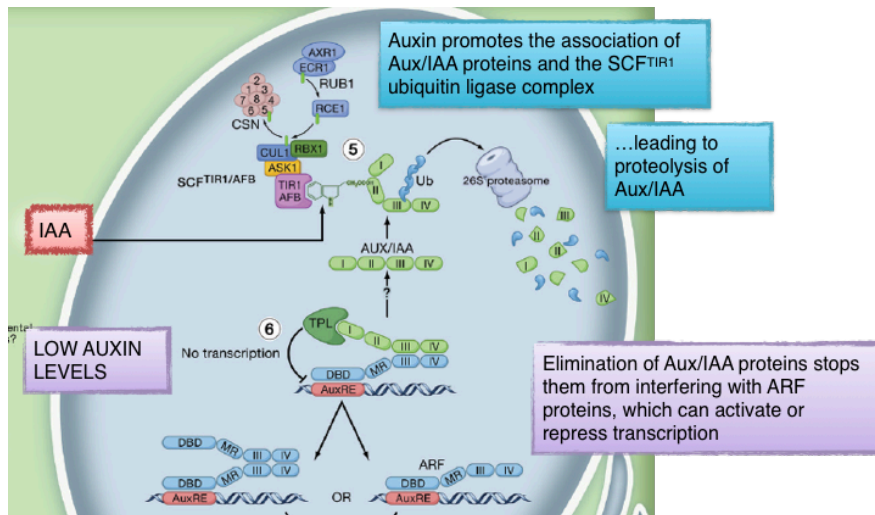

Figure 1.1.6 - Auxin signaling pathway

1.2.4. **Case studies: *pin1* and *monopteros***

Auxin is presented in nearly all aspects of plant development, from germinating seed to seed development, through all the development steps of the plant. Within this practical work we will use two mutants, *pin1* and *monopteros* (*mp*). PIN1 is one of the auxin efflux transporters described above. And MONOPTEROS is ARF5, one of the auxin response factors, activating transcription of genes after the activation of the signaling pathway.

Both MP and PIN1 act during the early phases of embryo development, in seeds, when a zygote will form an embryonic version of a plant (Figure 1.7).
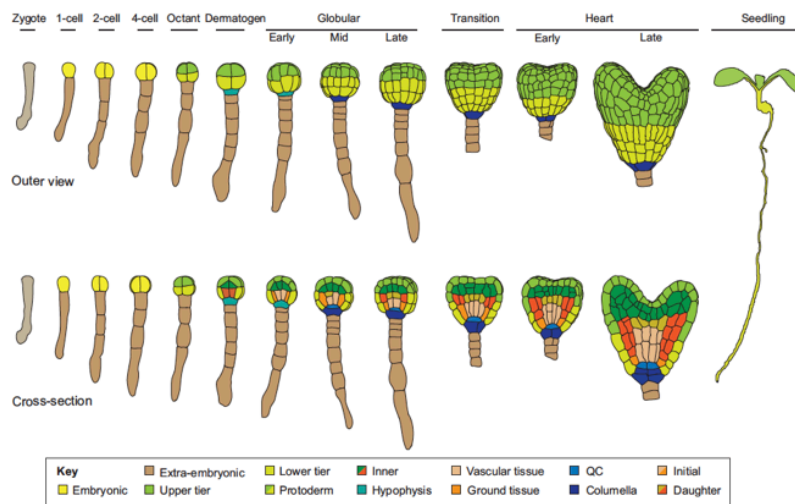
Figure 1.7 - Embryo development

PIN1 is important for the transport of auxin and the formation of the two embryonic leaves, the cotyledons. In a *pin1* mutant, auxin does not flow properly to the cotyledons, and the germinating seedlings will have defects in the number of cotyledons. PIN1 is also important for the formation and distribution of flowers along the stem (Figure 1.8). In a *pin1* mutant, nearly no flowers are formed. Therefore, the mutant line is maintained as heterozygous (see next chapter for the definition).

MP is transducing the auxin signal to trigger developmental program. During embryo development, MP is involved in the formation of a root. Therefore, when MP is absent (as in the *mp* mutant), the seedling won't have a proper root (Figure 1.8). Here again the mutant line is maintained as heterozygous.

Within the practical work, you will germinate seeds of the *pin1* and the *mp* mutants, score the phenotype of seedlings. Then you will extract the DNA of individual seedling, and genotype it for the presence of the mutation. You will interpret your data using the information given in Chapters 1, 2 and 3.

In addition, you will transfer seedlings of the pin1 mutant to soil to observe the phenotypes of the flowering *pin1* plants.

Figure 1.8 - The pin1 *and* mp *mutants*

1.3. References

Went, F.W. & Thimann, K.V., 1937. *Phytohormones*, New York,The Macmillan Company.

2. Mendel's law of genetics

2.1. Plant as model for studying the genetics

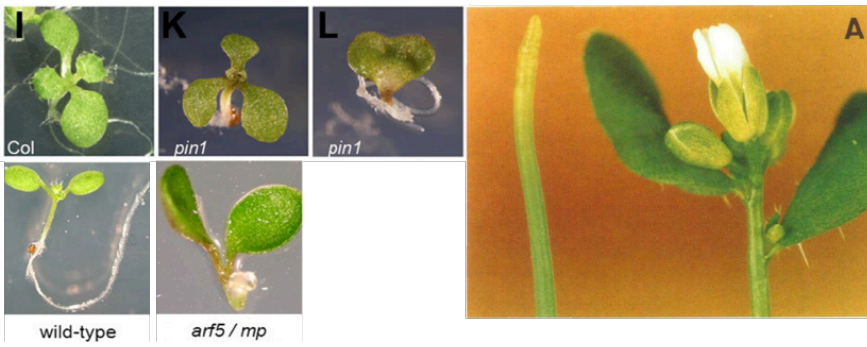The concept of genes and characters that are hereditary (inherited by the parents) has been initiated by Gregor Mendel in 1865. His observations have been done from crosses experiments in peas (*Pisum sativum*). Plants can be either **self-pollinated** or **cross-pollinated**. Self-pollination is obtained by the production of seeds with fertilization of ovules, inside the ovaries or carpel, female part of the flower, by its own pollen, inside the male parts of the flower or anthers. The pollen, male part of the flower, contains the sperm cells, fall on the stigma of its own flower, germinate a pollen tube which brings the sperm cells to the ovule (Figure 2.1). The experimenter can cross two plants (from the same species) by removing the anthers from one plant before they shed their pollen (emasculation), to prevent self-pollination. Pollen from another plant is then transferred to the stigma of the first plant.
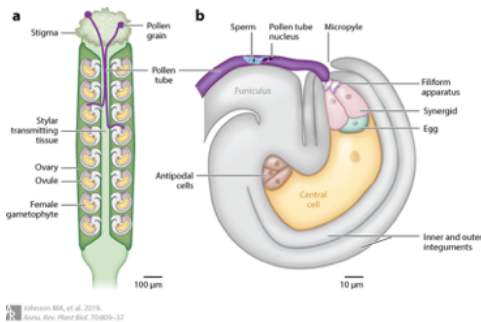

Figure 2.1 - The pollen tube journey from stigma to synergid. (a) In Arabidopsis thaliana, pollen grains are deposited on the stigma and germinate a pollen tube that enters the transmitting tissue of the style before exiting onto the interior surfaces of the ovary. The pollen tube then enters an ovule and contacts the female gametophyte. (b) The pollen tube (male gametophyte) grows along the surface of the funiculus and is attracted into the micropyle by the synergid cells. The male gametophyte consists of haploid pollen and sperm cells, both of which are derived from a single meiotic product. In addition to the synergid cells, the female gametophyte consists of an egg, a central cell, and three antipodal cells. The female gametophyte develops within the inner and outer integument cell layers of the ovule. The female gametophyte cells are haploid. Approximate scale bars are given.

After **crosses** of two pea family with different characters or variants (colour of the seeds, shape of the seeds, colour of the flowers), Mendel observed that the characters were inherited in the next generations. He noticed that the **segregation** of these characters (distribution of the parental variants within the progeny through several generations) follows a strict inheritance pattern. For each of the tested characters, Mendel firstly obtained **pure lines**, a population of breeds for a particular character that show no variation for multiple generation. With the observations of the segregation of the characters in the progeny a cross came the notion of **dominant** and **recessive** of a character when it will be observed or not in the first cross generation (F1). In genetics, the observation of a character is called **phenotype,** word derived from Greek meaning "form that is shown". This phenotype is given by the genetic information, the **genes**. Each gene is represented by two **alleles**, a gene pair. Each copy or allele is given by the **gametes**, one allele from the gamete of the mother (ovule), and one allele from the gamete of the father (sperm). If the organism resulting for the cross is **diploid** (2 alleles of one gene), the gametes are **haploid** (one copy of each gene). This allows the succession of generation between vegetative growth as diploid and the reproductive growth with the formation of gametes as haploid cells. Fusion of the two haploid gametes (fertilization) produces a new diploid organism, with a mixture of the genetic information from the two parents, a segregation of the alleles and the characters.

2.2. Analysis of segregation of characters

In one of his experiments, Mendel crossed two types of pea plants, one with yellow wrinkled seeds (P1), and one with green round seeds (P2), the parental generation P. The progeny of this cross is called filial generation (F). One plant is used as female (♀) and one as male (♂). The seeds of the obtained by this cross (F$_1$ generation) were yellow and round (Figure 2.2). We can conclude that the yellow colour is a dominant character and that the green colour character is recessive. The phenotype of a F$_1$ established by intercrossing two pure lines and identical to one of the parental phenotypes is defined as **dominant**. Similarly, the round aspect of the seed is a dominant character and the wrinkled aspect of the seed is a recessive character. The F$_1$ plants were self-pollinated to produce seeds in F$_2$ generation. It can be observed that the **recessive** characters became visible again. The recessive character was unexpressed in the F$_1$, masked by the dominant character. Mendel counted the number of F$_2$ plants with each phenotype (Table 2.1) and quantification of the inheritance of the phenotypes in the F$_2$ generation proposed a **3:1 segregation ratio** (Table 2.1). The ratio in F$_2$ generation for each character was always a 3:1 ratio, with 3/4 seeds with the dominant character and 1/4 seeds with the recessive character. We can also notice that the two types of characters (colour and aspect) are segregating independently always as a 3:1 ratio. A F$_2$ generation will be producing a ratio 9:3:3:1; 9 round yellow seeds (both dominant characters): 3 round green seeds (dominant recessive): 3 wrinkled yellow seeds (recessive dominant): 1 wrinkled green seed (both recessive).
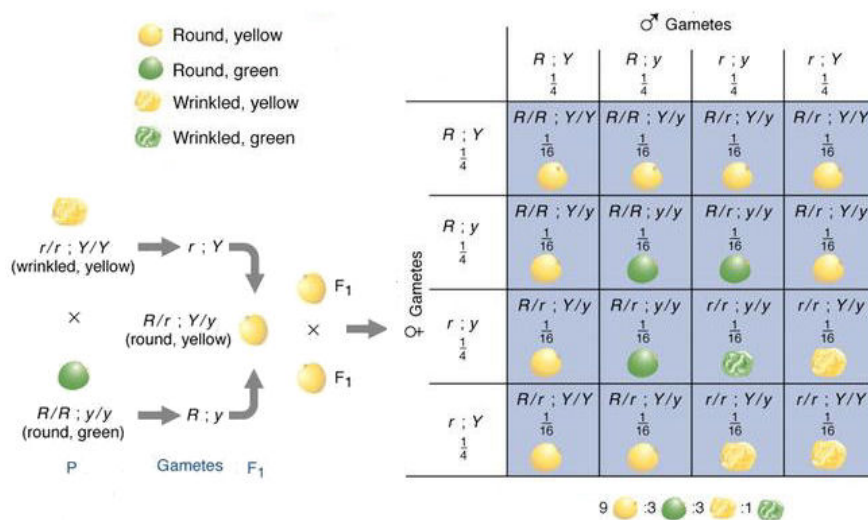

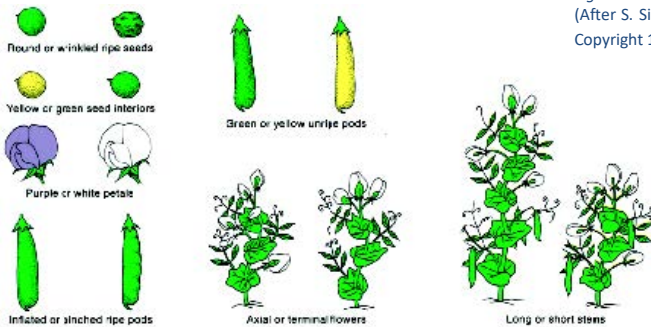*Figure 2.2 - Analysis of segregation of two unlinked characters*

cross	♀ parent	♂ parent	F$_1$ phenotypes	F$_2$ phenotypes	F$_2$ ratio
1	Round	Wrinkled seeds	All round	5 474 round; 1 850 wrinkled	2.96:1
2	Yellow	Green seeds	All yellow	6 022 yellow; 2 001 green	3.01:1
3	Purple	White petals	All purple	705 purple; 224 white	3.15:1
4	Inflate	Pinched pods	All inflated	882 inflated; 299 pinched	2.95:1
5	Green	Yellows pods	All green	428 green; 152 yellow	2.82:1
6	Axial	Terminal flowers	All axial	651 axial; 207 terminal	3.14:1
7	Long	Short stems	All long	787 long; 277 short	2.84:1

Table 2.1 – Results of all Mendel's cross in which parents differed in one character.

Further analysis testing a F$_2$ segregation (cross on one character, the seed colour), Mendel took a sample of 519 yellow F$_2$ peas and grew plants. These yellow-pea F$_2$ plants were selfed individually and colour of peas of F$_3$ generation were scored. Mendel found that 166 F$_2$ plants carried yellow peas and that 353 remained F$_2$ plants carried a mixture of yellow and green peas with a 3:1 ratio. Plants from green F$_2$ peas were also grown and selfed and found to bear only green peas. All F$_2$ green peas were pure-breeding. But of the F$_2$ yellow peas, 2/3 is like F1 yellow peas (producing yellow and green peas in a 3:1 ratio) and 1/3 are pure-breeding yellow parent. This study of individual selfings revealed that the 3:1 ratio is in fact a 1:2:1 ratio.

F1   all yellow (selfed)

F2   ⌈ 3/4 yellow   →   F3 (selfed) ⌈ 1/4 green (pure-breeding)

       ⌊   3/4 yellow (1/4 pure breeding; 2/4 segregating)

      ⌊ 1/4 green   →   F3 (selfed) all green   (pure breeding)

A 1:2:1 ratio is explained by the existence of **genes**, carrying the genetic information responsible for the inheritance of the characters and the phenotypic differences. The genes are in pairs. Each gene may have different forms, each corresponding to an alternative phenotype of a character. The different forms of one gene are called **alleles** (point 1, Figure 2.4). Each type of gene is present twice in each cell, constituting a **gene pair** (point 2, Figure 2.4). In different plants, the gene pair can be of the same alleles or of different alleles of that gene. The F$_1$ plants have one allele that is responsible for the dominant phenotype and another allele that is responsible for the recessive phenotype that will be

visible in the later generation. In our example, the gene "colour" has two alleles "yellow" and "green".

Each gamete carries only one member of each gene pair (point 3, Figure 2.4). The members of the gene pairs segregate equally into the gametes – 50% of the gametes will carry one member of a gene pair and 50% will carry the other (point 4, Figure 2.4). Fertilization of the gametes from each parent is random (point 5, Figure 2.4).

Figure 2.4 - Explanation of the 1:2:1 ratio
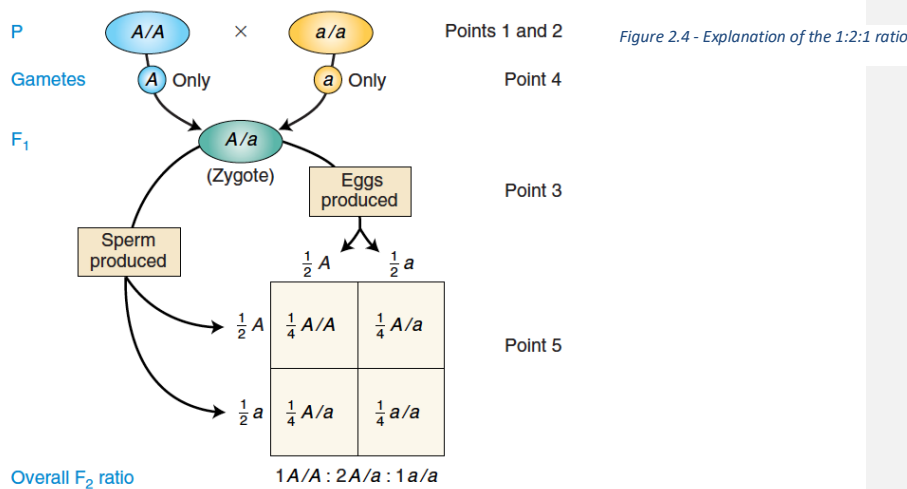
Symbolically the dominant allele will be represented in upper case (A in Figure 2.4, Y in Figure 2.5), and the allele responsive of the recessive phenotype will be represented in lower case (a, y). Therefore, an F1 plant (cross of a yellow seed plant and a green seed plant, pure lines) crossed with a plant grown from green seed, will produce an equal segregation (1:1) of yellow and green seeds.
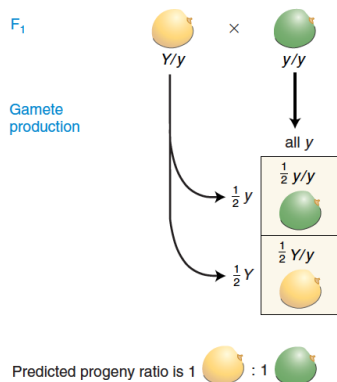
Figure 2.5 - Results of backcrossing
If Y stands for the allele that determines the dominant phenotype (yellow seeds) and y for the allele that determine the recessive phenotype (green seeds), we can predict an equal segregation of Y:y as 1:1 ratio for an F_1 plant crossed with the recessive parental pure line. This demonstrate the equal segregation of the alleles.

Y/y individuals are called **heterozygotes** (also **hybrids** in agronomy), and Y/Y are **homozygotes** for the Y allele, therefore pure line. Thus Y/Y are homozygous dominant, an y/y is homozygous for the

recessive allele. This is called a **genotype**. Note that a 3:1 phenotypic ratio in F_2 is in fact a 1:2:1 genotypic ratio of *Y/Y : Y/y : y/y*.

2.3. Cellular and molecular basis of the Mendelian genetics

Different alleles of a gene can provide a different phenotype. When analysed at the DNA level, they are generally found identical in most of the sequences and differ only at one or few nucleotides over the thousands of nucleotides that form the gene. To study such variation, it is helpful to have a standard. In genetics, this standard is the "**wild-type**" allele, the allele found in the wild, natural population, as opposite to the **mutant** allele.

If we consider the gene affecting the colour of the pea petal, we can say that the purple colour of the wild peas is caused by a pigment called anthocyanin, which is a chemical made in petal cells as the series of chemical reactions. Each reaction requires a specific enzyme. The structure (amino acid sequence) and activity of these enzymes are dictated by their nucleotide sequence of the respective gene. If the nucleotide sequence of any of the genes coding for the enzymes of this pathway changes as a result of a **mutation**, a new allele is created. These mutations can introduce more or less amino acids, or induce a stop codon in the gene sequence, resulting in a shorter protein, or exchange an amino acid. Any of these mutations may result in a loss 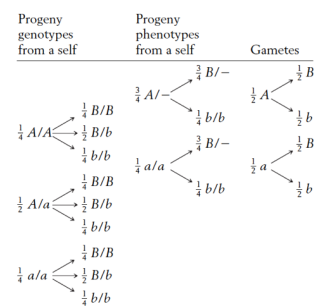of the enzyme function. If such mutant allele is homozygous, the pathway may be blocked and no purple pigment will be produced in petals, which then look white. If the plant is heterozygous, one functional copy remains to allow synthesis of enough pigment.

A/A → active enzyme → purple pigment

A/a → active enzyme → purple pigment

a/a → no active enzyme → white

2.4. Predicting progeny of crosses using the Mendelian ratios

An important part of genetics is to predict the types of progeny that will emerge from a cross, calculate their expected frequency and anticipate the number of plants that will need to be tested. We have illustrated segregation for 1 and 2 characters (genes). And we used the Punnett squares to calculate it (Figure 2.2). Punnet squares can be used to show hereditary patterns based on one gene pair, two gene pairs or more, that independently segregate. Such grid is graphic and easy to visualize the data up to 2 independent characters. But it may become laborious and time-consuming for more complex analysis. For 1 character, the square is with 4 compartments (2^1 types of gametes), for 2 characters, 16 compartments (2^2 or 4, types of gametes), for 3 characters, 64 compartments (2^3 or 8 types of gametes). A branch diagram may be then more appropriate and adaptable for phenotype, genotype and gametic proportion analysis.

It illustrates the segregation of a 2-character segregation *A/a ; B/b*. the dash (-) means that the allele is hidden, and the phenotype does not allow for knowing what the second allele is, dominant or recessive. Two gene pairs give 3^2, or 9, genotypes.

A 3-character segregation will give 3^3, or 27, genotypes (A, B, C) with 3 levels of branches.

If we consider an example of 5 characters. We have two plants of genotypes (*A/a* ; *b/b* ; *C/c* ; *D/d* ; *E/e*) and (*A/a* ; *B/b* ; *C/c* ; *d/d* ; *E/e*). From a cross between these two plants, we wish to recover a progeny plant of genotype (*a/a* ; *b/b* ; *c/c* ; *d/d* ; *e/e*), pentuple homozygous mutant. We wish to estimate the number of progeny plants we need to grow to have a reasonable chance of obtained the desired genotype. So, we need to calculate the proportion of progeny that is expected to be of that genotype. We assume that all the gene pairs will segregate independently. Let's consider the five pairs individually as it is separate crosses, and then probabilities will be multiplied.

From *A/a* x *A/a*, 1/4 of the progeny will be *a/a* (see Mendelian ratio, part 2.2).

From *b/b* x *B/b*, 1/2 of the progeny will be *b/b*.

From *C/c* x *C/c*, 1/4 of the progeny will be c/c.

From *D/d* x *d/d*, 1/2 of the progeny will be *d/d*.

From *E/e* x *E/e,* 1/4 of the progeny will be *e/e*.

Therefore, the overall probability of progeny of genotype *a/a* ; *b/b* ; *c/c* ; *d/d* ; *e/e* will be 1/4 x 1/2 x 1/4 x 1/2 x 1/4 = 1/256. We would need to examine ± 300 progeny to stand a chance to obtain one of the desired genotypes.

2.5. Using χ^2(Chi-square) test on Mendelian ratios

In practice, an experimenter is often confronted with results that are close but not identical with the expected ratio. But is it close enough? A statistical test to check such ratios against expectation is the χ^2(Chi-square) test.

In this test, we compare the observed number of items per categories to the numbers expected in those categories based on the hypothesis they follow a Mendelian segregation. The χ^2 (Chi-square) test quantifies the various deviations expected by chance of the hypothesis is true. The formula is:

$$X^2 = \Sigma \frac{(observed\ number\ per\ category\ - expected\ number\ per\ category)^2}{expected\ number\ per\ category}$$

Even if the hypothesis is true, we don't always expect an exact 1:1 ratio. However, if all levels of deviation are expected with different probabilities even if the hypothesis is true, how can we ever reject a hypothesis? The general scientific convention is if there is a probability of less than 5% of observing a deviation from expectations, then the hypothesis will be rejected as false. We estimate that 5% of the time we will mistakenly reject the hypothesis even if the hypothesis is true.

In practice, let's test the hypothesis that a plant is heterozygote. *A* stands in that case for red petals and *a* for white petals. We did a test-cross of a presumed heterozygote plant with a homozygote mutant line (*a/a*) and based on the hypothesis. Mendel's law predicts that 50% is *A/a* and 50%, *a/a*. Let's assume that the result of this cross gave 55 red and 65 white petal plants on a progeny of 120. These numbers differ from the precise expectations, which would have been 60 red and 60 white. The calculation is summarized in this table

Class	Observed	Expected	$(O-E)^2$	$(O-E)^2/E$	=
Red	55	60	25	25/60	0.42
White	65	60	25	25/60	0.42
				$\chi^2 =$	0.84

The χ^2 value is to compare to a table, which will give us the probability value we want. The lines in the table represent the different values of *degree of freedom (df)*. The number of degrees of freedom is the number of independent variables in the data. In our example, it is the number of phenotypic classes minus 1. So here df = 2-1 = 1. Looking at the 1 df line, we see that our χ^2 value of 0.84 lies somewhere between the columns marked 0.5 and 0.1; in other words, 50% and 10%. This probability value is much greater than the cut-off value of 5%, so we would accept the observed value as being compatible with the hypothesis.

Table 2-2 Critical Values of the χ^2 Distribution

df	0.995	0.975	0.9	0.5	0.1	0.05	0.025	0.01	0.005	df
1	.000	.000	0.016	0.455	2.706	3.841	5.024	6.635	7.879	1
2	0.010	0.051	0.211	1.386	4.605	5.991	7.378	9.210	10.597	2
3	0.072	0.216	0.584	2.366	6.251	7.815	9.348	11.345	12.838	3
4	0.207	0.484	1.064	3.357	7.779	9.488	11.143	13.277	14.860	4
5	0.412	0.831	1.610	4.351	9.236	11.070	12.832	15.086	16.750	5
6	0.676	1.237	2.204	5.348	10.645	12.592	14.449	16.812	18.548	6
7	0.989	1.690	2.833	6.346	12.017	14.067	16.013	18.475	20.278	7
8	1.344	2.180	3.490	7.344	13.362	15.507	17.535	20.090	21.955	8
9	1.735	2.700	4.168	8.343	14.684	16.919	19.023	21.666	23.589	9
10	2.156	3.247	4.865	9.342	15.987	18.307	20.483	23.209	25.188	10
11	2.603	3.816	5.578	10.341	17.275	19.675	21.920	24.725	26.757	11
12	3.074	4.404	6.304	11.340	18.549	21.026	23.337	26.217	28.300	12
13	3.565	5.009	7.042	12.340	19.812	22.362	24.736	27.688	29.819	13
14	4.075	5.629	7.790	13.339	21.064	23.685	26.119	29.141	31.319	14
15	4.601	6.262	8.547	14.339	22.307	24.996	27.488	30.578	32.801	15

What does the probability value actually mean? It is the probability of observing a deviation from the expected results at least this large on the basis of chance, if the hypothesis is correct. Now that the above results have "passed" the chi-square test because $p > 0.05$, it does not mean that the hypothesis is true, merely that the results are compatible with that hypothesis. However, if we had obtained a p value of < 0.05, we would have been forced to reject the hypothesis. We must be careful about the wording of the hypothesis, because often there are tacit assumptions buried within it. The present hypothesis is a case in point; if we were to carefully state it, we would have to say it is that the "individual under test is a heterozygote *A/a* and the *A/a* and *a/a* progeny are of equal viability." We must keep in mind that differences in survival would affect the sizes of the various classes (a mutation is lethal). The problem is that if we reject a hypothesis that has hidden components, we do not know which of the components we are rejecting. The outcome of the chi-square test is heavily dependent on sample sizes (numbers in the classes). Hence the test must use **actual numbers**, not proportions or percentages. Also, the larger the samples, the more reliable is the test.

3. Molecular genetics in Arabidopsis thaliana (mutations and their use)

I'll assume you know what a gene is, what a promoter is, and how genes are transcribed and translated into proteins. If you need to refresh your memory, please consult "An introduction to the Genetic Analysis" by Griffiths et al. The book can be found at the MUNI library (https://bit.ly/2HjXmSl). I will upload the 10th edition in IS (or link: https://bit.ly/39NGGiX).

3.1. Mutations – definition

Mutations of DNA can be obtained by various mutagen agents (irradiation [UV, X-ray, gamma rays], chemicals [EMS, ethyl methane sulphonate], insertion of pieces of DNA such as T-DNA and transposon [biotechnology, use of agrobacterium]). These mutations can introduce various modifications of the DNA structure: small or large deletion or insertions (**indels**), or an exchange of nucleotides, having consequences for the cellular function (Figure 3.1). If the modifications occur within the gene, they can be **anonymous**, meaning the change at the DNA level did not affect the sequence of the protein, either because it happens in introns or the codon change is **synonymous** (for example, GAT, GAC, GAA, GAG all code for Alanine) or the amino acid that was exchange is not important for the protein function (**silent mutation**). It can lead to unfunctional protein, because it introduces a stop codon (**nonsense mutation**), it exchanges an important amino acid (**missense mutation**), or the indel indices a **frameshift**, and disturbs the function, stability or behavior of the protein (**null mutation**). Mutations can also happen in non-coding regions, such as the promoters. In that case, it may change the regulation of the expression of the gene (level and pattern – when and where). Example in **Practical 1.**
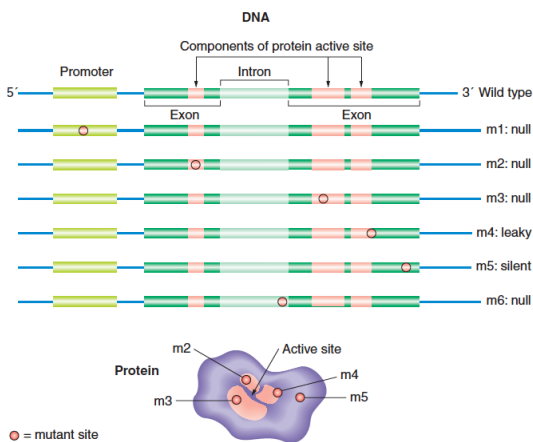
Figure 3.1 - Representative position of mutation sites and their functional consequences

*m5 – **silent mutation**, does not results in a defective protein and produce a wild-type phenotype*

*m1-3, m6 – **null mutations**, the encoded proteins is nonfunctional or the protein is not produced*

*m5 – **leaky mutation**, the protein has a remaining function (low level)*

Some mutations may create **recessive lethal alleles**, meaning that the homozygote mutant for this allele is not viable. No progeny will be produced. This can happen at fertilization, or later during embryo development (stops the growth and seed aborted) and during vegetative growth (no growth after germination or no progress to the next generation). In that can the recessive lethal alleles are maintained as heterozygotes. Example in **Practical 1.**

The hypothesis of "one gene – one enzyme" established by the study of Beadle and Tatum in the 1940s clarifying the role of genes and for which they obtain a Nobel prize, is based on their work on the

haploid fungus *Neurospora*. They first irradiated *Neurospora* to produce mutations and tested the cultures from ascospores for interesting mutant phenotypes. They isolated many auxotrophic mutants (Figure 3.2). Each mutation that generated the auxotrophic need was inherited by a single-gene mutation because of a wild-type backcross ratio of 1:1.
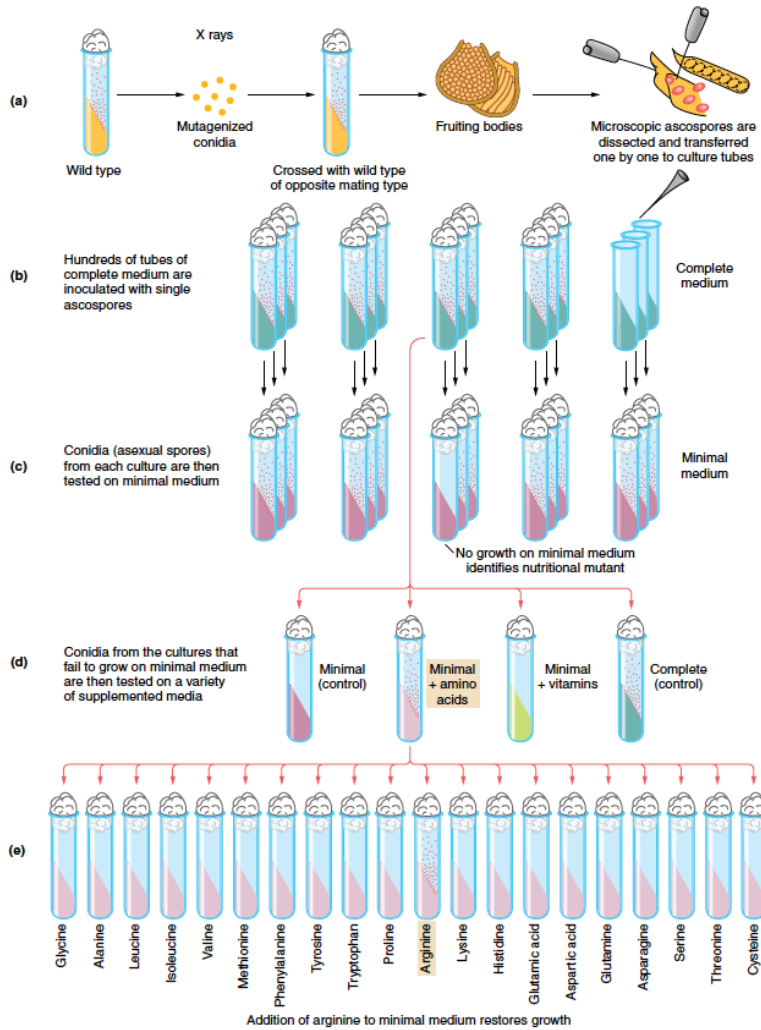

Figure 3.2- Experimental approach used by Beadle and Tatum for generating large number of mutants in Neurospora

3.2. Mutations – insertion of a transgene

Genetic engineering of plants, and thus the introduction of genetically modified organisms (GMOs), has been made possible with the identification of the infection mechanisms of a soil bacterium,

Agrobacterium tumefaciens. This bacterium causes what is known as *crown gall disease* (Figure 3.3). Infected plant produces tumor (or galls) by uncontrolled growth, at the base of the stem of the plant. To produce this tumor, the bacterium has a large circular DNA plasmid, the Ti (tumor-inducing) plasmid. When the bacterium infects the plant, a part of the Ti plasmid, a region called the **T-DNA** (transfer DNA) is transferred to plant cells and integrated to the genomic DNA of the host plant. The insertion is random. In its native form, the genes present in the T-DNA enabling the tumor growth, encode enzymes for the production of opines (important for bacteria growth) and for the production of two plant hormones, auxin and cytokinins (for production of the tumor).

Figure 3.3 - Infection by agrobacterium
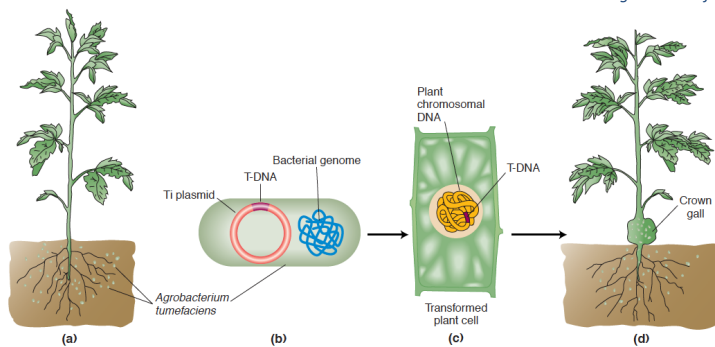
The Ti plasmid has been used as a vector for plant genetic engineering. From the Ti plasmid, we use the T-DNA transfer functions to incorporate a piece of DNA flanked by sequences of the T-DNA borders into the plant genome (disarmed Ti plasmid). The piece of DNA in the T-DNA may contain a selection marker (antibiotic) and any other genetic material that the researcher would need to be integrated into the plant genome. The selection marker serves to detect the plants would have been transformed. Typically, the transgenic plants grown on medium containing the antibiotic would contain the T-DNA. Segregation of the T-DNA follows a Mendelian segregation (Figure 3.4). By segregation analysis, one can determine how many T-DNA a plant has inserted into its genome. One insertion follows a 3:1 ratio. Two insertions would follow a 15:1 ratio (two characters) …
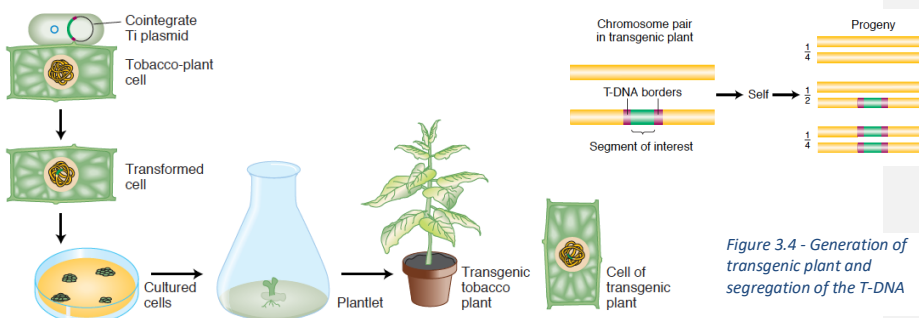

Figure 3.4 - Generation of transgenic plant and segregation of the T-DNA

Collection of insertional mutant lines have been generated where the T-DNA was randomly integrated. This T-DNA would contain mainly an antibiotic selection marker and, in some collections, a ubiquitous promoter that would promote the expression of the gene in proximity of the T-DNA (enhancer trap lines). The insertion of the T-DNA in the coding region of some genes or in the promoter of these genes may result in null allele by disturbing the gene sequence and therefore the formation of the coding protein. Example in **Practical 1.**

3.3. Mutations – detection

3.3.1. Detection of point mutation by restriction site differences

Point mutation, indel mutation, ..., alter the sequence of the DNA by creating a **SNP** (short nucleotide polymorphism). If lucky, this change may delete or create a restriction site recognized by a restriction endonuclease enzyme that would cut the piece of DNA (either the mutant or the wild-type allele) to allow discrimination between the two alleles. The piece of DNA is first amplified by PCR, the PCR product is incubated with the restriction enzyme and the reaction is analyzed by gel electrophoresis. The difference in the restriction pattern between the mutant and the wild-type alleles will be visible on the gel (Figure 3.5). This is referred as the **dCAPS** method (differential cleaved amplified polymorphic sequence). If the mutation does not create a differential restriction pattern, mismatches are included in one or the primers to create one linked to the mutation. Example in **Practical 1.**
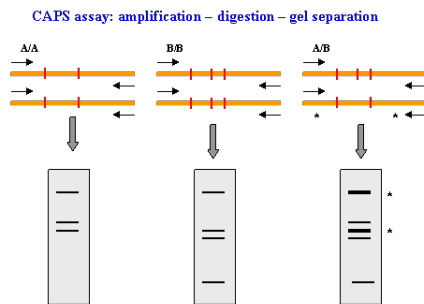
Figure 3.5 - CAPS assays
from https://www.ncbi.nlm.nih.gov/probe/docs/techcaps/

Unique sequence primers are used to amplify a DNA sequence from two related individuals, A/A and B/B, and from the heterozygote A/B. The amplified fragments from A/A and B/B contain two and three RE recognition sites, respectively. In the case of the heterozygote A/B, two different PCR products will be obtained, one which is cleaved three times and one which is cleaved twice. When fractionated by gel electrophoresis, the PCR products digested by the RE will give readily distinguishable patterns. Some bands will appear as doublets.

3.3.2. Detection of T-DNA

When its position is known, like in insertion line collections, the presence of the T-DNA can be detected by PCR amplification, using two sets of primers, followed by gel electrophoresis. One set would detect the presence of the wild-type (WT) allele (LP + RP) in wild-type homozygote plant and in heterozygote plant for the absence of the T-DNA. The second set detects the presence of the T-DNA, by amplifying from the border of the T-DNA with a T-DNA border primer (BP) and one gene specific primer (in Figure 3.6, RP). In that case, the electrophoresis gel will show a band when the T-DNA is present in heterozygote and homozygote plants for the insertion. Comparison of the two results (gene and border amplifications) will tell you if the T-DNA is present in the plant, as homozygote or heterozygote. Example in **Practical 1.**
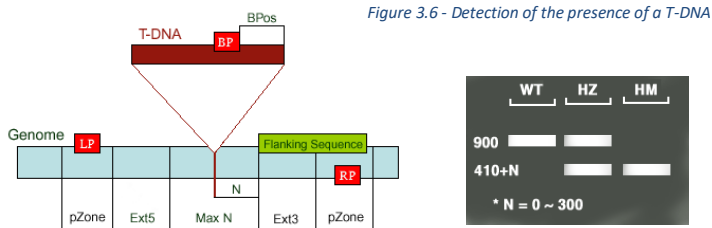
Figure 3.6 - Detection of the presence of a T-DNA

3.4. Forward and Reversed genetics

3.4.1. Forward genetics

Forward and reverse genetics are functional genetic methods. In forward genetics, the analysis starts with a phenotype of interest with the goal to identify the mutated gene and correlate the function of this gene to the altered development observed in the phenotype. In reverse genetics, the analysis starts with the mutation, in a known gene, to analyze the function of this particular gene (Figure 3.7).
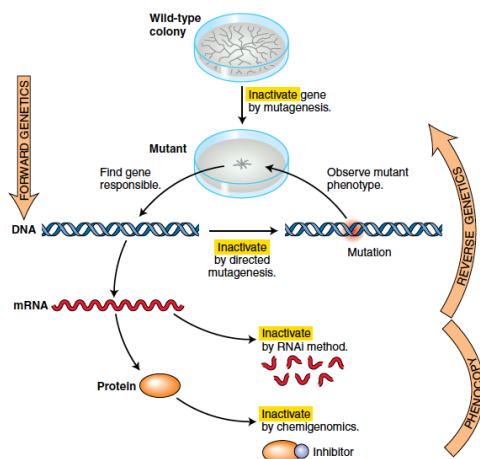
Figure 3.7 - Forward and reverse genetics

Typically, forward genetics starts with the wild-type genome, mutated randomly with a mutagen, and systematically surveys it for mutations that share some common phenotype. This method has been widely used in the 1980s and later and uncovered most of proteins involved in the production and signalling of hormones, in the development of flower (ABC model), and many others. This method is called **genetic screens**. When a plant with a phenotype of interest is identified, the position of the mutation needs to be identified. First the mutant is "cleaned" of unlinked mutations, mutations induced by the mutagen but not of interesting for the phenotype of interest, by backcrossing it to the original wild-type plant. Then, several methods are available, by methodically screening for SNPs after crossing the mutant plant with the wild-type plant of a different ecotype or cultivar. The goal is to link on of the SNPs to the phenotype, to identify the genomic region where the mutation may have

occurred. When identified, the genes located in this region are identified and sequenced for the presence of the mutation.

Modern methods including NGS methods where the full genome of the backcrossed mutant is sequenced, and the sequence compare to the wild-type one. SNPs are identified. The ones located in genes, creating missense, nonsense and frameshift are selected. Further studies will try to validate these mutations as the ones involved in the phenotypes of interest.

If the mutation is recessive, the mutant is complemented with the wild-type version of the allele. If the phenotype is restored, the mutant allele was identified. If the mutation is dominant, the mutant allele is cloned and introduced (using T-DNA technology) to the wild-type plant. If the wild-type plant with the mutant allele mimics the phenotype of the mutant, the mutant allele is identified. The function of the gene can be evaluated by various methods.

3.4.2. Reverse genetics

Reverse genetic analysis starts with a known molecule (DNA sequence, mRNA, protein) and attempts to disrupt this molecule in order to investigate the function of the wild-type allele. This starts with the search or the creation of the mutant. An insertional mutant can be identified in the different collections. A mutant can be created by novel methods such as CRISPR-Cas9 protocols. The gene can be silenced using other techniques such as RNA interference. Alternatively, the experimenter can over-express the wild-type allele or some altered version of this allele. Then the line is studied for phenotypes at the tissue level for developmental defects (form of the leaves, of the flower, growth of the root, …), at the cell level for defects such as cell division, differentiation, protein trafficking. The line can be also analyzed at the metabolic level for changes in the composition of targeted metabolites (hormones, lipids, sugars, secondary metabolites), at the transcriptomic level for changes in expression levels of targeted genes. All these data aim to provide information on the function of the protein of interest.

4. Practical 1 – Genotype to Phenotype: case studies of PIN1 and MONOPTEROS

You will analyze the relationship between phenotype and genotype using two mutant lines in auxin transport and signalling. PIN1 is one auxin efflux carrier and MONOPTEROS (MP) is a transcription factor in the signalling pathway.

> **Experimental objective**
>
> This experiment will give you an understanding of how mutation affects plant development and how to detect such mutation, using a model plant *Arabidopsis thaliana* (Arabidopsis).

4.1. Materials

4.1.1. Seeds

Seeds were sterilized for you by chlorine gas method
- *pin1 (salk line salk_047613)*
- *mp (mp-B4149)*
- Col wild type

4.1.2. Material

Petri dishes
Micropore tape
Aluminum foil
Stereomicroscope
Toothpicks
Marker pen
Tubes with the seeds
Tips
Tubes
Blue pestles
PCR strips
Electrophoresis material
Gloves
Pipettes (1000, 200, 10, 2ul)
Thermomixer

4.1.3. Solutions

1/2 strength MS for growing the seeds (will be prepared for you)
      for 1 Liter
      10 g Sucrose
      2,3 g MS Salts
      0,5 g MES
      adjust pH to 5,9 (KOH)
      8 g Agar (add separately to each bottle, 4 g per 500 ml bottle!)
Edward's buffer (DNA extraction) (will be prepared for you)
      200 mM Tris HCL pH 7.5

25 mM EDTA
250 mM NaCl
0.5% SDS
Autoclave it!
isopropanol
70% ethanol
MQ
primers
MP_1498S CTCTCAGCGGATAGTATGCACATCGG
MP_2082AS ATGGATGGAGCTGACGTTTGAGTTC
pin1-201 RP AATCATCACAGCCACTGATCC
pin1-201 LP CAAAAACACCCCCAAAATTTC
LBaI TGGTTCACGTAGTGGGCCATCG
enzymes
MseI (with reaction buffer)
Taq polymerase (with reaction buffer)
dNTP mix
Agarose
TAE buffer
Ethidium bromide

4.2. Experiment

4.2.1. Timetable

This experiment is designed to take ±fifteen days. We will start the experiment on Friday 9th, October and then remove the petri dishes from cold treatment on Monday 12th. The seeds will grow for 7 days. Phenotype will be scored. Individual seedling will be collected, and its genomic DNA extracted by the CTAB method. Genotype will be performed by PCR (and restriction for *mp*) and analyzed by gel electrophoresis. The two mutants have different types of mutations (point mutation induced by EMS, T-DNA insertion). You will learn how to genotype both and how they are different. The seedlings of the *pin1* mutant will be transfer to soil to observe the shoot phenotype.

Timetable

mutants	Prep Day 1	Prep Day 2-4	Day 1	Day 2	Day 3	Day 4
pin1	Sow the seeds	Cold treatment	Score the phenotypes; DNA extraction	PCR	Gel electrophoresis	Analysis
mp	Sow the seeds	Cold treatment	Score the phenotypes; DNA extraction	PCR	Restriction Gel electrophoresis	Analysis

4.2.2. Sowing

All this work is done in sterile conditions under flow benches in room 1S26

- You will be given 3 plates of MS medium

- Label your plates with the name of the seeds that will be sown (Col, *mp*, *pin1*)
- You may draw two lines with the marker pen at the back of the plate
- Take a toothpick and wet its tip by tapping it in the corner of the plate. 1 Toothpick per genotype to avoid mixing the seeds.
- Using the wet tip of the toothpick pick up one seed at a time from the tube and put it in its corresponding place on the Petri dish.
- Place 20 seeds for each genotype on the Petri dishes, following the lines you draw. Separate nicely the seeds along the line.
- Place the lid on the Petri dishes and wrap the edges of each Petri dish with Micropore tape completely sealing them shut.
- Wrap their stack completely with aluminum foil so that no light reaches the seeds.
- Label the wrapped Petri dishes using marker tape, with your name and date.
- Put the wrapped Petri dishes in the fridge (room 1S16) at 4°C-6°C. This process is called stratification (or vernalization). Stratification is important for breaking seed dormancy and synchronizing the germination of the seeds. Be extremely careful when preparing, transferring or working on Petri dishes with seeds. **Rough handling can cause seeds to shift from their intended position and ruin the experiment.**
- All Petri dishes should be kept in cold treatment until Monday 14th, October.

4.2.3. Data collection

- After the cold treatment is complete, remove Petri dishes from the fridge, remove the aluminum foil
- Place these dishes upright on one shelf on the cultivation room
- On Tuesday 22nd, score the phenotype of the seedlings. You will scan the plates for record in room 1S29. You may use the binocular microscopes in room A26, 2.08 for counting and observing the phenotypes.

4.2.4. DNA extraction

- Use a full seedling as starting material and transfer it to a 1.5 ml eppis
- Use 20 seedlings of *mp*, 20 seedlings of *pin1*, and 4 seedlings of Col. Distribute the samples among you.
- Grind the seedlings with a blue pestle. Change of pestle between each sample. DON'T TRASH THEM. They are washed and reused.
- Add 400 µl of the extraction buffer. Vortex briefly and spin down.
- The samples are left at room temperature until all the samples have been extracted.
- Centrifuge for 1 minutes at 13 000 rpm.
- Transfer 300 µl of the supernatant into a new tube.
- Add 300 µl isopropanol and mix well. Incubate for 2 minutes at room temperature.
- Centrifuge for 5 minutes at 13,000 rpm.
- Discard the liquid.
- Add 200 µl 70% ethanol
- Centrifuge for 5 minutes at 13,000 rpm.
- Discard the liquid.

- Air dry the pellet for at least 30 minutes (usually at 37°C or higher).
- Add 100 μl MQ water

4.2.5. Genotyping

The mp mutant

The *mp* mutant has a point mutation induced by EMS. The *mp-B4149* allele has a G to A mutation in its coding sequence at the splice acceptor site of the tenth exon. This mutation creates a T/TAA restriction site that can be cut by MseI. We will prepare one type of PCR reaction. The DNA fragment amplified by PCR will then be cut by the MseI restriction enzyme. MseI will cut the mutant allele but not the wild-type one. Because you have 20 + 4 samples. You will prepare 1 master mixes for 30 samples. You do more volume to compensate for pipetting inaccuracy.

You need:
- PCR buffer (10x concentrated)
- 10 mM dNTPs
- 10 uM oligo MP_1498S CTCTCAGCGGATAGTATGCACATCGG
- 10 uM oligo MP_2082AS ATGGATGGAGCTGACGTTTGAGTTC
- Taq polymerase (take out of the freezer at the last moment and keep it cold in one of the cold block)
- Genomic DNA

The master mix will be:

reagents/ Nr. of samples	1	10	30
MQ	18,5	185	555
10x Reaction Buffer	2,5	25	75
10mM dNTPs	0,5	5	15
10uM F Primer	0,5	5	15
10uM R Primer	0,5	5	15
***Taq* Polymerase**	0,12	1,2	3,6
total	25	250	750

- Mark the PCR strip at the beginning to recognize where the sample 1 will be. You need three strips, labeled with 1, 9, 17 in one extremity.
- The master mix is distributed in PCR strips, 22,5 ul per tube. Then add 2,5 ul of DNA, one sample per tube.
- DNA and mix are well mixed, and tubes are spin down.
- In room 2.21, there are the PCR machines. One machine has been booked for you. Put the three strips in the block of the machine. Start the machine.
- The program will be: 1x 95°C, 3'; 30x (95°C, 20"; 55°C, 20"; 72°C, 45"); 72°C, 5'
- We will stop the reactions for you and keep the samples in the fridge.
- The PCR reactions will be used as template for a restriction analysis with MseI. Each of you will prepare a master mix for one strip as follow:
  - MQ – 20 ul
  - 10x CutSmart buffer – 2.5 ul

- o MseI – 2.5 ul
- Mix 3 ul of the RE is added to 5 ul of PCR product in new PCR strips. Incubation for 30 minutes at 37°C in a PCR machine
- During the reaction time, you will prepare an electrophoresis gel. You wish to differentiate PCR fragments of 585 bp (uncut, wild type) and of 379 + 207 bp (*mp*). You need to prepare a 2% agarose gel.
- When working in the elfo room (2.18/2.19), wear lab coat and gloves
- Weight 4g of agarose and pour it an Erlenmeyer/bottle containing 200 ml TAE buffer. Pour the TAE from the barrel and measure it with one of the available cylinders.
- The agarose is melt using the micro-wave. Stay put to avoid over boiling. Be careful to not burn yourself (use the carry-over glove). All the agarose should be melted.
- Work in the chemical bench, prepare a chamber (you will be provided the one, the combs and the casting chamber). When the agarose cools a bit, add 3 ul of ethidium bromide, mix well and pour the agarose in the chamber. Let it solidify under the chemical flow.
- In the meantime, the restriction reaction finished. Get the strips from the machine, switch off properly according to instructions.
- To load the samples in the elfo gel, you will need to add loading buffer. It has two purposes: the blue color helps to visualize the progress of the migration; the glycerol renders the solution heavier and helps the solution to go down the wells. The loading buffer is 6x concentrated, meaning 1 ul for 5 ul of solution. Add 2 ul per sample.
- In the elfo room, place the gel in the appropriate running chamber. Make sure the buffer is clean and in the proper amount. You are ready to load the samples in the gel. One sample per well. Keep one well at one extremity to load a ladder. The ladder will indicate the size of DNA bands in the gel.
- Plug the cap, switch on the power supply. Set the voltage to 100, the timer to 30 minutes. Start.
- After 30 minutes, you collect the gel to take a picture using the BioRad machine. You will be instructed how to proceed.
- Print the gel picture.
- Analyze the size of the DNA fragments for each sample and interpret the results – what seedling is wild-type, heterozygous or homozygous for the mutation.

The pin1 mutant

The *pin1* mutant has a T-DNA inserted in his coding sequence. To detect it, we will prepare two types of PCR reactions, one to amplify the wild-type allele, one to amplify the mutant allele from the T-DNA border. Because you have 20 + 4 samples, you will prepare 2 master mixes for 30 samples.

Commented [HRB2]: To adjust according to the number of students

You need:
- PCR buffer (10x concentrated)
- 10 mM dNTPs
- 10 uM oligo pin1-201 RP AATCATCACAGCCACTGATCC
- 10 uM oligo pin1-201 LP CAAAAACACCCCCAAAATTTC
- 10 uM oligo LBaI TGGTTCACGTAGTGGGCCATCG
- Taq polymerase
- Genomic DNA

Each of the master mixes will be

reagents/ Nr. of samples	1	30
MQ	18,5	555
10x Reaction Buffer	2,5	75
10mM dNTPs	0,5	15
10uM RP Primer	0,5	15
10uM LP or LB Primer	0,5	15
Taq Polymerase	0,12	3,6
total	25	750

The WT mix is with pin1-201 RP and pin1-201 LP primers.

The T-DNA mix is with pin1-201 RP and LBaI primers.

- Mark the PCR strip at the beginning to recognize where the sample 1 will be. You need four strips, labeled with 1, 9, 17, 25 in one extremity (1, 9 for WT mix, 17, 25 for TDNA mix)
- The master mixes are distributed in PCR strips, 7,5 ul per tube. Then add 5 ul of DNA, one sample per tube.
- DNA and mix are well mixed, and tubes are spin down.
- In room 2.21, there are the PCR machines. One machine has been booked for you. Put the three strips in the block of the machine. Start the machine.
- The program will be: 1x 95°C, 3'; 30x (95°C, 20"; 55°C, 20"; 72°C, 1'); 72°C, 5'
- We will stop the reactions for you and keep the samples in the fridge overnight.
- You will prepare an electrophoresis gel. We wish to differentiate PCR fragments of ±1200 bp. We need to prepare a 1.2% agarose gel.
- When working in the elfo room (2.18/2.19), wear lab coat and gloves
- Weight 2.4g of agarose and pour it an Erlenmeyer/bottle containing 200 ml TAE buffer. Pour the TAE from the barrel and measure it with one of the available cylinders.
- The agarose is melt using the micro-wave. Stay put to avoid over boiling. Be careful to not burn yourself (use the carry-over glove). All the agarose should be melted.
- Work in the chemical bench, prepare a chamber (you will be provided the one, the combs and the casting chamber). When the agarose cools a bit, add 3 ul of ethidium bromide, mix well and pour the agarose in the chamber. Let it solidify under the chemical flow.
- To load the samples in the elfo gel, you will need to add loading buffer. It has two purposes: the blue color helps to visualize the progress of the migration; the glycerol renders the solution heavier and helps the solution to go down the wells. The loading buffer is 6x concentrated, meaning 1 ul for 5 ul of solution. Add 3 ul per sample.
- In the elfo room, place the gel in the appropriate running chamber. Make sure the buffer is clean and in the proper amount. You are ready to load the samples in the gel. One sample per well. Keep one well at one extremity to load a ladder. The ladder will indicate the size of DNA bands in the gel.
- Plug the cap, switch on the power supply. Set the voltage to 100, the timer to 30 minutes. Start.
- After 30 minutes, you collect the gel to take a picture using the BioRad machine. You will be instructed how to proceed.
- Print the gel picture.
- Analyze the size of the DNA fragments for each sample and interpret the results – what seedling is

wild-type, heterozygous or homozygous for the mutation.

4.2.6. **Observation of the shoot *pin1* phenotypes**

- The *pin1* seedlings remaining on plates will be transferred to soil. Done by us after the collection of samples for genotyping on 19/10.
- Plants are growing in the phytotron, room 1S08/1S09. We will perform the phenotyping of *pin1* mutant plants around the first week of December as you need flowering plants to assess the phenotype.
- Follow carefully the instructions for manipulating the plants. Wear only the lab coat available in the phytotron corridor (not yours). The plants will be available on the tables in the corridor.
- Score the phenotype (eventually take a picture for your record) of each plant.

5. Root development

5.1. Root system architecture

Plants are fixed in soil and need to access soil resources such as water and nutrients to survive in environments that are continually changing. To do so, plants have evolved extensive and highly plastic root systems to forage in the surrounding heterogenous soil. Therefore, roots are considered as a central component of plant productivity (e.g. biomass production). The root system architecture is flexible as dependent of the local growth conditions and type of soil. Its structure (physiology) correlates with nutrient uptake efficiency and stress (drought) resilience. However, the relative inaccessibility of the root system has caused root research to lag behind research into shoot development, physiology and architecture.

5.1.1. Root architecture correlates with soil composition

Root system development is an iterative process based on the repetition of four fundamental processes: (1) the production of roots, (2) their growth, (3) their growth direction in the soil domain (tropisms), and (4) their capacity to produce other roots (branching). The first organ to emerge from the seed is the primary (or tap) root. The primary root grows and changes direction in the soil in response to various stimuli (e.g., gravity, moisture, or mechanical impedance). After a certain time, it forms branches (or lateral roots); the primary root gives rise to secondary roots, which give rise to tertiary roots etc. These branch roots are known as "higher order" roots. For a given species (or genotype), lateral roots emerge from their parent at a fixed time interval. As a consequence, the distance between two lateral roots (or interbranch distance) is a function of that time interval and of the parent root's growth rate. The newly formed lateral roots emerge at a given angle with respect to their parent roots, called the insertion angle.

Once emerged, laterals extend into the soil and have the ability to change directions. Lateral roots can also form higher order branches, often with a different density than their bearing parent. This leads to the formation of a complex tree-like structure described as the root system architecture (Fig. 5.1). A direct consequence of these dynamics is that the root system as a whole is composed of a large range of root ages, types, and developmental stages, which leads to a great heterogeneity in functional properties within each root system.

Figure 5.1 - Various root architectures

The vertical placement of soil resources, such as water and nutrients, affects root form and function. In many soils, most organic matter and nutrients are found in the topsoil, or about the first 20 cm of soil. As plants grow and eventually die, nutrients are extracted from the whole soil profile but deposited on the top where shoot debris eventually decomposes and becomes incorporated into the soil, which serves to enrich the topsoil over time. In agricultural fields, fertilizers such as nitrogen, phosphorus, and potassium (NPK) are also applied to the topsoil. However, during dry periods, water can often be found deeper in the soil.

The spatial distribution of roots in soil largely determines their ability to forage for soil resources. For example, roots with shallow growth angles (closer to horizontal) will preferentially explore the topsoil where many nutrients are available. On the other hand, roots that grow steeper may be able, in certain environments, to reach deeper water pools during drought conditions.

Water uptake by the plant is a passive process, driven by differences in water potential along the soil, plant and atmosphere continuum. Water flows from areas of high potential (wet, low osmotic content) to low potential (dry, high osmotic content). Water evaporates from the leaves to air through pores called stomata. This process, called transpiration, creates a suction that pulls water from the soil, into the roots, up the stem, and out the leaves. The uptake capacity of a root segment (a short section of root) is influenced by its radial (from the soil to the xylem, across the root's width) and axial (along its length) hydraulic conductivities.

The radial conductivity is mainly influenced by the root diameter, or the number of cell layers water has to cross before reaching the xylem poles, the presence of hydrophobic barriers (endodermis and exodermis), and the presence and activation of aquaporins, a large family of water channel proteins. Root diameter is usually variable among different root types and, for dicots, increases as the root ages due to radial growth. Hydrophobic barriers are tissue-specific secondary structures that impede the passage of water through the apoplast (the space between cells), forcing it to flow through the symplast (the space within cells). These barriers form dynamically and degrade in response to environmental stresses. Aquaporins greatly facilitate the radial movement of water across roots by providing channels for water movement across membranes. Knocking down the production of certain aquaporins can reduce the uptake capacity of a plant by 60%. Aquaporins are not expressed uniformly within the root system, but rather their abundance depends on the root type, the root age, and the surrounding soil conditions. The root radial conductivity is then the combination of these three factors: root diameter, hydrophobic barriers, and aquaporins.

The axial conductivity, on the other hand, is mainly a function of xylem development, which is also highly related to root age, root type, and root diameter. Generally, thicker and older roots have larger and more numerous xylem poles, leading to a greater capacity to transport water.

5.1.2. Monocots versus dicots

Monocots and dicots have distinct root system architectures. In dicot root systems, the primary root and first order laterals generally form the backbone of the structure. In addition, some dicot species form basal roots that originate from the hypocotyl and can represent an important proportion of the total root system. Monocot root systems can also form basal roots, also known as seminal roots. In addition to the primary and seminal roots, monocots usually form nodal roots (also known as adventitious, crown or shoot-borne roots) that originate from the stem. As a monocot plant develops,

nodal roots emerge from successive shoot nodes, both from the main stem and from the tillers (shoot branches, if present). Through this process, in monocot root systems, nodal roots generally constitute the majority of first order (or axile) roots. Adventitious roots are also observed in some dicot species, but in a less systematic fashion. Nodal and seminal roots follow roughly the same developmental program as the primary root, although, in some species, some specific genetic determinisms have been identified. The primary and basal (seminal) roots are sometimes referred to as the embryonic root system, while the nodal roots are referred to as the postembryonic root system.

The second difference between monocots and dicots is that dicot roots undergo secondary growth, but monocot roots do not. Secondary growth involves cell divisions in mature root tissues that leads to a thickening of the older roots in the root system, sometimes accompanied by the deposition of water-impermeable suberin. The absence of secondary growth in monocots limits their water transport capacity through the xylem because there is no increase in size of the xylem vessels with increasing numbers of lateral branches. That limitation is overcome by the continuous production of new nodal roots that tend to be thicker than their predecessors.

5.1.3. Root structure

The structure of the Arabidopsis root is simple. A small number of stem cells at the tip of the root generate all of the cell types through stereotyped divisions followed by cell differentiation and regulated cell expansion (Fig. 5.2). Because root growth is indeterminate, these processes are continual, resulting in all developmental stages being present at all times.

*Figure 5.2 - Cell types in Arabidopsis root meristem*
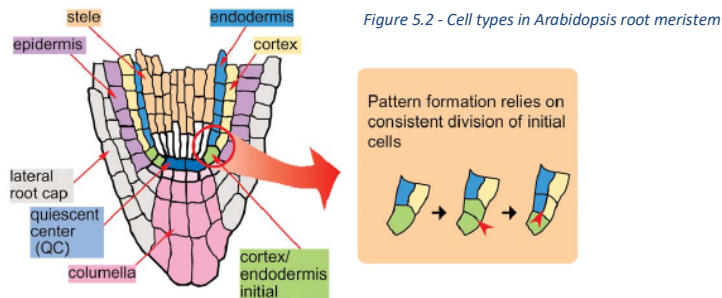
The radial symmetry of the root combined with a lack of cell movement means that clonally related cells are frequently found in cell files. These cell files can be traced back to their origins, which are four types of stem cells (or initial cells) at the root tip. The epidermal/lateral root cap initials give rise to the epidermis and the outer portion of the root cap known as the lateral root cap (Fig. 5.2). The central portion of the root cap, the columella, has its own set of initials. The ground tissue cells, the cortex and endodermis, are generated by division of the cortex/endodermal initials. Finally, the vascular tissue and pericycle have their own initials. Internal to and contacting all the initials is a small number of central cells that are mitotically inactive and are known as the quiescent center (QC). The loss of QC cells leads to the loss of stem cell status and progression toward differentiation of the contacting initial cells.

The radial organization of the root is generated by division of initial cells and subsequent acquisition

of cell fate. In a transverse root section, there are four radially symmetric layers (from outside in, epidermis, cortex, endodermis, pericycle) that surround the bilaterally symmetric vascular tissue (consisting of phloem, xylem and procambium) (Fig 5-3). The vascular tissue and surrounding pericycle are termed the stele.

Figure 5.3 - Root anatomy
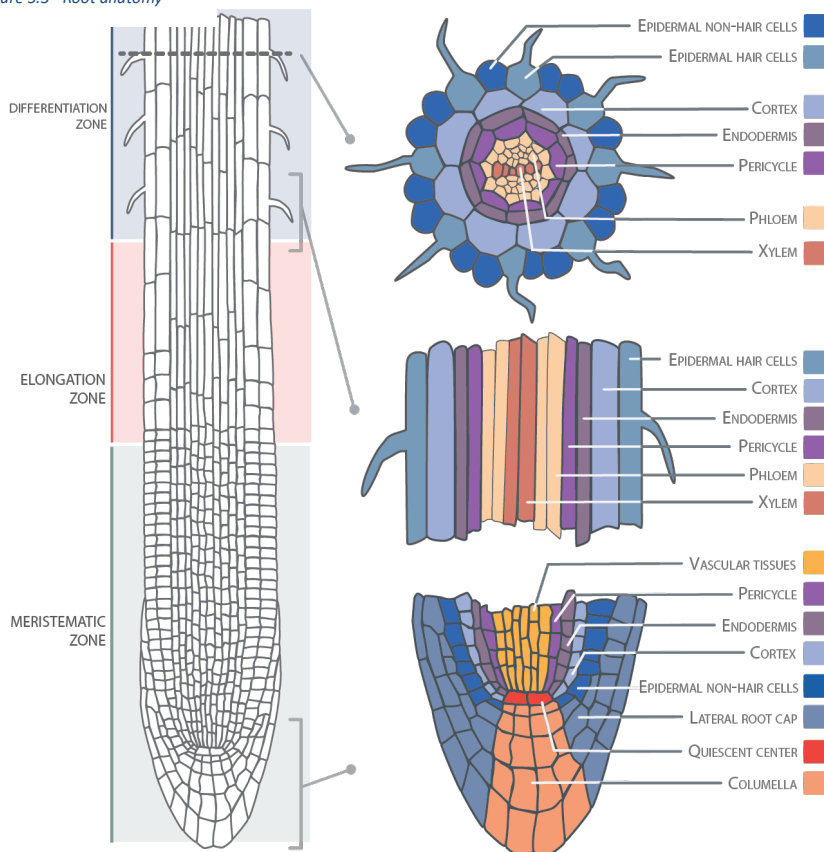
The root epidermis is composed of two cell types whose identity is regulated by positional information. Trichoblasts develop into hair cells and are located in the cleft between underlying cortical cells when (viewed in transverse section in Fig 5.3) while atrichoblasts remain hairless and are located over single cortical cells.

Upon germination, cells of the meristem begin to divide, and the root expands axially as a result of cell expansion. As the root continues to grow, the number of cells in the meristem increases and the rate of cell production increases. This increase in the number of cells can account for the doubling in the rate of root elongation between 6 and 10 days after germination. Cell expansion is also a strong

contributor to root growth. In the root region distal to the meristem (meristematic zone), cell division displace the progeny cells of the initial cells upward in the cell file, while cell expansion is limited. Then longitudinal expansion occurs in the elongation zone. In this zone, cell division is almost stopped. When cells are fully elongated, differentiation occurs as visible by the appearance of root hairs (Fig. 5.3).

Embryonic origin of the Arabidopsis root

During Arabidopsis embryo development, cell division occurs in stereotyped patterns. This facilitates the identification of founder cells for the primary root. The origin of the quiescent centre and the columella root cap can be traced back to a single cell, the hypophysis. This cell in turn derives from the basal daughter cell of the first zygotic division and is the only contribution of the basal cell to the embryo proper (Fig. 5.4).
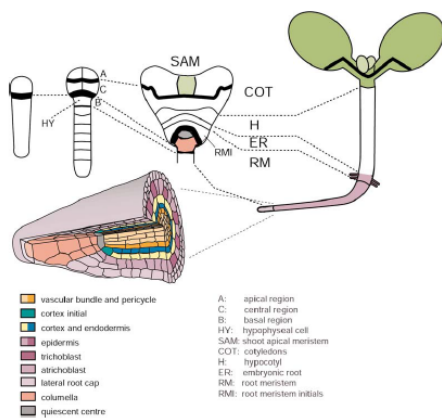

vascular bundle and pericycle
cortex initial
cortex and endodermis
epidermis
trichoblast
atrichoblast
lateral root cap
columella
quiescent centre

A: apical region
C: central region
B: basal region
HY: hypophyseal cell
SAM: shoot apical meristem
COT: cotyledons
H: hypocotyl
ER: embryonic root
RM: root meristem
RMI: root meristem initials

Figure 5.4 - Embryonic origin of the Arabidopsis root

The remaining cells that will form the root in the mature embryo derive from the apical daughter cell of the zygote. Their radial organization in protodermal, ground tissue and procambial layers is very similar to that of the entire embryo axis, except in the ground tissue. The boundary between root and hypocotyl is not evident from the anatomy of the embryo although it is clearly marked post-embryonically by differentiation characteristics of individual cell types such as root hair formation and by the chlorophyll content of ground tissue cells.

The radial arrangement of cells in the root is set up in the heart stage and maintained thereafter. The arrangement of initial cells around the quiescent centre, which is maintained in the mature root has been called the promeristem.

Lateral root formation

Lateral root primordia arise from pericycle cells opposite xylem poles at some distance from the primary root meristem. The initial cell division patterns that give rise to new primordia are very different from those occurring during primary root formation. At later stages of lateral root formation, the cellular organization becomes very similar to that of the primary root, although lateral roots display more variability in cell numbers and precise cellular organization. Eight stages of lateral root development were defined based on specific anatomical characteristics and cell divisions. Analysis of the cell division patterns revealed that a remarkable amount of organization and cell differentiation occur at very early stages of lateral root primordium development. In contrast, the lateral root meristem does not become active until after the primordium emerges from the parent root, and therefore does not appear to play a role in early pattern formation and organization. This is similar to what is seen during embryogenesis.

5.2. Root development and the role of auxin

Physiological experiments have demonstrated that the formation of entire root systems can be stimulated by auxins. Auxin is also required for specification of the meristem. Mutations in genes involved in auxin production, transport and signaling interfere with embryonic root formation. The *monopteros* (*mp*) mutants lack an embryonic root altogether (**Practical 1**). The *MP* gene encodes a member of the AUXIN RESPONSE FACTOR (ARF) proteins that can mediate rapid transcriptional responses to auxin. Several other mutants in auxin signaling display severe primary root defects.

As the primary root development strongly depends on the phytohormone auxin, it is expected that a similar important role for auxins in lateral root initiation. Indeed, genetic analysis of the formation of lateral roots has also revealed numerous links to auxins. Exogenous auxin addition leads to supernumerary roots.

Auxin is not homogenously distributed within tissues, notably in roots, due to the action of PIN proteins as auxin efflux carriers (see Chapter 1). An auxin gradient has been identified along the root longitudinal axis both by quantification and by expression analysis of the response reporter, DR5. The DR5 reporter is composed of an artificial promoter sequence made of inverted repeats of the DNA binding sequence of the ARF proteins (TGTCTC) driving the expression of a reporter gene (GUS or fluorescent proteins). The location and intensity of expression of the DR5 reporter is indicative of the intensity of the auxin signaling.

Cell-specific auxin measurements were performed to map auxin concentrations in the Arabidopsis root tip. This used cell sorting of fluorescent cells isolated from reporter lines with tissue-specific expression pattern. Auxin (and its metabolites) quantification in those cells was performed by GC-MS. These results indicated that auxin acts like a morphogen at the root apex. The highest concentrations were found at the QC, cells that remain quiescent. Intermediate concentrations were found in cells of the meristem, cells that divide frequently. Lower levels of auxin where found in cells of the elongation zone.

Auxin is involved in post-embryonic growth, such as *de novo* organogenesis of lateral roots. Auxin firstly accumulates at the location of organ initiation, and then an auxin gradient is established along the growth axis of the developing primordia with the maximum at its tip. Once a lateral or primary root meristem becomes functional, a stable auxin gradient form with its maximum at the quiescent center and young columella cells, which is required to maintain the pattern of the root meristem. In case that this maximum is lost, meristem cells start to differentiate, and the root tip is destroyed.

6. Practical 2 – The role of auxin in root development

In this practical we will test the effects of auxin on root growth.

We will use the seeds of the DR5::GUS line to monitor the effects of auxin application on auxin signalling and correlate it to the root phenotypes. We will use two concentrations of NAA, Naphthalene 1-acetic acid, a synthetic auxin. Depending its concentration, auxin may have opposite effects: inhibiting cell elongation at high concentration, favoring cell elongation at low concentration (Fig. 6.1). In this practical we will germinate seeds on 0.1 uM and 10 pM NAA.

Figure 6.1 - Effect of auxin concentration on cell elongation
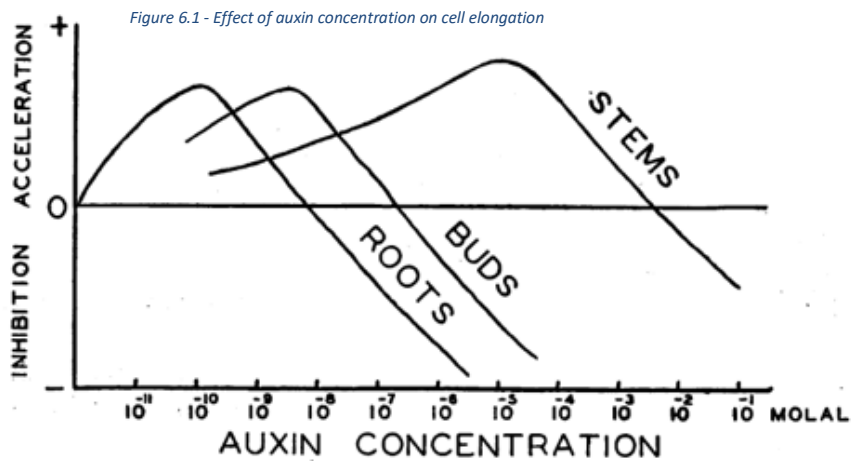
6.1. Materials

6.1.1. Seeds

Seeds were sterilized for you by chlorine gas method
DR5::GUS

6.1.2. Material

Sterile 50 ml tube
Petri dishes
Micropore tape
Aluminum foil
Stereomicroscope
Toothpicks
Marker pen
Tubes for the seeds
Well plates
Gloves
Pipettes (1000, 200, 10, 2ul)

37°C incubator
scanner
slides and cover-slides
microscope

6.1.3. **Solutions**

1/2 MS
10 mM NAA stock solution
GUS staining (solutions are prepared)
GUS wash buffer
      50 mM $NaPO_4$ pH7 (stock of 500 mM)
      5 mM Ferro-cyanide ($K_4Fe(CN)_6$) (stock of 50 mM)
GUS staining buffer
      50 mM $NaPO_4$ pH7 (stock of 500 mM)
      5 mM Ferro-cyanide ($K_4Fe(CN)_6$) (stock of 50 mM)
      0.05% Triton X-100
      X-Gluc added in the last moment in the required volume at 0.5 mg/ml final (stock 20 mg/ml in DMSO)
20% lactic acid in 20% glycerol (in PBS)

6.2. Experiment

6.2.1. **Timetable**

This experiment is designed to take ±fifteen days. We will start the experiment on Monday 9[th,] November and then move the petri dishes from cold treatment on Thursday 12th. The seeds will grow for 11 days. Phenotype will be scored at Day After Germination (DAG) 4, 6, 8 and 11. Seedlings of each treatment will be collected and stained for GUS on November 23rd. Microscopic observations will be on Monday 30th November and analysis of the results on Monday 7th December.

6.2.2. **Sowing**

All this work is done in sterile conditions under flow benches in room 1S26. You will have to calculate the volume of NAA to add for one plate for the given final concentrations. You will be provided with 200 mL of 1/2MS and will have to prepare 3 plates: control (no NAA added), high NAA (100nM), low NAA (100pM). One plate contains 50mL of medium.

- Calculate the volume of NAA to add for 50mL MS from a stock solution of 10mM for a final concentration of 100nM and 100pM. Propose how to have an accurate pipetting with the given stock solution.
- Label your plates with the treatment (control, 100pM NAA, 100nM NAA), the name of the seeds that will be sown (DR5::GUS), your name and the date
- You may draw one line with the marker pen at the back of the plate
- Work under sterile flow, using the 50mL-tube, pour MS into the control plate, then the low concentration NAA plate (by adding the proper volume of NAA into 50 mL MS in the falcon tube), and then the high concentration NAA plate
- Leave the plate open under the flow for ± 30 minutes to solidify
- Take a toothpick and wet its tip by tapping it in the corner of the plate.

- Using the wet tip of the toothpick pick up one seed at a time from the tube
- Place 20 seeds for each genotype on the Petri dishes, following the line you draw. Separate nicely the seeds along the line.
- Place the lid on the Petri dishes and wrap the edges of each Petri dish with Micropore tape completely sealing them shut.
- Wrap their stack completely with aluminum foil so that no light reaches the seeds.
- Label the wrapped Petri dishes using marker tape, with your name and date.
- Put the wrapped Petri dishes in the fridge (room 1S16) at 4°C-6°C. This process is called stratification (or vernalization). Stratification is important for breaking seed dormancy and synchronizing the germination of the seeds. Be extremely careful when preparing, transferring or working on Petri dishes with seeds. **Rough handling can cause seeds to shift from their intended position and ruin the experiment.**
- All Petri dishes should be kept in cold treatment until Saturday 9th, November.

6.2.3. Data collection

- Scan the plates to follow the growth of the root at DAG = 4, 6 , 8 and 11.
- The scans can be used to analyze the length of the roots, and roughly quantify emergence of the lateral roots.

6.2.4. GUS staining

- Prepare a 12-well plate marked with your name, and in the lid, the name of the treatment in the corresponding well
- Fill each well (=3) with 2 ml GUS wash buffer
- Collect ±10 seedlings per treatment per well
- Apply vacuum for 5 minutes
- Remove GUS wash buffer and replace by 1 mL of GUS staining buffer.
- Apply vacuum for 5 minutes
- Close the lid and wrap it in aluminum
- Incubate at 37°C for 30 minutes. Monitor if the staining is enough. You may incubate longer if necessary
- Remove the GUS staining buffer and replace by 2 ml of lactic acid solution
- Leave on the table to clear
- Mount on slides (1 slide per line per treatment)
- We will be looking at the microscope and take pictures. The manipulation of the microscope will be performed under supervision following the instructions of the teacher.