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Root–shoot Junction (Collet) Development
Vývoj spoje kořene a prýtu (krčku)

Bachelor's thesis

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Prohlášení:

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Poděkování:

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Abstract:

Although the root-shoot junction (collet) of adult plants is a well-identifiable part of the plant body, its development has, surprisingly till now, escaped serious research attention. The junction is a key region in the life of plants, as it connects two contrasting plant life environments and involves important changes in developmental programs - underground vs aboveground. The junction in angiosperms is first established during the embryogenesis phase of development in the form of the broad embryonic root-hypocotyl transition region, and it continues to develop further after seed germination during the individual's growth. The most important organ in this process is the hypocotyl, which exhibits considerable developmental plasticity, allowing extraordinary elongation in etiolated dark growth but also initiating formation of adventitious roots upon the deetiolation. During each stage of the junction's development, auxin signalling and polar auxin transport play a crucial role. Most of the research focuses on the development of the junction in the model organism *Arabidopsis thaliana*. The significance of the phylogenetic origin of the junction is also discussed from the perspective of the evolutionary origin of roots vs shoots and embryos evolution. This work aims to provide an overview of the ontogenetic and phylogenetic origin and development of the junction between the root and shoot.

Keywords:

root; shoot; root-shoot junction; collet; embryogenesis; clonal analysis; ontogenesis; response to environment; adventitious roots

Abstrakt:

Přestože předěl mezi kořenem a prýtem (krček) dospělých rostlin je dobře identifikovatelnou částí rostlinného těla, jeho vývoj překvapivě až doposud nebyl předmětem seriózního výzkumu. Předěl je klíčovým prvkem v životě rostlin, jelikož zde dochází k propojení dvou kontrastních životních prostředí rostlin a k důležitým změnám ve vývojových programech – podzemní a nadzemní. Předěl krytosemenných je prvotně založen již během embryonální fáze vývoje v podobě široké přechodové oblasti mezi kořenem a hypokotylem, a dále se vyvíjí po vyklíčení semen během růstu jedince. Nejdůležitějším orgánem při tomto procesu je právě hypokotyl, který vykazuje značnou vývojovou plasticitu, umožňující mimořádné prodlužování při etiolovaném růstu ve tmě, ale také dokáže iniciovat vznik adventivních kořenů při deetiolaci. Během všech fází vývoje předělu má zásadní roli signalizace auxinem a polární transport auxinu. Práce se z většiny zaměřuje na vývoj předělu modelového organismu *Arabidopsis thaliana*. Diskutován je také význam fylogenetického vzniku předělu z pohledu evolučního vzniku kořenů a stonků a evoluce embrya. Tato práce si klade za cíl poskytnout přehled o ontogenetickém i fylogenetickém vzniku a vývoji předělu mezi kořenem a výhonem.

Klíčová slova:

kořen; prýt; spoj kořen-prýt; krček; embryogeneze; klonální analýza; ontogeneze; odpověď na prostředí; adventivní kořeny

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Introduction

Vascular plants are organisms, that show a unique differentiation of two body regions with totally different characteristics. This difference is defined by the distinct environments that have evolutionary and developmental impacts on developmental programs, such as light, water, and accessibility of nutrients. There are two basic developmental programs for plant bodies: one for the upper part exposed to the light, transforming light energy into chemical bonds, and the other for the base, which anchors the whole plant in the soil and absorbs water along with necessary nutrients. These developmental programs meet at the root-shoot junction (RSJ, somewhere referred to as collet) – a very narrow developmental transition between underground and atmospheric environments. This bachelor thesis aims to describe and discuss known ontogenetic and phylogenetic processes defining this region.

Plant developmental research has traditionally focused on the root apical meristem (RAM) and shoot apical meristem (SAM), which are considered to have pivotal roles in the plant's organogenesis and growth. A search in the PubMed (<https://pubmed.ncbi.nlm.nih.gov/>) database reveals a substantial amount of literature focused on RAM and SAM, with 2619 results for RAM and 2645 results for SAM. In contrast, research into the RSJ has received considerably less attention, yielding only 99 results in comparison. These numbers underscore the disproportionate focus on RAM and SAM compared to the RSJ. While discoveries and research into RAM and SAM have driven plant developmental biology throughout their existence, the physical connection between these structures has remained relatively understudied within the scientific community.

In the developmental biology of plants, one major model organism has been selected - *Arabidopsis thaliana*. It has been widely used in all cell and molecular biology disciplines of plant science since its first sequencing of a whole nuclear genome as the first plant in history (2000) ('Analysis of the Genome Sequence of the Flowering Plant *Arabidopsis Thaliana*', 2000). Nevertheless, until that time, there have been more model organisms in plant developmental biology, including embryology, for example, the discovery of SERKs (Somatic Embryogenesis Receptor-like Kinases) was made on *Daucus carota* (Schmidt et al., 1997). The current advent of CRISPR/Cas DNA manipulation methods, along with genome sequencing, allows the use of many other plant species to address the mechanisms of plant life. It is necessary to stress here that plants are a huge group of organisms, and they differ in development in general as well as embryogenesis. The results of this bachelor thesis may not be relevant for every living plant.

Through RSJ, water flows with nutrients in one direction and metabolites and products of photosynthesis in the other. Roots and shoots exhibit differences in the composition of vascular tissues and RSJ serves as a transition zone. In *Arabidopsis's* primary root and hypocotyl, the vasculars are deposited in a central cylinder (Evert & Eichhorn, 2006). The xylem forms a central core plate (exarch localisation), which is accompanied by two phloem strands from both plane sides (Busse & Evert, 1999). The xylem and phloem are encased by a pericycle, which initiates lateral and adventitious roots (AR) (Evert &

Eichhorn, 2006). In contrast, the shoot displays a different vascular tissue composition. Collateral vascular bundles are formed and arranged around the circumference of the primary growing stem. The RSJ features a transition zone where vascular tissues undergo rearrangement, and in young seedlings, the main transition occurs at the border between shoot and hypocotyl. (Groff & Kaplan, 1988; Pazourek & Votrubová, 1997)

One of the best markers for above-ground tissues is a distinct cuticle, a layer of waxes on the surface of organs above ground. During embryogenesis, it covers all the cotyledons, hypocotyl, and the embryonic root too (showing that the cuticle is an ontogenetic default for the whole of the plant body) (Szczuka & Szczuka, 2003). So, the cuticle does not at first mark the junction. In the grown plants, obvious features and markers identify the RSJ; for example, previously discussed cuticle or production of photosynthetically active chloroplasts with chlorophyll in the shoot and the above-mentioned vasculars reorganisation. There is also a significant anatomical difference between hypocotyl and primary root, securing the initial RSJ, described in *Arabidopsis* (Y. Lin & Schiefelbein, 2001). This anatomical analysis found that the hypocotyl has one additional cortical cell layer compared to the primary root. The root-hypocotyl junction (RHJ) serves as a transition zone where the number of cortical cells is irregular.

The main question for the first chapter is, how far the RSJ is identified since the embryonic stage of plant development? When it is identified during embryogenesis, what are the molecular mechanisms of establishing the RSJ, and how can the plant move or reorganise the RSJ in reaction to the local conditions after germination – i.e., developmental RSJ plasticity? The first chapter will also describe the clonal analysis methodological method and its history, as a relevant scientific approach to the topic of this thesis.

The second chapter will discuss the phytohormone auxin as a major regulator of plant growth and development. It is also expected to be involved during embryogenesis and after the embryonic RSJ developmental plasticity. This developmental plasticity is mainly secured by adventitious roots, initiated from the hypocotyl after deetiolation. As expected, the maturation of RSJ after germination is necessary for every plant, which needs to react to the surrounding conditions, e.g. how deep it germinates. Deep germinating plants must find sunlight quickly – yet be firmly anchored in the soil. Reaction to this situation is etiolated growth, which is based on the fast lengthening of hypocotyls. I hypothesise that the etiolated hypocotyls can produce adventitious roots after deetiolation, to modify the RSJ to the proper place at the border between the ground and the atmosphere.

The final chapter is about the evolutionary point of view. The roots must have evolved during the colonisation of the land as it started to be necessary to be anchored in the ground and have a specific organ to gain water with minerals. It seems that all roots of today's terrestrial plants do not share a common ancestor and that there are separated branches in the root evolution (and therefore also simultaneously RSJ). In the second part, the chapter focuses on embryo evolution.

1. Establishment of Root-Shoot Junction During the Embryogenesis

The angiosperm embryo is an entity derived from organised zygote cell divisions. A plant zygote is formed by a fusion of haploid gametes (the male gamete is one of two sperm cells, and the female gamete is the egg cell) (Reiser & Fischer', 1993). Embryogenesis starts with only one diploid cell, the zygote, and ends with a full-grown embryo covered in seed (Goldberg et al., 1994).

The *Arabidopsis* embryo undergoes progressive developmental changes defined to allow comparative studies into: 2-cell stage, 4-cell stage, 8-cell stage, 16-cell stage (proembryo), globular stage, heart stage, and torpedo stage (showed in Figure 1) (Armenta-Medina et al., 2021). The stages are not sharply separated; embryogenesis is a continuous process. Therefore, there are defined transition stages (like early-, mid-, and late-globular), and sometimes, it can be hard to strictly classify the actual developmental stage (Ten Hove et al., 2015). The following chapter will describe and discuss embryo stages relevant to the thesis, i.e., the initiation of RSJ. Still, here I want to highlight the zygote's first division because it is crucial for the whole embryo and mainly root development. The zygote's first division is strongly asymmetrical, forming the upper smaller cell (apical cell) and the lower bigger cell (basal cell). This is the first time the embryo gets an apical-basal axis, which is crucial for the next phases of embryo development. The embryo proper will derive from the upper cell, while the lower cell will develop into the suspensor and the hypophyseal cell. (Armenta-Medina et al., 2021)

The embryo's ontogenetic progress produces all the basal body parts and tissues of a mature seed dormant embryo. The adult embryo is a structure formed by five different parts – RAM, embryonic root (ER, also sometimes referred to as a radicle), hypocotyl, two cotyledons and a small group of cells, which will form SAM (Barton & Poethig, 1993; Jurgens, 2001). By the cell production in both apical meristems, secondary meristems, and peripheral organ initiation regions, the plant body grows and forms these meristems in the post-germination phase of *Arabidopsis* plant life.

The ER is a particular part of the embryo which possesses the characteristics of the root anatomy, such as one layer of each epidermis, cortex, and endodermis, and a pericycle layer surrounding vascular tissues (Dolan et al., 1993). The difference between the embryonic and post-embryonic root is that the embryonic root cells mainly derive from cell divisions forming the embryo proper itself. In contrast, the postembryonic root derives from divisions of RAM and its initials (Scheres et al., 1994). Since the initials are formed from the basal cells of the embryo proper, and the quiescent centre and columella derive from the hypophyseal cell, the final RAM is a synthesis, composed of regions of different embryo parts. The root initials may be replaced during the post-embryonic growth, so the root can partially descend from the hypophyseal cell (Heyman et al., 2013; Kidner et al., 2000).

Hypocotyl (formed fully during the embryogenesis – there are no cell divisions after the germination), shows anatomical difference in comparison to the roots. The main difference is that hypocotyl has one more cortical layer. The number of endodermis cells in both hypocotyl and root is the same; it is always

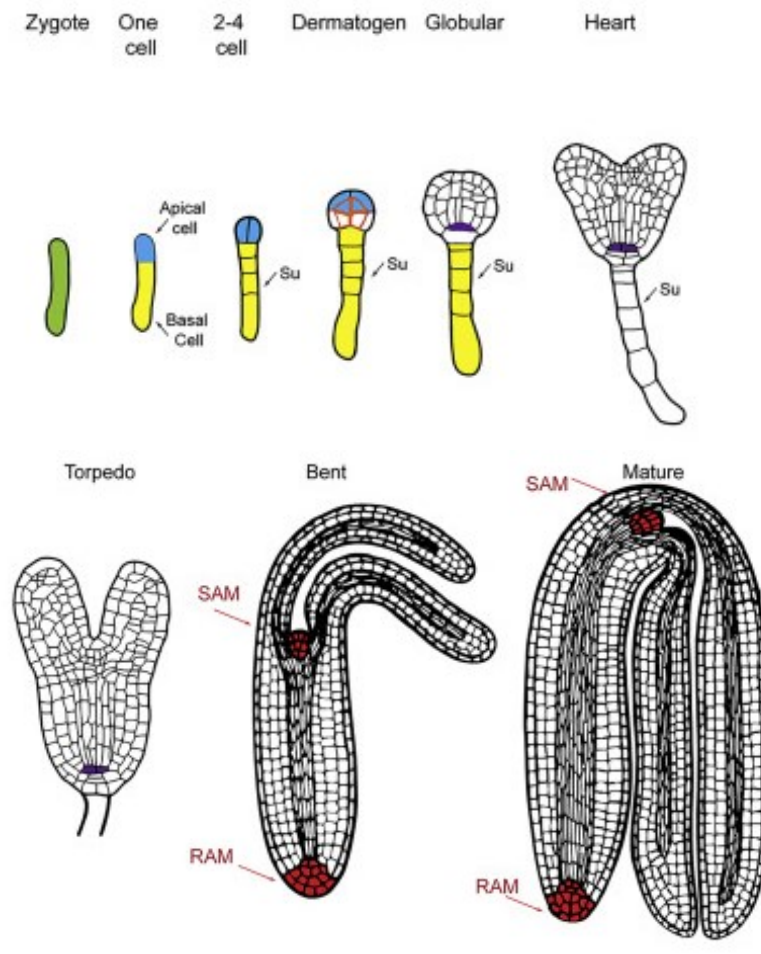


Figure 1: The selected developmental stages of *Arabidopsis* embryo development, dermatogen here is a 16-cell embryo, the proembryo, the typical bending in the bent embryo and the typical shape of the mature embryo, RAM = root apical meristem, SAM = shoot apical meristem, Su = suspensor. Adapted from (Armenta-Medina et al., 2021).

8 cells. The inner cortical layer (neighbouring endodermis) of hypocotyl and the only cortical layer of the root have both 8 cells. The unique outer cortex layer in hypocotyl has approximately 14.6 cells, and the epidermis of hypocotyl has a significantly higher number of cells – around 32.9 cells. In comparison, the root epidermis has 19.6 cells. Table 1 summarises these findings (Lin & Schiefelbein, 2001). Another difference is the number of cells in the vascular tissues inside the pericycle (Dolan et al., 1993). After the germination, the primary root elongates through the activity of RAM. However, the future SAM in *Arabidopsis* is until the lifting of cotyledons from the ground only a small group of cells and its development is finalised only after the germination (Barton & Poethig, 1993). Hypocotyls can also exhibit both photosynthetically active and nonactive growth – depending on the photomorphogenic light conditions. Root plastids do not differentiate into photosynthesising chloroplasts, while the hypocotyls grown on light are photosynthetically active and proplastids differentiate into chloroplasts. Both structures are connected via RSJ.

According to Compton (1912), the RSJ region was described in 1859 (but it was known even before – as a relevant plant region to farmers). It is a unique transition and joining structure which develops in a

plant's ontogenesis. In the literature, the collet is described as RSJ, but sometimes it is clearly used just for the RHJ, which is a primary, special type of RSJ. RHJ serves for a time of hypocotyl existence as the RSJ, but there are no changes in the composition of the vasculature (which occurs between hypocotyl and stem during the first stages of plant growth). After the secondary thickening of hypocotyl, the hypocotyl merges with the RSJ, and the RSJ is much wider and serves as a transition zone from root to stem, where the anatomy of both organs is reorganised, e.g. vasculature. (de Vogel, 1980)

The epidermis of the RHJ forms collet hairs (CHs, also called hypocotyl hairs) right after the germination. CHs are root hair-like structures, unicellular tip-growing extensions of the epidermis. CHs were apparently first described by Antonie van Leeuwenhoek in his letter to the members of the Royal Society on a willow seedling (Leeuwenhoek, 1693). CHs cover the RHJ, they firstly anchor the germinating plant in the soil and can also be the first effective water and nutrient-absorbing tissue (Parsons, 2009). The difference between CHs and root hairs is that the rhizodermis root hairs (trichoblast cells) of *Arabidopsis* are surrounded by cells that do not produce root hairs (atrachoblasts) (Gilroy & Jones, 2000). In contrast, CHs are formed by all epidermal cells of the RHJ.

The transverse cuts through the root and hypocotyl are shown in Figure 2. The anatomy of root and hypocotyl are much more similar than are the similarities between hypocotyl and shoot (Ragni & Hardtke, 2014).

However, the boundary or junction between hypocotyl and root, as defined by the second cortex layer of hypocotyl, is not precise—the transition zone cell number in this tissue is variable (see Table 1).

	Endodermis	1. Cortex layer	2. Cortex layer	Epidermis
Hypocotyl	8.0±0.0	8.0±0.0	14.6±0.9	32.9±2.4
Root-hypocotyl junction	8.0±0.0	5.0±2.2	11.5±2.7	27.0±4.3
Root	8.0±0.0	0.0±0.0	8.0±0.0	19.6±1.3

Table 1: The numbers of the cells of selected layers of hypocotyl, root-hypocotyl junction, and root counted on the transversal cuts, \pm = s.d., root-hypocotyl junction is here selected region containing CHs, the numbers in the root-hypocotyl junction indicates that in this region is slowly forming the second cortical layer, but it is not a sharp border. Adapted from (Lin & Schiefelbein, 2001).

1.1 Clonal Analysis

To see the developmental fates of embryo cells, clonal analyses are made on embryos, as well as all other phases and parts of the plant developmental process. Plants show great developmental plasticity (necessary for their sessile live strategy). Plants can dedifferentiate their cells (often in response to some stress or injury), as was demonstrated, e.g. in the RAM after its tip was cut out (Efroni et al., 2016). It is accepted that plant cells' developmental fates are mostly dependent on their position within the tissue context (Kidner et al., 2000).

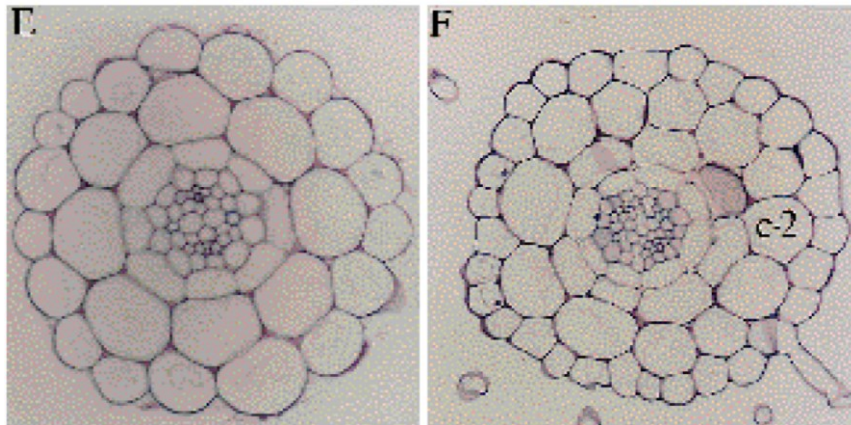


Figure 2: The comparison of transverse cuts through the primary root (E) and hypocotyl (F) of *Arabidopsis*. The second cortex cell layer is visible in the hypocotyl (c-2) as well as a higher number of cells in the epidermis of the hypocotyl. Adapted from (Dolan et al., 1993).

The methodological approach how to follow the cell fate and cell lineages is through clonal analysis. It is based on the idea of using or activating some visible marker in only one cell or one cell population in the whole organism. This enables to see descendants of the original cell or the original cell population in the following developmental phases.

1.1.1 The History of Clonal Analysis in Experimental Botany

The idea behind this method is quite old and was originally applied in experimental animal developmental biology, the first studies following the cell lineages appeared in the second half of the 19th century (Stent, 1998), probably with the very first made on *Clepsine complanata* embryos by Whitman (1878). To my knowledge, one of the first clonal analyses in plant biology was made on the currently rare plant model *Datura stramonium* in 1940 (Satina et al., 1940). The analysis was based on artificially induced periclinal chimeras with different ploidy levels in the SAM in every cell layer (L1/epidermis, L2 subepidermal layer – together tunica, and L3/corpus). That allowed experimenters to see descendants of these layers in the tissues of adult plants because higher ploidy cells have significantly higher volumes and increased nuclei. The chimeras were prepared by soaking the plants in the colchicine solution (anti-mitotic drug), leading to random disruptions of the mitotic spindles, to unseparated chromosomes and random increase in the ploidy level in cells of different layers. Chimeras with ploidy change in single layers were then selected (Satina et al., 1940).

Another method was invented around the same time as Satina et al.'s (1940) experiments. Brumfield (1943) published a study using X-rays to mutate root cell initials. It has been already known that X-rays cause mutations and chromosomal aberrations. The roots of *Crepis capillris* and *Vicia faba* were exposed to X-rays, and then they were cultivated long enough for the affected cells to undergo at least some divisions. After the mutated non-dividing meristem initials had been replaced by healthy cells, the

root was fixed and sliced, and chromosomes were stained. Cells with identical chromosomal aberrations were classified as a cell lineage by microscopy analyses.

With the increasing knowledge of plant genetics, easier and more efficient ways to perform clonal analysis in plants emerged. The discovery of transposable elements (McClintock, 1950) led scientists to another approach. Dawe & Freeling (1990) collected known and previously described *Zea mays* mutants with *Dissociator* (*Ds*) element inserted in some genes, crucial for seed coat colouring. In the presence of *Activator* (*Ac*) element, the *Ds* could spontaneously move from the gene. That leads, in some cases, to the restoration of the function of previously mutated genes. If this occurs in pigment production genes (for example, coding some enzyme in the metabolic pathway that leads to some anthocyanin product, as was used in the study), the descendants of the single restored cell would have different colouration from the rest of the plant's cells.

Over the last few years, studies using clonal analysis by heat shock activation have been reported. Two constructs are inserted into the plant in this case. The first one consists of a heat-shock promoter activated by high temperature. This promoter controls the expression of the Cre-Lox element, bound to the CyclinB1;1 destruction box (Smetana et al., 2019). This enables the expression of the construct only after the heat-shock signal. During the mitosis, the Cre element is degraded due to the presence of the cyclin destruction box. This stops the element from passing through cell lineage and random activation after the heat-shock signal (Tsuda et al., 2023). Cre-Lox element is a site-specific DNA recombinase from P1 bacteriophage, which cuts out the region with borders with unique sequences (Lin et al., 2004). This is how the second construct is activated. For example, there can be a GUS staining reporter gene under the control of the 35S promoter. Between the promoter and GUS, a region excised by the activity of Cre-Lox recombinase is inserted. Any other reported gene can be used, for example, some fluorescent proteins, or activation of expression of Gal4 transcription factor (TF), which binds to the UAS element (upstream activation sequence, a special enhancer, which is activated by Gal4) (Kakidani & Ptashne, 1988). This activates expressions of multiple other constructs under the control of UAS at the same time. It does not need to be used just for the expression of some marker gene but also for the expression of the gene of interest in its loss-of-function (LOF) mutant plant, which will result in mosaic complementation (Heidstra et al., 2004). This can be useful for some analysis if only a tiny population of cells/one-cell descendants are needed to express the gene of interest. For example, heat-shock-activated clonal analysis was recently used by Tsuda et al. (2023) and Smetana et al. (2019).

1.1.2 Clonal Analysis of Embryonic Development (based especially on Scheres et al. (1994))

Scheres et al. (1994) made a major publication related to this chapter – clonal analysis of *Arabidopsis* early embryo development. They used plants transformed with T-DNA construct carrying 35S::GUS (β -glucuronidase under 35S promoter) and maize transposon *Ac* element, which was inserted in it. With a

probability low enough, the transposon could be autonomously excised. That enables the activation of GUS expression in the single cell and all cells derived from this mother cell since the 35S promoter has the potential to be active in all plant cell types (Benfey et al., 1990). Interestingly, Scheres et al. (1994) noticed some problems with the expression under 35S promoter in the RAM, the expression in the RAM was very weak. This type of clonal analysis is possible only with a probability of excision of the transposon not too high because there could be more excisions and descendants of the other excisions would be unrecognisable. On the other hand, the probability cannot be too low because then there could be a very low frequency of activation events – i.e. useless for the observation of cell fate within the lineage.

In the globular stage, the embryo undergoes a series of transversal divisions, which further progress the diversification of the whole embryo proper to the “upper” and “lower” tiers. Later, the lower tier, defined as the basal hemisphere of the globular embryo, is in the early heart stage again diversified to the “upper” and “lower subtier” (Scheres et al., 1994). The upper subtier cells then form some parts of the abaxial side of cotyledons. Meanwhile, the lower subtier is destined to form hypocotyl and ER. Some ground meristem cells located in the heart stage go through two rounds of periclinal divisions until the torpedo stage. However, some of them go through only one periclinal division, suggesting that this is the first sign of development into either hypocotyl or ER. I would like to highlight that the formation of the second cortex layer precedes the formation of the endodermis in the hypocotyl region. From this data, it is unclear whether the cells divide more and will become part of hypocotyl or ER. The important question was whether the cells of the hypocotyl region could be further dividing after the finished embryogenesis or by the activity of RAM in late embryogenesis. The conclusion (based on staining cells with [³H] thymidine – reporting active DNA synthesis/S-phase - and observing the mitotic activity) is that the hypocotyl cells are already present in the embryo and are not derived from RAM, and they do not divide after the seed germination (Scheres et al., 1994).

Using above introduced clonal analysis approach, Scheres et al. (1994) constructed a cell fate embryonic map (in Figure 3) and identified significant developmental domains of the plant embryo. The embryonic hypocotyl and ER developmental domains overlap, indicating that there is no cell division and differentiation based on a defined lineage. The differentiation of the cell to either hypocotyl or root cell depends on the specific position and signal communication with the surrounding cells, and cell lineages of both tissues (ER and hypocotyl) might derive from the same pool of cells.

The sector E in Figure 3 shows the activity of RAM and initial stem cells. With varying apical ends of lineages in this sector, it seems that the initials start to be active in different stages of the embryo, or the activation event happened even before differentiation into initials, and initials are keeping the staining. Some of the root lineages also overlap with hypocotyl. It is possible that the whole sector G in Figure 3 has variable length of cell lineages on the basal end due to the replacement of the initial stem cell. This was reported by Kidner et al. (2000) and Heyman et al. (2013), who state that quiescent cells can rarely asymmetrically divide and give rise to new initials. If the original stem initial cells were randomly

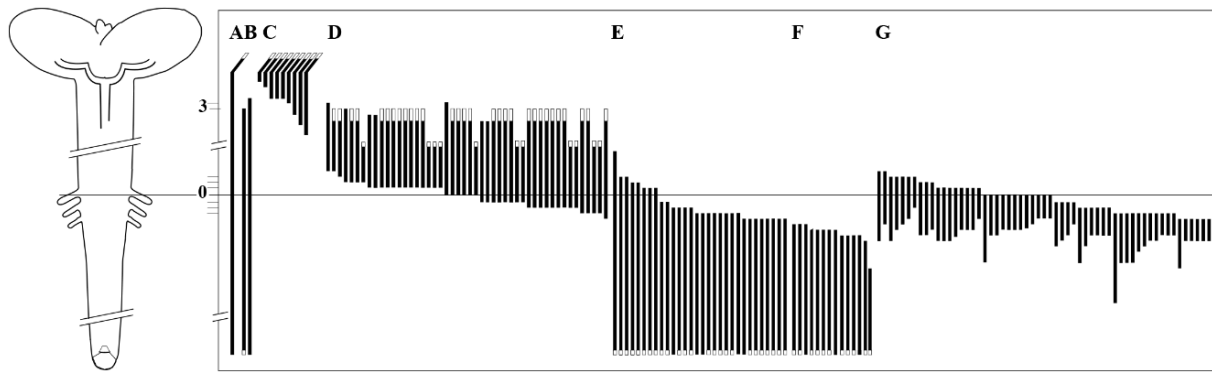


Figure 3: Embryonic fate map based on clonal analysis methodological approach. Visualisation of cells with the excision of the transposon then producing glucuronidase. A – cell’s descendants are present at all lengths of the plant. B – region specific for only hypocotyl and root. C – cotyledon shoulder cells region. D – a region of hypocotyl cells with a slight overlap to the root. E – root region. G – a region that overlaps both the embryonic root and hypocotyl. Black ends are experimentally observed, and white ends are expected. 0 marks the lower cell wall of the first root hair, and 3 marks the third epidermal cell on the hypocotyl. Adapted from (Scheres et al., 1994).

replaced, the cell lineage staining would be lost. To test this hypothesis, I propose a new experiment, where three constructs need to be expressed in the model *Arabidopsis* plant. The construct I (the scheme map of all proposed constructs is depicted in Figure 4) is based on the system of Collins et al. (2010), with the CRE element enriched with the CycB1;1 domain Smetana et al. (2019). This construct would have two promoters: one UBQ promoter, commonly used in experimental botany, and the second, the heat-shock inducible promoter as used by Smetana et al. (2019). After the heat shock, this would cause a random self-excision and, therefore, the expression of triple GFP. Large three GFPs linked together will stay in the cell and will not diffuse through plasmodesmata to neighbouring cells. Alternatively, depending on the experimenter's preferences, some strong nuclear-localization signal (NLS) might be used. After the excision, all the descendants of the cell would express the cell autonomously GFP. If this happens in some root initial cells, for example, ground tissue initial, the expression would be visible in cell lineage alongside the root in the cortex and endodermis. If there is some substitution of the initial cell, it would be detectable through the activity of the second and third construct (construct II and III). The Gal4 transcription factor in construct II is expressed under the promoter pAGL42, specific for the quiescent centre cells (Nawy et al., 2005). Therefore, the activation of the UAS enhancer through the presence of the Gal4 transcription factor is limited just to the quiescent cells (which would, since the beginning, shine red due to the activation of construct III, again there are two possibilities for how to prevent it from diffusing of RFP, NLS or linking more RFP together). Construct III could not be active in any other cell in the plant because the UAS is activated only by the Gal4 protein (Kakidani & Ptashne, 1988; Smetana et al., 2019), so the initiation has to come from the pAGL42 promoter activation. After the rare division of a quiescent centre cell, as reported by (Kidner et al., 2000), the cell descendent would keep the expression of RFP because there will be already present activated Gal4 TF. The results will show if the initial substitution/new cell line derives from the quiescent centre cells. If a line of green-

shining cells would switch to red shining, it would mean quiescent centre cell involvement in substituting the initials. This means that the three regions (E-G) in Figure 3 are potentially the same sector. Otherwise, if there were green line cells with no switch to red shining cells, that would prove that the quiescent centre cells do not substitute the initials, and the end of cell lineage has some other explanation.

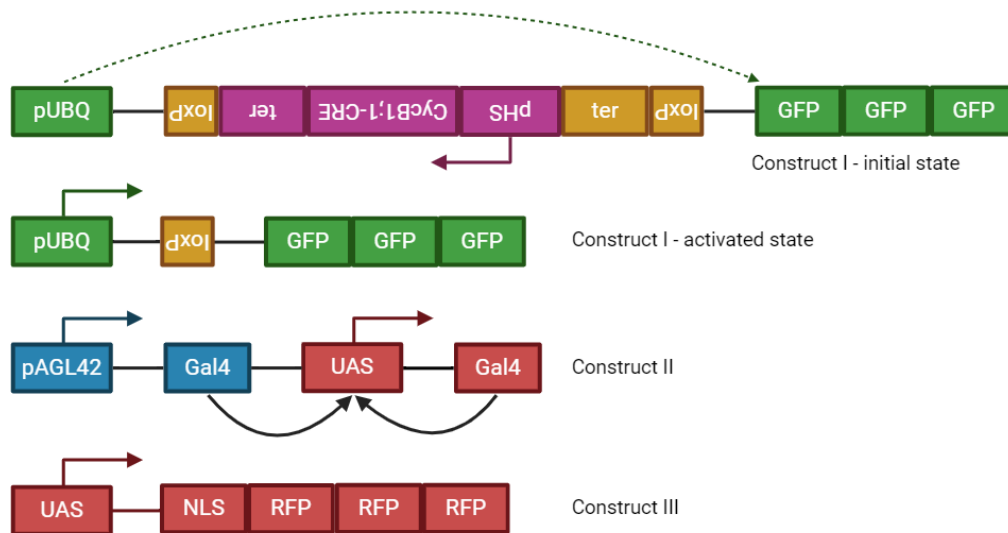


Figure 4: The illustration map of the three proposed constructs. IoxP = sites for CRE-dependent excision, GFP = green fluorescent protein, pHS = heat-shock promoter, RFP = red fluorescent protein, ter = some universal terminator, broken arrows = activity of promoters, rounded arrows = activation of promoters by transcription factors, and interrupted arrow = potential activity of promoter, created with <https://www.biorender.com/>.

1.2 Root-shoot Junction/Collet Ontogenesis

The mechanisms of RSJ/collet development are poorly understood because this topic is overlooked in plant developmental biology or plant anatomy. In two recent articles, the collet is studied with respect to endoreduplication and nuclear movements involved in CH initiation and growth. (Sliwinska et al., 2012, 2015).

Transcription factor GLABRA2 (GL2) is often used as a negative marker of root hair initiation, as GL2 is expressed specifically in rhizodermis atrichoblasts (Di Cristina et al., 1996). The signalling pathways of the developmental patterning of root hairs have been studied and are quite complicated, but a detailed description is unnecessary in the context of this thesis. In the developing embryo, changes in the expression of GL2 are already well documented (Lin & Schiefelbein, 2001). Since it is supposed that the epidermis of root and hypocotyl derive from the same population of cells (“lower subtier”) (Scheres et al., 1994), the determination of RSJ seems to start at the early-heart stage of plant embryo development, when the GL2 is for the first time expressed. Importantly, expression of GL2 and formation of the second cortex layer in future hypocotyl happens at the early torpedo stage, and the first indication of RSJ is a small domain lacking GL2 activity (H and I in Figure 5). This indicates that both processes are somehow connected, depending on the other. The oscillation in the expression of GL2 in epidermal embryonic

cells suggests that the position of the RSJ is already initiated in the heart-stage embryo. GL2 is thus a negative marker for RSJ. Figure 5 depicts the expression patterns of GL2 in the selected embryo stages and the seedlings. (Lin & Schiefelbein, 2001)

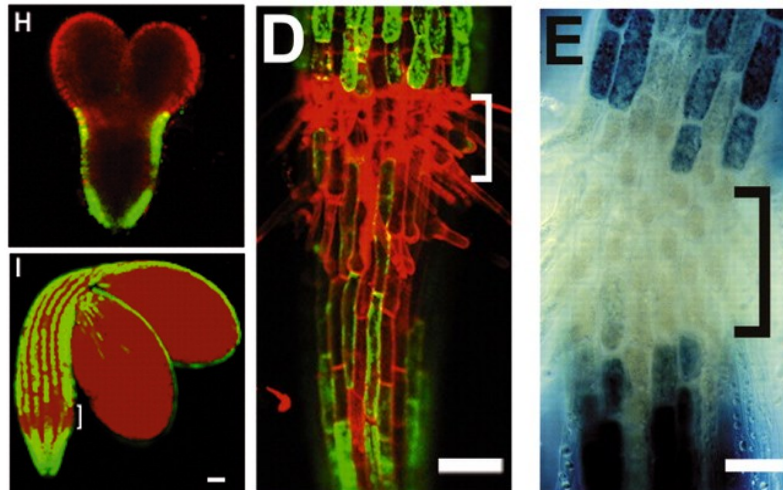


Figure 5: Expression of GL2 in selected developmental stages of *Arabidopsis*: H+I – GL2::GFP (green signal) production in torpedo stage (H) and surface scan of *Arabidopsis* mature embryo (I) with lesser expression in a future RHJ, bar = 40 μ m, red signal = autofluorescence. D + E – GL2::GFP (green signal) expression (D) and GL2::GUS (blue staining) in a 3-day-old seedling of *Arabidopsis* plant with no expression of constructs in the RHJ (marking the bar). Adapted from (Lin & Schiefelbein, 2001).

1.2.1 Developmental Deviation of RSJ in Secretory Pathway Mutants – GNOM and Exocyst

Vesicular transport is critical from the very beginning of an embryo's development and is also connected with polar auxin transport (PAT) dynamics regulation. This is well documented in one of the first *Arabidopsis* mutants *gnom* (*gn*). The mutant has knockdown Sec7 domain protein, functioning as a guanine-exchange factor (GEF) for Arf GTPase, contributing to the regulation of vectorial vesicle trafficking and recycling. LOF leads to the mislocalisation of PIN proteins (membrane efflux carriers for auxin) on the cytoplasmic membrane (PM), and *gn* embryos lose apical-basal polarity (Richter et al., 2010). A similar loss of basic organismal/embryonal polarity is also visible in KNOLLE, a SNARE protein of cytokinetic cell plate LOF mutants (Lukowitz et al., 1996).

Another example of vesicular trafficking's importance in plant development are *Arabidopsis* mutant plants in the exocyst tethering complex subunits, studied in our laboratory. The exocyst protein complex is formed of 8 subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Guo et al., 1999). The Exocyst complex is ancient in the Eukaryota domain of life (Koumandou et al., 2007); some of the subunits have many isoforms in plants (Hála et al., 2008). Specifically, Exo70 subunit isoforms are evolutionary multiplied in land plant genomes (Cvrčková et al., 2012). The exocyst works as a final component of a regulatory system in the cell to correctly deliver vesicles to the targeted domains at the PM. As already shown above in the *gn* mutant, the vectorial vesicle transport is crucial for the

development of plants (Busch et al., 1996; Richter et al., 2010). The *Arabidopsis* exocyst mutants *exo70A1*, *sec15b*, *exo84b*, and *sec8m3/LAT::SEC8* are all defective especially in normal growth (are dwarfed to different degrees), but surprisingly show also developmental deviations - formation of the ectopic RSJ (ERSJ) in the hypocotyls of etiolated plantlets (this phenotype was referred as twin-collet (TC)). This ERSJ is a novel type of developmental deviation, and it resembles the true RSJ by the formation of CHs and is formed/positioned on the hypocotyl above the true RSJ (Drdová et al., 2019). Sometimes, cells of the ERJS structure did not produce CHs and were only expanded and deformed (this phenotype was referred to as irregular-cells (IC)). Both phenotypes are shown in Figure 6 in the comparison with WT. The ERSJ also has deviations in the number of cortex cell layers. It is important to note that ERSJs were formed only in dark-cultured etiolated plants. Mutant's hypocotyls exemplified by *exo70A1* and *sec15b* mutants were significantly shorter than the hypocotyls of the WT plants. (Drdová et al., 2019).

This ERSJ seems to be developmentally a hybrid between the true RSJ and hypocotyl epidermis because the pGL2::GFP construct, normally active in root atrichoblasts and hypocotyl epidermis and non-active in RSJ (Figure 5 – D), is active in ERSJ cells producing CHs. On the other hand, the ERSJ has significantly fewer chloroplasts than the WT hypocotyls. This feature is similar to the true collet, which never possesses plastids with chlorophyll. (Drdová et al., 2019). Interesting data could come from the future analyses of GL2 expression in the embryos of the mutants, as did Lin & Schiefelbein (2001) in their study with WT. This will show if the ERSJ is also set in the embryonic stage as the true collet or, more probably, late after the germination since GL2 is active in the ERSJ hypocotyl hairs.

This ERSJ formation and shifted patterning of adventitious roots initiation described above both indicate that the hypocotyl is a developmentally plastic region maturing after germination in reaction to the actual specific environmental conditions. Currently, no other *Arabidopsis* mutant showing ERSJ phenotype has been observed, and the regulating molecular mechanisms of CHs development are still not known.

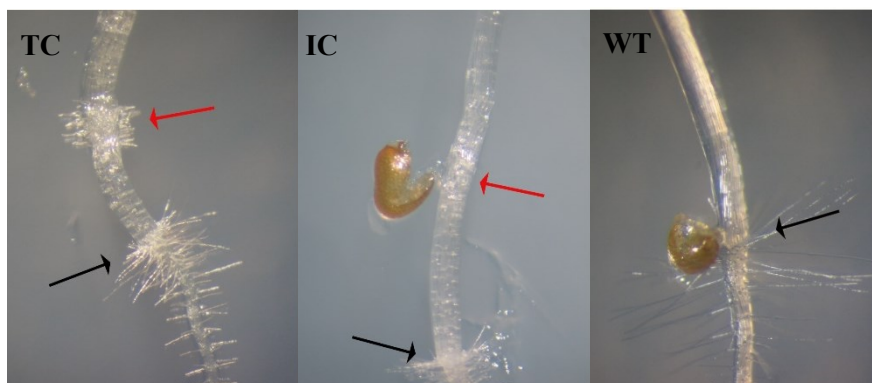


Figure 6: Comparison of the phenotypic deviations TC and IC of *exo70A1* LOF mutants, compared with the WT. TC shows an ERSJ with root hairs, and IC shows irregularly shaped cells in ERSJ. Red arrows indicate ERSJs and black arrows indicate true RSJs. Adapted from (Drdová et al., 2019).

1.3 Transcription Factors Involved in Apical-basal Polarity and RSJ Establishment

Transcription factors (TFs) are initiators and controllers of gene expression and tissue differentiation throughout a plant body. TFs bind to the cis-regulatory promoter of DNA, allowing them to activate or silence gene expression, and increase or decrease transcription. The composition of active TFs within one cell significantly contributes to its nature, shape, behaviour, and functional specialisation.

Plant multicellular bodies evolved a special way of intracellular communication that is not present in animals (nanotubules are not real analogues). Between most plant cells, there are cytoplasmatic connections called plasmodesmata. Many regulators, such as phytohormones, mRNAs, interfering RNAs, and small proteins, can move intracellularly through plasmodesmata. This increases the potential for intense regulatory dialogue between neighbouring plant cells. Since cells in plant bodies cannot move or migrate within the body (a crucial difference between plants and animals), the cells depend on their positional regulatory context within the body.

Unsurprisingly, some of the TFs are known to be involved in the development of root-shoot polarity and RSJ. I expect some major discoveries will be made in the future study of RSJ. The following groups of TFs are known to be involved in this process.

1.3.1 WOX Genes

WOX genes are plant-specific transcription factors found in all Viridiplantae species. The evolution of this protein family is discussed in the 4. chapter in relation to root evolution (Wu et al., 2019). The name derives from WUSCHEL-homeobox genes, based on the presence of the WUSCHEL homeobox domain in WOX genes. The group has 14 members in *Arabidopsis* (Haecker et al., 2004). They share the same homeobox domain but differ in the composition of other domains on both C- and N-end. (Dolzblasz et al., 2016)

Three members of the WOX family, WOX2, WOX8, and WOX9, are mainly involved in the apical-basal polarity establishment process (and later possibly RSJ development). WOX8 and WOX9 are members of the same subfamily of WOXes; WOX2, on the other hand, is more related to the WUSCHEL. (Wu et al., 2019)

WOX8 and WOX2 are already present in the embryo sac in the central cell and egg cell. Surprisingly, they are not present in any other cell of female or male gametophytes (Haecker et al., 2004). This is not stressed or discussed in any related publication, but it seems they are present just in cells, which fuse with male gametophyte cells (from the egg cell, the zygote is initiated after double fertilisation along with endosperm formed after the fertilisation from the central cell). The zygote is polarised (among the others) by the activity of zinc-finger TF WRKY2 with WOX8 or WOX9 (Ueda et al., 2011). In this function, WOX8 and WOX9 seem to be redundant, though in WT plants the WOX9 is firstly expressed in basal cell (Haecker et al., 2004). In both single LOF mutants *wox8* and *wox9*, the zygote cell divides

asymmetrically (as in WT), but in double LOF mutant *wox8wox9*, the asymmetry of the division is disrupted (Ueda et al., 2011). After the zygote's first division into the apical and basal cells, the expression of both WOX2 and WOX8 is divided between the apical and basal cells. The WOX2 is only expressed in the apical cell, while WOX8 is only expressed in the basal cell. At this stage, the expression of WOX9 starts exclusively in the basal cell (Figure 7). (Haecker et al., 2004)

WOX8 is only expressed in suspensor and hypophyseal cell throughout all stages of the embryo's development; therefore, WOX8 keeps its basal cell identity. (Haecker et al., 2004; Ueda et al., 2011)

WOX2 is expressed after the division of the zygote in the apical cell. In an 8-cell embryo, there comes a shift in the expression pattern because, since this stage, WOX2 is expressed only in apical four cells (Haecker et al., 2004). As was previously described, this region is destined to give rise later developing cotyledons and SAM (Scheres et al., 1994). These observations indicate that WOX2 is an early embryonic apical pole marker (see Figure 7). However, an exciting expression of WOX2 appears at the heart stage. In this stage, the expression of WOX2 decreases in the apical pole cells. However, a new expression domain is visible in the future RHJ (Figure 7– G, black arrow).

WOX9, sometimes called STIMPY (STIP) (Wu et al., 2005), is expressed in the basal cell after the first division of the zygote (Haecker et al., 2004). Then, the expression domain spreads to the neighbouring cells of the embryo proper, and the expression is stabilised in the lower tier defined in the globular stage by Scheres et al. (1994). The expression in the whole lower tier is stable until the transition from globular to the heart stage (Figure 7). After the transition to the heart stage, the expression domain of WOX9 is conspicuously restricted to the developing RSJ (Haecker et al., 2004). However, in a different report was observed a distinct and much broader expression of WOX9, all over the heart stage *Arabidopsis* embryo (Wu et al., 2007). The same methodological approach was used in both studies - in situ hybridisation for the observation of mRNA localisation (Haecker et al., 2004; Wu et al., 2007). Wu et al. (2007) also studied the WOX9:GFP construct activity, which resembled the domain of mRNA localisation. To resolve the details of WOX spatio-temporal activity regulation, more microscopy investigations of developing embryos are needed.

No published report addressed potential correlations between heart stage expression of WOX2 and WOX9. However, I suspect they could be involved in the same mechanism of embryonic RSJ establishment, and they might be used as markers. Another reason why WOX9 seems to be involved in ER establishment is based on the analysis of *Arabidopsis wox9* mutants. These mutants possess typically looking cotyledons and hypocotyls, but the root is totally missing (Wu et al., 2005). It seems they lack not only primary root but also CHs and RSJ.

I think following the expression pattern of WOX9, but also WOX2, in previously described exocyst mutants (*exo70A1* and *sec15b*) could lead to interesting results. Because of the expression of GL2 in the TC region, the authors of the study hypothesized that the development of TC happens postembryonically. However, another protein marker of embryonic RSJ development is documented here, and it is also solely expressed in the epidermal tissues. Therefore, if there would be any disruptions of expression

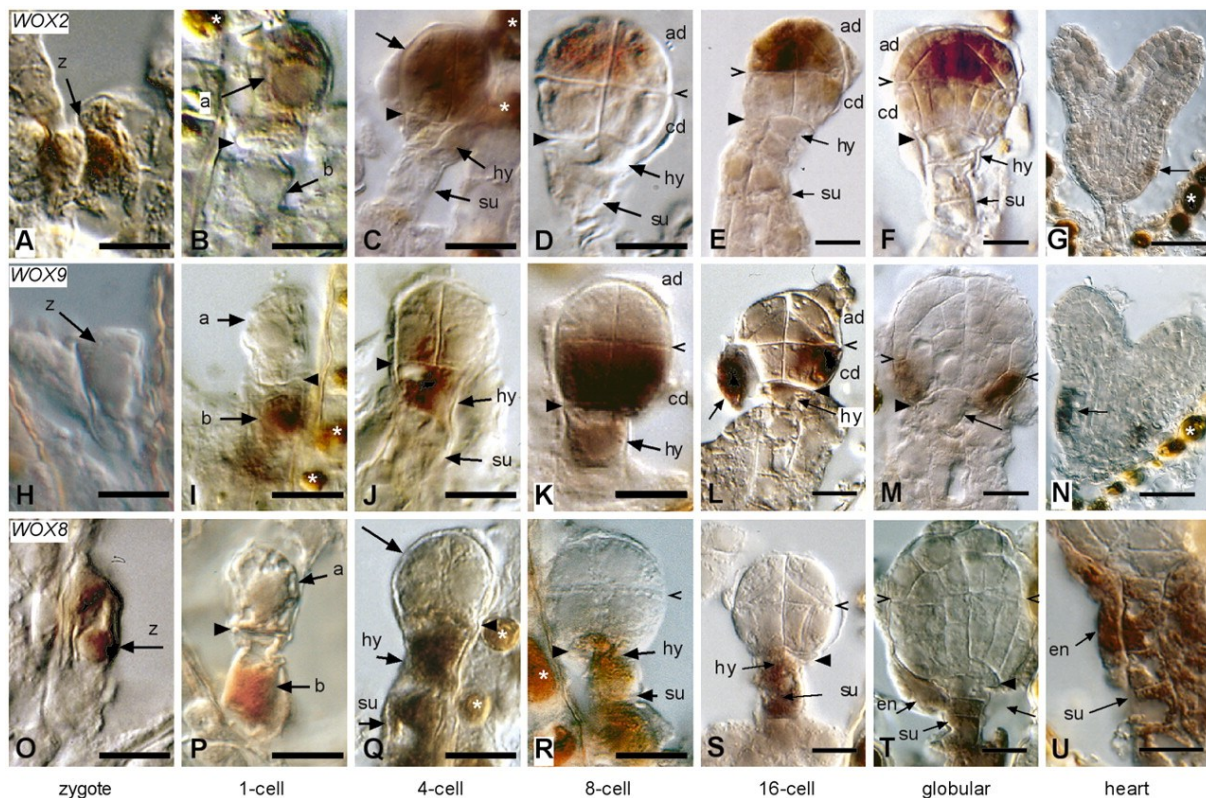


Figure 7: Expressions of mRNA (brown cells) of selected genes by in situ hybridisation, A-G *WOX2*, H-N *WOX9*, O-U *WOX8*, every column is the different embryo stage, brown colour marks expression of mRNA (white asterisks marking the naturally brown seed coats), in the picture is visible the zygote and suspensor localisation of expression of *WOX8* through all stages of embryo development (in the heart stage also in neighbouring endosperm cells), *WOX2* expression in the zygote, apical cell and upper region of globular stage embryo, and most importantly weak expression in the future embryonic RSJ in the heart stage, *WOX9* expression in the basal cell and the hypophysis, and lower region of globular stage, and its shift to the future RSJ in the globular and heart stage. Black arrowhead = boundary between apical and basal cells, in later stages between embryo proper and hypophyseal cell, open arrowheads = boundary between upper and lower region, A = apical cell, ad = upper region, b = basal cell, cd = lower region, en = endosperm, hy = hypophyseal cell, su = suspensor, z = zygote, bar A-T = 10 μm , bar U = 20 μm . Adapted from (Haecker et al., 2004).

in the exocyst mutant's embryos, it would clarify the embryonic establishment of TC. Also, it would link embryonic development with light signal perception since the TC and IC phenotypes occur only on dark germinating plants with etiolated hypocotyls.

Reading the literature for this thesis, I realised how poorly the rootless mutants are analysed. Cortical cell layers in the lowest region of the mutant seedling, which would indicate hypocotyl or embryonic root tissue, are not counted. The only case I came across was a study by Scheres et al. (1995) and by Willemsen et al. (1998), where in all reported mutants the number of cortical cell layers in roots and hypocotyls had been analysed. Many known rootless mutants (such as *monopteros* and others) have defects in the initiation or formation of RAM and root development, but the status of RSJ is unknown. To demonstrate this problem, I include here a small comparison of such mutants in Figure 8.

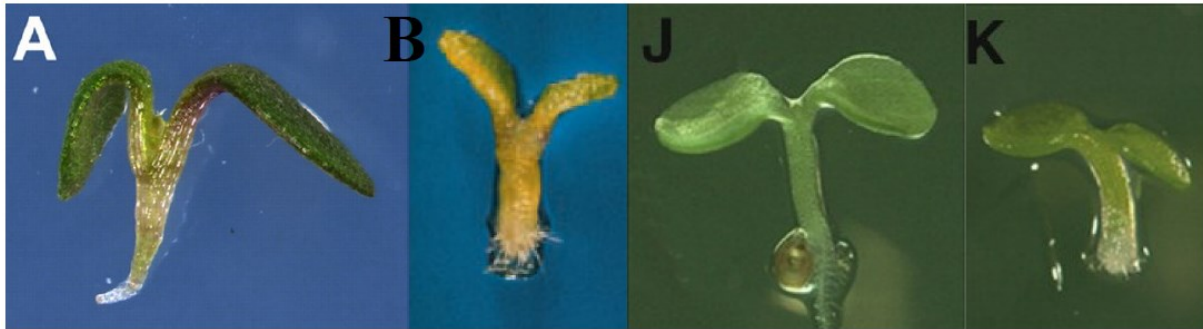


Figure 8: The comparison of selected *Arabidopsis* rootless mutant seedlings. A – *monopteros* mutant B – possible phenotype of a 7-days-old *hobbit* homozygous mutant, and J and K – possible phenotypes of 5-days-old *vox9* mutants (there are no quantitative statistics of the frequency of variances in the study), as we can see, it cannot be decided just from the pictures, if the mutant possesses the embryonic root and lacks just root apical meristem, or if it is genuinely rootless, in the study, from which the picture of *hobbit* mutant is adapted (Willemsen et al., 1998) there was the analysis of cross sections of *hobbit* root and hypocotyl, and it was discovered that there is present the region with only one cortical layer, which indicates a defect in forming the root apical meristem, this analysis is not published with *vox9*. Therefore, I cannot say if it lacks both embryonic and post-embryonic roots. A - adapted from (Cole et al., 2009), B - adapted from (Willemsen et al., 1998), and J and K - adapted from (Wu et al., 2005).

1.3.2 WIP Genes

WIP genes are a protein family of zinc-finger motive transcription factors. They bind to regulatory DNA regions (promoters) and also to other proteins, which impose the specificity of the regulatory action (Appelhagen et al., 2010). The family is defined by the presence of the WIP domain in the structure, which can be combined with other different protein domains, resulting in the variability and specificity of their activity. Analysis of WIP proteins showed that there are no similar proteins in fungi, bacteria, or animals, and therefore, it is a plant-specific group of transcriptional factors. There are six family members in *Arabidopsis*, and related proteins were also documented in *Oryza*, *Medicago*, *Zea*, *Triticum*, and other plants. (Sagasser et al., 2002)

From the six isoforms of *WIPs* present in *Arabidopsis*, *WIP2*, *WIP4*, and *WIP5* show stronger phylogenetic relations, compared to *WIP1*, *WIP3*, and *WIP6*, which form a separate clade. This functionally separates *WIPs* into two functional groups (Du et al., 2022). In the *Arabidopsis* root, mainly quiescent centre, three isoforms of *WIPs* are expressed: *WIP2* (also known as *NTT* – NO TRANSMITTING TRACK), *WIP4*, and *WIP5*. These genes function redundantly; they were analysed as single mutants of all three genes, and no significant differences from the WT were observed, but triple mutant *wip245* is rootless. Embryonically expressed *WIPs* are involved in the correct cell division orientation. (Crawford et al., 2015)

The other three *Arabidopsis* *WIPs* (and weak expression of *WIP2*) are expressed in the maternal tissues surrounding the developing seed (Du et al., 2022). It is still unknown if and how they can send the regulatory signal into the embryo (e.g., via suspensor). Some nutrients, molecules, and proteins are transported from maternal tissues through the suspensor into the embryo (Stadler et al., 2005).

WIPs could be differentiation master regulators in plant bodies (inducing and preventing the differentiation of basal parts of plant embryos). The analysis of WIP mutants shows redundancy of the isoforms. In hextuple mutant *wip123456* there is a loss of cell division orientation, but surprisingly, already in the triple *wip245* mutant, there is a loss of normal root development. The seedlings of both mutants are compared to the wild type depicted in Figure 9.

WIP1, WIP3, and WIP6 expressed in maternal tissues might work redundantly as a cell fate suppressor (as was described by authors of the study) (Du et al., 2022)), but I expect that there is a loss of cell differentiation. The WIP2, WIP4, and WIP5 could compete with the WIP1, WIP3, and WIP6, and the relation by both expressions could be the key to the proper development of the basal part of the embryo. The surprising missing root cell's developmental fate in the *wip245* mutant is significant since the heart stage of the embryo, the same stage when the first cells of the second cortex layer are formed and might be related to the above-mentioned antagonism. However, hypocotyl in the *wip245* seems damaged too. (Du et al., 2022)

The analysis of the *wip136* mutant and the precise molecular mechanism of interaction between both putative antagonistic modules are still missing.

The general presence of *WIP* genes in possibly all land plant species is supported by the analysis of *MpWIP* from liverwort *Marchantia polymorpha* and its involvement in developing air pore complexes (Jones & Dolan, 2017).

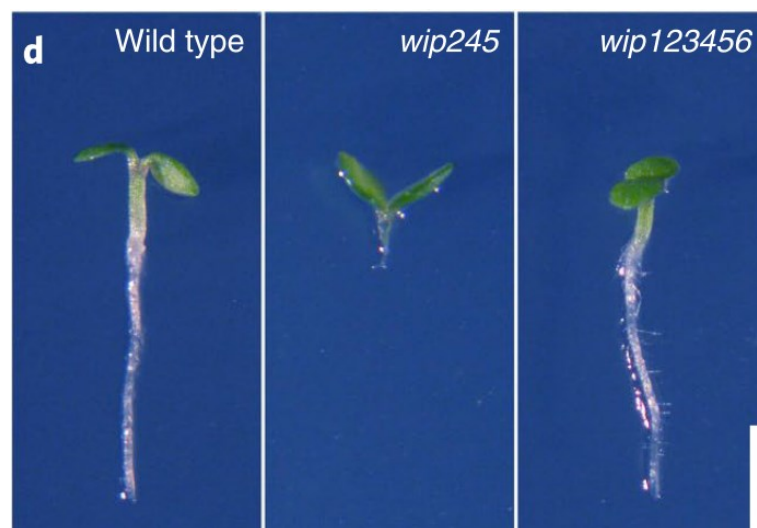


Figure 9: Comparison of phenotypes of 3-days-old *Arabidopsis* LOF triple mutant *wip245*, LOF hextuple mutant *wip123456* and WT, left – WT, middle – *wip245*, and right – *wip123456*, the loss of function of all three redundantly working WIPs expressed in embryo leads to the uncontrolled activity of maternally expressed WIPs and this leads to loss of cell differentiation in basal part of the embryo, in the hextuple mutant differentiation restores and only the orientation of cell division is disordered, but the phenotype is weak. Adapted from (Du et al., 2022).

2. Regulation of Root-shoot Junction Development by Auxin

Phytohormones are chemical compounds capable of modulating various processes within plant organisms. These signals can influence individual cells, tissues, or entire organs. Among the phytohormones, auxin was first discovered and studied by Charles and Francis Darwin in *The Power of Movement in Plants* (Darwin & Darwin, 1880). The auxins are a group of chemicals, with the most important member indole-3-acetic acid (IAA). Since the IAA discovery, auxins have been a widely studied chemicals. Auxin plays crucial roles in, e.g. phototropic or gravitropic growth, apical dominance maintenance, initiation of lateral and adventitious roots, and many more. Unsurprisingly, auxin plays also a pivotal role in embryo development and the post-germination root and hypocotyl development.

2.1 Symplastic Connectivity and Coordination of Embryo Development

Phytohormones can move from one cell to another in two different ways: through the symplast or apoplast. The apoplastic way usually needs some transporters at PM or the capability of the chemical to dissociate through PM. By the polar, oriented localisation of these transporters, the cell can regulate the direction of influx or efflux of the phytohormone (Wiśniewska et al., 2006). Phytohormones' second possible way is cell-to-cell movement through plasmodesmata, where the direction is not so selective (Mellor et al., 2020).

The experiments with expressing soluble cytoplasmic GFP(s) linked together in mid-torpedo embryos showed plasmodesmata function (Kim, Kobayashi, et al., 2005). Interestingly, 1x GFP, expressed under the STM (SHOOTMERISTEMLESS) promoter, which is active in mid-torpedo embryos in the future SAM region and on the base of the hypocotyl (overlapping the region of future CHs), is soluble all over the embryo (Kim & Zambryski, 2005). On the other hand, observing linked 2x GFPs showed that there was no movement of the construct into the cotyledons, and the movement into the root was significantly decreased (Kim, Kobayashi, et al., 2005). The movement of 3x GFPs was unique only for the hypocotyl, showing that the root and hypocotyl have some symplastic restrictions/barriers compared to the movement solely in the hypocotyl. The expression of 2x GFP in mid-torpedo in RAM showed that movement from root to hypocotyl of 2x GFP is restricted (in comparison to opposite movement from hypocotyl to root) (Kim, Cho, et al., 2005). This implies some symplastic barrier in the emerging RSJ.

2.2 Regulation by Auxin in Embryo Development with Focus on Embryonic Root and RSJ Development

The previously mentioned and described *gnom* mutant showed how important signal auxin is in the developing embryo (Richter et al., 2010). GNOM, as an ARF-GEF, regulates the polar localisation and recycling of PINs (most importantly PIN1). PIN proteins are transmembrane transporters for the efflux transport of auxin, therefore they are essential for the oriented transport of auxin within the tissue (Blilou

et al., 2005). Local expression of GNOM in the vasculature of the *gnom* background embryo restores ER formation. The same results the researchers got after expression in hypophysis only. However, the expression in hypophysis restored ER only in some embryos (Wolters et al., 2011).

PAT is crucial for plant development since the embryonic stages, as observed by Friml et al. (2003). The proteins facilitating PAT are mainly PINs (however there are some other adepts, like ABCB transporters). It is a comprehensive protein family with members having redundant/overlapping functions. In the embryonic PAT, PIN1, PIN3, PIN4 and PIN7 are involved. Its redundancy is documented by LOF single mutants in all embryonically expressed PINs, which do not show strong phenotypes. The significant phenotype characterised by not properly developed embryonic organs is visible on *pin1pin3pin4pin7* quadruple LOF mutant. The mutant shows fused cotyledons and defects in ER and RAM (Friml et al., 2003). PIN7 is crucial in the early development of the embryo because it is localised at the apical poles of the suspensor cell's PM and secures the auxin transport from seed envelopes to the embryo proper. However, in the 32-cell stage embryo, the PIN7 localisation changes to the basal pole of the suspensor cell, transporting auxin from the embryo (Friml et al., 2003). PIN1, on the other hand, is expressed in the embryo proper, securing PAT there. In the globular stage PIN1 is localised on basal PMs in the inner provascular cells, while in external cells, it is localised to the apical PMs (Friml et al., 2003). This secures PAT in the embryo proper, and the auxin-response maxima are formed. The auxin-response maxima are observable by using DR5-GFP, which is expressed in cells reacting to the presence of auxin (Benková et al., 2003; Ulmasov et al., 1997). Auxin maxima are one of the main signals for developing embryonic organs – cotyledons and ER with RAM. PIN4 has a unique localisation in hypophysis in the globular stage and in later stages in provascular initials (Friml et al., 2003). The minor role seems to have PIN3 during embryogenesis, with its localisation in RAM, but its mRNA is localised in the quiescent centre cells (Friml et al., 2003). The inability of proper embryonic organ establishment and development in *gnom* and *pin1pin3pin4pin7* documents, how important PAT is during embryogenesis. This conclusion is based on observations of PAT disruption by different approaches: LOF of transporters and their nonpolar localisation to PMs.

Auxin cell-to-cell movement is one part of the signalisation; the other part is the competence of cells to perceive and interpret the signal. Relevant to this chapter is a mutant named *monopteros* (*mp*, the name refers to a possible phenotype with lacking primary root and one cotyledon) (Mayer et al., 1991), which has LOF in MONOPTEROS (MP) or AUXIN RESPONSE FACTOR 5 (ARF5) (Hardtke, 1998). AUXIN RESPONSE FACTORS (ARFs) are a family of TFs, which start to be active and involve expression in the presence of auxin (Maraschin et al., 2009) through auxin-induced degradation of ARF repressors – Aux/IAA (Tiwari et al., 2003). The MP is in WT embryos located at the basal pole of the globular embryo (but not in hypophysis). Later in the heart stage, its expression changes to the quiescent centre, adaxial cotyledon side and future SAM. The *mp* seedlings lack hypocotyl and root, so the whole basal region of the globular embryo and, later, the lower tier of the heart embryo is affected. BODENLOS (BDL), or IAA12, is an Aux/IAA repressor of MP and is degraded in the presence of auxin.

However, mutation of BDL in *bdl* mutant, preventing BDL from ubiquitylation, affects its degradation in the presence of auxin, not the binding activity to MP (Hamann et al., 2002). Therefore, *bdl* shows the same phenotype as *mp* because the cells cannot react to the auxin through MP. Interestingly, there were prepared *bdl* mutants with stronger and weaker phenotypes. The analysis showed that in weaker phenotypes start to appear hypocotyls and RSJs with CHs and primary roots, respectively (Hamann et al., 1999). These findings show that RSJ is not just a border derived from the position of RAM and SAM, forming somewhere between, but it has a strictly defined position by some still not discovered mechanism.

In the previous chapter, the WOX genes were described with a focus on their functions in the ER establishment. Their activities are supported by the analysis of *mp*, *bdl* and *gn* embryos, with observed expression of WOX9 (Haecker et al., 2004). The involvement of WOX9 in the patterning of the RSJ also indicates the analysis of *mp*, *bdl*, and *gn* embryos and the expression of WOX9 in them (see Figure 10). Compared to WT, the expression of WOX9 in *mp* and *bdl* was identical, only in the hypophyseal cell. In *gn*, there was the expression of WOX9 in many epidermal cells, indicating that the developmental cell identity of this mutant was poorly specified. For comparison, in the WT, WOX9 expression occurs all over the basal region of the embryo and hypophysis in the early-globular stage. However, it is restricted just to the epidermal cells of the lower subtier in the mid-globular stage. The expression of WOX9 in hypophysis is lost in the transition from the early- to the mid-globular stage (see Figure 7 L-N).

I propose here another experiment on *bdl* mutants with weaker phenotypes, as were prepared by Hamann et al. (1999). If the expression of WOX9 were progressively spread to the basal region in these mutants, it would mean that the WOX9 is a key factor of hypocotyl and ER establishment. Moreover, it would mean that the establishment of hypocotyl and ER is directly linked to PAT and auxin signalisation. It would also be necessary to analyse WOX2 expression in these mutants to resolve this theory because WOX2 expression switches in the heart/torpedo stage from the apical domain to the epidermal cells of future RSJ (see Figure 7 M-N).

2.3 Auxin in Germination and Post-germination Development - Collet Hairs and Hypocotyl Adventitious Roots Formation

As sessile organisms, plants are bound to a single location for their entire existence. Therefore, sporophytes have developed many different mechanisms for dispersing seeds more or less randomly into the surroundings. However, this results in the distribution of seeds into various, unpredictable environments, to which germinating plants are immediately forced to react. One such adaptation appears to be the hypocotyl in species exhibiting epigeal germination.

The hypocotyl of epigeal germinating plants shows significant developmental plasticity. On the base of the hypocotyl at RHJ, CHs are formed, which ensure initial anchoring of the germinating plant in the

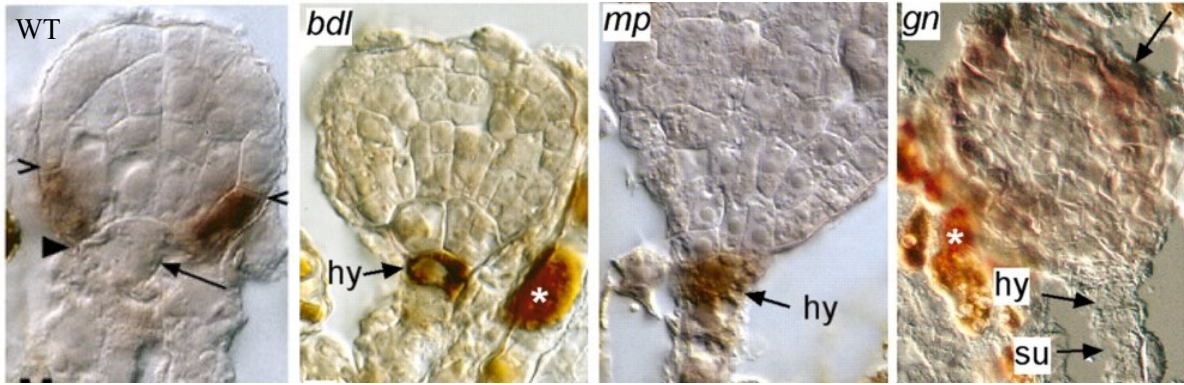


Figure 10: Globular stage expressions of mRNA (brown cells) of *WOX9* in selected mutants and WT of *Arabidopsis* by in situ hybridization. WT – localization of expression to the epidermal cells of lower region, *bdl* – localization of expression only to the hypophysis in *bodenlos* mutant, *mp* - localisation of expression just into the hypophysis in *monopteros* mutant, the same as in *bdl*, *gn* – localisation of expression in the cells all over the surface of *gnom* mutant embryo. Open arrowheads – border, dividing the embryo to the apical and basal regions, black arrowhead – border between hypophysis and embryo proper, black arrow and hy – hypophysis, su – suspensor, white asterisks – naturally brown coloured seed coats cells. Modified and adapted from (Haecker et al., 2004).

soil (especially important, for example, in floodplains germinating seeds, like *Populus* or *Salix* - Parsons, 2013). Hypocotyls also grow differently in light vs. dark conditions. In both cases, the growth is secured by a mere longitudinal expansion of its cells. However, the changing variable is the speed and extent of the growth/growth elongation, which is up to ten times faster in etiolated/dark-grown hypocotyls (e.g. Gendreau et al., 1997). Gendreau et al. (1997) also observed that hypocotyl cell elongation is accompanied by nuclei endoreduplication. Importantly, adventitious roots (ARs) are initiated along the hypocotyl, depending on the germination-growth conditions. Hypocotyl ARs are initiated endogenously from hypocotyl's central cylinder from the pericycle (Sukumar et al., 2013). PAT and auxin signalling very tightly control the initiation and growth of ARs, resulting in the formation of maxima in the pericycle, well documented, for example, by Sukumar et al. (2013). The ARs can be initiated by default in two ways in WT plants, by cutting the primary root or by de-etiolation of hypocotyls. Geneve & Heuser (1982) observed the ARs initiations after the different auxin exogenous treatments and Zeng et al. (2023) also show that etiolated hypocotyls of *Arabidopsis*, *Lycopersicon* and *Oryza* produce ARs not only upon the de-etiolation but also after treatment with auxin agonist hyssparin. The hypocotyl is, therefore, possibly, a part of root-to-shoot transitional structure with anatomy resembling roots and with the potential to produce ARs. In light conditions, the hypocotyls are photosynthetically active, while in dark conditions, they do not produce plastids with chlorophyll and grow by elongation faster. In this thesis, it was already shown that the embryonic RSJ is initially established during embryogenesis. The hypocotyls evolved as a developmentally plastic organ, which can serve as an AR formation domain in response to the environment after the germination. This is how plants can modify their RSJ in reaction to the environment.

The analysis of above already discussed exocyst subunits mutants (*exo70A1* and *sec15b*) showed possible disruptions in PAT in the hypocotyls. It was previously reported that the EXO70A1 subunit of the exocyst complex is involved in PAT organisation through the polar localisation of PIN1 and PIN2 to PM and their recycling (Drdová et al., 2013). Drdová et al. (2019) analysed the localisation pattern of PIN3 auxin transporter in the etiolated hypocotyls of WT and studied exocyst mutants. Also, the expression of DR5::GUS and DR5::GFP auxin activity reporters (already mentioned above) was studied to see any ectopic auxin maxima formations. Results show that disruption RSJ and hypocotyl patterning is related to changes in PIN3 PM localisation. In these regions, the PIN3 was more localised to the vacuoles. Also, DR5 reported ectopic auxin maxima in collet and ERSJ. The treatment with NPA (N-1-naphthylphthalamic acid), the chemical blocking PAT (Ching et al., 1956) led to weaker phenotypic deviations in mutants with TC and IC regions located significantly closer to the true collet. The disruption in PAT in the hypocotyls of the exocyst mutants results possibly in lower auxin activity in the hypocotyl. This shows that the auxin does have a crucial role in RSJ and hypocotyl development patterning.

Drdová et al. (2019) also show that the exocyst mutants (*exo70A1* and *sec15b*) have deviations in ARs initiations too. The observation of these mutants showed that, in contrast to the WT, the mutants are unable to form the ARs along the whole length of hypocotyl. The only place where ARs are initiated in the mutants is the true collet. This again indicates deviations in PAT and auxin gradient interpretation. I think exciting data could result from the exogenous treatment of etiolated hypocotyls of *exo70A1* and *sec15b* with some artificial auxin, or auxin agonist, as done for WT (Zeng et al., 2023). Interestingly, the same phenotype with ARs initiation from RSJ was recently observed by Li et al. (2023) in mutant *folb2* LOF mutant of dihydroneopterin aldolase, an enzyme responsible for folate synthesis. The plants have however also defects in RAM maintenance, primary root growth along with ARs formation. The analysis showed that *folb2* has disrupted PAT and decreased the expression of some PINs.

3. Evolution of Root-shoot Junction

RSJ evolved as a necessary transition zone along with the root vs. shoot evolution, however, not that much research has been done on this topic. Studies on this topic report that the common ancestor of land plants was rootless (e.g. Raven & Edwards, 2001). This suggests that roots evolved independently in different plant lineages, resulting in roots with different origins and characteristics (Hetherington & Dolan, 2017). Certainly, similar structures or organs developed through convergent evolution. This may lead to an assumption that if roots exhibit evolutionary origins, this should also be valid for the RSJ. This chapter aims to provide a brief overview of root evolution, with a focus on RSJ-related information. Along with plant body evolution, the development and patterning of the embryo also evolve and differ even in closely related monocots and dicots. Embryos are a shared characteristic (synapomorphy) within the Embryophyte clade (Delwiche & Cooper, 2015). Nevertheless, not all embryophytes have a primary root, as seen in bryophytes. As previously shown, primary roots and their meristem are possibly established during embryogenesis in most angiosperms. However, the highly reduced embryo of orchids – protocorm (Yeung, 2022) – and differences between monocot vs. dicot embryos indicate that the evolution of the whole plant body and the evolution of the embryo might be largely uncoupled in angiosperms. This part of the chapter focuses only on embryo development *sensu stricto*, meaning embryos of seed plants before germination and embryos of ferns and lycophytes as entities that initiate primary organs from the zygote divisions and are still attached to the mother organism.

3.1 Evolution of Roots

Firstly, it is essential to establish the definition of true roots. True roots exhibit specific characteristics as recognised in *Arabidopsis* and nearly all other vascular plants. These include a root apical meristem, comprising one or more basipetally located stem cells, which allows root elongation by cell production, positive gravitropic response and negative phototropic response, a root cap protecting the root apical meristem in some lineages, root hairs formation from rhizodermis and the endogenous initiation of lateral roots. (Raven & Edwards, 2001)

During the early stages of land plant evolution, the anchoring of their axes was facilitated by rhizoids. It is documented that in the earliest plants, rhizoids could grow out from various epidermal axes, giving rise to the first rhizoid-bearing axes (Eshel & Beeckman, 2013). For instance, in the Devonian vascular plant *Nothia aphylla*, vertical axes or rhizomes bore a distinctive rhizoidal ridge (Kerp et al., 2001). From this ridge, which served as a specialised organising centre located on the ventral side of the axis, rhizoids grew out and anchored the axes in the soil. Many plants from this period exhibited similar characteristics, although there were exceptions like *Asteroxylon mackiei*, which lacked any rhizoids (Edwards, 2003).

Rhizoid-bearing axes, or rhizomatous axes, are considered primarily surface organs, although, depending on the local soil conditions, they may also grow below the ground (Kerp et al., 2001).

Unlike the root hairs/rhizoids (Menand et al., 2007), the roots developed polyphyletically, supported by studies (Friedman et al., 2004; Hetherington & Dolan, 2018; Matsunaga & Tomescu, 2016; Raven & Edwards, 2001). Roots have evolved independently at least twice during evolution, separately in euphyllophytes and lycophytes during the Devonian period (Hetherington, Berry, et al., 2020). According to Fujinami et al. (2020), roots have independently emerged five times. Their analysis draws from both paleobotanical observations and molecular marker analysis. They suggest that there were two significant root development events in euphyllophytes, independent for seed plants and monilophytes, and at least three events in lycophytes, independent for Lycopodiaceae, Isoetaceae, and Selaginaceae. These findings are based on structural analysis of RAMs and their maintenance. Consequently, these conclusions would imply different characteristics of the RSJ in the aforementioned clades.

The branching of roots has also evolved independently multiple times, mirroring the independent evolution of roots themselves. In lycophytes, there is a common dichotomous branching pattern of roots, where the root apical meristem divides into two equal parts (Hetherington & Dolan, 2018). Conversely, euphyllophytes exhibit lateral root branching, where lateral roots branch endogenously, independent of the root apical meristem. Moreover, lateral root branching has emerged multiple times in various classes of euphyllophytes (Hetherington, Berry, et al., 2020).

In the early Devonian period, the lycophyte *A. mackiei* from Rhynie chert exhibited three distinct axes: leafy shoot axes, root-bearing axes, and rooting axes. *A. mackiei* belonged to the extinct order Drepanophycales, which experienced significant diversification during the Silurian and Devonian periods and is considered a sister family to extant lycophytes (Hueber, 1992). *A. mackiei* is a well-documented fossil that displays various features of a land-adapted plant. The leafy shoot axes produced adapted structures to terrestrial environments, etc., leaves (microphylls), xylem, stomata, and cuticle. Root-bearing axes branched from the leafy shoot axes anisotomously, with smaller and more slender axes exhibiting root-bearing characteristics. These axes also possessed land adaptation features, such as a cuticle on the body surface, sporadic stomata, and scale leaves. Root-bearing axes gave rise to rooting axes through anisotomous dichotomous branching. Dichotomous branching indicates a clearly common origin of root and shoot in lycopods (Fujinami et al., 2021). Rooting axes, although resembling roots, were not true roots; they lacked typical root structures such as root caps or root hairs (Hetherington et al., 2021). Hetherington & Dolan (2018) propose that the body structure of *A. Mackiei* indicates that the Lycophyta and Euphyllophyta share a rootless ancestor and that root caps, root hairs and endodermis developed subsequently in the Lycophyta clade. These findings point to the convergent evolution of roots Lycophyta and Euphyllophyta.

There are three extant Lycophyte families today: Lycopodiaceae, Selaginaceae, and Isoetaceae, each exhibiting distinct body structures. In Lycopodiaceae plants, roots are endogenously established, emerging from regions near the stem tips (Roberts & Herty, 1934; Stokey, 1907). Isoetaceae, on the other hand, have a unique body plan consisting of a corm serving as a stem, from which microphylls branch apically and rootlets branch basally (Paolillo, 1963; Yi & Kato, 2001). The rootlets of Isoetaceae emerge

endogenously from the corm and are considered the true roots (Hetherington, Emms, et al., 2020). In the Selaginaceae family, plants feature rhizophores, distinct root-like structures that are gravitropic and give rise to roots through endogenous branching. However, rhizophores lack root caps and emerge exogenously from dichotomous branching of the shoot apex. Furthermore, the molecular mechanism underlying rhizophore establishment and their gene expression patterns differ from those of roots (Mello et al., 2019). These characteristics suggest that rhizophores are not true roots, as they cannot produce leaves or leaf-based organs laterally (Banks, 2009; Imaichi & Kato, 1989).

This brief overview highlights the evolutionary trajectory of the most ancient vascular plant clades compared to their extinct sister clades. Since the beginning of continents colonisation by embryophytes, new roots and root-like structures have continually emerged. This process has inevitably led to the diversification of RSJ, as exemplified above by representatives of Lycopphyta.

Huang & Schiefelbein (2015) have observed a similarity in the genetic toolkit involved in root development across various rooting plants, despite their independent evolutionary origins. Their experiments on a range of species, including cucumber, soybean, *Arabidopsis*, tomato, rice, maize, and *Selaginella*, revealed the involvement of the same gene families in root development. This suggests that organisms from both euphyllophytes and lycophytes employ similar strategies to address common challenges. However, it is worth noting that younger gene families often play roles in root patterning specific to certain plant families or lineages, resulting in variations in root structure and organisation (Huang & Schiefelbein, 2015). In the future, questions related to the homologous vs. convergent evolution of roots in different clades will certainly be addressed.

The WOX gene family (presented in the first chapter in relation to ER development) plays a crucial role in patterning primary roots during embryogenesis. The family comprises three subfamilies, each with distinct evolutionary origins: the ancient clade (T1WOX), the intermediate clade (T2WOX), and the WUS clade (T3WOX) (van der Graaff et al., 2009; Wu et al., 2019). According to Wu et al. (2019), the WOX gene family is indeed an ancient protein family in land plants. It is probable that the T1WOX subfamily became independent earlier in the evolution than the other subfamilies, likely soon after the emergence of WOX genes. Members of the T2WOX subfamily are found in both lycophytes and euphyllophytes, but their proliferation occurred in seed plants after the divergence of monilophytes (Wu et al., 2019)

In Lycopphyte *Selaginella kraussiana*, there is only one member of T2WOX. This sole member, *SkWOX11C*, does not exhibit tissue-specific expression. Conversely, in the Monilophyte fern *Ceratopteris richardii*, there are two members of the T2WOX subfamily: *CrWOXA* and *CrWOXB*. *CrWOXB* is similarly expressed nonspecifically across various tissues, while *CrWOXA* expression specifically marks the root meristem cells (Nardmann & Werr, 2012). *AtWOX11* is also involved in root initiation, particularly in regulating the branching of adventitious roots from detached leaves in *Arabidopsis thaliana* (Zhang et al., 2023).

These observations suggest that members of the T2WOX subfamily may have played a pivotal role and were required for the establishment of roots in euphyllophytes. As mentioned earlier, there were at least two root origin events in euphyllophytes, and it appears that both involved T2WOX genes. The importance of the T2WOX subfamily for root origin and maintenance supports the absence of T2WOX or T3WOX genes in bryophytes; they likely lost the common ancestor (Wu et al., 2019). This loss could also be explained by the lack of root development in bryophytes, leaving additional WOX genes unnecessary, leading to the loss of the ancestor due to redundancy with T1WOX genes. If so this reinforces an idea of reductive moss evolution (Žárský, 2021).

3.2 Evolution of Embryos

The plant embryo, synonymous with early developing sporophyte, is a defining characteristic of the embryophyta clade, commonly known as land plants. The life cycle of land plants follows an alternation of generations pattern, wherein both generations—gametophyte and sporophyte—are consecutively present, although their size/prominence and independence can vary among different clades. For instance, Bryophyta exhibit a reduced sporophyte phase, while seed plants have a reduced gametophyte life stage. Upon transitioning from aquatic to terrestrial environments, plants encountered numerous challenges. It is documented that the zygote could remain attached to the mother's body for a period to overcome unfavourable conditions (Hemsley, 1994), e.g. zygote of *Coleochaete*, partially protected and supplemented by mother thallus (Haig, 2015). The first plant embryos arose from mitotically dividing zygotes. Embryogenesis occurring within or in a close connection with the mother's body provides the developing organism with nutrient support from maternal nutrients, increasing the likelihood of survival. Following the completion of embryogenesis, the embryo can disperse, and various techniques have been developed for dispersing embryos, later protected by seeds and seed coats.

Following the diversification of vascular plants, the inclusion of root primordium and RAM into the embryo became a progressive trend. Observations of embryogenesis in ancient plant clades corroborate this notion, indicating an evolutionary trajectory towards the integration of root structures into the embryonic development process.

Experiments on *Isoetes asiatica* and *Isoetes japonica* have revealed that the RAM of the first primary root originates from the three outermost layers of the embryo cells. The root cap arises from the epidermis of the embryo, with its layer number increased through additional periclinal division. Meanwhile, the rest of the initials of the root derive from the two inner cell layers. This process closely resembles the establishment of the SAM in *Arabidopsis*. The first primary root develops at the same time as the first leaf. Subsequently, another leaf and root emerge after the initial leaf is connected to the first primary root via vasculature. The positioning of the second primary root defines the location of the basal meristem, which gives rise to all lateral roots in the plant. The basal meristem, situated at the base of the

corm, is responsible for the bundle characteristic of the root system. Notably, the first two roots of the plant originate embryonically. (Yi & Kato, 2001)

During embryogenesis of the fern *Phlebodium aureum*, the very first organ known as the "foot" is formed approximately eight to ten days after fertilisation. From this specialised structure, the first leaf emerges through endogenous branching, followed by the development of the first root about two days later, also via endogenous branching. Subsequently, the stem develops after the leaf and root are connected by vascular tissues, with the stem emerging from the region apically to the first leaf. All of these structures can be analogously compared to the first embryonic organs of *Arabidopsis*: the first leaf resembling a cotyledon, the root resembling the radicle, and the foot serving as the hypocotyl. (Ward & Wetmore, 1954)

The embryogenesis of dicots and monocots exhibits also significant differences. While the embryogenesis of *Arabidopsis* has been extensively described, the main distinctions with monocots embryogenesis are listed here. In monocots, there is no strict pattern of cell divisions from the very first division of the zygote, unlike in dicots where a more universal and rigid pattern can be observed in young embryos. Additionally, after the division of the zygote, the basal cell does not elongate as it does in dicots. (Chen et al., 2017; Xiang et al., 2019). In monocots, the formation of the suspensor occurs too, but it appears to play a smaller role in embryo formation compared to basal and hypophyseal cells in dicots. Unlike in dicots, where the RAM is partially derived from the hypophyseal cell, in monocots, the embryonic root is established in the middle of the developing embryo (Armenta-Medina et al., 2021). Despite the differing embryogenesis processes, both dicots and monocots share *WOX5* expression, which serves as a molecular marker for the quiescent centre and RAM in dicots. Interestingly, in monocots, *WOX5* expression is also observed in the middle of the embryo, where the root later emerges (Nardmann et al., 2007; Sarkar et al., 2007). Additionally, monocotyledonous plants from the Poaceae family possess unique organs such as the coleoptile and coleorhiza (which cover both apical meristems), epiblast, and scutellum, and sometimes seminal roots primordia (Kruglova et al., 2022).

This brief overview of embryogenesis across different plant clades highlights the remarkable diversity compared to the relatively conserved structure of roots. Roots exhibit convergent evolution, resulting in similar structures across different plant lineages, with evolutionary innovations typically arising through the metamorphosis of pre-existing organs. In contrast, the structure of embryos varies between all previously mentioned clades.

Summary

Root shoot junction, also known as collet, is a specific region, where two developmental programmes of plant organs meet. This topic is poorly studied and needs more attention. This bachelor's thesis aims to provide a brief overview of the current literature concerning this topic.

The root-shoot junction is initiated during embryogenesis and it matures after seed germination during the plant's life. The root-shoot junction of adult plants is a border between root and stem and a very important vasculature transition zone. My thesis mainly focuses on the ontogenetic development of RSJ of the model angiosperm *Arabidopsis thaliana*. The transition region is initiated already during embryogenesis and is further specified after the germination post-embryonal life of the plant in response to specific environmental conditions. During the germination, collet/hypocotyl hairs are formed in this region. Since the hypocotyl and upper part of the embryonic root are thought to derive from the same embryonic domain, the junction needs to be specified by a molecular mechanism also involved in root development. I present possible transcription factor regulators participating in this process – WOX genes, specifically WOX9 and WOX2 proteins – actively transcribed during embryogenesis; in certain phases, their expression marks the embryonic root-hypocotyl junction. Along with them, I present another transcription factor protein family, WIP genes, also involved in hypocotyl and embryonic root development, and in the communication with maternal tissues.

The post-germination hypocotyl is a developmentally plastic organ; it can adapt to the local environment by etiolation vs. photomorphogenic development, photosynthetic activity or inactivity, speed of growth/expansion, or initiation of adventitious roots. *Arabidopsis* mutants with defects in exocyst tethering protein complex subunits participating in vesicular transport form an ectopic collet above the true collet.

Auxin is a major plant morphogenic molecule involved in development and growth. Polar auxin transport and auxin signalisation are discussed as crucial mechanisms for proper embryonic development and, therefore, also for the development of the root-shoot junction, as demonstrated by the analysis of the auxin-related mutants *monopteros*, *bodenlos*, and *gnom*.

The roots have developed several times independently in the evolution. Therefore, the root-shoot junction has developed with them. The WOX gene family seems to have contributed to the spermatophytes root origins, but further research will uncover many more regulators.

Abbreviations

Ac = *Activator* element

AR(s) = Adventitious root(s)

ARF(s) = Auxin response factor(s)

bdl = *bodenlos* mutant

CHs = collet/hypocotyl hairs

Ds = *Dissociator* element

ER = embryonic root

ERSJ = ectopic root-shoot junction (ectopic collet)

GEF = guanosine-exchange factor

GFP = green fluorescent protein

GL2 = GLABRA2

gn = loss-of-function *gnom* mutant

GUS = β -glucuronidase

IC = irregular-cells phenotype, ectopic collet region with irregularly shaped epidermal cells

LOF = loss-of-function

mp = loss-of-function *monopteros* mutant

NLS = nuclear-localization signal

PAT = polar auxin transport

PM = cytoplasmic membrane

RAM = root apical meristem

RFP = red fluorescent protein

RHJ = root-hypocotyl junction

RSJ = root-shoot junction, collet

SAM = shoot apical meristem

TC = twin-collet phenotype, ectopic collet region with ectopic collet hairs

TF(s) = Transcription Factor(s)

UAS = upstream activation sequence

WOX = Wuschel-homeobox domain protein family

WT = wild-type

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Reviews are marked with an asterisk. *

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During the preparation of this work, Chat GPT-3.5 was used to improve grammar and style. After using this tool, the content was reviewed and edited as needed, and I take full responsibility for the thesis’s content.