

Factors involved in asymmetric cell division
in the *Arabidopsis* root meristem

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"At first, it's unfamiliar, then it strikes root" – Fernando Pessoa

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FOREWORD

This thesis project is the result of a six-week internship at the department of Developmental Biology and Molecular Genetics of the University of Utrecht. The intent of the thesis is to examine how the *SHORT-ROOT* gene plays a role in ground tissue patterning in the *Arabidopsis thaliana* root meristem.

Apart from the labwork which is integrated in the thesis, the initial assignment was writing a thesis based on academic literature. However, working in the lab was very interesting and helpful for the coming years of masters. Together with the writing and reading of academic literature, it has been very instructive.

I want to thank my supervisors, Yuschen Long and dr. Ikram Blilou, of being great help during the development of this thesis.

The main goal in the field of developmental biology is addressing the question of how a single cell becomes a complex multicellular organism. Revealing the fundamental processes in the development of living organisms not only helps us to understand 'life' in general, but is also of agricultural and medical significance (e.g. Jones et al. 2008). One of the powerful tools available for studying developmental biology, is genetics. Understanding the process of development can be reached by examining the genetic mechanism by which it is directed (Dolan et al. 1993). In this thesis, an attempt has been made to reveal a small piece of the puzzle by focusing on a very specific process in the root of *Arabidopsis thaliana*, and show how genetics are applied to investigate developmental biology.

Arabidopsis thaliana, a small flowering plant, is the mostly used model organisms in plant research. Its root has a simple, yet well-defined pattern of tissue layers (Dolan et al. 1993). Single concentric layers of epidermis, cortex, endodermis and pericycle encircle the stele, while columella and lateral root cap compose the basal tissue of the root. These layers arise from the so-called stem cell niche, which is located at the tip of the root. The stem cells surround four mitotically less-active cells referred to as the quiescent center (QC) (Scheres et al. 1995), and together they form the niche.

One of the four sets of stem cells, called the cortex/endodermis initials, gives rise to the two layers of endodermis and cortex through two rounds of asymmetric cell divisions. Asymmetric cell division is one of the key issues in developmental biology, because it explains the cell fate diversity in a multicellular organism (Horvitz and Herskowitz 1992). The cortex/endodermis initial (CEI) divides asymmetrically in a new CEI, thereby regenerating itself, and a CEI daughter cell (Da). A subsequent asymmetric division of the Da results in two cells of which the inner cell adapts endodermal and the outer cortical cell fate. These cells add up to the two separate layers of endodermis and cortex.

The genes *SHORT-ROOT* and *SCARECROW* play a key role in patterning of the endodermis and cortex (Benfey et al. 1993, Helariutta et al. 2000). The endodermis is entirely absent when the gene *SHORT-ROOT* is mutated (Benfey et al. 1993); asymmetric division of the Da does not take place. Further investigations of *SHORT-ROOT* revealed that the gene encodes a transcription factor which seems to be able to move from the place where it is synthesized to an adjacent cell layer. There it initiates endodermal cell fate (Helariutta et al. 2000).

The *SCARECROW* gene has been shown to be essential for the asymmetric division of the Da as well (Scheres et al. 1995). Mutation of this gene inhibits division of the Da, and the resulting single layer has mixed identity (Di Laurenzio et al. 1996). *SCARECROW* has been found to be a direct target of *SHORT-ROOT* (Di Laurenzio et al. 1996). All of the above suggests that *SHORT-ROOT* is necessary for initiating endodermal cell fate, while *SCARECROW* is needed for the asymmetric division of the CEI (Heidstra et al. 2004). Mutation in either of the genes result in differentiation of the stem cell niche, indicating that both *SHORT-ROOT* and *SCARECROW* have a function in stem cell maintenance (Nakajima et al. 2001, Sabatini et al. 2003).

The main question of this thesis is: what is the role of *SHORT-ROOT* in defining endodermal cell fate and stem cell maintenance in *Arabidopsis thaliana*? This question can be divided in subquestions which will be discussed in separate chapters. The first chapter discusses the questions why

Arabidopsis thaliana is used as a model organism and how tools and techniques are applied to study developmental biology. Questions how the root is organized, and how asymmetric cell divisions play a role in root patterning are addressed in chapter two. The following chapter focusses on the question how *SHORT-ROOT* and *SCARECROW* interact, and what their roles are in asymmetric cell division, defining cell fates and stem cell maintenance. Chapter 4 discusses questions concerning other regulators of *SHORT-ROOT* movement. How does *JACKDAW* control *SHORT-ROOT* and *SCARECROW* action, and what is its role in the genetically separated process of epidermal patterning? The fifth and last chapter comprises the recently discovered gene *SCARECROW-LIKE23*. In this thesis, an experiment in order to characterize *SCARECROW-LIKE23* is described. Yet unpublished, some results indicate that *SCARECROW-LIKE23* acts in the same pathway of root patterning as *SHORT-ROOT* and *SCARECROW*.

Developmental biology engages all aspects of growth and development of organisms. Model organisms are often used to reveal biological processes involved. These organisms are comparable with, yet simpler than complex higher organisms. In this chapter, *Arabidopsis thaliana* will be introduced as a commonly used model organism in plant research, alongside some techniques used to study the development of this plant.

1.1 Model organisms

Two of the most widely used model organisms to study processes specific for the animal kingdom are the fruit fly *Drosophila Melanogaster* and the round worm *Caenorhabditis Elegans*. Amongst other advantages, their simple body plan, short generation time and the ease at which mutation and cloning is possible, make these organisms well suitable for this purpose (Fox 1986). The discoveries made by studying these organisms have revealed a tremendous amount of genetic information (Meinke et al. 1998)

However, these organisms alone do not give a comprehensive review of multicellular organisms. Studying these organisms alone is limited because they do not represent organisms from the plant kingdom. Plants have unique organization and physiology, flowering plants in particular (Meinke et al. 1998). Several important distinctions can be made. First of all, plants do not move. Immobility requires different characteristics compared to mobile organisms, such as differences in responses to the environment. Second, cells within the plant do not migrate. Cell lineages are premeditated from the early embryo stage on and therefore the plant needs a body plan that has no urge to be changed afterwards. The meristems, zones that stay embryonically active and can produce new organs, provide cell lineages that comply with those needs (Meyerowitz and Pruitt 1985).

1.2 *Arabidopsis thaliana*

Arabidopsis thaliana (its popular name is 'thale cress', see Figure 1) is a small flowering plant and member of the mustard family (*Cruciferae* or *Brassicaceae*). Its habitat is widely spread across different continents, and many different ecotypes are documented. The most common ecotypes used for experimental purposes are Landsberg and Colombia (Meinke et al. 1998). In natural conditions, the plant will reach a height of 20 cm, has small flowers, and a rosette of leaves just above ground. The plant produces fruits known as siliques, containing the seeds.

Arabidopsis thaliana (from now on referred to as simply '*Arabidopsis*') is a commonly used model organism in laboratories worldwide. Already in 1943, Friedrich Laibach has extensively studied the genetics of this plant. (Laibach 1943) A few decades later, a conference specifically held for *Arabidopsis* research introduced an era in which hundreds of researchers would work together to make significant advances in uncovering many aspects of *Arabidopsis* genetics. The founding of the *Arabidopsis* Genome Initiative in 1996 has led to the complete genomic sequence by the end of 2000. The genome of *Arabidopsis* is the first completed sequence of a plant. All the genomic information and the complete sequence is freely accessible on The *Arabidopsis* Information Resource (TAIR) website

(www.tair.com) and newly discovered genes and other information are collected in the database as well. Besides the world-wide cooperation and the fact that the entire genome sequence is known, *Arabidopsis* has many other advantages that make the plant an ideal model organism.

Growth conditions and reproduction cycle

Arabidopsis can be easily grown on moist soil, with fluorescent light as a source for photosynthesis and it takes little effort to keep the plant alive in laboratory conditions. The plant is small, which makes it possible to grow many plants in limited space. Also, seeds can germinate on sterile Petri-dishes with growth medium, making the analysis of the usually-underground root system conveniently accessible (Meyerowitz et al. 1995).

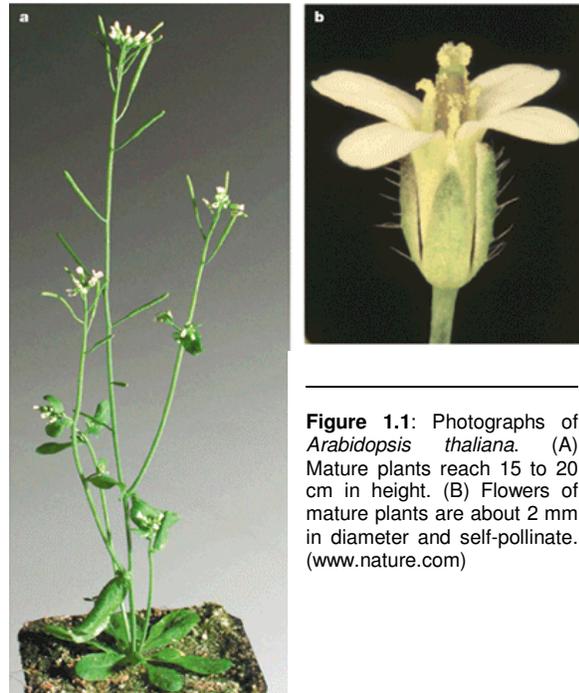


Figure 1.1: Photographs of *Arabidopsis thaliana*. (A) Mature plants reach 15 to 20 cm in height. (B) Flowers of mature plants are about 2 mm in diameter and self-pollinate. (www.nature.com)

The entire reproduction cycle, including germination of a seed, development of basic parts of the plant and maturation of the first seeds, is completed in approximately six weeks. The short generation time is a big advantage compared to other flowering plants. Moreover, a single plant can produce thousands of seeds. Plants carrying a heterozygous recessive mutation, produce progeny of which 1/4 will be homozygous for that mutation. Double heterozygous recessive mutations segregate already 1/16. Therefore, producing many seeds is an advantage concerning the preservation of genetic lines that are artificially made.

Genome

By the end of the year 2000, the first analysis of the entire genome of *Arabidopsis* was reported (*Arabidopsis* Genome Initiative, 2000). Over 25.000 protein-coding genes were found in the sequence of approximately 120 megabases, a relatively small genome compared to other species previously studied (see Table 1). This small genome contains largely unique sequences, and repetitive DNA is limited. This is an advantage for sequencing the genome. Large parts of genomic DNA which are similar in sequence, cause problems in mapping the sequenced fragments to the eventual genome sequence. A disadvantage of much repetitive DNA is that it can be responsible for relocation or replacement of genes (Brown 2007). When a gene of interest is not stable positioned in a genome, the process of cloning can be disturbed.

Table 1: Nuclear genome size in different species

Popular name	Scientific name	Genome size (Mb)
Wheat	<i>Triticum aestivum</i>	15,966
Garden pea	<i>Pisum sativum</i>	3,947
Corn	<i>Zea Mays</i>	2,292
Tomato	<i>Lycopersicum esculentum</i>	907
Thale cress	<i>Arabidopsis thaliana</i>	120

Genome size in millions of bases (Mb). *Arabidopsis thaliana* has one of the smallest plant nuclear genomes known so far. (Adapted from www.scielo.cl)

As mentioned previously, the sequence which is hosted by the The *Arabidopsis*

Information Resource is freely accessible, along with the vast information such as gene annotations, available clonal vectors, allelic polymorphisms and literatures of reference.

1.3 Tools and techniques used for analyzing *Arabidopsis* development

The function of a gene of interest is hard to address as long as the gene is fully functional. The function of a gene comes to light when the gene is not functional and a phenotype can be observed (Brown 2007, p. 149). To study a gene or a set of genes involved in a process, several techniques are available.

First, a gene of interest needs to be identified. **Forward genetics** are applied in order to identify genes that are responsible for a specific phenotype. An altered genotype is obtained by inducing random mutations, by radiation, by T-DNA insertions or adding chemical mutagens such as ethylmethane sulfonate (EMS). With sufficient population, the altered genotypes lead to observable phenotypes. Then, through a series of breeding of subsequent progeny, individuals with a selected phenotype are isolated. The next step in the process of analyzing genes of interest, is **reverse genetics**. Reverse genetics encompasses altering the function of a gene given the corresponding DNA sequence. The genome of *Arabidopsis* is fully sequenced, which makes it possible to apply reverse genetics by either looking for genes in the genomic sequence, or by genes that were found during forward genetics. **Cloning** the mutations in a host organism such as the bacterium *Escherichia coli* makes it able to preserve mutated DNA. Another way to apply reverse genetics is using **RNA interference** (RNAi). RNAi induces a loss-of-function effect by degradation of the RNA of a gene of interest. This method does not alter the genomic DNA-sequence, since the effect is post-transcriptional .

To further investigate a certain gene, **genetic constructs** are designed that alter the function of that gene. Besides 'loss of function', some of those genetic constructs comprise overexpression, tissue-specific expression and inducible expression. In overexpression, the gene is fused to an artificially inserted constitutively expressed promoter such as the 'cauliflower mosaic virus 35S promoter', which is continuously expressed in most plant tissues (Odell et al. 1985). This technique is useful because it shows the consequence of a gain-of-function mutation. A more specific expression is obtained when the gene of interest is designed to express in a tissue-specific manner, achieved by expressing the gene under the control of a tissue-specific promoter. Both overexpression and tissue-specific expression can also be induced on a moment of choice and the level of desire when fused to a promoter that inducible by controllable conditions. In this way, the difference between a phenotype with and without gene expression can be followed in time. Furthermore, using an inducible promoter allows the plant to develop without the effects of the mutation, until the induction of the mutation.

Genes are introduced into plant by *Agrobacterium tumefaciens* induced **transformation**. In short, this technique uses the integrative properties of bacterial transfer DNA (T-DNA) combined with *Agrobacteria*. These bacteria export the constructed DNA of interest from the bacterium into the plant genomic DNA (Gelvin 2003).

After finding and altering the gene of interest, the gene needs to be visualized. Several methods of visualizing the effects of the mutation are available. If the constructed gene is fused to a **reporter gene**, the resulting protein can be visualized. *Green Fluorescent Protein (GFP)* is the most commonly used reporter gene. Transcription of the reporter gene results in a protein that is able to be visualized using a microscope which can detect GFP (Prendergast and Mann 1978, see for an example Figure 1.2). Yellow Fluorescent Protein (YFP) is an alternative. Another frequently used reporter gene is *β -glucuronidase (GUS)*, a protein that is visible using a light microscope when stained with additional chemicals. Besides visualizing *GUS*, **staining** has a variety of applications. Cell type is determined by staining characteristics specific for a certain cell type. For example, the endodermis can be distinguished from other cell types in the *Arabidopsis* root by visualizing the Casparian strip.

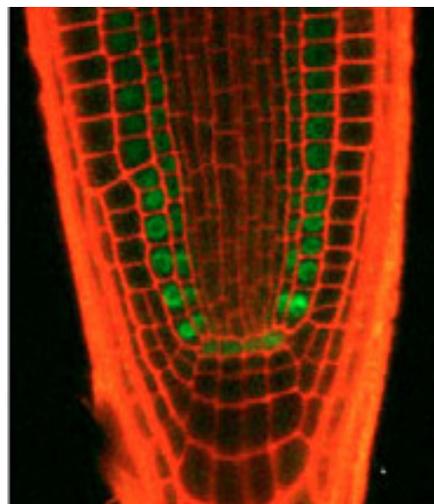


Figure 1.2: CLSM image of *Arabidopsis* root. Propidium iodide (red) marks the cell wall, GFP is fused to a protein of interest (Hassan et al. 2010).

To visualize the staining or proteins fused to reporters such as the afore mentioned GFP, **confocal laser scanning microscopy (CLSM)** is a widely used tool. These microscopes are able to section the whole tissue into specific micrometer-scale focal planes. By laser scanning, the microscope is able to detect fluorescence. Using fluorescent chemicals or proteins fused to reporters such as GFP, the sample can be visualized. For individual cells to become visible, Propidium iodide (PI) is used. Cells in plants are separated by cell walls, which allow PI to penetrate the space between the individual cell membranes. As a consequence, using the right settings, the space between cells become visible so size, number and location of cells can be determined (see Figure 1.2).

Genetic nomenclature

adapted from Leyser and Day 2003, p. 42

The system of genetic nomenclature differs amongst studied species. Here, the conventions of *Arabidopsis* are used. Four different notations can be distinguished concerning a single gene. Uppercase italics are used for the wild-type gene. For example: *SHORT-ROOT*, or abbreviated *SHR*. Lowercase italics refer to the gene when it is mutated. When a gene is expressed in a *shr* background, it means that there is no *SHORT-ROOT* present. The last two notations describe the protein the gene is encoding for: *SHORT-ROOT (SHR)* for the wild-type protein, *short-root (shr)* when no protein is present.

Organisms can have their genes mutated at different places within the sequence of the gene. Therefore, different mutant alleles are available. These are notated by the mutated gene, followed by a hyphen and the number given to the allele: *shr-2*.

Constructs of genes are described using the following symbols. When a protein is fused to a visible marker, for example green fluorescent protein (GFP) or beta-glucuronidase (GUS), a double colon is used (*SHR::GFP*, *SHR::GUS*). When a protein is expressed under the promoter of a different gene, the letter p is advancing the gene name. As an example: *pSCR::SHR* is a construct where *SHORT-ROOT* is expressed, when the cell actually expresses the *SCR* gene by inducing its promoter.

Mutation analyses are used to reveal the function of genes. Forward genetics (see previous chapter) are used to find unknown genes which are involved in a process that results in an observable altered phenotype. To assess the differences after altering the genotype, proper controls from a wild-type plant are needed: “*To understand how normal development proceeds and to identify precisely the defects caused by the mutations, a detailed knowledge of the development and structure of the system under analysis is necessary*” (Dolan et al. 1993). This chapter describes cellular organization of the *Arabidopsis* root meristem and the underlying mechanism which patterns the root.

2.1 Radial Organization of the *Arabidopsis* root

Arabidopsis has two apical meristems, laid down already early during embryogenesis. The shoot apical meristem (SAM) gives rise to the organs that are above ground, the other is called the root apical meristem (RAM) and gives rise to the root. In a mature seedling, the meristematic zone in the root constitutes about 250 µm at the very end of the root. There, the cells are small and overlaid by a root cap. More towards the SAM, the elongation zone is situated. The elongation zone is defined by cells being twice the size they had when they were still in the meristematic zone. The cells are already adapting fate and the process of differentiation is accomplished once the cells are in the last zone, the differentiation zone (Dolan et al. 1993, Scheres et al. 1995).

The root of *Arabidopsis* consists of single concentric layers of epidermis, cortex, endodermis and pericycle encircling the stele. The basal tissue of the root is composed of the columella and lateral root cap. The origins of the cell lines come from a particular set of stem cells called the initials (see Figure 2.1). Surrounding four mitotically less-active cells referred to as the quiescent center (QC), these initials comprise the epidermal/root cap initials, columella initials, cortex/endodermal initials and the stele/pericycle initials, each giving rise to corresponding tissues.

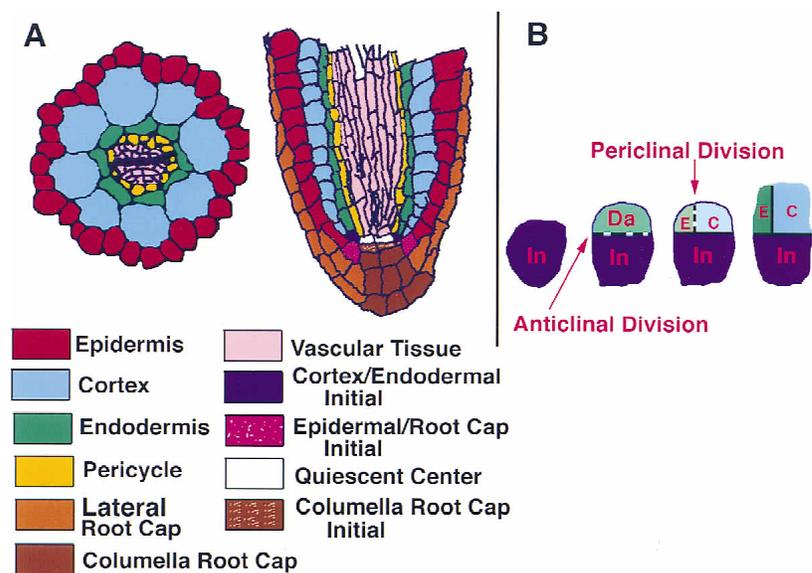


Figure 2.1: Cellular organization of the *Arabidopsis* root meristem. Transverse and longitudinal cross sections (A) show the organization of the concentric layers, as well as the stem cell niche. (B) Schematic representation of the asymmetric divisions of the CEI (In) and the CEI daughter cell (Da) (Dolan et al. 1993).

Epidermis and lateral root cap

The epidermis/lateral root cap initials give rise to the two separate layers of lateral root cap and the epidermis. The outermost layer of the root is the lateral root cap which extends to the elongation zone. Together with the central root cap on the tip of the root it is called the root cap which protects the fragile meristem from being damaged by friction while growing into the soil (Leyser and Day 2003, p. 7). The epidermis lies adjacent on the inside of the lateral root cap and is the outermost layer more towards the SAM. Cells in the epidermis are either trichoblasts or atrichoblasts. Trichoblasts develop root hairs which extend into the soil where they can take up minerals and water, while atrichoblasts do not. As described in chapter 4, the pattern of cells developing in either of the two is tightly regulated (Hassan et al. 2010, Galway et al. 1994).

Columella

The columella root cap initials give rise to the central root cap, referred to as the columella, which is part of the protective shield that surrounds the meristem.

Cortex and endodermis

In the meristematic zone, eight radially organized cells make up the endodermis, a single layer of cells. The radial and transverse cell walls of the endodermis cells contain a structure of cork, known as the Casparian strip. It blocks the passage of water and soluble materials, thereby enabling selective passage into the stele. Normally, the structure of cell walls enables non-selective passing but due to the Casparian strip, these materials have to cross the cytoplasm of the endodermal cells, making the endodermis able to regulate in- and efflux. The Casparian strip is a specific feature of the endodermis which can be used for cell type determination (Esau 1977, p. 215-242).

The cortex lies in between the endodermis on the inside and epidermis on the outside. It is a layer of morphologically rather unspecialized cells that function as storage for, among others, carbohydrates and plant oils. As the endodermis, eight radially organized cells make up a single layer of cortex in *Arabidopsis*. Both the endodermis and cortex trace their lineage back to the Cortex/Endodermis Initials (CEI), a ring of eight cells in total, that lies immediately outside the four QC cells.

Cells from the CEI undergo two subsequent asymmetric divisions (See Figure 2.1B). The first divides the CEI anticlinically into a new CEI and a CEI daughter cell (Da). As will be explained later in this chapter, another asymmetric cell division takes place, where the daughter cell divides periclinally into an endodermal and cortical cell, which together are called the ground tissue. Subsequent cell divisions of these cells will give rise to the eventual layers (Dolan et al. 1993).

Pericycle and vascular bundle

The vascular bundle, often referred to as the 'stele' could be described as a long cylinder running through the center of the root. Its basic function is transport of nutrients and other molecules such as hormones up and down the plant. Looking at the root in the horizontal plane, a few rings of cells make up the stele. Part of the stele is made up of end-to-end connected cells called sieve elements and has the function of translocating soluble materials throughout the plant. Cellular functioning of the sieve tube is provided by phloem companion cells, which are connected to the sieve elements by small holes in the cell-wall called plasmodesmata (Oparka and Turgeon 1999). Phloem primarily transports nutrients from the green parts of the plant to the root, while xylem, another part of the stele transports water and minerals from the root to the top of the plant.

The cells from the vascular tissue originate from stem cells pericycle/vascular initials that divide into a new initial cell and a stele cell. The latter adds up to form the eventual tissue. The outer most ring of cells in the stele is called the pericycle. This unique layer of cells is able to generate lateral roots by renewal of cell division of a pericycle cell, then growing through the adjacent cell layers to the outside of the root.

2.2 Stem cells and asymmetric cell division

Stem cells

A stem cell possesses two properties: self-renewal and the potency to differentiate. Given this description, stem cells divide per definition asymmetric. In the *Arabidopsis* root meristem, the initials can be considered stem cells (Sabatini et al. 2003). The CEI as well as the subsequent division of its daughter are asymmetric (Dolan et al. 1993). As shown in Figure 2.1B, The first asymmetric division is anticlinal (i.e., in the horizontal plane), while the second division is periclinally (i.e., in the vertical plane).

Asymmetric cell division can be regulated by intrinsic or extrinsic factors

There are two basic mechanisms underlying asymmetric cell division. The first is 'intrinsic', meaning that the information needed for the cell to execute the division is coming from the cell itself. The other mechanism, 'extrinsic', comprises a system by which information from the outside of the cell defines the asymmetry of the resulting daughter cells (see Figure 2.2). In the case of a stem cell niche, the latter is mostly the case, although a combination of the two is also possible (Hawkins and Garriga 1998, ten Hove and Heidstra 2008). The micro-environment providing the extrinsic factors, defines the stem cell niche (Spradling et al. 2001).

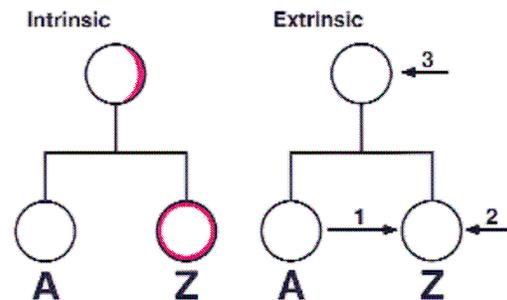


Figure 2.2: Schematic representation of intrinsic and extrinsic asymmetric cell division. Intrinsic factors are indicated in red and are unequally distributed in the progenitor cell, thereby creating daughters with different cell fates (A and Z). Extrinsic factors determine cell fate after symmetric division by signalling either (1) between daughter cells, (2) between a daughter and the surrounding cells, or (3) between the progenitor cell and its daughter cells (Hawkins 1998).

In the root meristem, asymmetric divisions are for the greater part regulated by extrinsic factors. Evidence lies in the presence of four regulating cells, the QC. The cells show little or no mitotic activity, but are thought to have an important role in maintaining the initials surrounding them (Scheres et al. 1995). The quiescent center was experimentally found when cells were stained with [³H]thymidine. This makes nuclei in the S-phase visible, an indication that a cell is dividing and hence 'active' (Dolan et al. 1993). The role of the QC was discovered using laser ablation experiments. During laser ablation, a precisely-positioned laser beam is pointed at a cell and the intensity of the laser beam, often provided by multiple lasers, kills the cell (Scheres 1995). When no QC is present, the root is unable to maintain its undifferentiated state. This defines the QC as an 'organizing center' (van den Berg et al. 1997). However, initials from the adjacent stele take the place of the ablated cells and take over QC fate, thereby restoring the QC.

This, together with the results of other laser ablation experiments, has led to the conclusion that position rather than cell lineage determines cell fate in the root meristem (Dolan et al. 2000, Leyser and Day 2003, p. 46). The apparent positional information and the role of the QC concerning initial maintenance, suggest that asymmetric division of the initials is controlled by extrinsic factors.

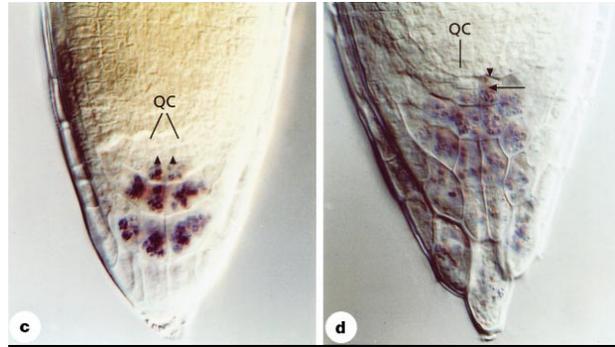


Figure 2.3: The QC inhibits differentiation of cells in the stem cell niche. Here is shown that when the QC is ablated, columella initials synthesize strach granules, an indication of columella differentiation. (C) Shows the wild-type root, (D) shows the root meristem after ablation of the QC. Because the root gets disorganized, the QC is defined as an 'organizing center' (Van den Berg 1997)

After clarifying the basic morphology of the root meristem, the underlying genetic mechanisms of *Arabidopsis* root radial patterning is described in this chapter. Cellular behavior is regulated by gene expression. Consequently, transcription factors are of great interest, because cell fate determination is partly dependent on the activity or synthesis of transcription factors (Leyser and Day 2003, p. 26). In this chapter, the putative transcription factors *SHORT-ROOT* and *SCARECROW* will be described. They play a key role in endodermis specification, asymmetric cell division of the CEI and stem cell niche maintenance. Some open questions are summarized in the discussion.

3.1 *SHORT-ROOT*

The simple radial patterning of the *Arabidopsis* root has a complex underlying genetic mechanism. In 1993, four mutant lines were identified by forward genetics that had abnormal root structures. *short-root* (abbreviated *shr*, see chapter 1 for nomenclature) was one of them (Benfey et al. 1993).

Mutant phenotype

The phenotype of the mutagenized root appeared to have two defects compared to the wild-type root. The first was that the meristem had lost its ability to stay undifferentiated: the QC lost its function as an organizing center (Sabatini et al. 2003). As shown in Figure 3.1, growth of the root ceased. From this defect, the term *SHORT-ROOT* was adapted (Benfey et al. 1993). The second defect was that only one cell layer originated from the CEI, instead of two in the wild-type plant. As described in chapter 2, after the asymmetric

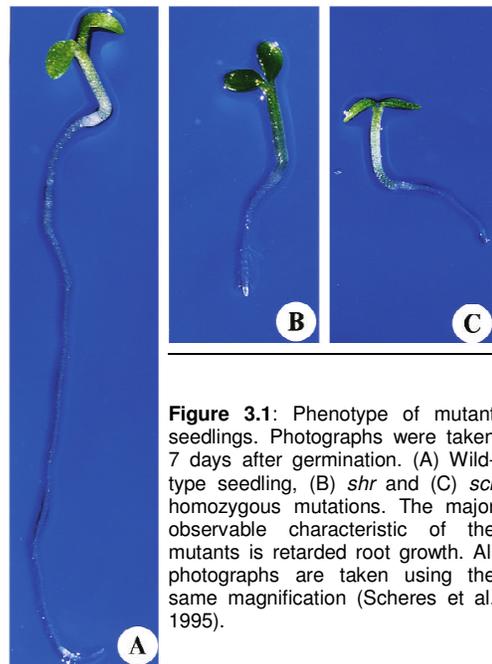


Figure 3.1: Phenotype of mutant seedlings. Photographs were taken 7 days after germination. (A) Wild-type seedling, (B) *shr* and (C) *scr* homozygous mutations. The major observable characteristic of the mutants is retarded root growth. All photographs are taken using the same magnification (Scheres et al. 1995).

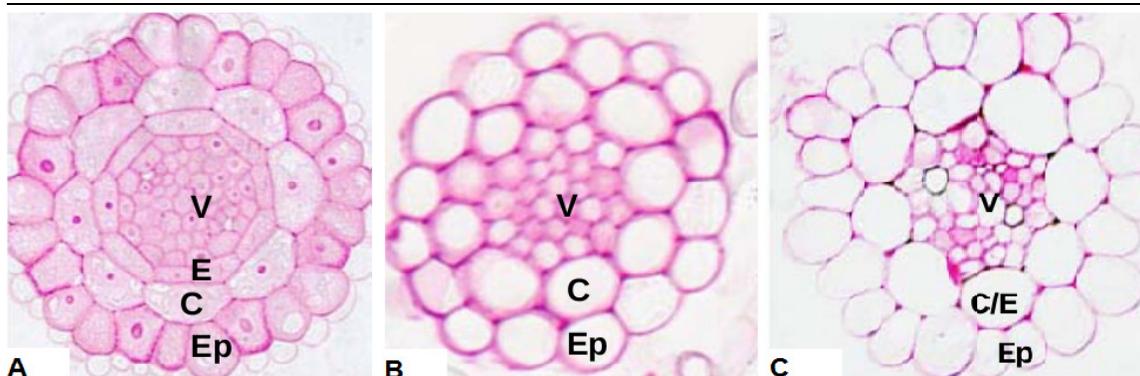


Figure 3.2: Cross sections of the *Arabidopsis* root in the elongation zone. (A) Wild-type root. The ground tissue consists of separate layers of cortex and endodermis. (B) Homozygous *shr* mutant. The the ground tissue consists of just one cell layer with endodermis identity. (C) The cell layer with both endodermis and cortex identity results from the *scr* mutation. V, vascular bundle; E, endodermis; C, cortex; Ep, epidermis (Welch et al. 2007)

division of the CEI daughter cell, two layers of tissue adopt different fates. After staining the Casparian strip markers specific for endodermis, which appeared to be absent, Benfey et al. concluded that this mutant was lacking the endodermis (see Figure 3.2). As a consequence, *SHR* is considered essential for endodermis specification and the asymmetric division of the CEI daughter cell. To test whether *SHR* is both necessary and sufficient for these processes, a construct was designed which constitutively expresses *SHR* (*35S::SHR*). Ectopically expressed *SHR* resulted in extra cell divisions of the root meristem, thereby initiating supernumerary cell layers in the root (Helariutta et al. 2000).

Characterization and function of *SHORT-ROOT*

In order to find the functions of a newly found gene, it is important to know where the protein executes its function. In order to visualize the protein distribution, a genetic construct was designed that fuses the *SHR* protein to GFP under the control of its own promoter (*pSHR::SHR::GFP*) (Nakajima et al. 2001). Fluorescence could be detected in the stele and the cell layer surrounding the stele (endodermis, CEI and QC, shown in Figure 3.3). The distribution of *SHR::GFP* within the cells of the vascular tissue is both nuclear and cytoplasmic, but in the endodermis the GFP was detected exclusively in the nuclei of the cells. Notably, GFP was found in both the nuclei of the cells resulting directly from the Da division, but only in the endodermis GFP was maintained (Nakajima et al. 2001).

Strikingly, the *SHR* gene is not expressed in the endodermis, nor in the initials or QC, shown by *in situ* hybridization of *SHR* in wild-type plants (Helariutta et al. 2000). As shown in Figure 3.4, no *SHR* transcript is detected in the layers surrounding the vascular tissue. A complementing experiment shows that expression of *pSHR::GFP* which only marks promoter activity, can only be detected in the stele tissue, but not in adjacent tissue layers (Figure 3.3, inset). All of the above suggests that *SHORT-ROOT* moves from the stele tissue into the endodermal layer (Helariutta et al. 2000, Nakajima et al. 2001).

Movement of *SHORT-ROOT*

Movement of transcription factors is not often encountered. Transcription factors are able to travel from nucleus and cytoplasm and vice versa, but on the whole they are not thought to travel between different cells. This is because transcription factors lack a signal peptide that directs intercellular movement. Several exceptions are documented, under which the *KNOTTED-1* transcription factor in maize (Prochiantz and Joliot 2003).

The greater part of moving transcription factors is most likely regulated, and not by random diffusion (Gallagher et al. 2004). A way for proteins to go from one cell to another in plants is through

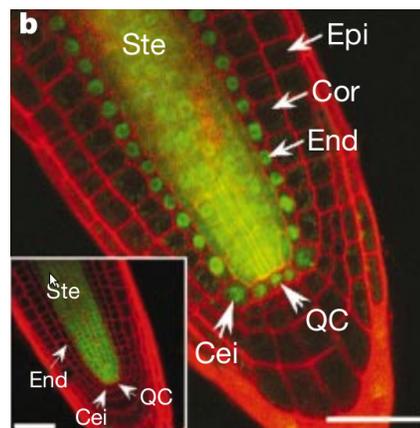


Figure 3.3: *SHR* protein localization. GFP is visible in the endodermis (End), quiescent center (QC) and cortex/endodermis initial (Cei). Inset shows transcriptional fusion of *SHR* to *GFP*, visualizing the transcription pattern of *SHR*. Ste, stele; Epi, epidermis; Cor, cortex; End, endodermis. Scale bars, 50 μ m (Helariutta et al. 2000, Nakajima et al. 2001).

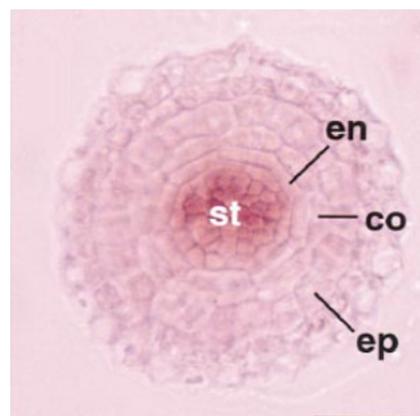


Figure 3.4: *In situ* RNA hybridization with a *SHR*-specific probe. *SHR* is only expressed in the stele. st, stele; en, endodermis; co, cortex; ep, epidermis (Helariutta et al. 2000).

plasmodesmata. Plasmodesmata are microscopic channels between cells composed of interconnected cell membranes. Size exclusion limit and other mechanisms make it possible to regulate protein trafficking. Movement of proteins using the plasmodesmata apparatus is called 'targeted movement', while movement through random diffusion through plasmodesmata or the cell membrane is called 'non-targeted' (Gallagher et al. 2004). An extensive study was published in 2004 by Sena et al. concerning the intercellular movement of SHR, where it was shown that movement of SHR is targeted instead of by random diffusion to the next cell layer. *SHR::GFP* was specifically expressed in the phloem companion cells (see chapter 2), where normally no *SHR* is expressed. No GFP was detected in the neighbouring layer (Figure 3.5A), despite the fact that clear connections exist between phloem companion cells and their neighboring sieve tube elements.

The question remaining, is how movement of SHR is restricted to just one cell layer. Different hypothesis exist to address this question. It could be possible that SHR is moving also to the cortex and further, but is rapidly degraded there. Another option is that a mechanism present in the endodermis layer is restricting movement of SHR. It is also possible that the connection between the stele and endodermis allows movement of SHR, whilst the connection between the endodermis and cortex does not. The results of various experiments are pointing to the second alternative, where a mechanism present in the endodermis restricts SHR movement.

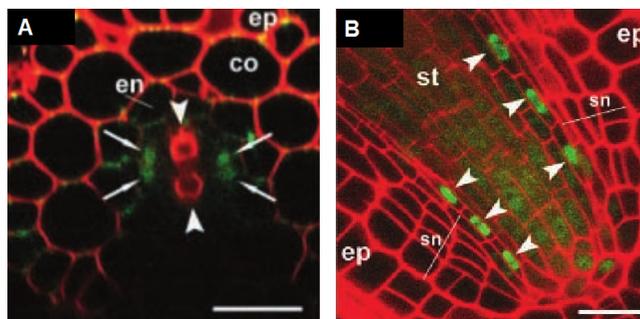


Figure 3.5: Transverse cross section (A) and longitudinal cross section of the root meristem showing the fusion protein *SHR::GFP*. Arrows in (A) indicate that *SHR::GFP* does not leave the phloem companion cells. (B) *SHR::GFP* moves only into the first of the supernumerary layers, which appear to be endodermis-like. Sn, supernumerary layers. Other abbreviations as in Figure 3.4. Scale bar, 25 μ m (Sena et al. 2004).

First, an experiment was executed to find out whether the one-layer-movement could be explained by the differences in symplastic connections

such as plasmodesmata of the cell layers involved. A prediction from this hypothesis would be that SHR moves from one endodermis layer to the other. Because in wild-type plants only one layer is present, plants were grown which had numerous layers of presumptive endodermis. Supernumerary layers were obtained by expressing *SHR* under the promoter of *SCR* (*SCRpro::SHR*), which is specifically expressed in the endodermis (Nakajima et al. 2001). Movement of *SHR* in these plants is also restricted to one cell layer (shown in Figure 3.5B). Therefore, difference in symplastic connections does not result in the restriction of movement (Sena et al. 2004). In addition, overexpression analyses show that *SHR::GFP* can accumulate in the cell layer adjacent to the endodermis (Helariutta et al. 2000). A mechanism that rapidly degrades SHR after movement from endodermis to cortex is therefore unlikely. More evidence is needed to exclude this option.

Another factor which is involved in defining two separate layers of ground tissue, is the gene called *SCARECROW*. The gene is a direct target of SHR and expressed in the endodermis, suggesting that a genetic mechanism at least partly located in the endodermis restricts movement of SHR.

3.2 SCARECROW

In 1995, the *scarecrow* (*scr*) mutation was first reported (Scheres et al. 1995). Named '*scr-1*', it had a phenotype of greatly reduced root length compared with wild-type (Figure 3.1C). It is, like SHR, a member of the GRAS family of genes and a putative transcription factor. Also, the discovery of this

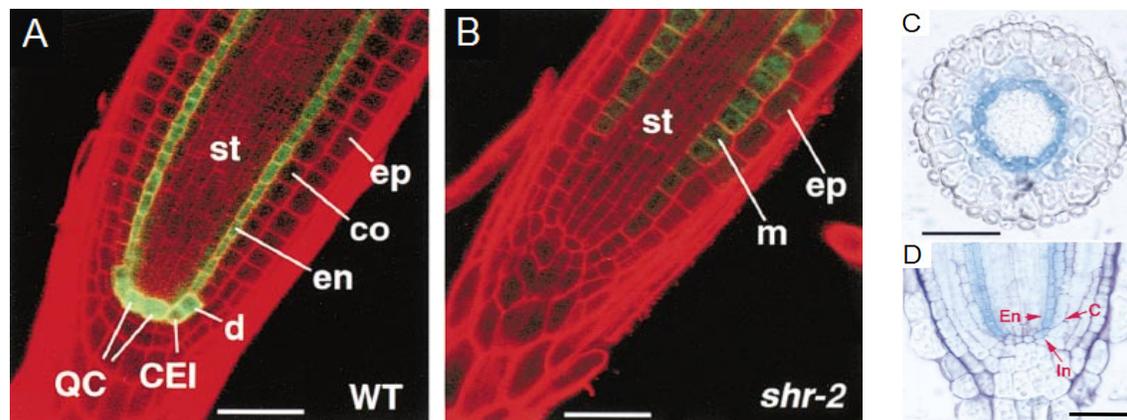


Figure 3.6: *SCR* is expressed in the endodermis, CEI, CEI daughter cell and the QC in wild-type plants. (A) Longitudinal cross section showing *SCR::GFP* in green. (B) *SCR::GFP* synthesis is greatly reduced in *shr-2* background (Helariutta et al. 2000). (C,D) In situ hybridization of a *SCR*-gene specific probe in transverse (C) and longitudinal (D) cross section (Di Laurenzio et al. 2006). d, CEI daughter cell; m, mutant cell layer; In, cortex/endodermis initial. Other abbreviations as in Figure 3.4. Scale bars 50 μ m.

gene led to the definition of a novel protein family, which are evolutionary conserved (Di Laurenzio et al. 1996). During embryonic development, *SCR* is expressed in the ground tissue of late heart-stage embryos. In this stage, the ground tissue is not yet divided into an endodermal and cortical cell layer. After division of the ground tissue into an endodermal and cortical cell layer, expression can be observed only in the endodermis, as in mature roots (Figure 3.6A). Using in situ hybridization of RNA, Di Laurenzio et al. showed that in the meristem of mature roots, *SCR* is specifically expressed in the endodermis, CEI and QC (Figure 3.6C and D).

Mutant phenotype

Mutating *SCARECROW* results in reduced root length and one layer of ground tissue. The phenotype differs from *shr* roots in that the effect on root length of the *scr* mutation is not as strong.

In addition, the identity of the layer differs between the two mutants. Whereas in the *shr* mutant the entire endodermis is missing, in *scr* plants the resulting single layer has mixed identity: both endodermis and cortex features can be made visible using corresponding markers, as depicted in Figure 3.7 (Nakajima et al. 2001).

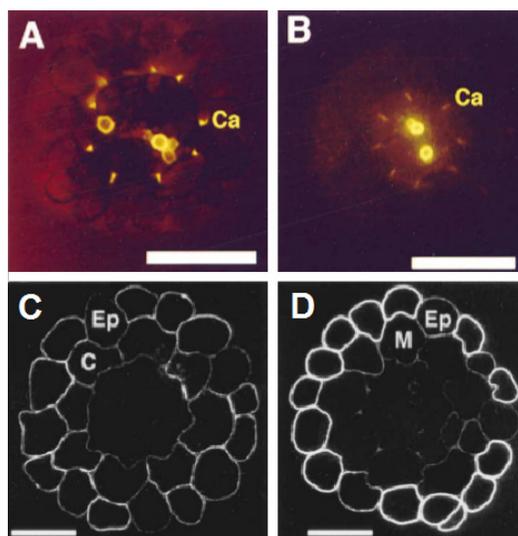


Figure 3.7: In *scr* roots, the mutant cell layer is of mixed identity. (A,B) Casparian strip staining of *scr-1* (A) and wild-type (B). (C,D) Immunostaining with the cortex and endodermis specific marker CCRC-M2. Wild-type (C) and *scr-1* (D). Ca, Casparian strip; C, cortex; Ep, epidermis; M, mutant cell layer. Scale bars (A,B) are 50 μ m, (C,D) are 30 μ m (Di Laurenzio et al. 1996).

Function of *SCARECROW*

One basic function of the *SCR* gene is stem cell maintenance. The QC plays a central role in positioning the stem cell niche (see chapter 2), and specific markers to identify the QC are lost in *scr* backgrounds (Figure 3.8). To test whether the expression of *SCR* in the QC is necessary and sufficient, a construct was designed in order to re-express *SCR* in the QC of a *scr* mutated plant.

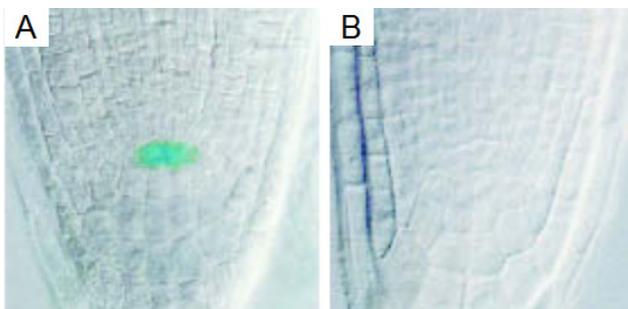


Figure 3.8: *SCR* plays a role in maintaining the QC. Staining with a marker specific for the QC (A) is lost in *scr* mutants (B) (Sabatini et al. 2003).

Staining by the specific markers was observed, suggesting that QC activity was at least partially restored. In conclusion, *SCR* plays a key role in maintaining the stem cell niche (Sabatini et al. 2003).

3.3 Restriction of *SHORT-ROOT* and interaction with *SCARECROW*

Another role of *SCR* is in defining separate endodermis and cortex cell fates, indicated by the absence of these two separate layers of ground tissue in *scr* roots. This section describes how *SCR* and *SHR* act together in a pathway that eventually defines separate cortex and endodermis cell fates.

***SHR* induces *SCR* expression**

Proper *SCR* expression requires expression of *SHR*. Plants carrying the *shr* mutation show greatly reduced expression of *SCR* in the endodermis (compare Figures 3.6A and B). Epistasis analysis places the *SHR* gene upstream of *SCR*: double mutants show the same phenotype as the *shr* mutant, whereas in a *scr* background, no effect on the amount of *SHR* is detectable (Helariutta et al. 2000, Nakajima et al. 2001).

***SCR* acts in an autoregulatory pathway**

Once *SHR* has moved from the stele into the endodermis, it triggers *SCR* transcription. Reciprocal pull down experiments using coimmunoprecipitation showed that *SHR* and *SCR* directly interact, and activation of *SCR* expression requires a complex of *SHR* and *SCR* (Cui et al. 2007). After a certain amount of *SCR* protein is reached, *SCR* acts in an auto regulatory pathway and becomes desensitized of *SHR* in the QC, while it remains dependant on *SHR* in the maturing ground tissue (Heidstra et al. 2004). This positive feedback loop induces a stable amount of *SCR* present in the endodermis (Heidstra et al. 2004).

***SCR* restricts *SHR* movement**

As mentioned previously, *SHR* moves from the stele into the endodermis, but not farther. A strong theory is that restriction of *SHR* to the endodermis is regulated on the level of subcellular localization. This hypothesis is supported by the fact that *SHR* fused to a strong nuclear localization signal (NLS) is no longer capable of moving (compare Figures 3.9C and D) (Cui et al. 2007, Sena et al. 2004).

The finding that SHR and SCR directly interact indicates that there is a role for SCR in the localization of SHR into the nucleus (Gallagher and Benfey 2009). As shown in Figure 3.9B, the fusion protein SHR::GFP becomes largely cytoplasmic in the mutant cell layer of *scr* roots, suggesting that SCR is necessary to translocate SHR to the nucleus (Cui et al. 2007, Sena et al. 2004). Further evidence for a role of SCR in restricting SHR to the endodermis comes from the finding that SHR::GFP can move farther than the endodermis when expressed in cloned endodermal cells carrying the *scr* mutation (Heidstra et al. 2004).

Due to the restriction of SHR to the endodermis, no renewed induction of SCR in the cortex is possible. Consequently, it effectively limits periclinal divisions in the ground tissue.

3.5 Summary

Both *shr* and *scr* mutations lead to a phenotype of retarded root growth, loss of the organizing center and one layer of ground tissue instead of two. Although the mutants resemble in phenotype, the identity of the single layer of ground tissue differs between *shr* and *scr*. In *short-root*, the absence of the Casparian strip suggests that the root is lacking the endodermis. Mutations of *scr* result in a cell layer with characteristics of both cortex and endodermis. *Scarecrow* mutations result in a cell layer with characteristics of both cortex and endodermis. In conclusion, *SHR* is involved in endodermis specification and together with *SCR* it plays a role in asymmetric division of the CEI daughter.

The mechanism behind these mutant phenotypes involves movement of SHR. In Figure 3.10, a model is depicted in which the following summary is schematically represented. After its movement into the endodermis, SHR activates transcription of *SCR*. Together with SHR, SCR promotes its own transcription, thereby initiating a stable feedback loop. Because SCR translocates SHR to the nucleus, SHR is not able to move farther than one cell layer. This mechanism sets a difference between the endodermis and cortex protein composition, thereby defining endodermal cell fate. Together with stem cell niche maintenance

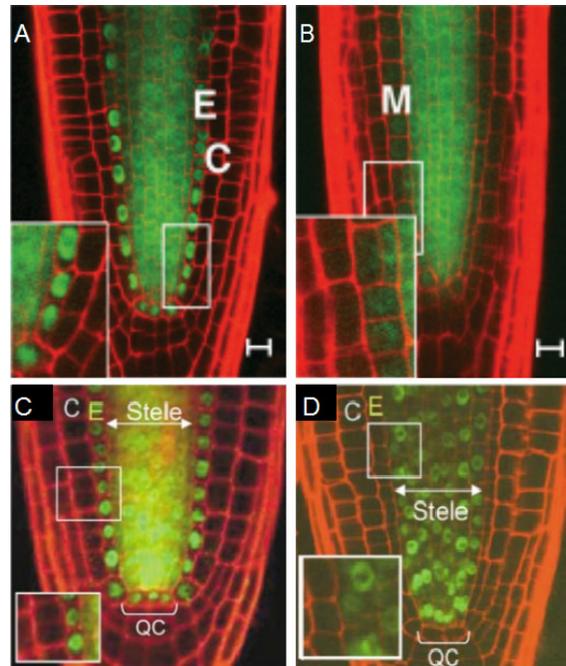


Figure 3.9: Involvement of SCR in nuclear localization of SHR. (A,B) In contrast with the wild-type situation (A), SHR::GFP is detected in the cytoplasm of cells in *scr* mutant cell layer (B) (Cui et al. 2007). (C) SHR::GFP accumulates in the endodermis as well. (D) Fusion to a strong NLS makes SHR incapable of moving (Gallagher et al. 2004). Scale bars in (A,B) are 10 μ m. E, endodermis; C, cortex; M, mutant cell layer.

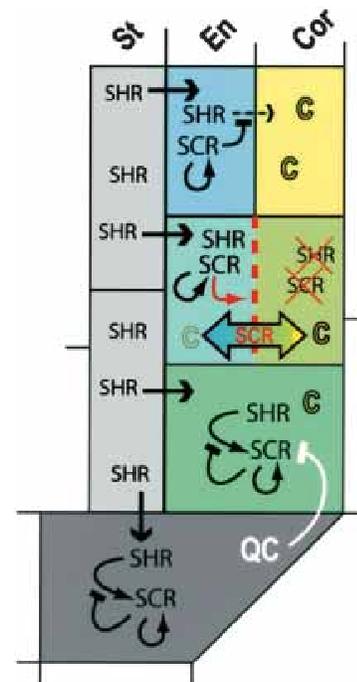


Figure 3.10: A model for SCR and SHR function in QC and ground tissue (Heidstra et al. 2004).

and QC specification, this is an important function of SHR and SCR in patterning of the *Arabidopsis* root.

However, there are many indications suggesting the existence of more factors involved in determining the different cell layers of the ground tissue (Cui et al. 2007, Heidstra et al 2004). Some of those factors will be described in the next chapters.

3.4 Discussion

The mechanism in which SCR and SHR act is complex, and there are questions still unanswered. Since in *scr* plants division of the CEI still takes place, SCR cannot account for the division by itself (Cui et al. 2007). Cell fate is determined rapidly after periclinal division of the CEI daughter cell, possibly by yet undiscovered additional factors. Future research has to reveal those factors (Heidstra et al. 2004). Furthermore, the influence of nuclear and cytoplasmic localization on movement, and the only conclusion that could be drawn is that both nuclear and cytoplasmic localization are required for SHR movement. This discrepancy is hard to address because the only conclusion could then be that both nuclear and cytoplasmic localization are required for SHR to move. A possible explanation is that by nuclear localization SHR gets modified in such way, that it promotes its own movement once cytoplasmic localized (Gallagher and Benfey 2009). However, to verify this hypothesis, more evidence is needed.

SHORT-ROOT and SCARECROW act in the same pathway. However, the regulatory pathway defining two separate layers of endodermis and cortex identity is more complex than implied in the previous chapter. SHR and SCR, despite being in the same pathway, also have their own distinct functions. And rapidly after periclinal division of the CEI daughter, cell fate is determined. Among others, these observations suggest that there are more factors involved in the genetics behind radial patterning (Heidstra et al. 2004, Levesque et al. 2006, Welch et al. 2007). In this chapter, two factors that interact with *SHR* and *SCR* are described.

4.1 JACKDAW

In 2007, Welch et al. described a gene called *JACKDAW* (*JKD*), acting in the same pathway as *SHR* and *SCR*. The gene came out of a screen where ~15.000 T-DNA inserted lines carried a promoterless gene expressing *GUS* in order to find expression patterns similar to that of *SCR* and to find mutations that affect asymmetric divisions in the ground tissue.

Mutant phenotype

Mutant lines were constructed using either T-DNA insertions or RNA interference (RNAi), the latter referred to as *jkd-i*. Plants carrying the *jkd* mutation differ from wild-type plants by displaying a slightly shorter root, and three extra layers of ground tissue, due to ectopic divisions of cortex cells (Welch et al. 2007).

Another feature of the *jkd* mutation is that the QC gets disorganized, indicated by differentiating columella cells (see Figure 4.1). More evidence for QC disorganization comes from the absence of expression of both the QC-specific promoter trap QC46 immediately after germination, and the expression of QC25, 8 days post-germination (dpg).

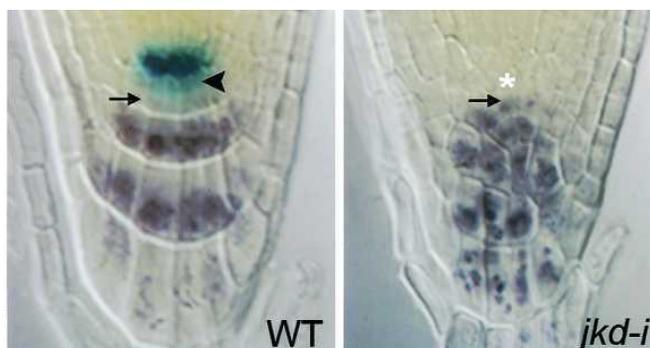


Figure 4.1: *JKD* is required for stem cell maintenance and QC identity. *QC25* expression in wild-type root (left) and *jkd-i* root (right). The absence of lugol staining marks columella initials in the wild-type root (arrowhead). Lugolstaining cells (arrow) designate differentiated columella cells. Defective QC cells are marked with an asterisk in *jkd-i*. (Welch et al. 2007).

Characterization

The *JKD* protein is a member of a large family of zinc finger proteins, defined by the protein *INDERMINATE*

(*ID1*) found in Maize. *ID* proteins harbor a highly conserved amino acid sequence consisting of a putative nuclear localization signal (NLS) and two C2H2 and two C2HC zinc finger motifs. The two C2H2 zinc finger motifs are known to have a function in DNA-binding, suggesting that *ID*-like proteins regulate gene transcription (Kozaki et al. 2004). Due to its homology to these *ID*-like proteins, *JKD* is suggested to be a transcription factor (Welch et al. 2007).

In situ hybridization shows that *JKD* mRNA accumulates in the QC, CEI, Da and to a lesser extent in the cortex and endodermis cells (see Figure 4.2A). When fused to *GFP* and driven by a 35S constitutively expressed promoter, the transcript 35S::*JKD*::*GFP* localizes nuclear.



Figure 4.2: *JKD* transcript accumulates in the ground tissue and the QC and depends on SHR and SCR. Whole mount *in situ* hybridization in wild-type (A), *shr* mutant (B) and *scr* mutant (C) roots 2 dpv. (Welch et al. 2007).

***JKD* expression depends on SHR and SCR**

JKD expression in post-embryonic roots depends on SHR and SCR. In both *shr* and *scr* mutants, initiation of *JKD* transcription is reduced, but the reduction is stronger in *shr* (compare Figure 4.2A with 4.2B and C). In *shr* and *shr* embryos initiation of *JKD* transcription is not affected, indicating that a mechanism independent of *SHR* and *SCR* initiates *JKD* transcription until maturation of the root.

In the QC, *JKD* affects *SCR* expression and *SHR* localization

From embryonic early heart stage onward, *SCR* expression in the QC is absent in *jdk* mutants. Moreover, QC identity is lost earlier during development and roots are shorter in *jdk scr* double mutants than in *scr* single mutants. This is a strong indication that *JKD* regulates *SCR* and additional factors required for QC and stem cell maintenance. *JKD* has been found to act largely, but not exclusively through *SHR*, since the *jdk shr* double mutant shows significant, yet slight reduction in root length compared to the *shr* single mutant (Welch et al. 2007).

In wild-type roots, *SHR* localizes nuclear in the QC, which is shown by visualizing pSHR::*SHR*::*GFP*. In the background of *jdk* mutants, pSHR::*SHR*::*GFP* can be observed both in the nucleus and cytoplasm. Comparison of *SHR* localization in the *jdk* mutant with *SHR* localization in the *scr* mutant suggests that *JKD* controls the nuclear localization of *SHR* in the QC mostly through its effect on *SCR* expression (Welch et al. 2007).

***JKD* affects expression range of *SHR* in the ground tissue**

The inner cells of the three extra cell layers resulting from periclinal divisions in *jdk* mutants take on endodermis characteristics, suggested by the expression of Yellow Fluorescent Protein under the control of the *SCR* promoter (Figure 4.3). The ectopic periclinal divisions in the cortex are therefore asymmetric. The expression of new *SCR* in these cells associates with an expanded *SHR* domain (Welch et al. 2007). Furthermore, the *jdk* mutant showed increased number of cells in the endodermis (Figure 5.4B), revealing its requirement for restricting cell number in the ground tissue.

***MAGPIE* oppositely controls *JKD* activity**

In a search for *JKD* homologs expressed in the ground tissue, Welch et al. (2007) identified a gene independently discovered as a direct *SHR* target (Levesque et al. 2006, Cui et al. 2007). *In situ* hybridization of *MAGPIE* transcript, together with the results of RT-PCR analysis confirms that expression of *SHR* is required for

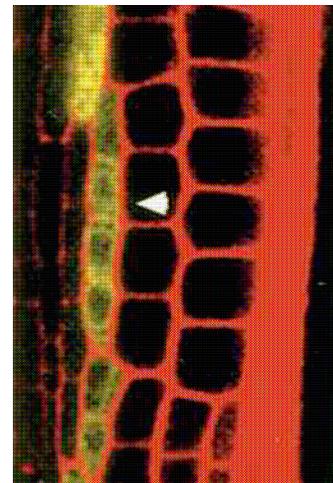


Figure 4.3: Mutation of *jdk* results in ectopic periclinal divisions (arrowhead), of which the inner cells show endodermal characteristics. Longitudinal confocal cross section of pSCR::YFP in a *jdk-i* root (Welch et al. 2007)

MAGPIE expression. *MAGPIE* (MGP), as JKD a member of the C2H2 zing finger proteins, accumulates in the nuclei of ground tissue and stele cells, but is absent from the QC. *MGP* oppositely controls JKD activity, as indicated by a restored ground tissue pattern in the *jkd mgp* double mutant (see Figure 4.4C, Welch et al. 2007).

MGP* and *JKD* physically interact with *SHR* and *SCR

SHR and *SCR* proteins are thought to form a complex and together regulate transcription of *SCR* and *MGP* (Cui et al. 2007). It has been hypothesized that *JKD* also takes part in the nuclear protein complexes, indicated by the data resulting from independent protein-protein interaction experiments (Levesque et al. 2006, Cui et al. 2007, Welch et al. 2007). Figure 4.5 depicts a model which could explain these results.

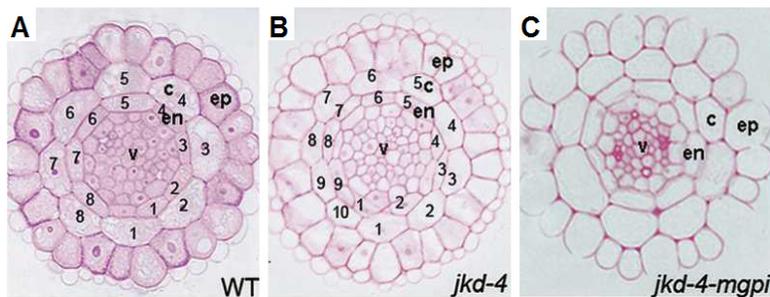


Figure 4.4: MGP oppositely controls JKD activity. Root tissue sections from 10-day-old WT (A), *jkd* single mutant (B) and *jkd mgp* double mutant (C) roots. The *jkd* mutant displays extra cells within the endodermal layer (B), a phenotype which is restored to wild-type in the double mutant (C). v, vascular bundle; en, endodermis; c, cortex; ep, epidermis (Welch et al. 2007).

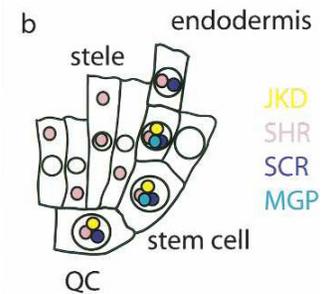


Figure 4.5: A model describing JKD, MGP, SHR and SCR action. Three different zones of protein composition can be distinguished (Welch et al. 2007).

4.2 JKD acts non-cell-autonomously in epidermal patterning

In the ground tissue, JKD regulates cell type specification and stable boundary formation by counteracting the occurrence of supernumerary SHR–SCR-mediated asymmetric cell divisions (Welch et al. 2007). Recently, however, it was shown that JKD also regulates epidermal patterning in the *Arabidopsis* root meristem, which is genetically separated from controlling cell division in the ground tissue (Hassan et al. 2010).

Epidermal patterning

In the wild-type root, cell types in the epidermis at defined positions are specified in a predictable manner. Trichoblasts (T cells) are located in a cleft between two underlying cortical cells and differentiate into root-hair (H) cells, whereas atrichoblasts (A cells) are positioned over a single cortical cell and differentiate into non-hair (N) cells (see section 2.1). Genes acting in the pathway that specifies N fate are predominantly expressed in N cells comprise the homeodomain transcription factor protein *GLABRA2* (*GL2*), the WD40-repeat protein *TRANSPARENT TESTA GLABRA* (*TTG*), the bHLH transcription factors *GLABRA3*, *ENHANCER OF GLABRA3* (*GL3* and *EGL3*) and *WEREWOLF* (*WER*), a MYB transcription factor. H fate is promoted by the redundant action of MYB-like proteins *CAPRICE* (*CPC*), *TRIPTYCHON* (*TRY*) and *ENHANCER OF CPC* (*ETC*). Mutations in genes of N fate determinants show an increased amount of root-hair cells, while cell lines harboring

specific mutants or combinations of mutants of H fate determinants adopt N fate (reviewed in Hassan et al. 2010).

The network regulating the bias of these two cell fates is conducted by response to extracellular cues, most likely originating from the cortex. The leucine-rich repeat receptor-like kinase (LRR-RLK) called SCRAMBLED (SCM) is required for this bias, shown by random distribution of A and T cells in the *scm* mutant. Kwak et al. (2005) proposed a model describing negative regulation of WER by SCM, which enables T cells to adopt H fate, whereas WER abundance in A cells is relatively high, resulting in differentiation into N fate. However, SCM action is not required in patterning during embryogenesis, which suggest that there are more factors involved in this pathway.

JKD mediates epidermal patterning

JKD plays an important role in epidermal patterning. Supportive evidence comes from the finding that in *jdk* plants, H cell distribution becomes randomized (Figure 4.6B). In the wild-type plant, *GL2*/*WER*/*CPC* genes are preferentially expressed in the precursors of A cells. JKD acts through these genes, because *GL2*/*WER*/*CPC* in the root epidermis was no longer restricted to A cells in the *jdk* mutant. Epistasis analysis placed *JKD* upstream of *GL2*, *WER*, *CPC* and *SCM*. These data indicate that the post-embryonal decisions of cell fate is modulated by JKD and that JKD acts upstream of the entire currently known root-hair patterning network (Hassan et al. 2010).

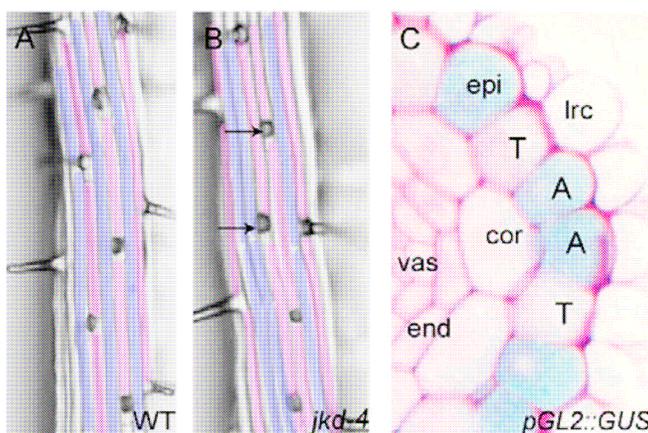


Figure 4.6: *JKD* is required for proper epidermal patterning. (A,B) External view of roots of wild-type plants (A) and plants carrying the *jdk* mutation (B). Root-hair files are shown in pink, non-hair files are in blue. Arrows in (B) indicate root hairs originating from consecutive hair files. (C) Expression of *pGL2::GUS* reporter gene in a transverse root section shows the organization of cells in the epidermis. c, cortex; end, endodermis; epi, epidermis; lrc, lateral root cap; vas, vascular bundle (Hassan et al. 2010)

JKD-mediated epidermal patterning is independent of SHR action

As shown by Welch et al. (2007), mutation of *jdk* results in extra cortex layers. However, in circumference the number of layers is the same as in the wild-type plant. Therefore, the positions of the cortical clefts relative to the epidermal cells surrounding the cortex remain unchanged in *jdk* mutants, suggesting that the mutant phenotype in epidermal patterning does not result from the changes in the cortex layer. Furthermore, the *jdk* mutation shows a patchy *GL2* expression, in contrast to the wild-type situation where *GL2* is restricted to the A cells (see Figure 4.6C), which suggests that *JKD* acts early in development, and not through alteration of cortical cleft position. In addition, *SHR* overexpression does not result in misexpression of *GL2*. All these data together suggest that the misplaced N and H cells are a direct consequence of the *jdk* mutation rather than a result of the extra divisions taking place in the cortex.

A model illustrating the role JKD in epidermal patterning

Hassan et al. (2010) proposed a model which could explain the available data as follows (summarized in Figure 4.7): Located in the cortical cleft, a trichoblast has a larger contact surface spanning than

atrachoblasts which are located over a single cortex cell. T cells are therefore expected to receive more signals from the cortex. This then leads to more inhibition of transcriptional regulation of WER in the T cells, mediated by SCM. The cells adopt H fate, due to CPC/TRY/ETC dependent lateral inhibition, which could explain the predominant effect of JKD in H cell positions.

In the *jdk* mutant, signals dependant on JKD are reduced and release WER repression, affecting the relative abundance of WER and CPC. This could lead to N fate in the H position, and as a more rare secondary effect, H fate in the N position (Hassan et al. 2010).

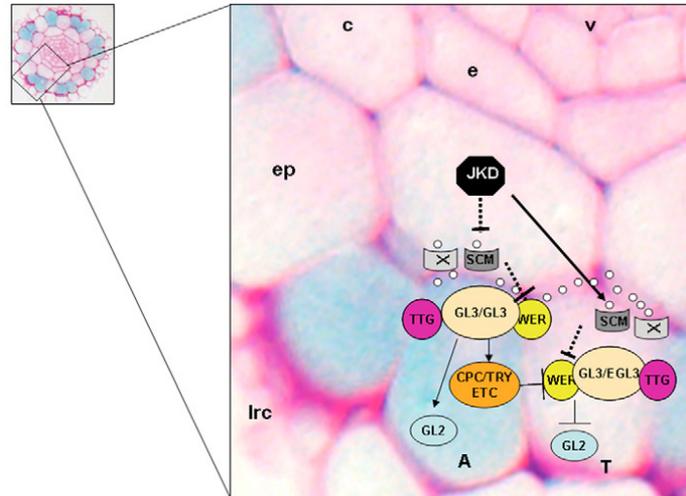


Figure 4.7: Model which explains available data of the role of JKD in epidermal patterning. JKD (black octagon) triggers a signal (white circles) that binds to the receptor kinase SCM (grey) and or to unknown factors (X). The cell located at the cleft will receive more input, which leads to SCM activation, partial WER repression and GL2 inhibition. Located over a cortical cell, cells will receive relatively less JKD-mediated input, possibly by JKD inhibiting SCM. This repression results in WER release, thereby activating GL2 expression. v, vascular bundle; e, endodermis, c, cortex; ep, epidermis; lrc, lateral root cap; A, atrachoblast; T, trichoblast. (Hassan et al. 2010).

The mechanisms of JKD regulating SHR and SCR action, QC maintenance and epidermal patterning are unknown. To dissect the function of JKD, a yeast two-hybrid screen was carried out to identify other interactors of JKD. One of the targets found was SCARECROW-LIKE23 (SCL23, unpublished results).

5.1 Introduction

SCARECROW-LIKE genes are part of the GRAS family of genes, as reviewed in Pysh et al. (1999). Several SCL proteins are putative interactors of SHR, many of them closely related to SCR (Pysh et al. 1999, Lee et al. 2008). Based on the phylogenetic tree, SCARECROW-LIKE23 is the closest homolog of SCR (Figure 5.1). At first, no expression of *SCL23* was found in the root meristem (Lee et al. 2008). Later, however, the results of RT-PCR analysis indicated that *SCL23* is expressed in the root (unpublished results). Homozygous mutations of the *scl23* gene do not result in an obvious morphological phenotype (see results and discussion, Figure 5.4), which suggests that functionally redundant proteins act together in the pathway (Lim et al. 2008). However, when *SCL23* expression is reduced using RNAi, a phenotype similar to the *jdk* mutation can be observed. (unpublished results). In this thesis, we tried to characterize the loss-of-function phenotype of the *scl23* mutant in different mutant backgrounds. We found at least one interesting phenotype which could link SCL23 to the SHR-SCR-JKD pathway.

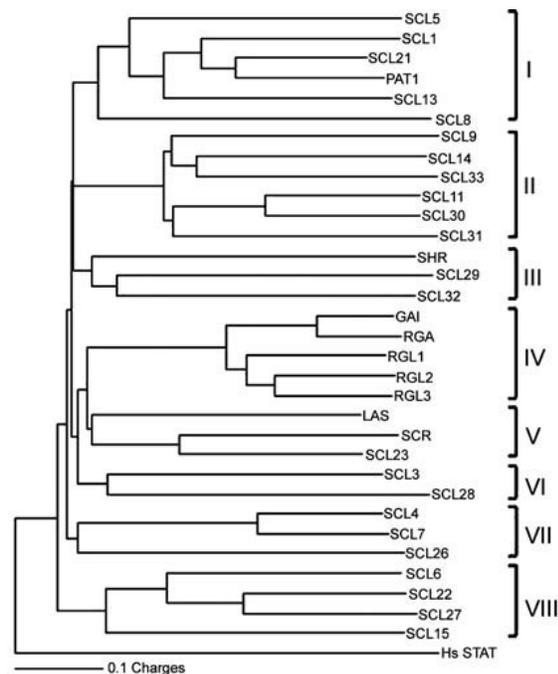


Figure 5.1: *SCL23* is the closest homolog to *SCR*. Phylogenetic tree constructed using the neighbor-joining method (Lee et al. 2009).

5.2 Results and discussion

Characterizing the *scr scl23* double mutant

Because *SCL23* and *SCR* are the closest homologs, we looked at the effect of *scr scl23* double mutants on ground tissue patterning. Plants harboring the *scr-3* and *scr-4* single mutations (see materials and methods) were crossed to plants harboring the *scl23* mutation. F2 plants with shorter root phenotype presumably carrying the *scr* homozygous mutation were selected and checked for *scl23* homozygosity. Of 62 plants, 18 plants largely suggested *scl23* homozygosity (see Table 1 of supplementary information).

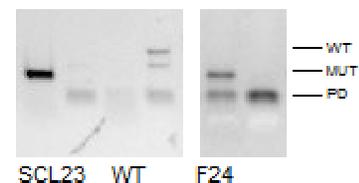


Figure 5.2: F24 seems to be homozygous for the *scl23* mutation. Bands correspond to wild-type (WT, upper), mutant (MUT, middle) and primer-dimers (PD, lower) fragment lengths (gene-ruler not shown).

Figure 5.2 shows the result of genotyping one of the putative double mutant candidates, line F24.

Of these samples, seeds were collected and 8 days post-germination phenotype was observed. The fact that no consistent results were obtained with *scl23* genotyping due to unknown technical issues, along with the lack of *scr* genotyping experiments, makes drawing firm conclusions impossible. However, one sample coming from the cross *scl23* x *scr-4*, referred to as F24, showed dramatically altered root morphology. Although not statistically confirmed, F24 had largely reduced root length comparing to *scr-3* mutant as shown in Figure 5.3, indicating an enhanced defect on stem cell maintenance.

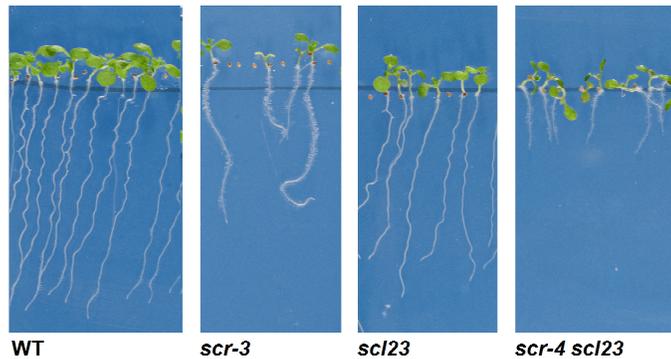


Figure 5.3: The presumed *scr-4 scl23* shows a phenotype of greatly reduced root length. Photographs taken 8 days post-germination. Samples WT, *scl23* and *scr-4 scl23* are originating from the same plate, *scr-3* is a control grown in the same conditions on a different plate.

Further characterization of this sample with confocal microscopy revealed that the presumed double mutant displayed drastically reduced meristem size and thinner stele with fewer cell files comparing to WT, *scl23*, and *scr-3* mutants (Figure 5.4). Moreover, F2 also only harbored one ground tissue layer. To test whether the remaining ground tissue layer has endodermis characteristics, roots were treated in order to visualize the Casparian strip, the hallmark of endodermis. As depicted in Figure 5.5, F24 showed no Casparian strip, which suggests that the remaining ground tissue layer has lost endodermal fate.

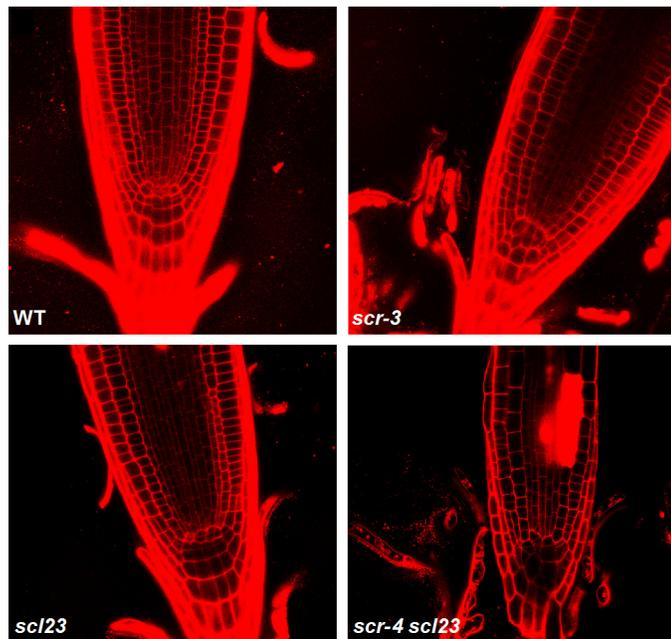


Figure 5.4: The *scr-4 scl23* double mutant shows a stronger altered phenotype compared to wild-type and the *scr-3* single mutant. Confocal microscopy images showing propidium iodide (PI)-stained cell wall in red.

All aforementioned phenotype of F24 seems to suggest that the presumed double mutant of *scr-4 scl23* superficially mimics *shr* with shorter root than *scr* and total loss of endodermis. However, further experiments are still required with appropriate controls such as *scr-4* and *shr-2* mutants.

Besides *scr scl23*, we also tried to generate *scl23 shr* and *scl23 jkd* double mutants. Yet no results are available with the still ongoing genotyping of such lines.

RT-PCR analysis

As mentioned earlier in the chapter, two lines of *SCL23i*, B3 and B6, displayed a phenotype similar to that of the *jkd* null mutant (unpublished results). However, the supposed *scl23* knock out line showed no observable phenotype. These could be an indication that the effect of altering *scl23* expression is level dependent. To this end, RT-PCR analysis was carried out in order to examine how much *scl23* mRNA is reduced in *scl23* mutant and the RNAi lines. In addition, mutants of *shr-2*, *scr-3* and *jkd-4* were also included to test whether *SCL23* expression is dependent on SHR, SCR or JKD. The mRNA was extracted with a yield of 300 – 800 ng/ μ L, but probably due to technical issues, we did not succeed in obtaining consistent controls (See

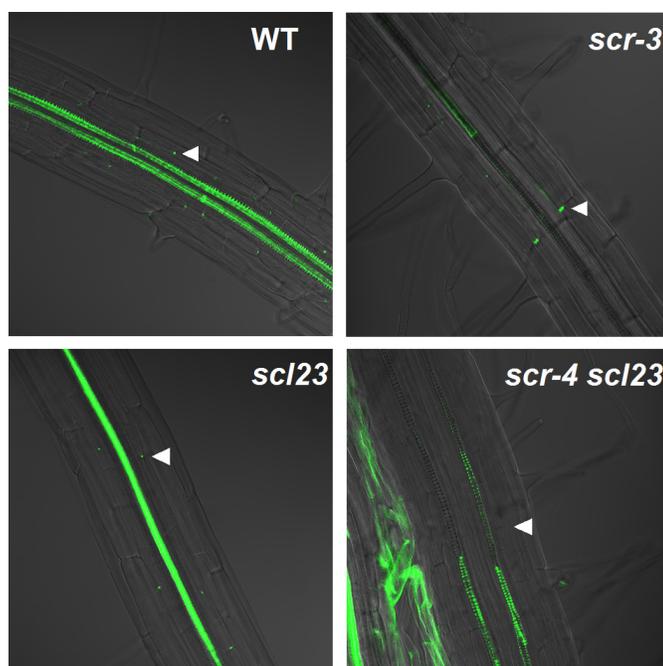


Figure 5.5: Visualization of the Casparian strip (see materials and methods). Arrowheads indicate transverse cell walls where the Casparian strip is situated (in WT and *scr-3* / *scl23* single mutants) or where the Casparian strip is absent (*scr-4 scl23* double mutant).

Figure 1 in supplementary information). We were unable to identify what caused these inconsistencies, but it could be a contamination issue, since negative control samples containing water instead of DNA template gave a band of the size that corresponds to the calculated fragment size of *scl23* cDNA. Further optimization of the RT-PCR is required.

5.3 MATERIALS AND METHODS

Growth conditions and genetic lines: Lines carrying the *jkd-4*, *scr-3*, and *scr-4* mutations were obtained as described in Welch et al. (2007). The *scl23* mutation was obtained from the SALK T-DNA collection (<http://signal.salk.edu>, accession number SALK 054051). Plant growth conditions are as described in Sabatini et al. (1999).

Genotyping: DNA was extracted by taking 3 – 4 small leaves from 1-week-old seedlings, using procedures as described in Welch et al. (2007) for DNA extraction. For the polymerase chain reactions the following primers were used: for mutant analysis forward (5'-CTTAGCTTACTCCTCCAATGC-3') and Lba-insertion (5'-TCAAACAGGATTTTCGCCTGCT-3') primers and for wild-type analysis forward and reverse (5'-CATCATATAACCGGTGCTGC-3') primers. Using gel electrophoresis (1% agarose gel, 100V), samples were checked for their genotype under UV light.

Casparian strip staining: Sample roots were transferred to small Petri-dishes containing 0.24M HCl in 20% methanol and incubated at 57 °C heat for 15 minutes. This solution was replaced with 7% NaOH in 60% ethanol for 15 minutes at room temperature. Roots were then rehydrated for 5 minutes

each in 40%, 20%, and 10% ethanol, and infiltrated for 15 minutes in 5% ethanol, 25% glycerol. Roots were mounted in 50% glycerol on glass microscope slides for observation.

Confocal microscopy: All confocal images were obtained on 8-day-old roots, treated with propidium iodide. A Leica SP2 microscope was used for the roots treated with PI, for visualization of the Casparian strip a Zeiss LSM-710 microscope was used.

RT-PCR: mRNA was extracted using Spectrum™ Plant Total RNA Kit (SIGMA). After concentration leveling and DNase treatment cDNA was synthesized using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Using nanodrop, concentrations were measured and all samples were diluted until a concentration of 145 – 155 ng/μL. Polymerase chain reactions for genes of interest were executed using forward and reverse primers as described in the preceding paragraph, for controls standard actin-forward and actin-reverse primers were used.

The first three chapters of this thesis describe what is currently known about the role of *SHORT-ROOT* in ground tissue patterning and stem cell maintenance, and how this data was obtained using *Arabidopsis thaliana* as a model organism. As indicated by the fourth and fifth chapter, the pathway by which *SHR* is acting, comprises many other factors such as *SCARECROW*, *JACKDAW*, *MAGPIE* and possibly *SCARECROW-LIKE23*. In this final section, the data will be summarized and discussed.

Regulation of SHR movement involves complex mechanism

As summarized in section 3.4, a complex mechanism regulates *SHORT-ROOT* movement; it specifically moves one cell layer and triggers cell division there. Several previous literatures suggested that *SCARECROW* restricts the movement of *SHR*. However, *SHR* movement is not entirely dependent on *SCR* action, supported by several data. First, in the *scr* plant, *SHR* is able to move from the stele into the mutant cell layer, but not into the epidermis, suggesting additional factors that play a role in restricting *SHR* movement. Second, the finding of the *JKD* family members reveals that there are other factors involved. This is supported by data from double mutant analysis: the double mutant of *jdk* and its close homolog *bald-ibis* (*bib*) shows supernumerary layers, while the triple mutant of *jdk*, *mpg* and a *JKD* homolog *nutcracker* (*nuc*) has only one layer of ground tissue. Together, these data suggest that there are more factors involved apart from *SCR* in ground tissue patterning which can regulate the action range of *SHR*.

However, supportive for the hypothesis that *SCR* regulates *SHR* action is the phenotype resulting from the RNAi mutant of *scr*. As shown in Cui et al. (2007), *SCRi* showed supernumerary cell layers in the ground tissue with extended *SHR* movement range, suggesting that sufficient amount of *SCR* is required to fully restrict *SHR* action in only one layer outside the stele. This phenotype, however, might also be the effect of unspecific knock-down of *SCL23*, as is discussed in the following section.

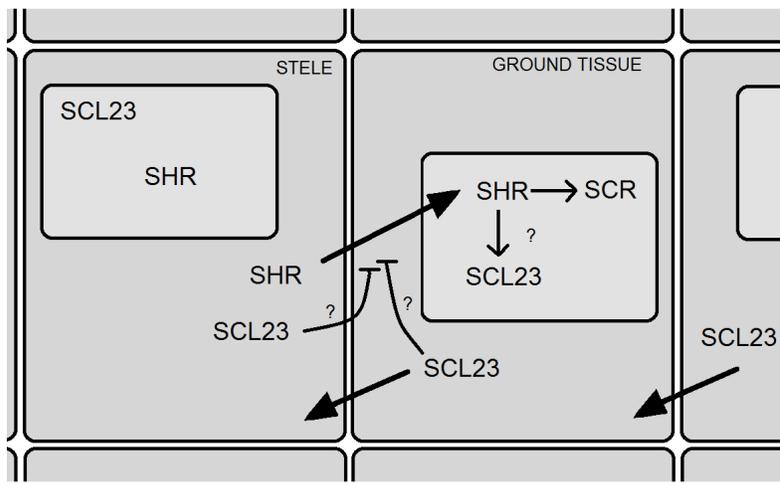
SCL23 might be an additional player in the classic SHR-SCR pathway.

Being the closest homolog to *SCR*, *SCL23* interacts directly with *JKD*, *SCR* and *SHR* in both yeast two-hybrid and bimolecular fluorescence complementation (BiFC) assays. Single mutant of *scl23* showed no observable phenotype, indicating redundancy to other *SCARECROW-LIKE* genes. To identify the role of *SCL23*, overexpression and RNAi lines were constructed and phenotype was observed. Different from the single *scl23* mutant, the *SCL23i* resulted in one additional layer of ground tissue, a phenotype similar to that of *jdk* mutants, while the overexpression of *SCL23* resulted in reduction of ground tissue layers to one only. All together, these data suggest a role for *SCL23* in restricting *SHR* action range. Further research has to reveal how *SHR* action is affected by the *scl23* mutation by visualizing *SHR* expression pattern alteration.

The discrepancy between the single *scl23* mutant and *SCL23i* could be explained in at least two ways. The first possibility is that the effect of *scl23* knockdown is level dependent, where reduced level of *SCL23* might loosen its restriction to *SHR* movement and null mutant represses *SHR* action totally. Since we were not able to get proper RT-PCR results, further optimization of the RT-PCR experiment is needed in order to examine this possibility. The second alternative is that knocking down *scl23* using RNAi unspecifically affects the level of *scr* as well. To investigate this possibility, constructing

specific miRNA for *scl23* and *scr* might reveal whether the phenotypes of *SCL23i* and *SCRi* are the effect of unspecifically knocking down each other.

The absence of an observable phenotype of single *scl23* indicates that there are redundant factors. To test this hypothesis, we tried to isolate the double mutants of *scl23* with its closest homolog *scr*. The presumed *scr scl23* shows an enhanced phenotype compared to the single *scr* mutation. The ground tissue consists of only one layer without endodermal fate. The role of SCL23 could be that it affects SHR function in the ground tissue layer by specifying endodermal fate. Another option is that SCL23 restricts SHR movement. This is supported by expression analysis and protein interaction analysis of *SCL23*. Protein interaction experiments of SCL23 indicate that the interacting complex with SHR is localized both in the nucleus and cytoplasm of *Arabidopsis* mesophyll protoplasts. Further expression pattern analyses indicate SCL23 is localized both in the nucleus and cytoplasm in the stele, which coincides with SHR localization (unpublished results). The function of SHR is dependant on its localization as shown in chapter 3. There is a possibility that SCL23 regulates SHR action range via altering SHR subcellular localization. Furthermore, there is evidence that SCL23 is a mobile protein which seems to move in the opposite direction of SHR (see the model below), making the regulation of movement and radial patterning more complicated and interesting.



Model based on the available data. SHR moves from the stele into the nucleus of the ground tissue cells (thick arrows) where it induces SCR and possibly SCL23 action (small arrows). SCL23 moves from the epidermis into the ground tissue, to the nucleus as well as the cytoplasm; where the restriction of SHR movement might also be level dependent to SCL23.

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SUPPLEMENTARY INFORMATION

Table 1: Results of genotyping for *scI23* homozygosity. (M) mutant and (WT) wild-type lanes with strong band in dark grey, no band in white and questionable band in light grey. PCR1 and PCR2 refer to two separate cycles of genotyping (Y, candidate for homozygosity; ND, not determined; Smear, unclear band). Seeds column indicate if seeds could be collected.

Cross	Plant	M	WT	PCR 1	PCR2	Seeds	Cross	Plant	M	WT	PCR 1	PCR2	Seeds	Cross	Plant	M	WT	PCR 1	PCR2	Seeds						
ScI23 x scr3	11	Dark	Dark				ScI23 x Jkd-4 "1"	C1	Dark	Light	Y			ScI23 x shr-2 "4"	Z1	Dark	Dark									
	12	Dark	Dark					C2	Dark	Dark	Y	Y				Z2	Dark	Dark		Y	ND					
	13	Dark	Dark					C3	Dark	Dark						Z3	Dark	Dark		Y	ND					
	14	Light	Dark					SCL	Dark	Light						Z4	Dark	Dark		Y	ND					
	15	Dark	Dark					WT	Dark	Dark	Y															
	21	Dark	Dark					ScI23 x Jkd-4 "2"	D1	Dark	Dark					ScI23 x shr-2 "2"	G5	Smear	Dark							
	22	Dark	Dark						D2	Dark	Dark							G6	Dark	Dark		Y	ND			
	23	Dark	Dark		Y				D3	Dark	Light							G7	Dark	Dark		Y	ND			
	25	Dark	Dark						D4	Dark	Dark							SCL	Dark	Dark		Y	ND			
	26	Dark	Dark						D5	Dark	Dark							WT	Dark	Light						
	32	Dark	Dark						D6	Dark	Dark	Y	Y		Y											
	33	Dark	Dark						D7	Dark	Dark	Y														
	34	Dark	Dark						D8	Dark	Dark															
	35	Dark	Dark						D11	Dark	Dark															
	36	Dark	Dark						D12	Dark	Dark															
	37	Dark	Dark						D13	Dark	Dark	Y	Y		Y											
	38	Dark	Dark						D14	Dark	Dark															
	41	Dark	Dark		Y				D15	Dark	Dark	Y	Y		Y											
	42	Dark	Dark						D16	Dark	Light															
	43	Dark	Dark						D17	Dark	Dark															
	44	Dark	Dark						D18	Dark	Dark															
	45	Dark	Dark						D19	Dark	Light															
	SCL	Dark	Dark						D20	Dark	Dark	Y	Y		Y											
	WT	Dark	Dark						D21	Dark	Dark															
46	Dark	Dark				D22	Dark		Light																	
47	Dark	Dark		Y	Y	D23	Dark		Dark																	
A11	Dark	Dark				SCL	Dark		Dark																	
A12	Dark	Dark		Y		WT	Dark		Dark	Y																
A13	Dark	Dark		Y		ScI23 x scr-4 "1"	F1		Dark	Dark																
A14	Dark	Dark		Y	Y		F2	Dark	Dark																	
A21	Dark	Dark		Y			F3	Dark	Dark																	
A22	Dark	Dark		Y	Y		F4	Dark	Dark	Y																
A23	Dark	Light																								
A24	Dark	Dark		Y																						
A31	Dark	Dark																								
A32	Dark	Dark																								
A33	Dark	Dark																								
A41	Dark	Dark		Y																						
A42	Dark	Dark		Y	Y																					
A43	Dark	Dark		Y																						
A44	Dark	Dark																								
A45	Dark	Dark																								

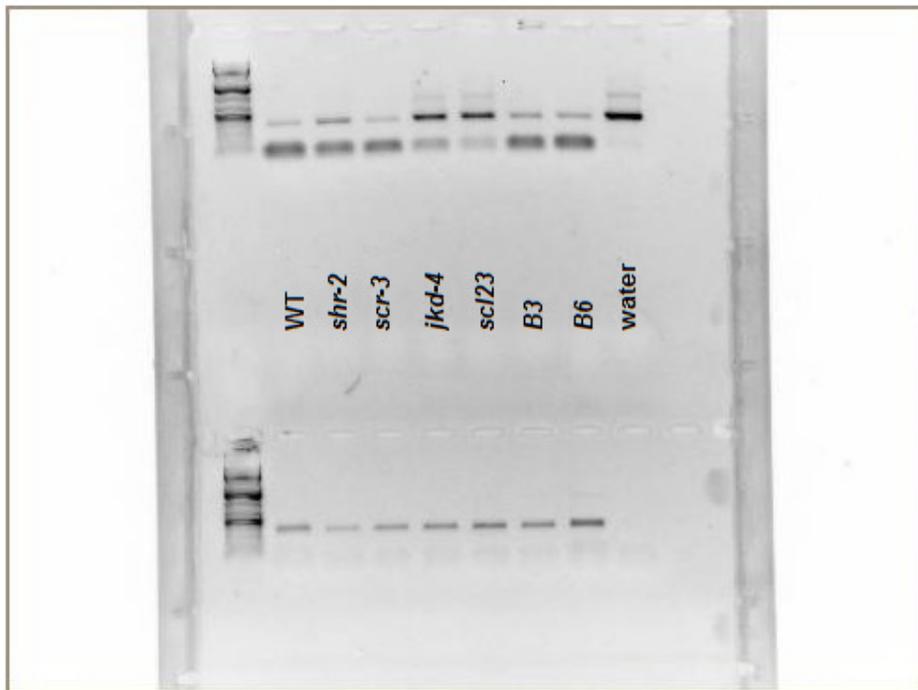


Figure 1. Typical gel resulting from the TR/PCR analysis. Size of the bands correspond to calculated fragment size of *sc123* cDNA (upper row of bands) and actin cDNA (lower row of bands). Although actin levels are leveled, bands of *sc123* cDNA stayed inconsistent and even a clear band in the water sample is visible.