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Receptor Kinase Signaling in *Arabidopsis* Root Meristem Maintenance

Receptor kinase signalering in *Arabidopsis* wortel meristem handhaving

(met een samenvatting in het Nederlands)

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht,
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door

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"Aquest moment no tornarà mai més.

I tanmateix, aquest moment què importa,

si encara hi ha l'eternitat després

i del passat el record em conforta?"

Màrius Torres

Als meus pares

Cover
RCH1 expression in a WT cell and in a root ectopically expressing LLP1 in the RCH1 domain.

Paranimfen
Maruxa Martínez Campos
Sabrina Sabatini

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Lay-Out
Pieter van Dorp van Vliet, Beeldverwerking & Vormgeving

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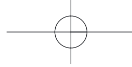
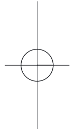
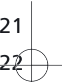
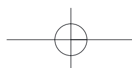


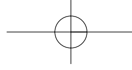
Chapter 1

General Introduction









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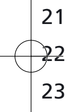
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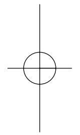
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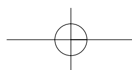
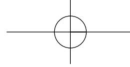


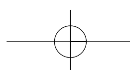


INTRODUCTION

The beauty of life diversity among higher plants and animals as we know it today is the result of over a billion years of evolution, during which multicellularity evolved independently in plants and animals from different unicellular ancestors. These, in turn, derived from a common ancestor, likely a eukaryote that possessed mitochondria and aerobic metabolism. It probably possessed also receptor proteins in its cell membrane to respond to environmental signals via the regulation of intracellular signaling networks, leading ultimately to changes in gene expression. All these characteristics were inherited by the progenitors of plant and animal kingdoms (Leyser and Day, 1998).

Sequence information suggests that most basic cellular processes are shared by all life forms and conserved to the present; for example replication, transcription, translation and the basic structure of proteins, nucleic acids and membranes. Furthermore, the basic organization and functions shared by all eukaryotic cells, but not prokaryotes, must have been present at least 2 billion years ago, before single-celled eukaryotes diverged. This conservation would include their larger size, their dynamic membranes capable of endocytosis and exocytosis, their membrane-bounded organelles (most prominently the nucleus), mitosis and meiosis, sexual reproduction by cell fusion, actin and tubulin based cytoskeletons, a cdk/cyclin-based cell cycle and histone/DNA chromatin complexes (Gerhart, 1999). However, before multicellularity emerged some divergence already occurred: plants acquired chloroplasts and autotrophic metabolism, making them able to produce their own organic compounds using energy from the sun by the process of photosynthesis, and had a cell wall, while the progenitor of multicellular animals was heterotrophic and wall-less (Leyser and Day, 1998).

An important consequence of multicellularity is the need of the cells in an organism to communicate among each other to achieve coordinated development. Already in prokaryotes, in a process called quorum sensing, groups of bacteria communicate with one another to coordinate their behavior and function like a multicellular organism. Quorum sensing regulates bioluminiscence, virulence factor expression, biofilm formation, sporulation and mating through



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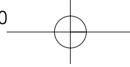


the production, release and subsequent detection of and response to threshold concentrations of signal molecules (Bassler, 2002). Since multicellularity evolved independently in each kingdom, plants, animals and fungi use different mechanisms for cell-cell signaling, which have however some similarities and share some common components. For example, innate immunity in higher eukaryotes involve a highly conserved regulatory pathway mediated by a set of interacting homologous domains present in proteins found in all eukaryotes (Cao et al., 2001). In plants, like in animals, a core set of signaling pathways is used repeatedly in many different developmental contexts. The reiteration of core pathways in both plants and animals suggests that development evolved through duplication and innovation on basic pathways that were recruited early in evolution of the respective lineages (McCarty and Chory et al, 2000).

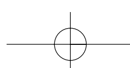
In this chapter, I will first address how animals have solved the need for communication among cells during development, considering particular cases where receptor kinases act as signaling molecules in *Drosophila* stem cell maintenance and axis formation. Secondly, I will discuss the signaling pathways involved in innate immunity, which are homologous in animals and plants, and its evolutionary implications. Finally, I will address how plants have solved their need for cell-cell communication during development and which common and different features exist in comparison to the animal kingdom.

CELL-CELL SIGNALING IN ANIMAL DEVELOPMENT

The elaboration of the basic body plan in animals occurs mainly during embryogenesis. An almost complete but smaller version of the adult is present when embryonic development is nearly completed, and post-embryonic development consists mainly of further growth and completion of the existing organ systems.

The crucial role of cell-cell signaling in early development was revealed in 1924 when the vertebrate organizer was discovered by Spemann and Mangold. They surgically removed the cells from an early gastrula and grafted it into the opposite side of another embryo of the same age which then developed two body axes (Spemann and Mangold, 1924). Molecular characterization of the signaling components within these organizing cells that were able to organize a body axis had to await several decades.

When molecular tools became available, information about the nature of the signals started to emerge, and it became clear that receptor kinases play a major role in transducing those signals. In animal development there are two major pathways involving receptor kinases to relay the signal to downstream components: the receptor serine/threonine kinase (TGF-β) pathway and the receptor tyrosine kinase (RTK) pathway.



In the TGF-β pathway, the transmembrane receptor consists of two proteins (receptor type I and II), and the cytoplasmic tails of both are serine/threonine kinases. Upon ligand binding, they form a heterodimer in which type II kinase phosphorylates the type I tail, which then phophorylates a receptor-regulated Smad protein (R-Smad), which associates then with a common-mediator Smad protein (co-Smad). This complex translocates to the nucleus to activate the transcription of target genes, some of which encode inhibitory Smad proteins that antagonize the R/Co-Smad complex. In *Drosophila* early development this pathway is used in the establishment of dorsoventral compartments and later on in the development of appendages, eyes, wings and the gut (Raftery and Sutherland, 1999).

In the RTK pathway, the receptor dimerizes upon ligand binding and each member cross-phosphorylates its partner. This phosphorylation can lead to the interaction with the initial components of at least four major transduction series. One of these components is the RAS protein, a small G-protein which in turn activates a series of intermediates, including many MAP kinases. The other components are PI3 kinase, the phospholipase PLCγ and a phosphotyrosine phosphatase. An immense variety of ligands and RTK receptors feed into this complex pathway, which is involved in a wide variety of processes such as anteroposterior and dorsoventral polarity specification in the oocyte, eye development, and tracheogenesis in *Drosophila* (for review Gerhart, 1999).

Besides these developmental signaling pathways, the Toll-Dorsal pathway, involving a leucine rich repeat (LRR) receptor coupled via other connector proteins to a Ser/Thr cytoplasmatic kinase plays a crucial role in development but also in innate immunity. Interestingly, this pathway is conserved among animals and plants, which suggests it might have been already used by the common eukaryotic ancestor for defense against pathogens, which has been recruited at later stage for developmental processes.

The molecular genetic analysis of *Drosophila* development has been essential in the identification of components of the above signaling pathways and in the elucidation of their roles. I will discuss three examples in which receptor kinases are involved in key developmental processes in *Drosophila*: germ stem cell maintenance and axis specification.

The TGF-β pathway in *Drosophila* Germ Stem Cell Maintenance

Early separation into generative and somatic cells is typical of animal development. Germ cells retain their developmental competence and differentiate in the gonads to form the gametes. The germ cells arise from stem cells, which are defined by their ability to self-renew and to generate differentiated

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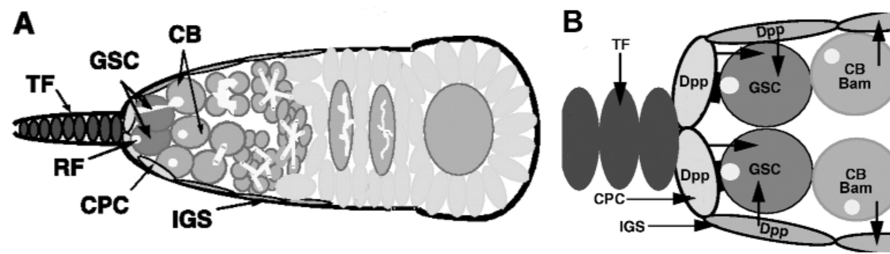
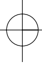


Figure 1. Germarium structure and stem cells.
(a) Diagram of the *Drosophila* germarium in cross section indicating terminal filament (TF), germline stem cells (GSC), cystoblast (CB), cap cells (CPC), inner germarium sheath cells (IGS). (b) Model of the signalling between the CPC and IGS expressing *Dpp* and the GSC (see text for details; modified from Xie and Spradling, 2000).

progeny. Post-embryonically, animals use stem cells to build and replenish particular organ systems, such as their hematopoietic, nervous systems and gonads. The *Drosophila* ovary is a good example in which stem cells remain active during much of adult life, and it can be studied at the cellular and molecular level (Fig 1).

Near the beginning of each developing egg string (or ovariole), about two germ line stem cells (GSCs) reside within the ovary whose progeny differentiates into cystoblasts. These stem cells are surrounded by three differentiated somatic cell types -terminal filament, cap and inner sheath cells- which help make up the anatomically simple tubular structure known as the germarium (Fig 1a). The TGF-β homologue DECAPENTAPLEGIC (DPP) is specifically required to maintain female germline stem cells and promote their division. It has been shown that overexpression of *DPP* blocks germline stem cell differentiation and produces ovarian stem cell tumors, while reduction of *DPP* promotes stem cell differentiation (Xie and Spradling, 1998). *DPP* is expressed in both the cap cells and the inner sheath cells, from which it could signal to the contacting GSC (Xie and Spradling, 2000; Fig 1b).

Major components of the *DPP* signaling pathway include *SAXOPHONE* (*SAX*) and *THICK VEINS* (*TKV*) which encode type I serine/threonine kinase transmembrane receptors, whereas *PUNT* encodes a type II serine/threonine transmembrane receptor. DPP binds both type I and type II receptors to allow the constitutively active PUNT kinase to phosphorylate and activate type I kinases, which phosphorylate the R-Smad protein MOTHERS AGAINST DPP (MAD). Association of Phospho-MAD with the co-mediator Smad protein MEDEA (MED) leads to the translocation of the heteromeric MAD-MED complex into the nucleus where it can bind to cis-acting elements in target genes and activate or repress transcription (Affolter et al, 2001 for review of DPP signal transduction).



The *BAG-OF-MARBLES* (*BAG*) gene encoding a novel protein, is highly expressed only in the stem cell daughter (Mc Kearin and Spradling, 1990). The loss of BAM protein in cystoblasts prevents their differentiation, causing germ line tumors, while the forced expression of BAM in germline stem cells causes them to differentiate (Ohlstein and McKearin, 1997). Thus DPP signaling might negatively regulate BAM protein levels in germline stem cells.

Together these data show that the TGF-β signaling pathway plays a key role in stem cell maintenance in the germ line of *Drosophila*.

The RTK Pathway and *Drosophila* Axis Specification

One of the first steps in the elaboration of the body plan of an organism is the specification of axes which provide positional information. This information will allow cells which initially have the same developmental potential to express different sets of genes according to their coordinates.

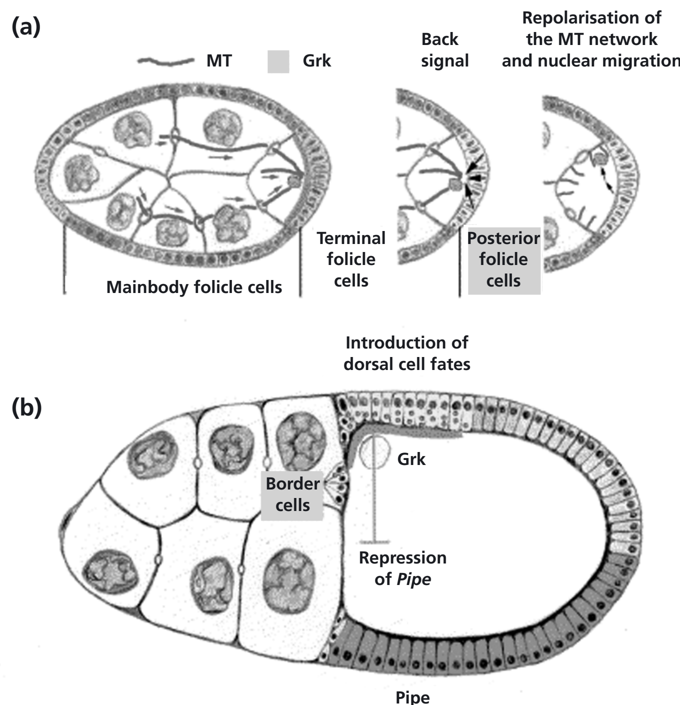
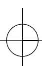


Figure 2. *Drosophila* Axis Specification.
(a) The first Grk signal induces the terminal folicle cells to adopt posterior fate, leading to the production of a yet unidentified signal back from the posterior follicle cells that causes repolarisation of the microtubules. The oocyte nucleus moves then along them, towards the anterior-dorsal corner of the oocyte. (b) GRK protein associated with the nucleus signals again to induce dorsal cell fates, in part by repressing *PIPE* expression in dorsal regions, thereby restricting the region in which a ventralising signal is produced (modified from Riechmann and Ephrussi, 2001).



Axis specification in *Drosophila* is initiated through the interaction between the egg cell and its surrounding follicle cells. GURKEN (GRK), a TGF-α homologue, and the RTK pathway, through its receptor TORPEDO, play a central role in the interactions of follicle cells with the oocyte, as the oocyte develops anterior-posterior and dorso-ventral polarity (Gonzalez-Reyes et al, 1995; Fig2).

The oocyte is polarised by two signaling events both involving GRK. The first GRK signal specifies the anterior-posterior axis. During early oogenesis, the oocyte nucleus localizes at the posterior side of the egg, where it localizes GRK which is then secreted from the oocyte and signals through TORPEDO in the folicle cells at the posterior end of the egg chamber, inducing this follicle cells to adopt posterior fate. In turn, these posterior follicle cells respond by sending an as yet unidentified signal back to the oocyte, resulting in the repolarisation of its microtubules (MT), which causes the oocyte nucleus to travel along them towards an anterior corner of the egg (Fig 2a). This MT reorganization is also responsible for the localization of *BICOID* mRNA at the anterior pole and *OSKAR* mRNA at the posterior pole, defining the anterior-posterior axis of the embryo. *BICOID* mRNA is translated after fertilization to produce a morphogen gradient that patterns the anterior region of the embryo, while *OSKAR* recruits *NANOS* mRNA, which when translated acts also as a morphogen forming a gradient that patterns the posterior regions (Riechmann and Ephrussi, 2001).

The second GRK signaling event specifies the dorsal-ventral axis. Following the arrival of the oocyte nucleus at an anterior region of the egg, GRK signaling occurs in the neighboring follicle cells, which defines the dorsal pole (Fig 2b), controlling dorsal-ventral patterning mainly by restricting the expression of the protease *PIPE* to the ventral follicle cells and more locally by specifying the fate of the dorsal follicle cells (Peri et al, 2002). PIPE then induces ventral cell fates in the embryo via activation of the Toll signaling pathway (for review Riechmann and Ephrussi, 2001).

The Toll-Dorsal Pathway in Dorso-Ventral Patterning in the Embryo

In *Drosophila* embryos, ventral patterning depends on the Toll-Dorsal pathway. Activation of the TOLL receptor leads to the formation of a broad nuclear gradient of the transcription factor DORSAL, that specifies different thresholds of gene expression (Reviewed by Belvin and Anderson, 1996).

TOLL is a large transmembrane receptor protein with an extracellular domain containing LRRs, and an intracytoplasmic region containing an interleukin-1 receptor homologous domain (TIR domain; Hashimoto et al, 1988). LRRs are a common signal transduction motif thought to be involved in pro-

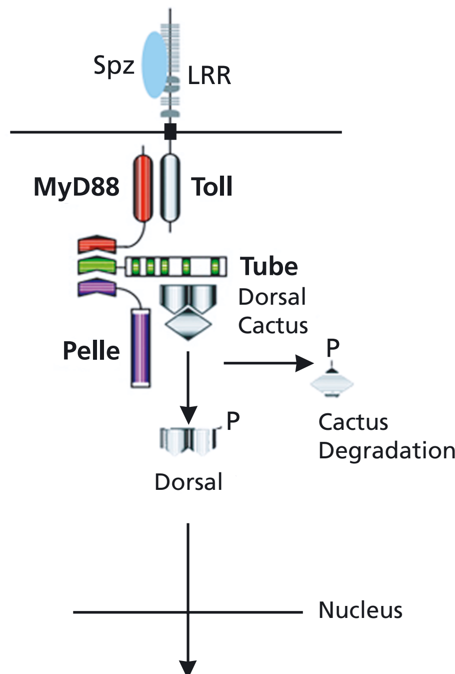


Figure 3. Model for the signal transduction of the Toll pathway.
Upon SPZ binding to the TOLL receptor, the PELLE Ser/Thr kinase, connected to the receptor through MYD88 and TUBE, gets activated. This results ultimately in CACTUS degradation and translocation of DORSAL in to the nucleus where it activates target genes (Modified from Sun et al, 2002).

tein-protein interaction. Each repeat is composed of an α-helix and a β-sheet, with the β-sheet creating a surface that mediates protein-protein interaction (Kobe and Deisenhofer, 1995).

TOLL is evenly distributed throughout the membrane of the precellular embryo, and its restricted activation results from the localized processing of its ligand, SPÄTZLE (SPZ) in ventral regions. SPZ cleavage is achived by the sequential activation of three serine proteases, among them PIPE which is repressed in dorsal follicle cells by GRK signaling (see above).

DORSAL is initially present throughout the cytoplasm, where it is retained by an inhibitory protein, CACTUS. Binding of extracellular SPZ ligand to TOLL, activates the Ser/Thr cytoplasmatic kinase PELLE (Towb et al, 1998) which is connected to the TOLL receptor through the adaptor proteins MYD88 and TUBE (Fig 3; Sun et al, 2002). Activated PELLE phosphorylates multiple substrates, eventually resulting in CACTUS phosphorylation and degradation, and translocation of DORSAL into the nucleus, where it directs expression of ventral specific genes and repression of dorsal-specific genes (Fig 3; Belvin et al, 1995; Bergman et al, 1996; Reach et al, 1996; Shen and Manley, 1998; Belvin and Anderson, 1996 for review).

Innate immunity in animals and plants

In addition of its central role in dorsoventral patterning, the Toll-Dorsal pathway is involved in innate immunity, which is an ancient form of defense against microbial infection. Interestingly, the innate immune system is mediated through similar signaling cascades in insects (TOLL-PELLE), mammals (TOLL likes) and plants (PELLE related; Fig 4b), suggesting an early evolutionary origin of eukaryotic pathogen defense systems, which were possibly then recruited for development. In *Drosophila* and mammals the innate immune response to pathogens activates the Toll-like LRR receptors, which in turn activate intracellular Ser/Thr kinase, PELLE and IRAK. This leads to the inactivation of a repressor, CACTUS and IκB, which allows the translocation of the transcription factors DIF and NF-κB into the nucleus, to activate the transcription of genes involved in the defense response (Fig 4b). Specificity of the immune response in *Drosophila* seem to be achieved by the use of different kind of proteases for SPZ activation than the ones used for dorsal-ventral patterning (Lygoxygakis et at, 2002).

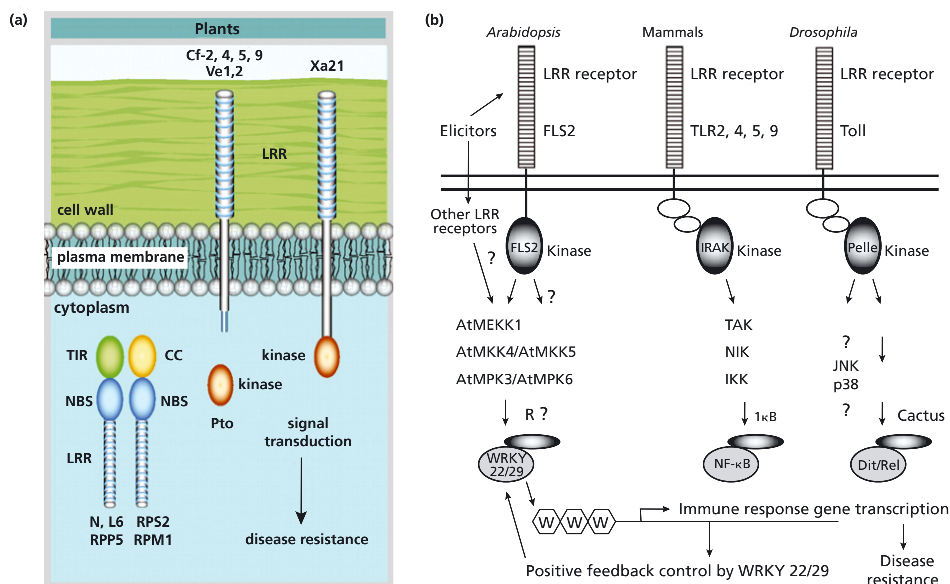


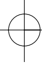
Figure 4. Innate immunity signal transduction pathways.
(a) Classes of R proteins involved in plant disease resistance (modified from Staskawitcz, 2001). (b) Homology in innate immune signalling in plants, mammals and *Drosophila*. Upon ligand binding a LRR receptor signals through a Ser/Thr kinase which ultimately leads to the translocation of a transcription factor(s) in to the nucleus and activation of transcription of target genes. A putative repressor (R) could control WRKY22 and WRKY29 activity because their overexpression bypasses the requirement of elicitors (modified from Asai et al, 2002).

The genetic bases of plant resistance is often controlled by single resistance (R) genes evolved to recognize organisms expressing specific avirulence (avr) genes. Several classes of R genes have been identified in plants (Fig 4a), and as in animals, LRR proteins constitute the predominant structural bases for pathogen perception. The R genes include transmembrane proteins containing extracellular LRRs, e.g. Cf2, 4, 5 and 9, Ve1 and 2; transmembrane proteins containing extracellular LRRs and a cytoplasmic serine-threonine kinase e.g. Xa21 and FLS2; and cytoplasmic serine-threonine kinases, e.g. Pto; where the kinases show homology to the animal IRAK/Pelle kinase (Staskawicz et al, 2001). It has recently been shown that plant receptor kinases form a monophyletic group with the Pelle family (Shiu and Bleecker, 2001).

The NB/LRR (nucleotide binding site/LRR) class of proteins is the most prevalent, and it can be subdivided into two subclasses based on conserved N-terminal motifs: one class contains a coiled-coiled (CC) domain containing a putative leucine zipper domain (such as RPS2 and RPM1), whereas the other class contains significant homology with the TIR domain present in Toll and TLR (such as N, L6 and RPR5). NB/LRR proteins appear to be cytosolic receptors that sometimes associate with the plasma membrane, where they may be capable of directly or indirectly perceiving pathogen effectors as these enter the plant cell (Fig 4a; Staskawicz et al, 2001).

Taking the FLS2 receptor as an example, there are also some common features in the downstream signaling events in plants and animals. In plants one of the elicitors of the innate immune response is flagellin, a highly conserved component of bacterial flagella. Flagellin leads to the activation of the FLS2 receptor, a RLK containing an extracellular domain with LRRs and an intracellular Ser/Thr kinase (Gómez- Gómez and Boller, 2000). Activated FLS2 induces a MAPK signaling cascade leading to the activation of the WRKY 22 and WRKY 29 transcription factors that activate their own transcription as well as the transcription of genes involved in the immune response (Fig 4b; Asai et al, 2002). In addition of the MAPK signaling cascade, flagellin signaling also induces a MAPK-independent pathway which remains to be unraveled.

In summary, host defenses in higher eukaryotes involve a highly conserved LRR kinase-mediated signaling pathway with a set of homologous proteins found in all eukaryotes. Unlike in animals where TGF-β and RTK are the major developmental signaling pathways, plants have adapted the ancient LRR-kinase signaling pathway as the predominant form for a vast variety of developmental processes as I will discuss in the next section.



CELL-CELL SIGNALING IN PLANT DEVELOPMENT

Multicellularity in plants developed from ancestral cells independently with different properties. Among the more obvious are photoautotrophic growth, absence of mobility, and the presence of a semirigid cell wall (McCarty and Chory, 2000).

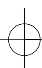
Since plants are sessile and so they have little choice over their immediate growth environment they need the ability to modify development to cope with an environment of enormous variability. Numerous environmental factors such as temperature, light, touch, nutrients, water and gravity serve as signals for the activation of endogenous developmental programs. There is substantial evidence that key elements of pathways related to stress, defense, sugar and osmotic responses are at least partially conserved in plants, animals, and fungi. These conserved pathways regulate processes that are basic to unicellular as well as multicellular organisms (McCarty and Chory et al, 2000). In contrast, the signaling pathways that underlie much of multicellular development use novel combinations of conserved domains.

Plant Receptor Kinases

Plant receptor-like kinases (RLK) belong to a large gene family with more than 400 members in *Arabidopsis* (compared with only 25 in *Drosophila* or 70 in humans; Becraft, 2002), probably as a consequence of the plants need to modify its development according to their immediate environment.

In *Arabidopsis*, there are over 21 different classes of extracellular domains. Since plant RLKs form a monophyletic group with the animal Pelle family, this suggests that most likely kinase domains from this group were recruited multiple times by fusion with different extracellular domains (Shiu and Bleecker, 2001). In addition, the distribution pattern of RLKs on *Arabidopsis* chromosomes indicates that the expansion of this gene family is partly a consequence of tandem duplication events and large-scale duplications of chromosomes. Overall, this indicates that plant RLKs evolved by sequential recruitment and fusion of various domains to an ancestral kinase and further expansion of certain classes through duplication events (Shiu and Bleecker, 2001).

Of the 21 different classes, the most common extracellular motif is the leucine-rich repeat (LRR), present in more than half of the RLKs. All known plant RLKs contain a serine/threonine kinase consensus sequence, but at least two: PRK1 (pollen receptor-like kinase1) and SERK (somatic embryogenesis receptor-like kinase) have dual specificity, PRK1 being able to phosphorylate on serines and tyrosines, while SERK phosphorylates serines, threonines and tyrosines (Becraft, 2002).



Although for the majority of plant RLKs no functional information is available, different members of this family are known to function in a wide variety of developmental processes and in innate immunity. RLKs have been implicated in a plethora of processes such as pollen-pistil interactions in the self-incompatibility response in *Brassicaceae* (Kachroo et al, 2002), gametophyte development (Zhao et al, 2002; Lee et al, 1996), somatic embryogenesis (Schmidt et al, 1997), hormone signaling (Bishop and Koncz, 2002), cell morphogenesis (Kohorn, 2001), organ shape (Torii et al, 1996) and meristem maintenance (Clark et al, 2001).

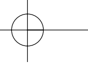
I will next consider two well-studied signaling pathways in which LRR-RLKs have a prominent role: first the brassinosteroid signal transduction pathway controling multiple processes involved in plant growth and development e.g. cell expansion and xylem differentiation, and second the CLAVATA pathway controlling stem cell maintenance in the shoot apical meristem.

Brassinosteroid signaling pathway

Steroid hormones play essential roles in plants as well as in animals. In plants, many steroids have been identified, brassinolide (BL) being the most bioactive form of the growth-promoting plant steroids termed brassinosteroids (BRs). Like their animal counterparts, BRs have been shown to regulate gene expression, stimulate cell division and differentiation and modulate reproductive biology. But BRs are also able to mediate some plant specific responses including promotion of cell elongation in the presence of a cell wall and coordination of multiple developmental responses to darkness and light (Clouse and Sasse, 1998).

In animals, steroid hormones generally pass freely across the plasma membrane into the cells, where they bind members of the nuclear receptor superfamily of ligand-dependent transcription factors in the cytoplasm, and the active complex is then translocated into the nucleus to promote or repress transcription of hormone-responsive genes (Beato et al, 1995). Plants lack close homologues of animal nuclear steroid receptors indicating that steroid signaling in plants is mediated by alternative mechanisms (Wehling, 1997).

To find genes involved in the BR signal transduction pathway, several genetic screens have been performed looking for BR insensitive mutants, which display a characteristic dark green dwarf phenotype. The first such mutant to be identified was *bri1* in *Arabidopsis*, from which multiple alleles were isolated. *BRI1* was cloned and shown to encode a protein with homology to LRR-RLKs (Li and Chory, 1997). *BRI1* is ubiquitously expressed, with high levels of expression in meristems, root, shoot and hypocotyl of seedlings and lower levels later in development (Friedrichsen et al, 2000). BR-insensitive

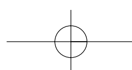


mutants encoding for *BRI1* orthologs (Bishop and Koncz, 2002) have been identified in rice (Yamamuro et al, 2000), pea (*lka*; Nomura et al,1999) and tomato (*cu-3*; Koka et al, 2000) highlighting the conservation of BRI1 function in BR perception in higher plants.

To test whether the extracellular LRR region of BRI1 was essential for BL sensing, a chimeric receptor was constructed containing the extracellular LRR and transmembrane domains of BRI1 and the serine/threonine kinase domain of XA21, a rice disease resistance receptor (He et al, 2000). The chimeric receptor initiated plant defense responses upon treatment with BL suggesting that the extracellular domain of BRI1 plays a direct role in brassinosteroid perception. In addition, the number of BL binding sites depends on the level of BRI1 protein, the BL binding activity co-immunoprecipitates with BRI1 and requires a functional BRI1 extracellular domain, and treatment of *Arabidopsis* seedlings with BL induces autophosphorylation of BRI1, all suggesting that BRI1 acts as a BL receptor (Wang et al, 2001).

Recently a second LRR-RLK involved in BL signaling, BAK1 (BRI1 Associated receptor Kinase 1), has been identified by yeast-two hybrid screens as an interactor of BRI1 (Nam and Li, 2002) and in an activation tagging screen for suppressors of *bri1* (Li et al, 2002). Overexpression of *BAK1* results in elongated organ phenotypes (reminiscent of *BRI1*-overexpressors) and rescues a weak *bri1* mutant, while a *bak1* null allele displays a semidwarf phenotype and has reduced sensitivity to BR. Expression of a dominant negative *bak1* mutant allele causes a severe dwarf phenotype, resembling the phenotype of null *bri1* alleles. *BAK1* and *BRI1* share similar gene expression and subcellular localization patterns and interact in vitro and in vivo, suggesting that BRI1 and BAK function together, most likely through heterodimerization, to mediate plant steroid signaling (Fig 5). BRS1 (*bri1* suppressor dominant 1), an upstream component in BL signaling, was identified in a gain-of-function screen for suppressors of a weak *bri1* allele. *BRS1* encodes for a presumed secreted type II carboxypeptidase, which when overexpressed could suppress extracellular *bri1* mutations but not the intracellular ones, suggesting that BRS1 processes a protein involved in an early event in BRI1 signaling (Fig 5; Li et al, 2001a).

Downstream components of the signaling pathway are also being unraveled. BIN2, a GSK3/SHAGGY like kinase, has been shown to be a negative regulator of BL signaling (Li et al, 2001b; Li and Nam, 2002). Interestingly, GSK3/SHAGGY like kinases belong to a class of cytoplasmic serine/threonine kinases highly conserved and widely found among eukaryotes, often acting as negative regulators of signal transduction pathways controlling metabolism and developmental events (Fig 5). Two novel homologous proteins that are potential targets for BIN2 are BES-1D (bri1 EMS-suppressor 1-D) and BZR 1-D (brassinazole resistant 1-D), containing nuclear localization signals and consen-



sus sites for phosphorylation by GSK-3 kinases. They are found in the cytoplasm and their nuclear localization is rapidly induced by brassinosteroids (Yin et al, 2002; Wang et al, 2002). But while BES1 appears to promote mainly the activation of genes involved in cell expansion, BZR1 seems to inhibit primarily the activation of genes coding for enzymes involved in BR biosynthesis. The Kinase-Associated Protein Phosphatase (KAPP) and the Rho-like GTPase ROP2 have also been found to act downstream of BRI (Schumacher and Chory, 2000). KAPP has been previously observed to negatively regulate the CLAVATA signaling pathway by dephosphorylating a pressumably active receptor (Williams et al, 1997), suggesting that KAPP might function as a negative regulator of the BL signaling pathway. ROP2 has been suggested to act as a signal transducer in the BL signal transduction pathway, maybe by transducing signals to MAPK, as biochemical analysis of *BRI1* antisense rice plants showed that MAPK activity was induced by BL treatment (Li et al, 2001c; Sharma et al, 2001).

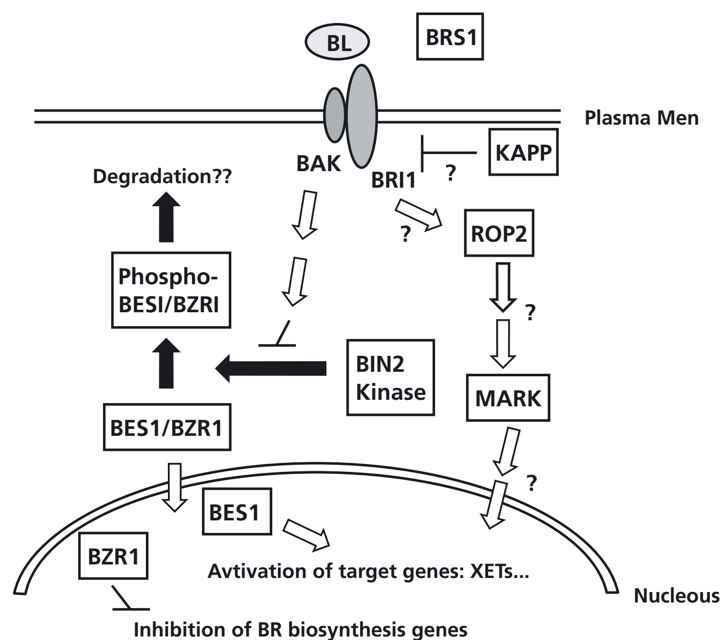
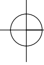


Figure 5. Models for BL signal transduction.
BL ligand binding to the cell surface receptors BRI1 and BAK1 initiates a signaling cascade that inactivates BIN2, and allows accumulation and nuclear localization of BES1 and BZR1. BES1 mainly induces activation of genes involved in cell expansion, while BZR1 is involved in repression of genes involved in BR biosynthesis. The BRS1 carboxypeptidase might be involved in the processing of proteins which act as helpers in ligand binding. KAPP would be responsible for dephosphorylation of the active receptor, while ROP2 transduces signals to downstream components like MAPK. Steps that have a positive effect on the signaling are with open arrows, while those with a negative effect are shown with black arrows.



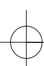
In addition to signaling components, some target genes of the BL signaling pathway have been identified. BR biosynthesis genes and transcription factors have been shown to be repressed upon BL treatment and acumulate in *bri1* mutants (Choe et al, 2001; Müssig et al, 2002). Others, like xyloglucan endotransferases (XET) have been found to be up-regulated (Xu et al, 1996; Klahre et al, 1998) demonstrating that the induction of XETs correlates with cell wall loosening during BL-induced growth responses. Recently, three highly redundant bHLH transcription factors, *BEE1*, *2* and *3* have also been identified as early response genes required for full BR response (Friedrichsen et al, 2002).

Taken together, one possible model would be that BL binds to the BRI1-BAK1 receptors, may be with the help of proteins processed by BRS1. This lead to the repression of the BIN2, which would allow for the accumulation of BES1/BZR1 and its translocation into the nucleus. There, BES1 would mainly activate genes involved in cell expansion, while BZR1 would repress genes involved in BR biosynthesis. In the absence of ligand, BIN2 would be active, and phosphorylate BES1 and possibly BZR1, may be marking them for degradation. Other components of the activated pathway include KAPP, possibly involved in dephosphorylation of the active receptor(s), and ROP2 which could transduce signals to downstream components like MAPK (Fig 5; Becraft, 2002; Bishop and Koncz, 2002; Clouse and Sasse, 2002 for reviews).

Shoot Meristem Maintenance: the CLAVATA pathway

One of the main differences between plant and animal development is that in plants organogenesis goes on after embryogenesis through the entire life span of the plant, thanks to the activity of the meristems where a constant population of stem cells is maintained. The shoot meristem will generate all the aboveground organs and the root meristem will give rise to all the underground parts.

In the shoot apical meristem (SAM), cells are organized into the outer tunica layers and the inner corpus layers. In many species, including *Arabidopsis*, the tunica consist of two clonally distinct cell layers, named L1 and L2 where cell divisions are exclusively anticlinal while below the tunica, in the corpus or L3 layer, cell divisions are not strictly oriented. Even though this separation into clonally distinct cell layers may suggest cell-lineage dependant cell fate specification, studies using genetic mosaics have shown that the position of a cell and not its clonal origin, determines its fate (Irish and Sussex, 1992). A second level of organization divides the SAM into the central zone (CZ), which contains the stem cell population of slowly dividing cells, and the peripheral zone (PZ), where cells divide more frequently and are incorporated into lateral organs.



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Arabidopsis mutants that either lack stem cells or accumulate ectopic stem cells have uncovered a signaling pathway involved in stem cell maintenance in the shoot meristem. Loss-of-function mutations at the *CLAVATA* (*CLV1*, *CLV2* and *CLV3*) loci cause the progressive accumulation of undifferentiated stem cells as development proceeds (Clark et al, 1993; Clark et al, 1995; Kayes and Clark, 1998). Genetic analysis has shown that *CLV1*, *CLV2* and *CLV3* function together to restrict the size of the stem cell population in shoot and floral meristems, although *CLV2* also functions more broadly to regulate other aspects of development.

The three *CLV* genes have been cloned and shown to encode a LRR-RLK (Clark et al, 1997), a LRR receptor-like protein with a short cytoplasmic tail (Jeong et al, 1999), and a small secreted protein (Fletcher et al, 1999), respectively. *CLV3* is expressed in the stem cell population at the central zone of the meristem, primarily in the L1 and L2 layers, while *CLV1* is found mostly in an underlying domain in the L3. Expression of *CLV2* can be found in

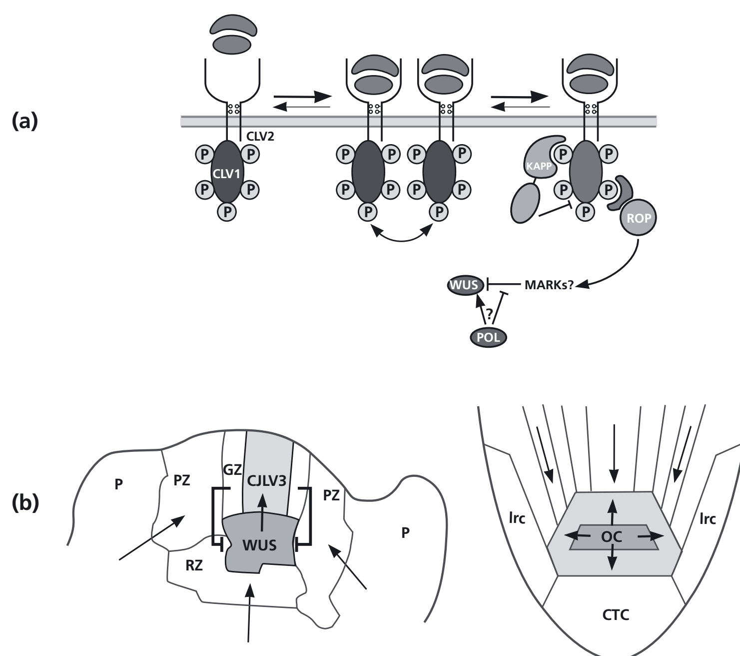

Figure 6. Models for stem cell maintenance.
(a) CLV3, may be as a multimer or with the help of another protein, binds to the extracellular domain of CLV1/CLV2 heterodimer. Ligand binding leads to autophosphorylation of CLV1, which then recruits downstream components as KAPP and ROP. The signaling cascade ultimately leads to repression of *WUS* expression. POL acts as a negative regulator of the pathway, downstream of CLV1 (modified from Clark, 2001). (b) In the shoot meristem the organizing center, where *WUS* is expressed, acts to promote stem cell fate in the cells above it. In turn, the stem cells, through the action of CLV3, act via the CLV1 signaling pathway to inhibit the domain of *WUS* expression. In analogy, in the root meristem the QC has also been shown to promote the stem cell activity of the cells surrounding it (modified from Lenhard and Laux, 1999).

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whole meristems and also in other tissues, consistent with the broader domain of action of this gene.

CLV3 has been shown by biochemical and genetic approaches to act as the ligand for CLV1 as part of a multimeric complex (Trotochaud et al, 1999 and 2000; Rojo et al, 2002). In wild type, the CLV1 protein can be found as part of two protein complexes. As a 450 kDa protein complex, assumed to be the active complex, which requires the presence of functional CLV3 and CLV2 protein for its stability. Or as a part of a 185 kDa complex, supposed to be the inactive form, which possibly contains CLV1 and CLV2. Two other proteins have been identified as components of the 450 kDa complex: KAPP, a phosphatase which was previously shown to be a negative regulator of the CLV pathway (Williams et al, 1997; Stone et al, 1998), and a Rho/Rac-GTPase-related protein (ROP) which on the basis of the role of such proteins in animal signal transduction, it has been suggested it may respond to the CLV1 activation by activating a MAPK signaling cascade (Fig 6a; Trotochaud et al, 1999).

Another key component involved in shoot meristem maintenance is *WUSCHEL* (*WUS*), which encodes a putative homeodomain transcription factor (Mayer et al, 1998). *WUS* seems to be involved in promoting stem cell activity throughout development, as *wus* mutant shoot and floral meristems terminate prematurely after the formation of few organs (Laux et al, 1996). After embryogenesis, *WUS* is expressed in a small group of cells that lie beneath the *CLV3* expression domain and partially overlap with the *CLV1* domain in the central L3 cells, forming what has been named the organizing center.

wus clv double mutants have the same phenotype as *wus* single mutants, indicating that WUS might act downstream of CLV (Schoof et al, 2000). In addition, in *clv3* mutants, the *WUS* expression domain expands upwards and laterally, while in *CLV3* overexpressing plants, that form arrested meristems and phenocopy the *wus* loss-of -function mutation, *WUS* mRNA is not detected (Brand et al, 2000). This suggests that the CLV pathway represses *WUS in vivo*. *WUS* expression under the *CLV1* promoter recreates the *WUS* expression domain seen in *clv1* mutants, and mimics a *clv* phenotype, suggesting that the accumulation of stem cells in *clv1* mutants is a consequence of *WUS* misexpression. When *WUS* was expressed in the *AINTEGUMENTA* (*ANT*) expression domain (in nascent organ primordia) it prevented differentiation of incipient organs, forming a large mass of stem cells instead (Schoof, 2000). Together these results point to the existence of a negative feedback loop, where CLV3 acts (non-cell autonomously) from the stem cell population to negatively regulate *WUS* expression, while WUS from the organizing center activates *CLV3* expression in the center of the meristem. In such system, the balance between WUS and CLV3 controls the size of the stem cell population in the SAM (Fig 6b).

In a genetic screen for suppressors of *clv3* and *clv1* intermediate alleles, the *poltergeist* (*pol*) mutant was identified as another downstream component of the CLV pathway (Fig 6a; Yu et al, 2000). The single *pol* mutant does not have any phenotype, but in the double mutant combination with *clv* the plants have fewer stem cells in the shoot and floral meristem than the *clv* single mutants. On the other hand, *pol* mutations enhance *wus* phenotypes in a dosage dependent fashion. *POL* have been recently shown to encode a protein phosphatase 2C (PP2C) broadly expressed throughout the plant (Yu et al, 2003). Interestingly, analysis of *pol clv wus* triple mutants and *pol wus* double mutants revealed that in a *pol* background CLV1 can function in the absence of WUS. Thus, POL functions in both a WUS-dependent and a WUS-independent CLV pathway promoting stem cell identity (Yu et al, 2003).

Recent evidence suggest that the CLV pathway controlling meristem maintenance is conserved in other species. A mutant of maize, *fasciated ear2* (*fea2*), causes shoot and floral meristem enlargement and massive over-proliferation of the ear inflorescence meristem. FEA2 protein is closely related to the *Arabidopsis* CLV2 LRR receptor-like protein, and localizes to the plasma membrane (Taguchi-Shiobara et al, 2001). *OsLRK1*, a *CLV1*- related gene from rice has also recently been isolated, and rice plants expressing antisense copies of this gene have extra floral organs but are not affected in the SAM (Kim et al, 2000). This suggests that the components of the CLV pathway are also present in monocots, which might mean that this may be the conserved pathway in angiosperms to regulate meristem size, at least in flowers. (Sharma and Fletcher, 2002 for review on CLV pathway).

Aim of this thesis

The earliest vascular plants were rootless with leafless shoot axes. The first roots evolved among an extinct group, the Lycopsids, and they were modified lateral appendages possessing a single leaf trace. The Trimerophytes is the group from which the remainder of vascular plants originated, and they were also rootless. Consequently roots evolved first among the Lycopsids and on at least one further occassion during the evolution of vascular land plants (Dolan and Scheres, 1998).

Some common mechanisms are known to act in both shoots and roots. In roots as in shoots, despite regular cell lineages, cell fates are regulated by positional signaling (van den Berg et al , 1995). In addition, cell fate in root epidermal cells is regulated by genes also used in leaf epidermis specification. Furthermore, the quiescent center is involved in the control of stem cell maintenance, in analogy with the role of the *WUS*-expressing organizing center in the SAM. All these three processes are mechanistically reminiscent of corre-

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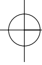
sponding processes in the shoot (Dolan and Scheres, 1998). One obvious possibility would be that roots might be derived from ancestral shoot structures.

In the root meristem the processes of cell proliferation and specification take place in their most simple form. In the centre of the root meristem a pool of stem cells or initials surround four mitotically less active cells, the quiescent center (QC). Within the meristem, cell files are extended in such a way that they are continuous with pre-existing tissues, leading to the propagation of a simple radial cellular pattern that is initially laid down in the embryo (Dolan and Scheres, 1998). By laser ablation experiments it was shown that the QC inhibits the differentiation of the stem cells surrounding it in a contact-dependent manner, and that progression to differentiation depends on signals from more mature cells (van den Berg, 1995 and 1997). This scenario is analogous to the situation in the SAM, where an organizing center also controls the differentiation status of the stem cell population (Fig 6b).

We addressed which molecular mechanisms control root meristem maintenance, and whether a CLV-like pathway is involved in controlling the balance between cell division and cell differentiation. Whether or not a CLV-like pathway is responsible for both shoot and root meristem maintenance in higher plants may help us understand the process of organ evolution in seed plants.

Two approaches have been taken to address these questions. First, we performed a suppressor screen on transgenic plants ectopically expressing a *CLV3*-like gene in the root meristem, which causes progressive root mersitem differentiation. Mutations in two genes were isolated, and phenotypic analysis undertaken. One of the genes was cloned and shown to encode a Zn^{2+}-carboxypeptidase. Second, we used a reverse genetic approach to isolate and study *CLV1*-like receptors which are expressed in the root meristem. Initially, two LRR-RLKs were isolated, *RCH1* and *RCH2* (*ROOT CLAVATA HOMOLOGUE 1* and *2*). Functional loss-of-function analysis revealed no obvious phenotypes neither in the single mutants nor in the double, possibly due to genetic redundancy. We extended our study to the next 3 closest members, which altogether form a clade, and further functional and expression analysis was performed.



Chapter 2

SOL1 and SOL2 implicate a novel CLV-like pathway in the control of *Arabidopsis* root meristem differentiation.

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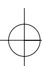
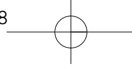
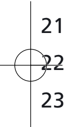
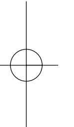
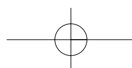










SOL1 and SOL2 implicate a novel CLV-like pathway in the control of *Arabidopsis* root meristem differentiation.

ABSTRACT

The balance of cell division and cell differentiation in the *Arabidopsis* shoot apical meristem is controlled by a negative feedback loop between the cells expressing the *WUSCHEL* gene and the overlaying stem cells expressing the CLAVATA3 (CLV3) ligand of the CLV1-CLV2 receptor complex. Here we show that ectopic expression of *LIGAND LIKE PROTEIN 1* (*LLP1*) encoding a CLV3 homologue in roots, promotes differentiation or restricts cell division in the root meristem, without primarily affecting quiescent center specification or stem cell maintenance. From a screen aimed to identify components of a root CLV-like pathway, we isolated mutations in two loci, *SOL1* and *SOL2*, that suppress the ectopic *LLP1* expression phenotype. *sol2* plants display floral phenotypes reminiscent of *clv* weak alleles, suggesting that components of the pathway are shared in roots and shoots. *SOL1* was cloned and found to encode a putative Zn^{2+}- carboxypeptidase which may be involved in ligand processing.

INTRODUCTION

Stem cells have the ability to renew themselves and to give rise to daughter cells which differentiate. In seedlings there are two main populations of stem cells, one within the shoot apical meristem (SAM) which gives rise to all above-ground organs, and one in the root meristem which forms all the underground parts (Nakajima and Benfey, 2002; Clark, 2001).

Post-embryonic plant development depends on strict regulation of stem cell maintenance, cell division and cell differentiation at the meristems. In the SAM the current view is that stem cell maintenance depends on cell-cell communication between an organizing center and the stem cells above it. The organizing center, specified by the putative homeodomain transcription factor *WUSCHEL* (*WUS*), signals in a non-cell-autonomous manner to overlying cells to specify them as stem cells (Mayer et al, 1998), which express the small secreted protein CLAVATA 3 (CLV3; Fletcher et al, 1999). CLV3 likely inter-

acts and activates the heterodimer receptor complex formed by the leucine rich repeat (LRR)-receptor kinase CLAVATA1 (CLV1), and the LRR-receptor CLAVATA 2 (CLV2) resulting in transcriptional repression of *WUS* (Trotochaud et al, 1999; Rojo et al, 2002; Brand et al, 2000; Schoof et al, 2000). Thus, the available data support a model where the size of the stem cell population in the SAM depends on a negative feedback loop. WUS promotes stem cell fate by activating *CLV3* expression, and CLV3 acts via the CLV1/CLV2 receptor complex to limit the domain of *WUS* expression.

In the *Arabidopsis* root meristem the stem cells or initials surround a group of mitotically less active cells, the quiescent center (QC), and they can be unequivocally identified by anatomical features (Dolan et al, 1993). Laser ablation experiments suggested that the QC inhibits the differentiation of the surrounding stem cells by short-range non-cell-autonomous signals (van den Berg et al, 1997). Recently Sabatini et al (2003) have shown that the putative transcription factors *SCR* and *SHR* are required for distal specification of the QC, which in turn regulates stem cell fate of the immediately surrounding cells. Daughter cells that become disconnected from the QC differentiate according to positional cues (van den Berg et al, 1995).

Since the role of the QC in roots seems to be functionally equivalent to the role of the *WUS*-organizing center in the SAM, we wondered whether a CLV-like pathway might operate in roots to control root meristem maintenance. We ectopically expressed *LLP1*, a *CLV3* homologous gene, in the root meristem and we observed root meristem differentiation in analogy to the effect in shoots upon *CLV3* overexpression. Our data suggest that a novel CLV-like pathway is involved in root meristem maintenance. To isolate the signaling components involved in this pathway, we performed a suppressor mutagenesis screen. We report on two new loci involved in this signal transduction pathway, one of them encoding a putative Zn^{2+}-carboxypeptidase of the type implicated in ligand processing events in animals.

RESULTS

Ectopic expression of *LLP1* in root meristems causes meristem differentiation

LLP1 is a small potentially secreted protein belonging to the CLE family of which also CLV3 is a member. *LLP1* corresponds to *CLE19* from the Cock and McCormick classification (2001). Overexpression of the *Brassica napus BrLLP1* in *Arabidopsis* under the 35S CaMV promoter causes root meristem differentiation among other phenotypes (Chun-Ming Liu, unpublished results). In analogy, when *CLV3* was overexpressed under the 35S promoter, the shoot meristem ceased organ initiation after emergence of the first leaves (Brand et

al, 2000). This similar effect suggested that a CLV-like pathway might also act in roots to maintain the balance between cell division and cell differentiation in the meristem. However, the effect on root meristem maintenance by over-expressing *LLP1* in the whole plant could be indirect, for example as a consequence of long-range shoot-to-root signaling.

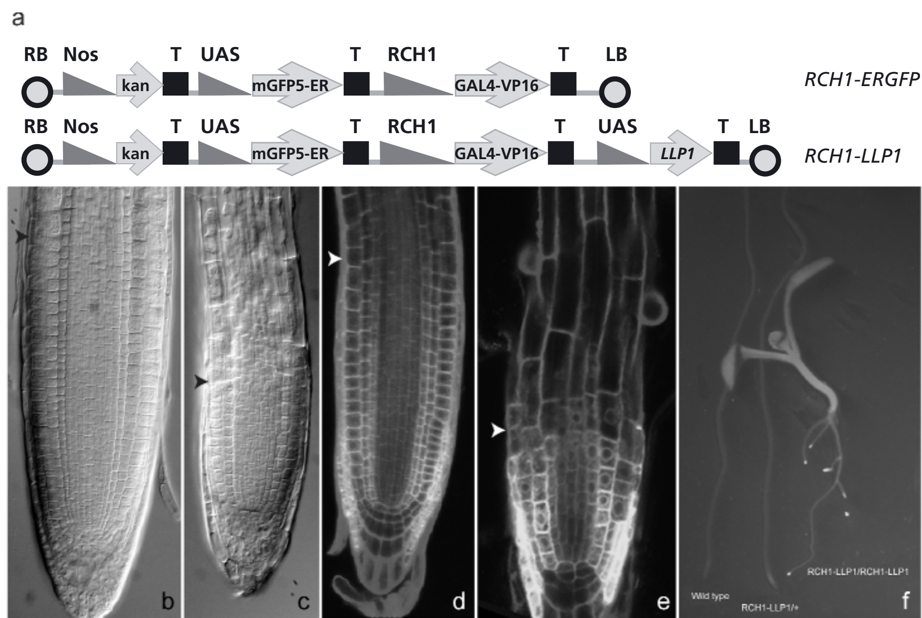
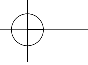


Figure 1. Ectopic expression of *AtLLP1* in the root meristem causes root meristem differentiation.
(a) *RCH1-ERGFP* and *RCH1-LLP1* constructs (promoters in green, coding regions in orange) (b-c) Root meristem boundary (arrowhead) of four-day-old roots from WT and RCH1-LLP1. (d-e) Confocal image of a one- week-old WT and RCH1-LLP1 root (meristem boundary marked with an arrowhead). (f) Image of one-week-old seedling roots as viewed under a GFP binocular, from left to right WT, heterozygous RCH1-LLP1 and homozygous RCH1-LLP1.

To address whether *LLP1* expression in the root meristem only is sufficient to cause a root meristem differentiation phenotype, we expressed the *Arabidopsis AtLLP1* transgene under the control of the *RCH1* promoter, which is highly expressed specifically in the root meristem (Patent number PCT/EP01/14154; Fig 1d). We used the *UAS-GAL4VP16* transactivation system, whereby the *RCH1* promoter is fused to *GAL4VP16* which in turn promotes the transcription of ER-GFP by binding to upstream *UAS* elements, resulting in the *RCH1-ERGFP* root specific binary vector (Fig 1a). *AtLLP1* under the control of the *UAS* promoter was then cloned into this vector creating the *RCH1-LLP1* binary vector (Fig 1a). *Arabidopsis* plants were transformed with the *RCH1-LLP1* vector and transgenic plants were selected for root GFP expression.



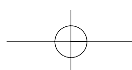
23 independent lines carrying the transgene showed similar phenotypes as the homozygote single insertion line described below. In this single insertion line, which we will call RCH1-LLP1 hereafter, we observed that the ectopic expression of *LLP1* in the root meristem caused a progressive differentiation of the meristem in a dose dependent manner. Heterozygous plants, which were distinguished by lower GFP expression in the root meristem, behave as wild type roots, while homozygous RCH1-LLP1 plants have short roots with high GFP expression (Fig 1e, f), indicating that two doses of RCH1-LLP1 are required to affect root growth in this line and that the RCH1-LLP1 construct is inherited as a recessive trait. We decided to use this single insertion line for detailed analysis. Homozygous RCH1-LLP1 roots progressively lose cells in the meristematic zone (Fig 1c and e), indicated also by the formation of root hairs closer to the root tip, and eventually in some seedlings the root meristem is fully consumed while this never happens in control roots (Fig 1b, d).

To assess whether this phenotype is due to ectopic expression of the AtLLP1 protein, we mutated codon 3 of *AtLLP1* in the *RCH1-LLP1* construct into a stop codon, creating the *RCH1-LLP1stop* vector (see matetial and methods). The roots of plants transformed with this vector have a wild type appearance, indicating that the defective root growth phenotype in RCH1-LLP1 plants is caused by ectopic expression of the LLP1 protein (data not shown). We concluded that the *CLV3* homologue *LLP1* induces differentiation phenotypes when ectopically expressed in roots (like overexpression of *CLV3* does in shoots), consistent with the hypothesis that LLP1 may overactivate an endogenous CLV like pathway in the root meristem involved in root meristem maintenance.

Ectopic LLP1 induces meristem differentiation rather than failure in stem cell maintenance

Two causes for enhanced root meristem differentiation can be envisioned: loss of stem cell maintenance by lack of QC activity or specification (van den Berg et al, 1997; Sabatini et al, 2003) and loss of division potential or more rapid differentiation of stem cell daughters. In the first case, primary defects in the QC region would be expected, while in the second case meristem size would decrease before QC and stem cells would show defects.

To asses whether QC specification is rapidly affected in RCH1-LLP1 plants, we introduced the QC markers QC25 and QC184 in RCH1-LLP1 (Sabatini et al, 1999; Fig 2a, b, data not shown). RCH1-LLP1 roots still express these markers one week after germination when root meristem size is already significantly reduced compared to WT (Fig 2a, b; Fig 1e), suggesting that QC specification is not primarily affected.



To analyze whether stem cells are maintained in RCH1-LLP1 roots, we stained for starch granules that mark differentiated columella cells, to check if the columella initials remain devoided of starch granules and hence retain their stem cell status. One week after germination, no starch granules could be detected in the columella stem cells of 73% (n=26) of the RCH1-LLP1 seedlings analyzed (Fig 2a, b arrow), suggesting that stem cell status is maintained for a prolonged period.

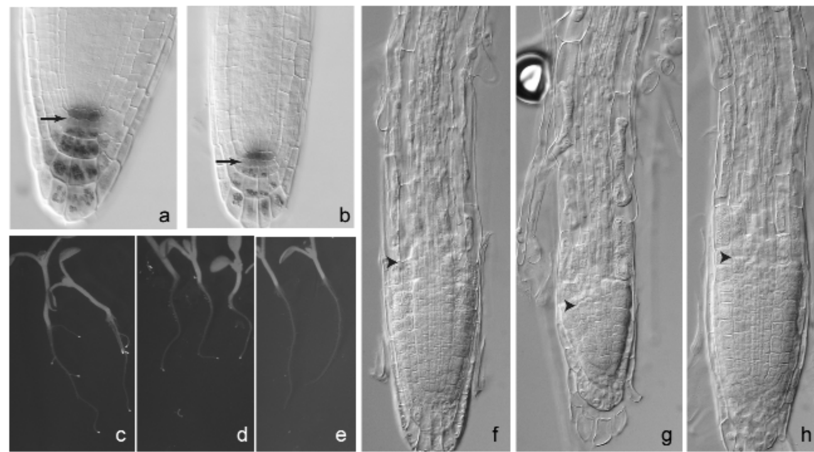


Figure 2. QC specification and stem cell status is not affected in RCH1-LLP1 roots. (a-b) Double labeling of QC and differentiated columella cells visualized by the QC25 marker and amyloplast staining in one-week-old WT and RCH1-LLP1 roots. The columella initials (arrow) do not show signs of differentiation. (c-e) Six-day-old seedlings of homozygous RCH1-LLP1, double homozygous *RCH1-LLP1,shr-1* and homozygous *shr-1*, respectively. (f-h) Root meristem boundary (arrowhead) of six-day-old roots from homozygous RCH1-LLP1, double homozygous *RCH1-LLP1,shr-1* and homozygous *shr-1*, respectively.

The expression of both QC markers together with the maintenance of columella stem cells one week after germination in RCH1-LLP1 seedlings, when progressive differentiation of the meristem actively occurs, indicates that ectopic expression of *LLP1* in the root meristem enhances differentiation or reduces cell division by a mechanism different from interference with QC specification and/or stem cell maintenance.

The LLP1 induced differentiation pathway is independent of SHR and SCR

In *shr* and *scr* mutants, root growth ceases prematurely as we also observed in RCH1-LLP1 plants (Fig 2h; Benfey et al, 1993; Scheres et al, 1995). *SHR* and *SCR* are both members of the GRAS family of putative transcription factors, and are required for QC specification and stem cell maintenance (Di Laurenzio et al, 1996; Helariutta et al, 2000; Sabatini et al, 2003). In *scr* and *shr*

mutants, the QC25 marker is never expressed and the columella initials differentiate. Our observation that QC and stem cells are intact in RCH1-LLP1 roots suggests that LLP1 acts in a different pathway or downstream of SHR and SCR.

To investigate whether LLP1 acts downstream in the same pathway as SHR and SCR in the control of root meristem maintenance, we introduced *RCH1-LLP1* in *shr-1* and *scr-1* mutants. *shr-1* mutants homozygous for *RCH1-LLP1* could be identified by short roots with a smaller domain of high GFP expression (characteristic of two doses of RCH1-LLP1) and absence of lateral roots as in *shr* mutants of this stage (Fig 2c, d, e). Double homozygosity was confirmed by genotyping (see material and methods). It is of note that *RCH1-LLP1,shr-1* double homozygotes show additive phenotypes, the root meristem differentiates faster than in either single homozygote as seen by the decreased number of meristematic cells (Table 1; Fig 2f, g, h arrowhead) and the closer proximity of root hairs to the tip. Similar results were observed in *RCH1-LLP1,scr-1* double homozygotes (data not shown).

Table 1 . Meristem size measurements.

	Average number of cortex cells per file in the meristem*
RCH1-LLP1/RCH1-LLP1 *shr-1/shr-1*	1.8 ± 1.5
RCH1-LLP1/RCH1-LLP1 +/+	6.8 ± 0.4
+/+ *shr-1/shr-1*	6.3 ± 0.4

*Values represent the average number of cortex cells per file in the meristem showing no signs of rapid elongation ± standard deviation. Measurements were performed 6 days after germination, using 9 roots from RCH1-LLP1/RCH1-LLP1 *shr-1/shr-1* plants, 5 from +/+ *shr-1/shr-1* and 4 from RCH1-LLP1/RCH1-LLP1 +/+. Per root one cortex cell file was analyzed.

Our results suggest that control of the differentiation of meristematic cells by ectopic *LLP1* expression in the root meristem is independent of the SHR/SCR pathway that specifies the QC and thereby stem cell identity.

Mutagenesis screen for suppressors of *RCH1-LLP1*

To find molecular components involved in the root meristem differentiation phenotype caused by ectopic expression of *AtLLP1* in the root meristem, an ethylmethane sulfonate (EMS) mutagenesis was performed in the RCH1-LLP1 background to identify suppressors. 8100 mutagenized RCH1-LLP1 M0 seeds were divided in 10 pools, and a minimum of 11000 M2 seedlings were

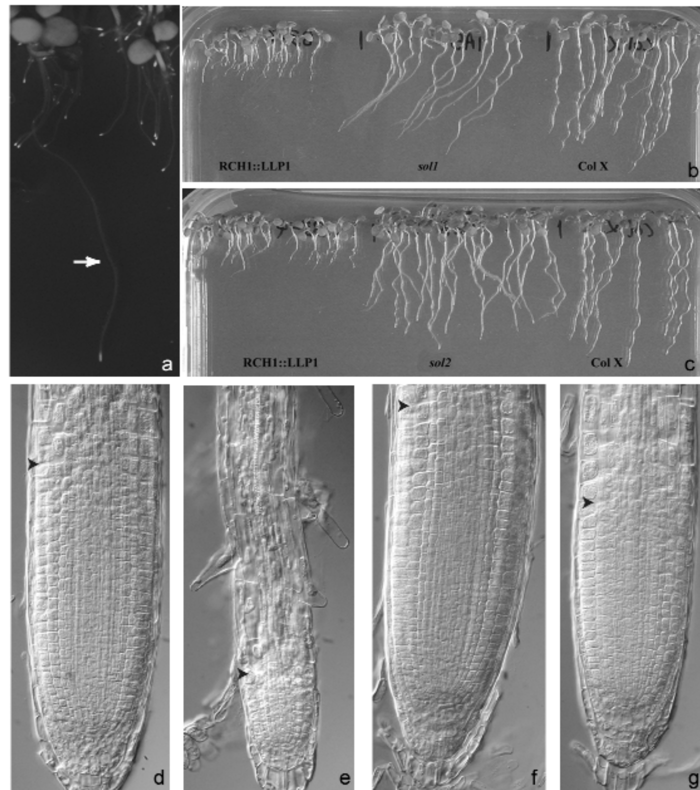


Figure 3. The *sol1* and *sol2* mutants suppress *RCH1-LLP1* induced root meristem differentiation.
(a) Screening for suppressors. A suppressor mutant (arrow) has a long root with high GFP expression, among other RCH1-LLP1 seedlings which posse's short roots with high GFP. (b-c) One-week-old *sol1* and *sol2* seedlings have a root length comparable to WT and much longer than RCH1-LLP1 of the same stage. (d-g) Root meristem boundary (arrowhead) of one-week-old WT, RCH1-LLP1, *sol1* and *sol2* roots, respectively.

screened per pool for recovery of root length with high GFP (indicating full activity of the transgene; Fig 3a arrow). Putative mutants were checked in the M3 generation for re-segregation of the suppressor phenotype and put in complementation groups by pairwise crossing. Mutations at two novel loci suppressed RCH1-LLP1, and were named *suppressor of RCH-LLP1 1* and *2* (*sol1* and *sol2*). Hereafter, *sol1* and *sol2* refer to the mutants in the *RCH1-LLP1* homozygous background unless stated otherwise. Four alleles from *sol1* were isolated from 4 independent families, while 2 alleles coming from 2 different families were recovered for *sol2*.

Both *sol1* and *sol2* are able to fully suppress both the root length and meristem differentiation defect seen in the RCH1-LLP1 plants up to one week after germination (Fig 3b-g, Fig 4a-b). At this stage the meristem size of *sol1* and *sol2* resembles the wild type (Fig 3d, f, g arrowheads, Fig 4b), even though *sol2* meristems tend to be slightly smaller (Fig 3d, g arrowhead, Fig 4b). After

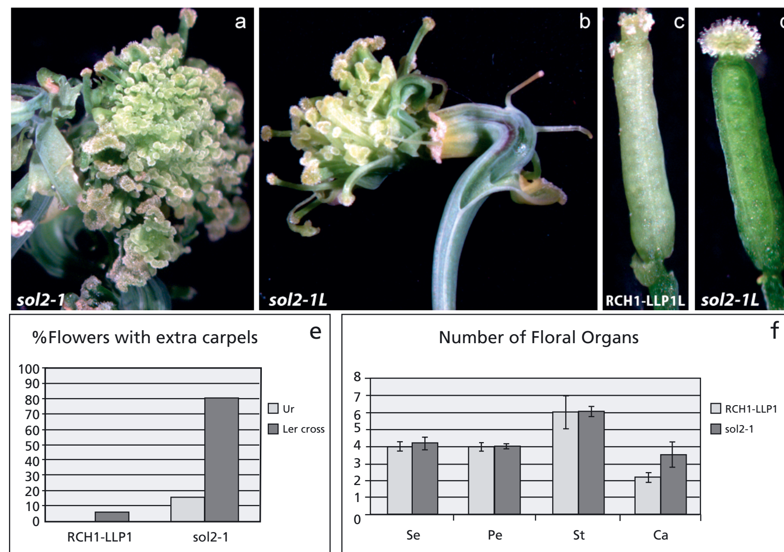


Figure 4. Root growth and meristem size in *sol1* and *sol2* roots in the RCH1-LLP1 background.
Root length (a) and root meristem cell number (b) of seedlings grown on 1/2 GM of RCH1-LLP1, WT, and the different *sol1* and *sol2* alleles.

one week *sol2* seedlings slow their root growth while *sol1* root growth accelerates (Fig 4a). This difference in growth becomes more clear 15 dpg when all 4 *sol1* alleles have longer roots and all roots of the 2 different *sol2* alleles are shorter than controls of the same age without the *RCH1-LLP1* construct (Fig 4a). However, roots of all *sol1* and *sol2* alleles grow longer than RCH1-LLP1 roots (Fig 4a). As a measure for meristem size, we counted the number of cortex cells in a single file extending from the QC up to the first rapidly elonga-ting cell, and we observed that while all *sol1* alleles contain more meristematic cells in the 15 dpg meristem, all *sol2* alleles contain less (Fig 4b). These results suggest that the differences in root growth rate between these alleles correlate with the amount of cells in the root meristem.

sol2 flowers have extra carpels, reminiscent of *clv* mutant defects

We noticed that 45 % of *sol2-2* flowers and 15 % of *sol2-1* flowers contain extra carpels in the last whorl (Fig 5e). In addition, terminal flowers occasionally contain less whorl 1 to 3 floral organs and accumulate a central mass of carpels (Fig 5a). In some cases when this extreme accumulation of carpels occurs, fasciation of the main stem was also observed (data not shown). Both the floral and suppression phenotypes of *sol2* co-segregated (see Material and Methods).

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When the *sol2-1* allele was crossed to the Landsberg-*er* ecotype, and selected for the presence of both the suppressor mutation and the *RCH1-LLP1* construct in homozygosity in the F2 generation (named *sol2-1L* hereafter), the penetrance of the floral phenotype was higher. We found extra carpels in the last whorl in up to 80 % of *sol2-1L* flowers, while only 5 % of the RCH1-LLP1 crossed to L-*er* (RCH1-LLP1L) showed this phenotype (Fig 5c-e). On average *sol2-1L* has 3.4 (±0.8) carpels per flower, while 2.1 (±0.4) is the average carpel number for RCH1-LLP1L (Fig 5f). In addition, we again observed extreme accumulation of carpels in some terminal flowers (Fig 5b).

The higher penetrance of the *sol2* floral phenotype in a mixed ecotype background was not due to the *erecta* mutation present in L-*er* plants, since *er* is linked to the position where *RCH1-LLP1* is inserted and hence counter selected for. This was confirmed by phenotypic analysis of the *sol2-1L* and RCH1-LLP1L plants.

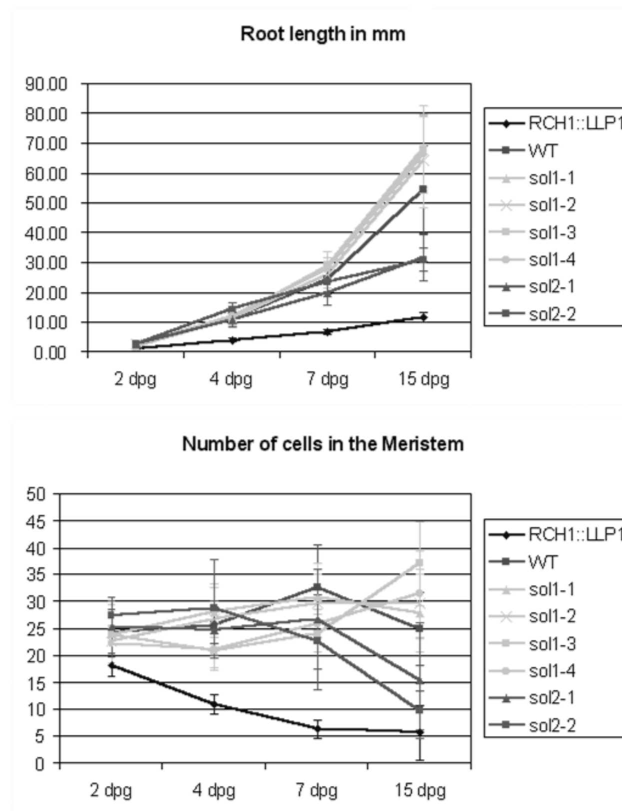
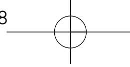


Figure 5. *sol2* mutants are affected in flower development.
(a) Accumulation of carpels in terminal flowers in *sol2-1*. (b) Accumulation of carpels also occurs in *sol2L* terminal flowers. (c-d) Carpels in the fourth whorl of a RCH1-LLP1L and a *sol2L* flower. (e) Percentage of RCH1-LLP1 and *sol2-1* flowers with extra carpels in Utr and L-*er* backgrounds (f) Average number of organs in each whorl in RCH1-LLP1 and *sol2-1* flowers in the L-*er* background.



The presence of extra carpels in the fourth whorl of *sol2* flowers suggests that stem cells may accumulate in the flower meristem of these mutants as observed in *clv* mutants. This observation is consistent with the hypothesis that *sol2* is affected in a CLV-type signaling pathway. *SOL2* was mapped to the bottom arm of chromosome II, which does not contain neither predicted LRR-RLK nor LRR-receptors (data not shown).

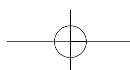
SOL1 encodes for a putative Zn^{2+}-carboxypeptidase

To investigate the molecular basis of the *sol1* mutations, we isolated the affected gene by map-based cloning. *sol1* mapped to a single locus on chromosome 1 between markers nga128 and nga111 (Fig 6a). Fine mapping located the gene in a region covered by 6 BACs, in between bp 92082 of BAC F5H11 and bp 10734 of BAC F17M19 (Fig 6b).

Since our aim was to isolate components of a putative CLV like pathway in roots, we sequenced candidate genes in this region which might be involved in signal transduction. LRR-RLKs present on these BACs revealed no mutations. In addition, a putative carboxypeptidase predicted at locus At1g71696 was chosen for analysis since an unrelated carboxypeptidase, BRS1, had been linked to brassinolide signaling, which involves the LRR-RLK BRI1 (Li et al, 2001a). We sequenced the At1g71696 gene from the *sol1* alleles with the corresponding wild type as control, and detected different mutations in all four alleles (Fig 6c).

Thus, *SOL1* encodes a putative Zn^{2+}-carboxypeptidase which belongs to the group of regulatory carboxypetidases, and shows the highest homology to a predicted carboxypeptidase from rice and the carboxypeptidase domains of animal carboxypeptidase D (EC 3.4.17.22; Aloy et al, 2001) and carboxypeptidase E (Fig 6e). SOL1 contains all the conserved residues present in these type of proteins: the triad H, E, H involved in Zn^{2+} binding (Fig 6d, e asterisk), the R and Y involved in substrate binding (Fig 6d and e squares) and the E involved

Figure 6. *sol1* mutations reside in a putative Zn^{2+}-carboxypeptidase.
(a) *sol1* mutations map between markers nga128 and nga111. (b) *sol1* is located within a genomic region spanned by BACs F15H11, F23N20, F3I17, F26A9, F14O23 and F17M19. (c) Structure of *SOL1*: white boxes represent exons. Nucleotide sequence change for each mutant allele is depicted. (d) SOL1 protein sequence. The predicted signal peptide cleavage site is marked with an arrowhead, and the putative transmembrane domain is underlined. The carboxypeptidase conserved residues are depicted: the triad H, E, H involved in Zn^{2+} binding with an asterisk, the R and Y involved in substrate binding with a square and the E responsible for the catalytic activity with a circle. (e) Alignment of the carboxypeptidase conserved region from SOL1, *Oriza sativa*, the first and second domains of *Anas platyrhynchos* Carboxypeptidase D (CPD) and the *Homo sapiens* Carboxypeptidase E (CPE). The conserved residues crucial for carboxypeptidase activity are depicted as in d. (f) Reverse Transcriptase (RT) PCR reaction using RNA from roots, shoots, leaves, flowers, siliques and whole seedlings for wild type plants, and seedlings for *sol1* alleles. Specific primers for *SOL1* (upper panel) and *ACTIN8* (lower panel) were used.



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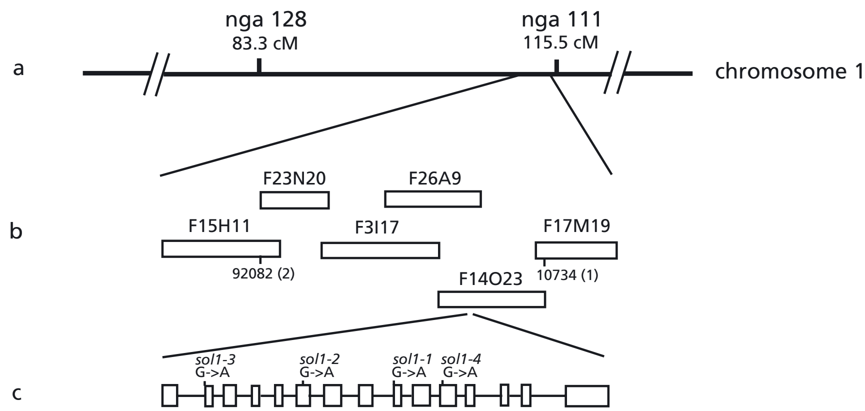
a

b

c



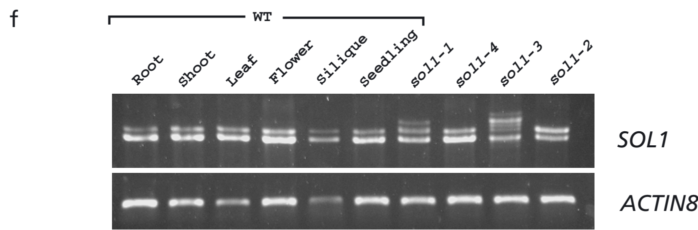
d

MSKLRFFQSLLISTVICFFLPSINARGGHSDHIHPGDGNYSFHGIVRHL
FAQEEPTPSLELTRGYMTNDDLEKAMKDFTKRCSKISRLYKGFLERAFS
IGKSVNGFPLWVIEISDRPGEIEAEPAFKYIGNVHGDEPVGRELLLRLA
NWICDNYKKDPLAQMIVENVHLHIMPSLNPDGFSIRKRNNANNVDLNRD
FPDQFFPFNDDLNLRQPETKAIMTWLRDIRFTASATLHGGALVANFPWD
GTEDKRKYYYACPDDETFRFLARIYSKSHRNMSLSKEFEEGITNGASWY
PIYGGMQDWNYIYGGCFELTIEISDNKWPKASELSTIWDYNRKSMLNLV
ASLVKTGVHGRIFSLDKGKPLPGLVVVKGINYTVKAHQTYADYHRLLVP
GQKYEVTASSPGYKSKTTTVWLGENAVTADFILIPETSSRGNQLRSSCD
CSCKSCGQPLLTQFFTETNNGITLTLFVVVVFLCFLLQRRVRFNLWKQR
QSSRRSITV.

e

SOL1
O.sativa
CPE-H.sapiens
CPDII-A.platyrhynchos
CPDI-A.platyrhynchos

f



in catalytic activity (Fig 6d, e circles; Aloy et al, 2001). It is predicted to be a secreted protein, with a transmembrane domain and a small cytoplasmatic tail (Fig 6d).

By RACE PCR we determined the transcriptional start and stop of *SOL1* and by RT-PCR we analyzed its expression. Two different *SOL1* cDNA variants were found to be expressed in WT (Fig 6f), one containing all predicted 15 exons and 14 introns, while the second is a splicing variant in which intron 3 is not spliced out, resulting in a stop codon which likely causes a translational stop after amino acid 89. In this second splicing variant, the predicted protein would only contain the first 3 exons, an hence none of the carboxypeptidase conserved domains. In addition, we found two 3' UTR va-riants, a long one consisting of 210 base pairs, and a shorter version of 120 base pairs. All mutations in the different *sol1* alleles are single base pair substitutions: *sol1-1* and *sol1-3* mutations are in exon-intron boundaries, the *sol1-4* substitution is predicted to yield an amino acid substitution (G to D in amino acid 297) in a conserved residue, and in *sol1-2* the mutation is predicted to result in a translational stop after 148 amino acids.

We isolated RNA from roots, shoots, leaves, flowers, siliques and whole seedlings and performed Reverse Transcriptase (RT) PCR reactions to determine the expression of *SOL1* in these tissues. We detected both *SOL1* splicing variants described above in all these tissues (Fig 6f). RT-PCR for *sol1* alleles showed that different splicing variants are formed in *sol1-1* and *sol1-3*, as predicted, since the mutations in these alleles are in exon-intron boundaries (Fig 6f). In addition, we detected both WT splicing variants in *sol1-4* and *sol1-2* alleles, however in *sol1-2* the unspliced variant appears to be more abundant than in WT (Fig 6f). Sequencing of the different RNA's formed in each mutant should reveal whether a true null allele is among these. Or that alternatively splicing can still lead to formation of a mature protein with minor deviations from the WT sequence.

DISCUSSION

Two lines of evidence suggest that a CLV like pathway is involved in root meristem maintenance. First, ectopic expression of *AtLLP1* in the root meristem causes the meristem to differentiate, in analogy to the induction of shoot meristem termination by overexpression of *CLV3* (Brand et al, 2000). Second, *sol2* contains extra carpels in the fourth whorl, reminiscent of the phenoypes of weak *clv* alleles (like *clv1-7*; Clark et al, 1993). In addition, the floral phenotype observed in *sol2* suggests that some of the components of the pathway might be shared among roots and shoots. Cloning of *SOL2* will uncover the molecular identity of this potentially shared component in the future.

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clv1 mutants do not show any root phenotype, suggesting that not CLV1 itself but homologous root-expressed receptors are involved in transduction of the LLP1 signal in roots. Why didn't we uncover root CLV1-like receptors as suppressors in our screen? In *Arabidopsis* there are more that 400 receptor like kinases (RLK), but only in very few cases a function has been identified (Shiu and Bleecker, 2001), suggesting there is high level of redundancy among the RLK members. In addition, in a reverse genetic approach we identified CLV1-like receptors specifically expressed in the root meristem, but loss-of-function mutations in these genes revealed no phenotype, not even in double mutant combinations (Chapter 3). These results support the notion of a high level of redundancy among LRR-RLKs in roots.

Root meristem size is tightly regulated implying stringent control of the balance between cell division and cell differentiation. It has recently been shown that this balance is impaired in *scr-1* and *shr-1* mutants due to stem cell differentiation caused by QC mis-specification (Sabatini et al, 2003). The root meristem differentiation defect observed in RCH1-LLP1 is not caused by QC mis-specification or failure in stem cell maintenace, as QC25, QC184, and columella markers are primarily properly expressed. In addition, our studies with *scr-1* and *shr-1* show that ectopic LLP1 acts through a novel pathway independent of SHR and SCR. To our knowledge, this is the first indication of a pathway in roots promoting differentiation without affecting QC activity and/or stem cell maintenance, suggesting a new level of meristem regulation.

Interestingly, the CLV pathway in the SAM is involved in control of meristem size by regulating expression of *WUS* in the organizing center and therefore stem cell function. The role of the *WUS*-expressing organizing center could be considered analogous to the *SCR*-expressing QC. No *WUS* expression occurs in the shoot apex of *35S::CLV3* plants (Brand et al, 2000). Also in RCH1-LLP1 arrested meristems starch granules eventually appear in the columella initials indicating loss of stem cell identity. Double mutant studies indicated that WUS acts downstream of the CLV signaling, while the double homozygote *RCH1-LLP1,scr-1* indicates that the root pathway acts independently from SCR. This suggests that the CLV pathway in the SAM and the putative root CLV-like pathway control meristem maintenance through different mechanisms. Once the critical components of a root CLV pathway are isolated, it will be interesting to investigate the evolutionary relationships between root and shoot meristem maintenance mechanisms.

The *sol1* suppressor in the RCH1-LLP1 background grows faster than control roots and this correlates with a greater amount of cells in the meristem, suggesting that SOL1 is involved in either suppression of cell division or promotion of cell differentiation. We cloned *SOL1* and showed that it encodes a putative regulatory Zn^{2+}-carboxypeptidase with a possible ortholog in rice but no homologues in *Arabidopsis*. In animals, there are 5 members of the sub-

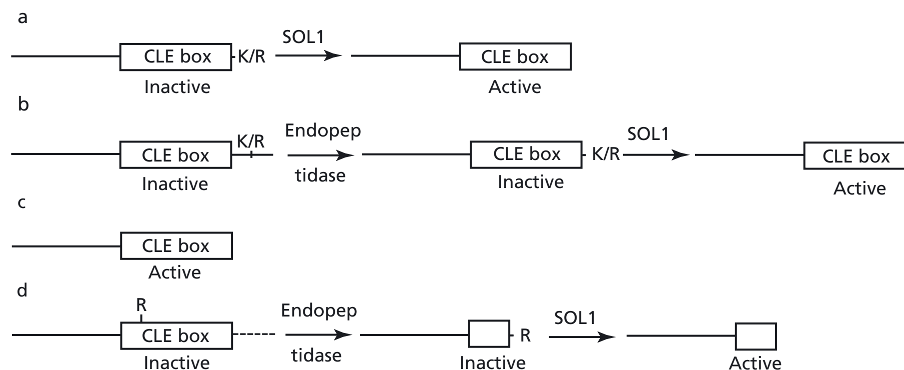

Figure 7. Model for SOL1 action.
(a) SOL1 cleaves terminal K and R residues from CLE proteins (like LLP1) to activate them. (b) A putative endopeptidase cleaves the terminal peptide after the CLE box present in some CLE proteins, up to the internal K or R, which would then be removed by SOL1, activating the CLE protein. (c) Some CLE members do not have any amino acids after the CLE box, and they might not require carboxypeptidase cleavage for activation. (d) An endopeptidase cleaves the CLE peptides up to the conserved R present in the CLE box of all CLE members, which is then removed by SOL1 for activation.

family of regulatory carboxypeptidases (Reznik and Fricker, 2001). SOL1 shows the highest homology to the carboxypeptidase domains of animal CPD and CPE, which have been shown to cleave terminal R and K residues, and are known to be involved in neuropeptide and prohormone processing (Nillni et al, 2002).

Neuropeptides and peptide hormones are biosynthesized as precursors that must undergo an endoproteolytic cleavage followed by removal of C-terminal basic amino acids by carboxypeptidases E or D to become active (Nillni et al, 2002). Similarly, it can be envisaged that SOL1 may process inactive CLE peptides with terminal R and K residues, to a bioactive form. From the 24 *Arabidopsis* CLE members (Cock and McCormick, 2001), seven, including LLP1, contain a terminal R or K after the CLE box, which might be cleaved by SOL1 (Fig 7a). Eight CLE members, including CLV3, contain a terminal small peptide sequence after the CLE box with internal R or K residues. These peptides might be first cleaved by an endopeptidase and then the terminal R or K residues might be removed by SOL1 to make them active (Fig 7b). The other CLE members do not have any amino acids after the conserved CLE box, and they might be constitutively active (Fig 7c). Alternatively, all members contain a conserved R in the CLE box in favor of a two step activation process as described for animal neuropeptides (Fig 7d). Biochemical activity assays with SOL1 protein will be required to test this model.

One prediction of the model is that a *clv3* phenotype should occur in *sol1* mutants, while this is not the case. It is of note that currently it is not known whether any of the four *sol1* alleles is a null. Thus, there may be residual SOL1

carboxypeptidase activity sufficient for activation of CLV3. Alternatively, the serine carboxypeptidase BRS1 might be functionally redundant with SOL1. BRS1, which has homology to yeast Kex1p, has been proposed to act in ligand processing in the brassinosteroid signal transduction pathway (Li et al, 2001a). In yeast, Kex1p is required together with Kex2p for the excision of signaling peptides from their inactive precursors (eg. α-mating pre-hormone and K1 killer toxin; Dmochowska et al, 1987; Fuller et al, 1989). Kex2p is an endoprotease that specifically cleaves on the carboxyl side of pairs of basic amino acids, and after its action Kex1p cuts the amino acid from the C terminus of processed intermediates. Thus, it is plausible that even though BRS1 and SOL1 belong to structurally different classes of carboxypeptidases, they might be redundant at the functional level. Double mutant combination of *brs1,sol1* will clarify this point.

In the near future, analysis of complete loss-of-function mutations in *SOL1* and cloning of *SOL2* should give more insight into either function and the role they play in a CLV-like pathway controlling root meristem maintenance.

MATERIAL AND METHODS

Plant growth conditions, plant lines and mutagenesis

Seeds were sterilized in 5 % sodium hypochloride, imbibed for 2-5 days at 4 °C in the dark in sterile water containing 0.1 % agarose, and germinated on plates containing 0.5x Murashige and Skoog (MS) salt mixture, 1% sucrose and 0.5 g/l 2-(N-morphilino) ethanesulfonic acid (MES) pH 5.8, in 0.8 % agar. Plates were incubated in a near vertical position at 22 °C and a cycle of 16 hours light/8 hours dark. Starch granules and β-glucoronidase activity were visualized as described (Willemsen et al, 1998).

QC25 and QC184 promoter trap lines were selected from the INRA T-DNA collection (Bechtold et al, 1993) and described in Sabatini et al (2003).

scr-1 and *shr-1* mutants were kindly provided by Philip Benfey (Duke University, USA). Double homozygous combinations of RCH1-LLP1 and *shr-1* were selected for presence of high GFP (indicative of full activity of the RCH1-LLP1 transgenes) and absence of lateral roots as seen in *shr-1* mutants 6 dpg. Double homozygotes were confirmed by genotyping for the *shr* mutation using the primers: SHR-1F [5'- ATTCATCACGTTGGAGATTTATCT-GAGTTT 3'] and SHR-1R [5'-ACCAAACACCTTCTTTATATCTCC TCAACA-3]' which amplify a 517 bp region of the *SHR* gene, containing a 50 bp deletion in *shr-1* mutants.

Double homozygous combinations of RCH1-LLP1 and *scr-1* were selected for high GFP expression (indicative of RCH1-LLP1 homozygosity)

and closed cotyledons indicative of *scr-1* homozygosity, and confirmed in the next generation for re-segregation of the same phenotype in all the seedlings.

For mutagenesis experiments the *Arabidopsis thaliana* ecotype Utr (Willemsen, 2003) homozygous for a single copy of *RCH1-LLP1* was used. 8100 dry seeds were divided in two pools and mutagenized with freshly made 5 or 10 mM ethyl methane sulphonate (EMS) in water, for 24 hours at 22 °C. Seeds were sown on soil and grown in a plant chamber at 22 °C, 75 % humidity with a 16 hours light and 8 hours dark cycle. Between 11400 to 20000 M2 seedlings from 10 independent pools were analyzed per pool.

Complementation analysis was done by pair-wise crossing of the suppressors. If the F1 of the cross showed the phenotype of RCH1-LLP1, suppressors were considered to be from different complementation groups, and when the RCH1-LLP1 phenotype was suppressed we grouped them into the same complementation group.

Map based cloning

Homozygous *sol1* plants (containing *RCH1-LLP1* in homozygosity) were crossed to L-*er*. In the F2, *sol1* mutants still containing the *RCH1-LLP1* homozygous (selected for long roots and high GFP expression) were selected and DNA was isolated using a CTAB method (Lukowitz et al, 2000).

We initially mapped the *SOL1* gene to chromosome 1 between nga 128 (83.3 cM) and nga 111 (115.5 cM). Primers for further mapping were designed using information from the CEREON collection (http://www.arabidopsis.org/) and Primer 3 software (http://www-genome-wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The interval was narrowed down to 270 kb spanning four BACs (F23N20, F3I17, F26A9 and F14O23).

We sequenced the *sol1* alleles using the Big Dye Terminator (Genpak Ltd.) on an ABI PRISM 310 Genetic Analyzer. The primers used to PCR the *SOL1* genomic region for sequencing were: CARB-F1: [5'-AAAGTTTCATGTCCGTTTTGGAAGAAG-3']; CARB-R1 [5'-TTTCATTAAGCGCT ATGAACAAAAATTAGA-3']; CARB-F2 [5'- CTAATTTCGACTGTGATCTGCTTTTTCCT-3']; CARB-R2 [5'- TATTACCAAAGGAAATCCATTGACACTCTT-3']; CARB-F3 [5'- TATTTCTCTCTTTGAGGG TTTTCTGGAAC-3']; CARB-R3 [5'-ATCTAGCTACCATCATGAAAATCACAGCAT-3']; CARB-F4 [5'-GATGGCTTTTCAATCAGGAAACGTAATAA-3']; CARB-R4 [5'- CATTGGTGATTCCTTCCTCAAATTCTT-3']; CARB-F5 [5'-ATACTATTATGCATGTCCTGACGATGAGAC-3'] and CARB-R5 [5'-GCTCGTATCATAATCTTATAACAGTGGACAA-3'].

For predictions of the signaling peptide and transmembrane domain we used SignalP V2.0 and TMHMM v 2.0 software.

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SOL2 was roughly mapped to the bottom arm of chromosome II within a region spanning from BAC F11C10 to the telomere.

Plant vectors and transformation

For the *RCH1-ERGFP* construct, a 2.2 kb fragment upstream of the *RCH1* gene was cloned in front of a *GAL4VP16* transcriptional activator gene, in the pGreen vector carrying a kanamycin resistance cassette (Hellens et al, 2000). In this vector, the *ERGFP* gene cloned behind the *UAS* promoter was introduced to form the *RCH1-ERGFP* vector (Fig 1a).

To form the *RCH1-LLP1* construct, the *AtLLP1* gene cloned behind the *UAS* promoter was introduced into the *RCH1-ERGFP* vector (Fig 1a). The *Brassica LLP1* cDNA is smaller than the predicted CLE19, thus we used the CLE19 coding sequence starting at the second methionine which corresponds to the *BrLLP1* cDNA region. The primers used to clone the *Arabidopsis LLP1* are ArDD3B-F [5'- AATGAAGATAAAGGGTTTGATGA-3'] and ArDD3-R [5'-AGTTACCTGTTGTGGAGTGG-3'].

To create a stop codon at the beginning of *AtLLP1* in the *RCH1-LLP1* contruct the QuickChange™ Site-Directed Mutagenesis Kit from Stratagene was used. The stop codon was introduced two codons away from the expected ATG of *AtLLP1*, using the primers DD3-StopF: [5'-GACCAAAAATAGA-CAAATGAAGATATAGGGTTTGATGATATTGGC-3'] and DD3-StopR [5'-GCCAATATCATCAAACCCTATATCTTCATTTGTCTATTTTTGG TC-3'].

Plants were transformed by the floral dip method (Clough and Bent, 1998).

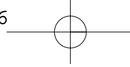
Root length and meristem size analysis

Root length of WT Utr, RCH1-LLP1, *sol1-1*, *sol1-2*, *sol1-3*, *sol1-4*, *sol2-1*, *sol2-2* seedlings were measured 2, 4, 7 and 15 days after germination as described (Willemsen, 1998).

Meristem size was expressed as the number of cells in cortex files proximal to the QC that did not yet rapidly elongate.

Floral organ counts

We established co-segregation of floral and suppression phenotypes based on the following two observations. First, independent *sol2* alleles showed the same floral phenotype. Second, all *sol2* plants with suppressor phenotype showed the flower defects even when outcrossed twice to RCH1-LLP1.



Only the first ten flowers of any given plant were analyzed. Values represent the mean value ± standard deviation of the mean of indicated floral organs. 60 flowers from RCH1-LLP1 and 120 from *sol2-1L* were counted for each mean calculated.

Microscopy

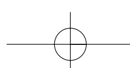
Plant material for light microscopy was prepared as described in Scheres et al (1994). Images were taken on a Zeiss Axioskop 2 microscope with a Nikon DXM1200 digital camera. For confocal microscopy a Leica SP2 was used. Files were assembled in Adobe PhotoShop VI (Adobe System Inc. Mountain View, CA, USA).

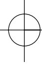
RNA isolation and RT-PCR

RNA of *SOL1* variants was obtained using the Purescript® RNA isolation kit from BIOzym. Chromosomal DNA contamination was removed upon Dnase I (Promega) treatment. cDNA was made using SuperScript™ III reverse transcriptase protocol from Invitrogen. The primers used to PCR the full length cDNA were cDNA-F [5'-CGAAGGAGAAACAGTTATCACATAG-GAATA-3'] and Carb-cDNAL-R [5'-CTCAATTGTTTGGATTTTGGTT-GTTCTTAT-3'].

The transcriptional start and stop of the *SOL1* RNA was determined by 5' and 3' RACE-PCR. For the 3' RACE we made cDNA using primer RACE-T [5'-CATCTAGAG GATCCG AATTC(T) $_{16}$-3']. We then amplified the 3' ends in two rounds of nested PCR using primers RACE-A [5'-CATCTA-GAGGATCCGAATTC-3'] and CARB-F4; and RACE-A and CARB-F5 primers. PCR products were isolated from gel and sequenced. For the 5' RACE we made cDNA using primer RACE-R3 [5'-TCTCCAGGCCT-GTCTGAAAT-3] after which terminal transferase was used to add a polyA tail. Two rounds of nested PCR reactions were then performed with primers RACE-T and RACE-R2: [5'-CCATTAGACACTCTTTCCGATG-3'] and RACE-A and RACE-R1 [5'-CTTGGTGAAATCCTTCATTGC-3']. The products of this last PCR were isolated from gel and sequenced.

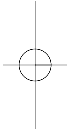
Reverse Trancriptase (RT) PCR was performed using total cDNA obtained as previously described, and for the amplification the primers cDNA-F and Carb-cDNAL-R were used for *SOL1*, and Act8f [5'-ATGAAGAT-TAAGGTCGTGGCA-3'], Act8r [5'-TCCGAGTTTGAAGAGGCTAC-3'] for *ACTIN 8*. The cDNAs were amplified during 32 cycles for *SOL1* and 23 cycles for the ACTIN8.

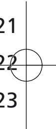


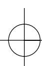


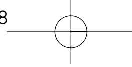
Acknowledgements

We thank Frits Kindt, Ronald Leito, Piet Brouwer and Wil Veenendaal for photography and assistance with Adobe Photoshop; Maarten Terlou for help with root length measurements; Philip Benfey for making available seeds of *shr-1* and *scr-1* mutants. B.S. is supported by an NWO-PIONIER grant.









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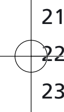
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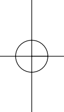
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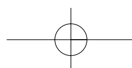

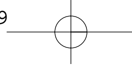

Chapter 3

Expression and functional analysis of the *ROOT CLAVATA1 HOMOLOGUE1* (*RCH1*) clade of root expressed leucine-rich-repeat receptor kinases

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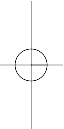

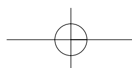

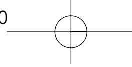

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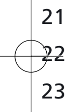
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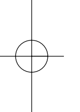

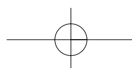

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Expression and functional analysis of the *ROOT CLAVATA1 HOMOLOGUE1* (*RCH1*) clade of root expressed leucine-rich-repeat receptor kinases.

ABSTRACT

The leucine-rich-repeat (LRR) receptor like kinase (RLK) *CLAVATA1* (*CLV1*) is expressed in the shoot apical meristem and required for shoot and floral meristem maintenance. Here we describe the isolation of the *CLV1*-like receptors *RCH1* and *RCH2* that are specifically expressed in *Arabidopsis* roots. *RCH1* is expressed in the root meristem only, posing the question whether this gene has a role in root meristem maintenance. *RCH1* and *RCH2* belong to subfamily LRRXI of the RLK family, and form a clade together with *M3E9.30*, *MDA7.8* and *F12G12.7*. *RCH2*, *M3E9.30* and *MDA7.8* are all expressed in the proximal portion of the meristem at the transition to the elongation zone, suggesting a role for these genes in cell-cell communication in this region. However, loss-of-function studies of single and double mutant combinations for these genes did not reveal any phenotype, suggesting a high level of redundancy in this family.

INTRODUCTION

Organ formation in plants continues post-embryonically through the activity of the meristems. Stem cells in meristematic regions are able to renew themselves and give rise to differentiating progeny cells. During embryogenesis, two meristems are formed at opposite poles of the embryo. The shoot apical meristem (SAM) will give rise to all aboveground organs, and the root meristem is responsible for the formation of all of the root system. It has been shown that in both shoot and root meristems the position of a cell and not its clonal origin determines its fate underlining the importance of cell-cell communication in plant development (Irish and Sussex, 1992; van den Berg et al, 1995).

More than 400 RLKs have been identified in *Arabidopsis*, but only in very few cases a role has been reported (Shiu and Bleecker, 2001). One of the RLKs with a known function in stem cell homeostasis is CLAVATA1, a LRR-RLK involved in shoot meristem maintenance. *clv1* mutants have enlarged

shoot and floral meristems indicating that CLV1 is involved in promotion of cell differentiation or inhibition of cell division in these meristems (Clark et al, 1993; Clark et al, 1995). CLV1 has been shown to form a complex with CLV2, a LRR receptor without kinase domain, which is predicted to become activated upon binding its ligand, CLV3 (Trotochaud, 1999; Jeong et al, 1999). The CLV signaling pathway ultimately leads to transcriptional repression of the putative homeodomain transcription factor *WUSCHEL* (*WUS*), which in turn acts to promote stem cell fate and *CLV3* expression in the stem cells (Laux et al, 1996; Mayer et al, 1998). The current view is that a negative feed back loop between CLV3, through the CLV1/CLV2 complex, and WUS controls the size of the stem cell population in the shoot (Brand et al, 2000; Schoof et al, 2000).

Root meristem maintenance also depends on a population of stem cells (or initials), which surround four mitotically less active cells, the quiescent center (QC). It has previously been shown that the role of the QC is to promote stem cell fate in the surrounding cells (van den Berg et al, 1997; Sabatini et al, 2003), in analogy to the role of the *WUS*-expressing organizing center in the SAM. Since there are similarities between shoot and root meristem maintenance, we sought to investigate whether CLV1-like receptors in roots are required for root meristem size control. We identified the LRR-RLKs *ROOT CLAVATA1 HOMOLOGUE 1* and *2* (*RCH1* and *RCH2*), specifically expressed in the root meristem and in the transition from meristematic to elongation zone respectively, the sequence of which are closely homologous to *CLV1*. Single and double mutant loss-of-function analysis and *RCH1* gain-of-function mutants did not reveal their role in root development. We extended our study to the three closest homologues of *RCH1* and *RCH2*, and analyzed their expression profile and function by loss-of-function analysis of single and double mutant combinations.

RESULTS

Root CLAVATA1 Homologues

To isolate *CLV1*-like genes specifically expressed in the root meristem, we designed degenerated primers against the LRR and the kinase domain of the consensus sequence obtained by alignment of different receptor genes. We created three degenerated primers for the LRR region (LRR 1, 2 and 3) and three for the kinase domain (KINR1, 2 and 3), based on sequences of *CLV1*, *HAESA*, *XA21*, *CF2*, *CF9*, *ERECTA* and *INRPK1* for the LRR, and on *CLV1*, *HAESA*, *ERECTA*, *PTO*, *XA21*, *TMK1* and *SERK* for the kinase, with preference for the *CLV1* sequence (Fig 1a).

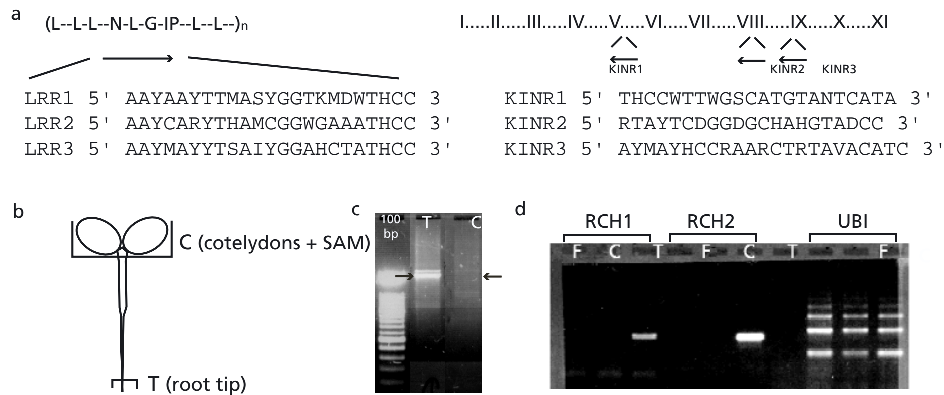

Figure 1. Differential RT-PCR strategy to isolate root *CLV1*-like homologues.
(a) Design and sequence of degenerated primers for the LRR and the kinase consensus with preference for the *CLV1* sequence. (b) Schematic drawing of the regions in the seedling used for mRNA isolation: cotyledons with the SAM (C) and the root tip including the root meristem (T). (c) Example of agorose gel electroforesis with cDNAs from C and T amplified under one of different conditions used. In this case specific RNA's could be amplified only from root tip. (d) RT-PCR expression analysis of *RCH1* and *RCH2* using RNA from flowers (F), cotyledons and SAM (C) and root tips (T). Specific primers for *RCH1*, *RCH2* and *UBIQUITIN* (control) were used.

Table 1. **Isolation, identity and RT-PCR expression analysis of cDNA clones from the differentia root-cotyledon (T,C) RT-PCR. Clones are ordered according to the frequency at which they were isolated.**

Clones	Gene	Frequency	Expression
1-2	*RCH2*	28	T
3-2	*CLV1*	18	T+C
2-7	*RCH1*	6	T
2-8	*BRI1*	5	T+C
1-9	*MDA7.8*	3	T+C
1-5	*T1N24.22*	2	T+C
2-2	*M3E9.30*	2	T+C
3-4	*MPA24.5*	1	T+C
1-5	*HAESA*	1	T+C
1-20	*T32A17.160*	1	T+C
2-4	*T3F20.25*	1	T+C
2-12	*MBM17.3*	1	T+C
4-20	*MNI5.4*	1	T+C
4-24	*MMG15.8*	1	T+C

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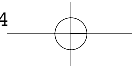
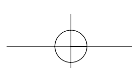


We isolated RNA from Cotyledons and SAM (C) and root tips (T; Fig 1b) that was reverse transcribed to make cDNA, followed by PCR amplification using all combinations of LRR and KINR primers. In total we isolated 14 different clones, of which 2 seemed root specific. We sequenced all and we compared the sequences to the database to determine their identity. As expected, some clones corresponded to genes with known function as *CLV1*, *HAESA* and *BRI1* (Table 1).

The two cDNA clones amplified from root RNA only were novel genes, which we named *ROOT CLAVATA1 HOMOLOGUE 1* and *2* (*RCH1* and *RCH2*). We confirmed their root specificity using gene specific primers for each gene in a reverse transcriptase (RT) PCR reaction with independent RNA isolates from root tips, cotyledons and SAM, and flowers (Fig 1d).

By RACE-PCR we determined the full size RNA's of both genes. Both *RCH1* (accession number AJ550162) and *RCH2* (accession number AJ550163) contain an open reading frame predicted to encode LRR-RLKs, with extracellular LRRs flanked by pairs of conserved C residues (Fig 2 asterisk), a transmembrane domain (Fig 2 double underlined) and all the conserved cytoplasmic Ser/Thr kinase motifs (Fig 2 underlined) present in CLV1. They contain one intron, in the same position as in the *CLV1* gene, in the region encoding the conserved domain VIII of the kinase. RCH1 and RCH2 are 60% identical and 75% similar to each other, while CLV1 shows 32.6% and

Figure 2. RCH1 and RCH2 are highly homologous to each other and to CLV1. Protein alignment of RCH1, RCH2 and CLV1. The conserved pair of C's flanking the LRRs are marked with an asterisk, the predicted transmembrane domain is double underlined and the conserved domains in the kinase region are underlined and indicated with Roman numbers.



32% identity and 49% and 48% similarity with RCH1 and RCH2 at the protein level, respectively.

As *RCH1* and *RCH2* were the only root-specific close homologues of the *CLV1* LRR-RLK and we are interested in *CLV1*-like genes which might be involved in root meristem maintenance, we chose them for further analysis.

RCH1 and *RCH2* Expression Patterns

To test whether the expression of *RCH1* and *RCH2* was consistent with a role in root meristem maintenance, we constructed promoter fusions for *RCH1* and *RCH2* using the *ER-GFP* and the β-*Glucoronidase* (*GUS*) genes as reporters, respectively. In both cases several independent transformants were analyzed, all showing the same expression pattern.

For *RCH1* we used the GAL4-*UAS* transactivation system to express *ER-GFP* under the control of a 2.2 kb promoter region of *RCH1*. *RCH1* is specifically expressed in the root meristem, in all tissues except in the columella (Fig 3a and b). Its expression is high in endodermis, cortex, epidermis and lateral root cap while it is low in the QC and vascular bundle (Fig 3a). *RCH1* expression is progressively lower in the elongation zone and undetectable in the differentiation zone (Fig 3b). In embryos *RCH1* expression is detected from heart stage onwards, initially only in a subset of epidermal and lateral root

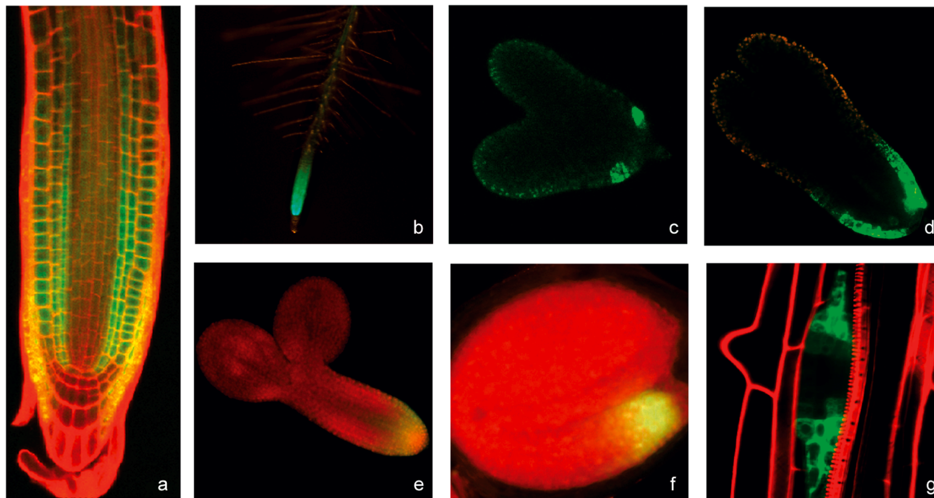


Figure 3. *RCH1* is specifically expressed in the root meristem from heart stage embryo onwards.

(a) GFP expression marking the root meristematic expression domain of *RCH1* in a one-week-old seedling. (b) GFP binocular image showing *RCH1* expression in the meristem, fading away in the elongation zone and absent from the differentiation zone that is apparent for the presence of root hairs. (c-f) *RCH1* expression domain in heart stage, early and late torpedo stage, and bent cotyledons stage embryos, respectively. (g) *RCH1* expression in a lateral root primordium.

cap cells (Fig 3c, d), while later on in embryogenesis, at late torpedo and bent cotyledon stage its expression extends to all the root embryonic meristem except the columella, as in seedlings (Fig 3e and f). In addition, *RCH1* is also expressed in lateral root primordia (Fig 3g).

We introduced the *RCH1* promoter fusion into the expansion mutants *lion's tail*, *cobra* and *sabre* to test whether *RCH1* is expressed in the non elongated cells of these mutants outside the meristem or if its expression correlates with meristematic activity. These mutants have highly reduced elongation, and expansion is proportionally greatest in the epidermis of *cobra*, in the stele of *lion's tail*, and in the cortex of *sabre* (Benfey et al, 1993; Hauser et al, 1995). *RCH1* is still expressed in the meristematic cells of these mutants even though these cells are expanded, and it is not expressed in non-elongated cells outside the meristem, suggesting that *RCH1* expression is always correlated with the dividing cell population (data not shown).

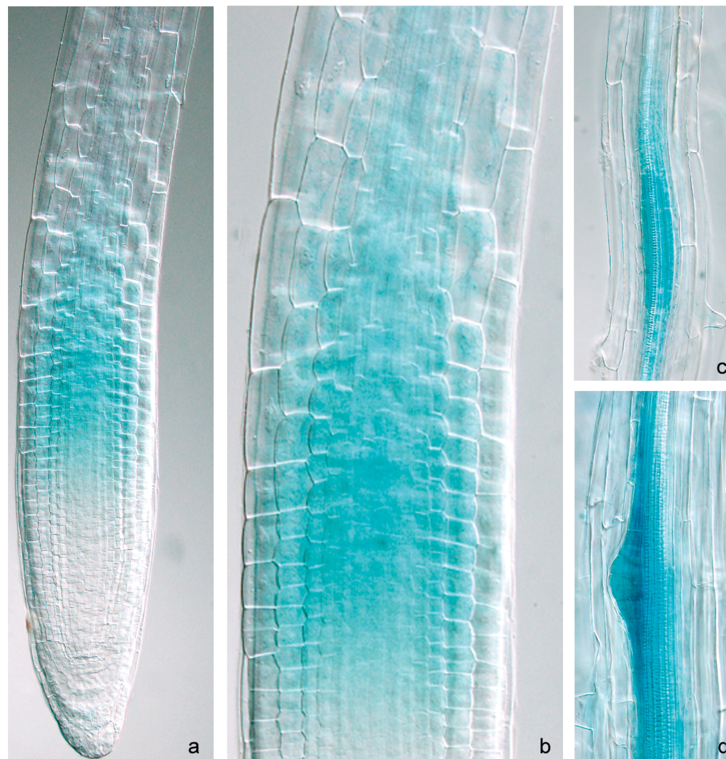


Figure 4. *RCH2* is specifically expressed in roots, in the transition from meristematic to elongation zone.
(a-b) *RCH2* expression in the transition from meristematic to elongation zone in a five- days-old seedling. (c-d) *RCH2* expression in early lateral root primordia.

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To study the expression pattern of *RCH2*, we cloned a 2.3 kb region upstream of *RCH2* in front of the *GUS* reporter gene. *RCH2* is expressed in a more proximal position than *RCH1* in the root meristem, in the transition from meristematic to elongation zone (Fig 4a, b). In addition, *RCH2* is expressed early in lateral root formation (Fig 4c), and high expression is observed in lateral root primordia (Fig 4d). The expression patterns of *RCH1* and *RCH2* were confirmed by whole mount in situ hybridization (data not shown).

The specific expression of *RCH1* in the root meristem from embryogenesis onwards and its high homology to *CLV1* suggests a possible role for RCH1 in root meristem maintenance. In addition, the *RCH2* expression domain suggests a possible role for this gene in cell-cell communication among cells in the transition from meristematic to elongation zone.

***RCH1* and *RCH2* loss-of-function mutants**

To study possible functions of RCH1 and RCH2 in the root meristem, we screened the *En*-1 mutagenized *Arabidopsis* seed collection described in Baumann et al (1998), and the SIGnAL T-DNA collection to find loss-of-function mutants for these genes. Two different *En* insertions were identified for *RCH1*: *rch1-1* with an insertion 115 bp upstream of the predicted ATG and *rch1-2* with the insertion at the beginning of the kinase domain (Fig 5c). In addition, a T-DNA insertion was present in the *RCH1* LRR region in the SALK_038309 line, which was renamed *rch1-3* (Fig 5c). Two *En* insertion lines were recovered for *RCH2*: *rch2-1* with the insertion at the beginning of the kinase domain, and *rch2-2* with the insertion at the end of the kinase region (Fig 5c). Homozygous seedlings for all the alleles of both genes were analyzed, but no phenotype was apparent for any of them.

To test for conditional phenotypes, we studied the response of the different *rch1* and *rch2* alleles to a concentration range of metabolites (sugar, nitrate), hormones and cell cycle regulators as well as their response to tropism and light (Table 2). In all the cases all alleles tested behaved as the WT controls (data not shown).

Since *RCH1* and *RCH2* are highly homologous to each other (60 % identity at the protein level) and their expression domain overlaps in the proximal part of the root meristem it is possible that both genes act redundantly in this region and have separate roles redundant with other RLKs in the non-overlapping domain. To investigate this possibility we created a double mutant using the *rch1-1* and the *rch2-1* alleles. However, *rch1-1/rch1-1,rch2-1/rch2-1* plants were indistinguishable from WT controls, even when tested for conditional phenotypes (Table 2).

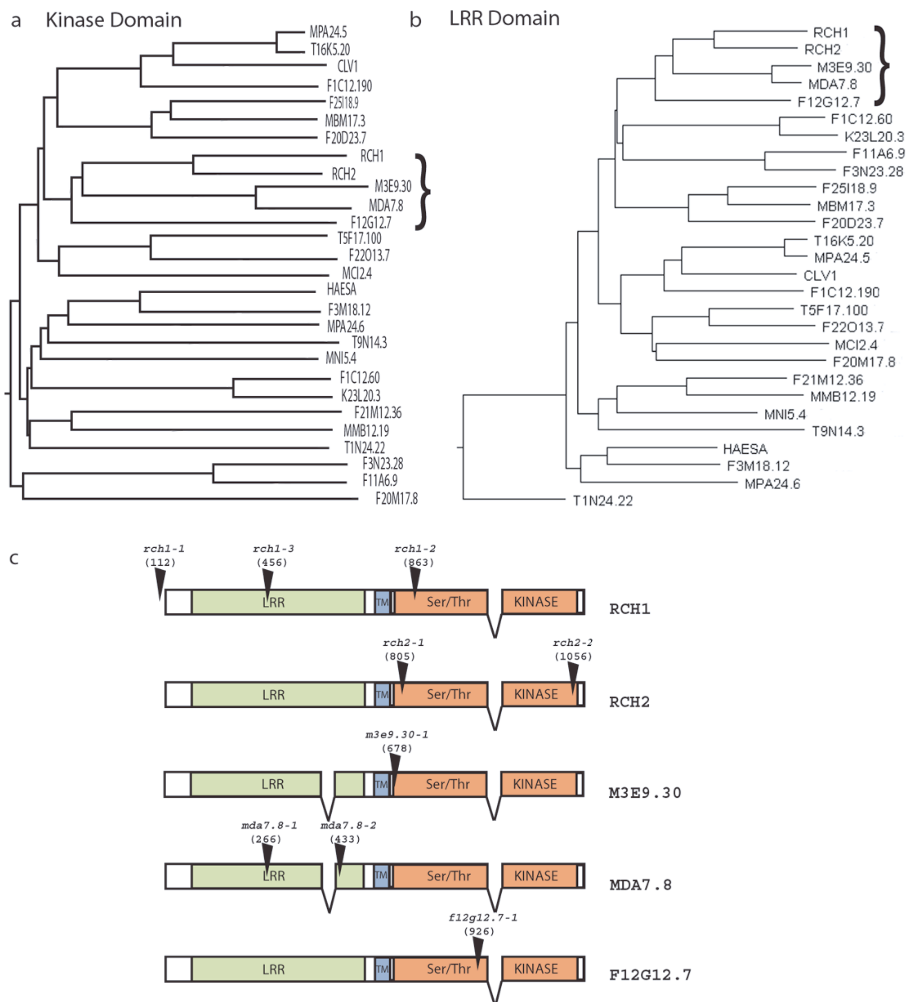

Figure 5. The RCH1 clade belongs to the subfamily LRRXI of RLKs.
(a) Phylogenetic tree of the kinase domain of the subfamily of LRRXI (modified from Shiu and Bleecker, 2001). (b) Phylogenetic tree of the LRR region of all LRRXI members. (c) Schematic representation of all RCH1 clade members. Insertion mutants are indicated with an arrowhead, the position of the insertion in the protein sequence is represented by the number below the allele name, except for *rch1-1* in which the number represents base pairs before the predicted translation start.

Analysis of other members of the RCH1 clade

RCH1 and RCH2 belong to the LRRXI subfamily of plant RLKs from the Shiu and Bleecker (2001) classification, based on the phylogenetic relation of all RLKs using the kinase domain (Fig 5a). We aligned the LRR region of all LRRXI RLKs and constructed a phylogenetic tree (Fig 5b). In both kinase

Table 2. Conditional phenotype tests for *rch1* and *rch2* single and *rch1*,rch2 double mutants. X marks the condition tested.

	rch1 mutants		*rch2* mutants		Double mutants
	rch1-1	*rch1-2*	*rch2-1*	*rch2-2*	*rch1-1,rch2-1*
Metabolism					
1% sucrose	X	X			X
3 % sucrose	X	X	X		X
0 Nitrate	X	X	X	X	X
0.1 mM Nitrate	X	X	X	X	X
0.6 mM Nitrate	X	X	X	X	X
6 mM Nitrate	X	X	X	X	X
60 mM Nitrate	X	X	X	X	X
Hormones					
1 µM IAA	X	X	X		
10 µM IAA	X	X	X		
1 µM 2,4-D	X	X	X		X
10 µM NPA	X	X	X		
50 µM NPA	X	X	X		
10 µM TIBA	X	X	X		
30 µM TIBA	X	X	X		
10 µM Etaphon	X	X	X	X	X
100 µM Etaphon	X	X	X	X	X
10 µM ACC	X		X		
100 µM ACC	X		X		
10 µM AVG	X		X		
100 µM AVG	X		X		
10 µM GA3	X		X		
5 µM Zeatin	X	X	X		X
10 µM ABA	X	X	X		X
Cell Cycle					
70 µM GSH	X	X	X		
1 mM BSO	X	X	X		
5 mM BSO	X	X	X		
Tropisms					
Touch response (45º)	X	X	X		X
Gravitropism (90º)	X	X	X		X
Others					
Darkness	X		X		
Continuous light	X		X		
Sand	X	X	X		X

and LRR trees, RCH1 and RCH2 form a clade together with 3 other proteins: M3E9.30, MDA7.8 and F12G12.7. We searched for ESTs from these genes and found ESTs from roots for *M3E9.30* and *MDA7.8*. For *F12G12.7*, ESTs had been recovered only from aboveground organs and siliques. The fact that at least *M3E9.30* and *MDA7.8* are expressed in roots and that they are highly homologous to each other and to the other members of the clade, suggests that they might have redundant functions with RCH1 and RCH2.

As in situ and promoter fusions of RCH1 and RCH2 revealed identical expression patterns, we decided to create promoter fusions to study if these

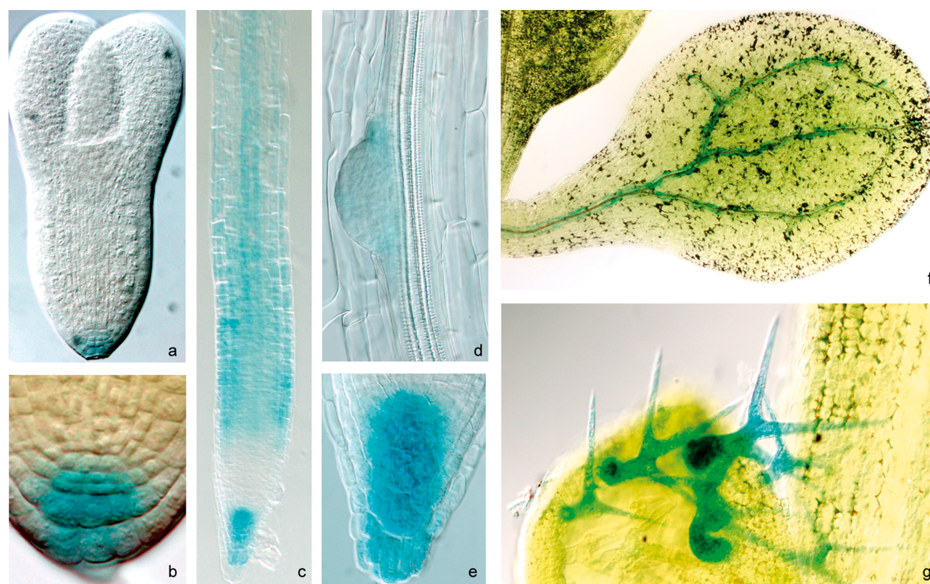

Figure 6. *M3E9.30::GUS* expression analysis.
(a-b) *M3E9.30::GUS* is expressed in torpedo stage embryos in the distal cell types: QC and columella. (c, e) Expression of *M3E9.30::GUS* in six-day-old seedlings in QC, columella (e) and in all tissue types in the transition from meristematic to elongation zone (c). (d-g) *M3E9.30::GUS* is expressed in lateral root primordia (d), vascular tissue of cotyledons and leaves (f) and trichomes (g).

genes have overlapping expression domains with *RCH1* or *RCH2*. Therefore we cloned the 2.2 kb and 2.1 kb upstream regions of *M3E9.30* and *MDA7.8* respectively, in front of the *GUS* reporter gene (*M3E9.30::GUS* and *MDA7.8::GUS*, hereafter).

The *M3E9.30::GUS* is expressed during embryogenesis in the QC and columella cells from torpedo stage onwards (Fig 6a, b). In seedlings, high expression in the QC and columella is also observed (Fig 6c, e). In addition,

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the *M3E9.30::GUS* is expressed in the proximal root meristematic region, in the transition from meristematic to elongation zone, in a similar domain as *RCH2* (Fig 6c). The *M3E9.30::GUS* is also expressed, as *RCH2* and *RCH1*, in young lateral root primordia (Fig 6d). Aboveground, expression in the vascular tissue of the leafs (Fig 6f) and trichomes (Fig 6g) was observed but only after prolonged incubation with GUS (over-weekend), indicating that *M3E9.30::GUS* is lower expressed in these cell types.

MDA7.8::GUS expression in the root is confined to the transition from meristematic to elongation zone like *RCH2* and *M3E9.30*, with the highest expression in vascular bundle and endodermis, lower expression in cortex and absent in the epidermis (Fig 7a, b). But unlike *RCH2*, *RCH1* and *M3E9.30*, *MDA7.8::GUS* is not expressed in young lateral root primordia. Aboveground, *MDA7.8::GUS* is expressed in the vascular bundle of cotyledons and leaves and in stipules (Fig 7c, d). In flowers a low expression was detected at the position where the filament joints the anthers, and in the ovules expression was observed at the micropyle region (Fig 7e–g).

The overlapping expression domain of *RCH2*, *M3E9.30* and *MDA7.8* in the transition from meristematic to elongation zone and their high homology, suggests a possible redundant role for these genes in this region.

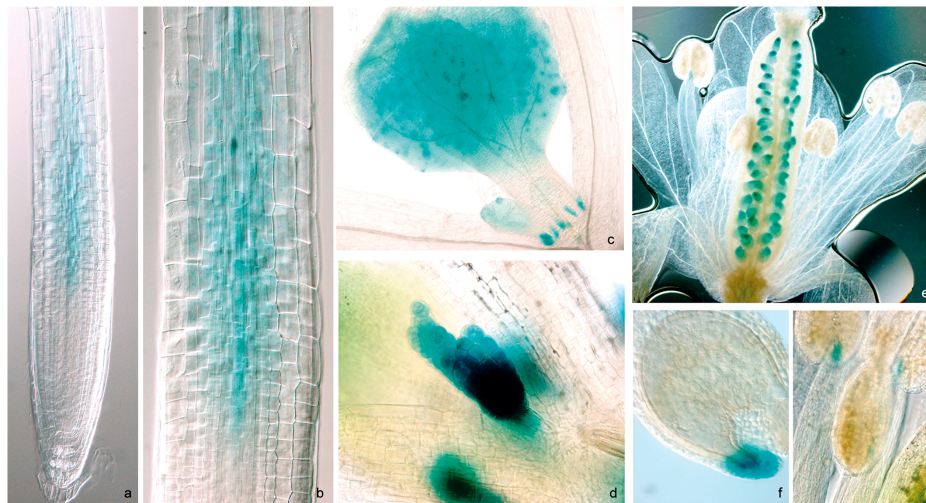

Figure 7. *MDA7.8::GUS* expression analysis

(a-b) Six-day-old roots showing *MDA7.8::GUS* expression in the transition from meristematic to elongation zone, which is high in vascular tissue, low in ground tissue and absent in the epidermis. (c-d) *MDA7.8::GUS* expression in cotyledons (c) and stipules (d). (e-g) In flowers *MDA7.8::GUS* is expressed in the micropilar region of the ovule (f) and in the position where the filament joints the anther (g).

Analysis of RCH1 family loss-of-function mutant combinations

To study the function of *M3E9.30*, *MDA7.8* and the putative role of *F12G12.7* in the root, and their relation with *RCH1* and *RCH2*, we looked for T-DNA insertions in these genes using the SIGnAL and the Syngenta GARLIC collections (McElver et al, 2001).

For *M3E9.30* we obtained the Garlic_1220b_B03 (renamed *m3e9.30-1*), which contains a T-DNA insertion at the beginning of the kinase domain (Fig 5c). For *MDA7.8* we found two insertion lines, the Garlic_18b_F04 and the SALK_008060 both supposed to contain the T-DNA insertion at the LRR region, and renamed *mda7.8-1* and *mda7.8-2*, respectively. For *F12G12.7*, the T-DNA SALK _014726 line from the SIGnAL collection containing an insertion in the kinase domain was used for analysis, and renamed f12g12.7-1.

By PCR we confirmed the insertion site and determined which plants contained the T-DNA insertion except for *MDA7.8* (see material and methods). No phenotype was observed in homozygous plants.

We next created double mutant combinations using one of the alleles for each of our genes of interest (Table 3). Double mutant combinations of these genes were obtained and confirmed by PCR, but again no phenotypes were observed.

Table 3. Double mutant combinations.

Double mutant combinations
rch1-1 x rch2-1
rch1-3 x m3e9.30-1
rch1-3 x f12g12.7-1
rch2-1 x m3e9.30-1
rch2-1 x f12g12.7-1
m3e9.8-1 x f12g12.7-1
irk x rch1-3

The lack of phenotypes for the single and double mutants of the 4 members that we were able to analyze, reinforces the notion that there is a high level of redundancy among LRR-RLKs, and that multiple mutant combinations might be required to unravel the function of these genes.

RCH1 gain-of-function

To try overcoming the problem of genetic redundancy we chose to study the effect of *RCH1* gain-of-function. We created transgenic plants containing the genomic *RCH1* coding region behind the *35SCaMV* promoter (*35S::RCH1* hereafter). 35 independent transgenic plants were selected and RNA was isolated from the leaves of all these transgenic plants to determine expression levels on a Northern blot. While *RCH1* is not expressed in the leaves of WT plants, different levels of expression were detected in the leaves of transgenic plants confirming overexpression of *RCH1* in these plants (Fig 8). EtBr staining of RNA in the gel used for Northern blotting was taken as loading control (data not shown). No phenotypes were observed even in the

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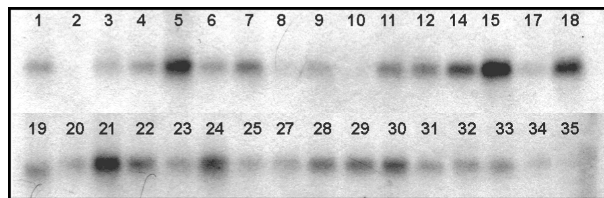

Figure 8. Northern blot of *RCH1* overexpression lines.
Northern blot from leaf RNA isolated from transgenic *35S::RCH1* lines 1-35.

higher overexpressor lines like 5 or 15. This suggest that RCH1 is not a limiting component of a signaling pathway whose overactivation gives a visible phenotype.

DISCUSSION

We studied a clade of five members from the LRRXI family of RLK with high homology to *CLV1* (from Shiu and Bleecker classification, 2001), and show that at least four of the five members of the clade: *RCH1, RCH2*, *M3E9.30*, *MDA7.8* are expressed in roots, and *RCH1* and *RCH2* are specifically expressed in roots only. The specific expression of *RCH1* in the root meristem makes it a likely candidate for playing an important role in root meristem homeostasis. *RCH2*, *M3E9.30* and *MDA7.8* expression in the transition between meristematic and elongation zone suggests a role for these genes in cell-cell-communication in this region.

Our loss-of-function studies of single and double mutant combinations among four members of the clade did not reveal the function of any of these genes. In the *Arabidopsis* genome more than 400 RLKs have been identified (Shiu and Bleecker, 2001), but only few LRR-RLKs have been reported to reveal their function in forward or reverse genetic screens. This suggests high redundancy among family members. Our loss-of-function study for this clade reinforces this idea, since double mutant combinations even with genes having clear overlapping expression patterns, like *RCH2* and *M3E9.30*, did not reveal any phenotypes. *MDA7.8* is also expressed in a similar pattern as these two genes, but unfortunately no loss-of-function mutations in this gene are available at the moment. When this becomes available, multiple mutant combinations might be needed to uncover their functions.

In addition, *RCH1* seems to be the only member of this clade expressed in the full root meristem region (except in the columella). *F12G12.7* expression data should help clarify if this gene is expressed in a similar domain. But double mutant combinations between these two genes did not reveal any phenotype suggesting that perhaps other LRR-RLKs, may be outside the LRRXI

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clade, can function redundantly with *RCH1*. One such candidate gene is *IRK*, which is a member of the LRRVII subfamily, of which promoter fusions revealed completely overlapping expression domain with *RCH1* in the root meristem (Dr Takemura, personal communication). No phenotype was observed in double mutants *irk,rch1-3*, hence no redundant function could be shown among these two members of different subfamilies. Alternatively, the loss of RCH1 function might induce the other members of the clade to expand their expression domain into the whole meristematic region. There are examples for this among the MADS-box family of transcription factors: although *AGL8* expression does not overlap with that of *AP1*, in *ap1* mutants *AGL8* expression expands into the *AP1* domain and can partially compensate for loss of *AP1* function (Martienssen and Irish, 1999). Introducing the promoter fusions of each gene in the loss-of-function mutants for other members and more combinations of mutant alleles should help to clarify this point.

Overexpression of *RCH1* in the whole plant using the *35SCaMV* promoter did not induce any phenotypes, suggesting that the levels of this receptor are not limiting in the RCH1 signaling pathway. This observation is not unexpected, as in the SAM the levels of the *CLV3* ligand and of the homeodomain transcription facor *WUSCHEL* seem to control the size of the SAM, while the *CLV1* receptor levels do not seem to be limiting (Brand et al, 2000; Schoof et al, 2000).

The question remains if besides a putative role for the RCH1 clade members in root development, a role in more general signal transduction mechanisms could be plausible. Although we cannot exclude a role in general signal transduction, we do not consider this likely since different conditional tests affecting the metabolic status, the hormone levels and the light conditions did not yield to any variations as compared to WT controls.

Lastly, a role in disease-resistance could be envisaged as many LRR-RLKs in plants are involved in pathogen recognition. However, no mutations in any of the *RCH1* clade members have been reported from screens with different pathogens. This may suggest that this clade of LRR-RLKs is not involved in disease-resistance signaling. However, there may be a wide variety of pathogens in the wild, which might be difficult to test under laboratory conditions, and hence this possibility cannot be excluded.

MATERIAL AND METHODS

Plant growth conditions and plant lines

Seeds were sterilized in 5 % sodium hypochloride, imbibed at 4 °C in the dark in sterile water containing 0.1 % agarose for 2-5 days, and germinated on

plates containing 0.5x Murashige and Skoog (MS) salt mixture, 1% sucrose and 0.5 g/l 2-(N-morphilino) ethanesulfonic acid (MES) pH 5.8, in 0.8 % agar. Plates were incubated in a near vertical position at 22 °C and a cycle of 16 hours light/8 hours dark. β-glucoronidase activity was visualized as described (Willemsen et al, 1998).

For all experiments the Col0 ecotype was used.

Growth conditions for conditional phenotypes tests

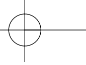
For the sucrose response test, seeds were germinated on 1/2 MS plates: containing 0.5x Murashige and Skoog (MS) salt mixture, and 0.5 g/l 2-(N-morphilino) ethanesulfonic acid (MES) pH 5.8, in 0.8 % agar, in which 1% or 3% sucrose was added to the medium.

For the nitrate response seeds were germinated on medium which composition for 1000 ml was: 33.22 ml calcium chloride (10g/l), 17 ml potassium phosphate (10g/l), 35.35 ml magnesium sulfate (10g/l), 62 ml boric acid (100mg/l), 250 μ>l of cobalt chloride 6 H_2O (100mg/l), 250 μl cupric sulphate 5 H_2O (100mg/l), 16.9 ml manganese sulphate H_2O (1g/l), 2.5 ml natrium molibdat (100 mg/l), 8.3 ml potassium iodide (100mg/l), 86 ml zinc sulphate (100mg/l) and 30 ml NaFe EDTA (30 mg/l) and 31.24 g of sucrose. The pH was adjusted to 5.6-5.8 with KOH. In addition, 165 ml, 16.5 ml, 1,65 ml, 165 μl and none amonium nitrate (10g/l); 190ml, 19 ml, 1.9 ml, 190 μl and none potassium nitrate (10g/l) and none; 117 ml, 128.7 ml, 129.8 ml and 130 ml of potassium chloride (10g/l) were added to the 60 mM, 6mM, 0.6 mM, 0.1 mM and 0 nitrate medium, respectively.

To test the response of the different alleles to different concentrations of hormones and cell cycle regulators we added the appropriate concentrations to the 1/2 MS medium, before pouring the medium into the plates. The concentrations used are described in Table 2.

Designing degenerate primers

To RT-PCR members of the LRR receptor kinase gene family we compared the sequences of the *CLV1*, *HAESA*, *XA21*, *CF2*, *CF9*, *ERECTA*, *INRPK1*, *PTO*, *TMK1* and *SERK* genes and their encoded proteins. The degenerate LRR primers (LRR1, 2 and 3; Fig 1a) were designed against the NxLxGxIP encoding region of the LRR consensus xLxxNxLxGxIPxxLxx LxxLxxL, with preference for the *CLV1* sequence. Degenerate kinase primers were designed for different conserved regions of the kinase domain also with bias for the *CLV1* sequence (primers KINR1, 2, and 3; Fig 1a).



RNA isolation

For RNA isolation the root tips and the hypocotyl/cotyledon part including the shoot apical meristem of *Arabidopsis thaliana* (Col0) plants, 4 days after germination, were collected (Fig 1b).

Total RNA was isolated as described in Pawlowski et al (1994) and chromosomal DNA contamination was removed upon Dnase I (Promega) treatment. The amount and quality of RNA was determined using spectrophotometry and agarose gel electrophoresis.

Differential RT-PCR

Reverse transcription was performed using 5 µg of root tip (T) and hypocotyl/cotyledon (C) total RNA in a 20 µl reaction volume with 0.5 µg oligodT$_{12-18}$ (Pharmacia) and 200 U Superscript II (LifeTechnologies). Following first strand cDNA synthesis the samples were treated with RNase H (LifeTechnologies) and subsequently diluted to a total volume of 100 µl with water.

Amplification reactions were performed with all degenerate LRR and KIN primer pairs. For the PCR reaction 2.5 µl of the T or C cDNA sample was amplified in a total volume of 50 µl containing 100 µM dNTPs, 100 ng LRR and KIN primer, 1 U Taq polymerase (Roche) and its accompanying buffer. The cDNAs were amplified during 40 cycles (94°C, 1 min; 45-55°C, 1 min; 72°C, 3 min).

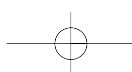
Cloning and sequencing

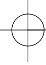
The amplified T and C cDNAs from the same reaction conditions were separated and compared with agarose gel electrophoresis (Fig 1c).

The root specific fragments, e.g. fragments present in T and not in C, were isolated from gel, cloned into the pGEM-T vector (Promega) and transformed into *E.coli* (strain DH5α).

Several PCR fragments of the same size but from different members of the LRR receptor kinase gene family may be generated using degenerate primer pairs. Therefore, 24 randomly picked colonies obtained after transformation were used in a colony PCR. Aliquots of the amplified inserts were separated on agarose gel followed by Southern transfer to a Supercharge N+ membrane (Schleiger and Schluel) to create a "colony blot". An aliquot of one insert was labeled with ^{32}P-dCTP and hybridized to the colony blot to determine the number of clones it represented in the pool of 24. This procedure was repeated until all clones were identified.

A single representative of each pool of clones was sequenced and the sequence was analyzed using the BLAST program (Table 1).





For predictions of the transmembrane domain we used TMHMM v 2.0 software. The different conserved domains of the kinase were determined by comparison to the CLV1 sequence (Clark et al, 1997).

RT-PCR

The relative expression levels of *RCH1* and *RCH2* in root tip (T), hypocotyl/cotyledon (C) and flowers (F) were determined using RT-PCR. Primers specific for the *RCH1*: RCH1F: [5'-CGATCAGACACAAGAACA T-3'] and RCH1R: [5'-AGCAATGGTGTTGGAAGAA-3'] and *RCH2*: RCH1F and RCH2R: [3'-AGCAACGGTATTAGAACAC-5'] were used. The PCR samples were analyzed with agarose gel electrophoresis. *Ubiquitin* (*Ubi*) mRNA was used as an internal control. Polyubiquitin genes consist of multiple units and the *Ubi* primers hybridize with the ends of each single unit. For RT-PCR we used the *Ubiquitin* primers UBIF: [5'-TGCAGATCTTYGT-GAAGAC-3'] and UBIR: [5'-GACTCCTTCTGGATGTTG-3'].

Independently isolated total RNA was reverse transcribed and for the PCR reaction 2 μl of the cDNA sample was amplified in a total volume of 50 μl as described above. The *RCH1*, *RCH2* and *Ubi* cDNAs were amplified during 26, 26 and 20 cycles (94°C, 30 sec; 56°C, 30 sec; 72·C, 30 sec), respectively.

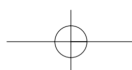
RACE-PCR

RACE-PCR was performed as described in chapter 2 of this thesis. For the 5' RACE we used the gene specific primers: RACE27F1 [5'-TTACTTG-CACCATGACTGTG-3'] and RACE27F2 [5'- ACATTGGAGATTTCG-GACTC-3'] for *RCH1* and RACE12F1 [5'- ATCTTCGCTTGATTGGGAT C-3'] and RACE12F2 [5'-AAAGCCAACAACATCT TGATC-3'] for *RCH2*.

For the 3'RACE we used primers RACE27R2 [5'-TATCAGGTAT CTTGCCAGTC-3'] and RACE27R3 [5'-AGAGACATCAAGTACTTGA AG-3'] for *RCH1* and RACE12R2 [5'-GTGAGAAGATTGTCGAAGAG-3'] and RACE12R3 [5'-TCTTGAGTTTCGAACATTTGC-3'] for *RCH2*.

LRR Tree

The sequences of all LRRXI as classified in Shiu and Bleecker (2001) were obtained from the MIPS database. To select the LRR domain, we used the PlantsP prediction of the LRR region, with the exception of *RCH2* and *F12G12.7* (http://plantsp.sdsc.edu). For *RCH2* the PlantsP predicts a smaller gene than what we confirmed by RACE-PCR and we selected the LRR region by alignment to the RCH1 protein. For F12G12.7 the PlantsP



does not predict any LRR region and we selected this region by comparing the F12G12.7 predicted protein to RCH1.

LRR protein domains were aligned using Lasergene software (Madison, WI).

Promoter fusions

For *RCH1* a 2.2 kb fragment upstream of the *RCH1* gene was cloned in front of a *GAL4VP16* transcriptional activator gene, in the pGreenII vector carrying a kanamycin resistance cassette (Hellens et al, 2000). In the same vector, the *ERGFP* gene was cloned behind the UAS promoter to form the *RCH1-ERGFP* vector.

For *RCH2*, *M3E9.30* and *MDA7.8* we cloned a 2.3kb, 2.2 kb and 2.1 kb promoter region respectively, in front of the β-Glucoronidase (GUS) reporter gene in a pGreenII vector which contains a basta resistance cassette.

Plants were transformed by the floral dip method (Clough and Bent, 1998).

Insertion Mutants

En insertion mutants for *RCH1* and *RCH2* were obtained by screening the *En* mutagenized *Arabidopsis* seed collection described in Baumann et al (1998). For *RCH1* we used primers FS27F3: [5'-GCGTTAAACTTGAGCT GGAATTCATTAGATGG-3'] and FS27R3: [5'-CTGTTATGAGAGAT-GTTCAGAGAAACCAAG-3']. For *RCH2* primers FS12F2: [5'-TACTT GATGTTTCAGCTAACCAGTTTTCAG-3'] and FS12R2: [5'-CTTC-CCAGTGAGTC TATTGCTACTCAAGTT-3'] were used.

For a *M3E9.30* KO the T-DNA insertion line Garlic_1220b_B03 from the Syngenta GARLIC collection was used, which was renamed *m3e9.30-1*. To genotype for the insertion we used the *M3E9.30* genomic primers GM3E9-F: [5'-GGAATAGGATTGTTGACAGAGCTTACAAAG-3'] and GM3E9-R: [5'-GAGGACCCAATAAGACATTCATAGCTTTAAC-3'] and primer GARLIC-LB3 which lies in the T-DNA insertion: [5'- TAGCATCT-GAATTTCATAACCAATCT

CGATACAC-3'].

For a *MDA7.8* KO the T-DNA insertion line Garlic_18b_F04 from the Syngenta GARLIC collection was used and renamed *mda7.8-1*, but we were not able to confirm the T-DNA insertions. The primers used for genotyping were GARLIC-LB3 in the T-DNA insert and ANTMDA7-F: [5'-ATCC-CGGGGGCGCGCCTGATTTAGCTGATAATTCTCTCTCTGGTGA-3'] and ANTMDA7-R: [5'- ACGGATCCATTTAAATTTAACACTTAGCT-GAAGCTCTTGGAGATTT-3'] for *MDA7.8*.

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T-DNA insertions from the SALK collection were also obtained for *RCH1*, *M3E9.30*, *MDA7.8* and *F12G12.7*. Insertion mutant information was obtained from the SIGnAL website at http://signal.salk.edu . For a *RCH1* KO the SALK_038309 line was used and renamed *rch1-3*. The primers used for genotyping were ANTRCH1-F: [5'-ATCCCGGGGGCGCGCCAAGCTCAAATAGTCTTGTTGGTGAAATACC-3'] and FS27R3: [5'- CTGTTATGAGAGATGTTCAGAGAAACCAAG-3'], and SalkLB: [5'- GCGTGGACCGCTTGCTGCAACT-3']. For a second *MDA7.8* KO the SALK_008060 line (renamed *mda7.8-2*) was used for analysis but also here no T-DNA insertion could be confirmed. The primers used for genotyping were MDA7i-F: [5'-TCAGACAATAGCTCTTTACACGTCACTCTT-3'] and FSMDA7R: [5'-AGGTGATTGCAGCTGAGATTTAGAGA-3'] in the MDA7 genomic region and SalkLB in the T-DNA insert. For a *F12G12.7* KO the SALK _014726 line was used and renamed *f12g12.7-1*. The primers used for genotyping this line were SalkLB for T-DNA insertion and G-F12-F: [5'-CTCCATCAACAGCAGAAGATTTCTCATAC-3'] and G-F12-R: [5'-CAAATTTCCCCCAATTTCTTGTAGTAATG-3'] for the *F12G12.7* genomic region.

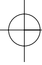
RCH1 Overexpression

To make the *35S::RCH1* construct, the full genomic region of *RCH1* (including 50 bp before the predicted ATG) was subcloned in the 35S cassette from the pGreen vector series, containing the *35SCaMV* promoter and the *35S* terminator (Hellens et al, 2000). The 35S Cassette containing the *RCH1* genomic region was cloned into the pGreenII vector containing a basta resistance cassette, and transformed into plants using the floral dip method (Clough and Bent, 1998).

For the Northern blot, RNA was isolated from leaves of 35 independent transgenic plants as described above. We performed the Northern blot as described in Ausubel et al (1999), loading 20µg of total RNA for each sample and using *RCH1* as a probe.

Microscopy

Plant material for light microscopy was prepared as described in Scheres et al (1994). Images were taken on a Zeiss Axioskop 2 microscope with a Nikon DXM1200 digital camera. For confocal microscopy a Leica SP2 was used. Files were assembled in Adobe PhotoShop VI (Adobe System Inc. Mountain View, CA, USA).



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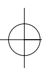
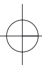
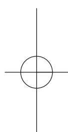
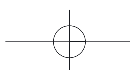


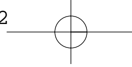


Chapter 4

Summarizing discussion







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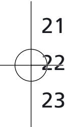
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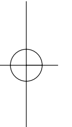
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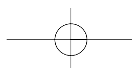

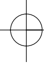

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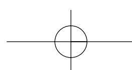
SUMMARIZING DISCUSSION

Post-embryonic plant development depends on the activity of a population of stem cells residing at opposite poles of the seedling in the shoot and root meristems. Clonal analysis in shoots and laser ablation experiments in roots have demonstrated that positional cues and not the clonal origin of a cell determines its fate, underlining the importance of cell-cell communication in plant development (Irish and Sussex, 1992; van den Berg et al, 1995). The research described in this thesis was aimed to identify signaling components required for root meristem maintenance.

In the shoot apical meristem (SAM) the current view is that a negative feed-back loop between the CLV3 small peptide ligand and the homeodomain transcription factor WUS, through the CLV1/CLV2 receptor complex, maintains the population of stem cells (Brand et al, 2000; Schoof et al, 2000). The *WUS*-expressing "organizing center" in the SAM acts to promote stem cell fate in the overlaying cells. In analogy, the QC in roots promotes stem cell fate in the cells surrounding it (van den Berg et al, 1997; Sabatini et al, 2003). This suggests that similar signaling pathways might act in both meristems to control meristem maintenance.

In chapter 2 of this thesis we investigated whether a CLV-like pathway acts in the control of root meristem homeostasis by ectopically expressing a *CLV3*-like gene (*LLP1*) in the root meristem ("RCH1-LLP1" plants). Ectopic *LLP1* expression causes root meristem differentiation, suggesting that LLP1 acts by overactivating an endogenous root pathway. The LLP1 homology to CLV3 prompted us to think that LLP1 functions through a root LRR-RLK with homology to CLV1. We performed a suppressor mutagenesis screen on RCH1-LLP1 plants aimed at the identification of the root LRR-RLK(s) through which LLP1 might act as well as other components of the presumed endogenous root meristem maintenance signaling pathway.

Suppressor screens on transgenic plants ectopically expressing a certain component might have the advantage of sensitizing the pathway through which the component is presumed to act. This can lead to isolation of novel



mutants that would have been missed in forward genetic screens, for example mutants with mild phenotypes in a WT background. However, a disadvantage of such strategy is that ectopic expression creates an artificial situation and the uncertainty remains as to whether the ectopically expressed component utilizes an analogous endogenous pathway. Identification of the molecular identity of the suppressors and analysis of their function in a WT background should clarify this point.

In our screen for suppressors of RCH1-LLP1, we identified two loci, *SOL1* and *SOL2*. *SOL1* encodes for a Zn^{2+}-carboxypeptidase likely to be involved in ligand processing. *SOL2* has not yet been cloned, although initial mapping located this gene into a region at the bottom of chromosome II that does not contain any predicted LRR-RLKs or LRR receptors. Why did we not identify the receptor of the presumed root CLV-like pathway? We consider genetic redundancy among this class of receptors as the most likely explanation. However, other possibilities cannot be excluded. First, our screen might not have been saturating, although we recovered multiple alleles for each suppressor (four for *sol1* and two for *sol2*). Second, LLP1 might not overactivate a LRR-RLK, but act by blocking an endogenous LRR-RLK forming a poisoned complex or by blocking an unrelated pathway. However, due to the homology of LLP1 to CLV3 and the dependence of the phenotype on correct translation of this protein we expect LLP1 to act analogous to CLV3, and hence through LRR-RLK(s).

Identification of *SOL1* as encoding a putative Zn^{2+}-carboxypeptidase with homology to carboxypeptidases D (CPD) and E (CPE) from animals involved in neuropeptide and prohormone processing, suggests a role for SOL1 in ligand processing (Nillni et al, 2002). This raises the question whether SOL1 has a role in processing endogenous root ligands, and hence in root meristem maintenance, or whether in *sol1* mutants the suppression phenotype is merely caused by failure to activate the ectopic LLP1. The presence of potential carboxypeptidase cleavage sites in all CLE members and the expression of SOL1 throughout the plant (in all tissues tested) would support the idea of a general role for SOL1 in ligand processing. Biochemical and loss-of-function *sol1* studies (in WT background) will be necessary to test this possibility.

Strikingly, mutations in all four *sol1* alleles are sufficient to fully suppress the root phenotype induced by LLP1 ectopic expression, but no other phenotypes were detected, e.g. the *clv3* like phenotypes that would be expected from the proposed model. One explanation is that different thresholds of SOL1 activity might be required for sufficient activation of different ligands. Testing whether there is any carboxypeptidase activity left in the different *sol1* alleles, and analysis of a complete loss-of-function *sol1* in the future should help to clarify this point. *SOL1* is a single copy gene in *Arabidopsis*, although redun-

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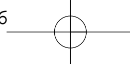
dancy of SOL1 with different types of carboxypeptidases cannot be excluded. One such candidate might be the Ser-carboxypeptidase BRS1, which was identified in an activation tagging screen to isolate novel components of the signaling pathway that involves the LRR-RLK BRI1 (Li et al, 2001a). *BRS1* has many homologues in *Arabidopsis*, and shows homology to Kex1p carboxypeptidase involved in signal peptide processing in yeast. Analysis of *sol1,brs1* double mutants can test whether non-structural redundancy occurs between these two genes.

A second component of the putative CLV-like root pathway is *SOL2*. Mutations in *sol2* lead to floral phenotypes which resemble those of weak *clv* alleles, suggesting that some components of a CLV pathway might be shared between shoots and roots. The penetrance of the *sol2* phenotype seems to be enhanced in a L-*er* background. Interestingly, *clv* mutant phenotypes are also stronger in a L-*er* background, indicating that there is a modifier in L-*er* able to modulate both *sol2* and *clv* mutations.

Preliminary analysis of *sol2* mutants in a WT background revealed the same floral phenotype as *sol2* mutants in the RCH1-LLP1 background, but no root phenotype. Even though the nature of the mutations in *sol2* is unknown at the moment, if *sol2* mutants would be nulls it could mean that SOL2 is a non-limiting or redundant component in root but limiting in shoot signaling. Cloning of *SOL2* will unravel the nature of this possibly shared component, and help to clarify similarities and differences of the (presumed) CLV pathways in the control of both shoot and root homeostasis. Double mutant analysis of *sol2,clv* and *sol2,wus* should clarify if *SOL2* is a component of the shoot CLV pathway.

In chapter 3 we addressed whether *CLV1*-like LRR-RLKs operate in roots. We identified a clade of five genes (the RCH1 clade), from which *RCH1* is specifically expressed in the root meristem, while three of the other four members are expressed in the transition from the meristematic to the elongation zone. Single and double mutant analysis and *RCH1* overexpression did not unravel the function of these genes.

Both our suppressor screening study (chapter 2) and the reverse genetic approach failed to identify the receptor(s) for a putative root CLV-like pathway. Currently, it cannot be formally excluded that there is no such CLV-like receptor involved in root meristem maintenance. However, the existence of such a receptor is likely since ectopic expression of LLP1, a CLV3-like ligand, in roots has profound effects on root meristem maintenance, suggesting that it acts by overactivating an endogenous pathway involving CLV-like receptors able to respond to LLP1. Another perhaps more plausible explanation for our inability to identify the receptor(s) is genetic redundancy among them. Two lines of

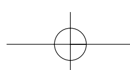


evidences support this idea. First, single and double mutant analysis of the RCH1 clade did not yield any phenotypes. And second, we did not identify a receptor through which LLP1 might act in our suppressor screen.

Indeed, in *Arabidopsis* there are more than 400 receptors but only in very limited cases a function has been identified (Shiu and Bleecker, 2001). What could be the advantage for the plant in retaining redundant genes? Presumably, duplicated genes have been retained over evolutionary time due to unique functions which might be difficult to asses under laboratory conditions (Martienssen and McCombie, 2001). Finding the right experimental conditions is crucial to detect an effect under these circumstances. Kimura's theory of neutral evolution predicts that in large populations, small selection coefficients are sufficient to fix a gene that conveys a selective advantage. This predicts that there should be genes or genetic functions that have only a very small effect on the fitness of an individual, but are nonetheless important for long-term fitness within a population (Tautz, 2000). Being generally sessile organisms, plants have to respond to local environmental conditions by changing their physiology or redirecting their growth. It could be envisaged that a wide variety of receptors which are partially redundant but each conferring specificity to respond to a particular range of environmental stimuli such as light, pathogens, temperature, water, nutrients, touch or gravity might confer a selective advantage. This could be the case for RCH1 and RCH2 which show 60% identity and 75% similarity at the protein level, suggesting they are not products of a recent duplication event. It seems difficult to imagine that the 40% difference at the protein level would not yield to acquisition of new function(s).

With the availability of the rice genome sequence and the sequencing of genomes of other plant species on the way the level of expansion of different clades can be compared. In addition, comparison of expression patterns from conserved genes in different species should uncover which genes are truly orthologous. For example, *RCH1* and *RCH2* in *Arabidopsis* are specifically expressed in roots. Preliminary database searches identified putative rice homologues of RCH1 and RCH2 (data not shown), and it would be interesting to compare their expression patterns to assess whether the root specificity is conserved and therefore likely to be a relevant feature. Furthermore, such comparative studies should uncover whether receptor kinase redundancy is a common theme in the plant kingdom.

How could we overcome genetic redundancy when assessing the developmental role of redundant receptors? Large scale reverse genetics can be an option. It would involve expression pattern profiling, and selection of receptors with overlapping expression domains in the region of interest. Multiple combinations of loss-of-function alleles of these genes may ultimately uncover their

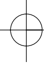


function. In addition, dominant-negative or dominant-active versions of receptors of interest, such as the RCH1 clade of receptors might provide a useful tool to determine their functions.

Even though we did not yet succeed in the identification of the putative CLV-like root receptor(s), two types of evidence indicate that a CLV-like pathway acts in roots in the control of root meristem maintenance. First, ectopic expression of a *CLV3*-like gene in roots (*LLP1*) causes root meristem differentiation (like *CLV3* overexpression phenotypes in the shoot). Second, the *sol2* suppressor has flower phenotypes reminiscent of *clv* weak alleles.

Interestingly, we have shown that the putative root CLV-like pathway appears to promote cell differentiation or restrict cell division without primarily mis-specifiying the QC and/or affecting stem cell status. In addition, the root pathway does not act through SCR which has recently been shown to be required for QC specification and hence stem cell maintenance, in analogy to the role of WUS in the organizing center (Sabatini et al, 2003). Thus, the putative CLV-like root pathway may control root meristem homeostasis by a different mechanism than the CLV pathway in the SAM.

Besides *shr* and *scr* mutants, the *root meristemless 1* and *2* (*rml1,2*) and the *hobbit* (*hbt*) mutants are also disturbed in control of cell division in the root and it can be questioned whether these genes act in parallel or in the same pathway as the presumed CLV-like root pathway. The *root meristemless 1* and *2* mutants have roots in which post-embryonic cell divisions are absent or limited respectively, and the root meristem differentiates soon after germination (Cheng et al, 1995). *RML1* encodes γ-glutamylcysteine synthetase, the first enzyme of glutathione (GSH) biosynthesis (Vernoux et al, 2000). *rml1* mutants can be rescued by GSH application, and WT roots treated with BSO (an inhibitor of GSH biosynthesis) show a similar phenotype as *rml1* roots. Because the oxidized form of GSH (GSSG) could not rescue *rml1* mutants, GSH might directly affect the redox state of developmental regulators (Vernoux et al, 2000). In animals, some transcription factors have been shown to change their ability to bind the DNA in a redox-dependent manner (Abate et al, 1990; Mihm et al, 1995). In addition, Jiang et al (2003) have shown in maize that commencement of more rapid cell divisions in the QC is preceded by changes in the overall redox status of the QC, which become less oxidizing. In the maize QC, GSH is reported to be about 10x lower in concentration than in the proximal meristem. Interestingly, location of the auxin maximum correlated with oxidative stress in the QC and it was suggested that auxin might provide positional cues by virtue of its ability to influence, on a localized scale, the redox status of tissues (Jiang et al, 2003). It is possible that in *rml1* post-embryonic cell division is blocked in the meristem because of oxidative stress,

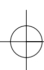


which might interfere with auxin accumulation or responsiveness in the meristem, but more direct evidence is needed to test this hypothesis.

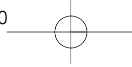
Several scenarios are possible to explain the similar phenotypes encountered in *rml1* and RCH1-LLP1 roots: the putative CLV-like root pathway might negatively regulate RML1 transcription and hence deplete the level of GSH in the meristem; RML1 might modulate components of the pathway post-transcriptionally, or both pathways may be independent. Combinations of *rml1* mutants with RCH1-LLP1 together with application of GSH and BSO to RCH1-LLP1 roots should help clarify if they act in the same pathway controlling restriction of cell division rather than promotion of differentiation in the root meristem. In addition, it will be interesting to test if the QC and stem cell status is affected in *rml1* mutants. Double mutants *rml1,scr* and *rml1,shr* should clarify if the corresponding genes act in the same pathway.

Mutations in the *hobbit* (*hbt*) gene interfere with postembryonic cell division and differentiation of the distal cell types: QC, columella and lateral root cap, which depend on distal accumulation of auxin (Willemsen et al, 1998; Sabatini et al, 1999). *hbt* mutants show reduced auxin sensitivity and accumulate the AXR3/IAA17 repressor of auxin responses (Blilou et al, 2002). *HBT* encodes a homologue of the CDC27 subunit of the anaphase-promoting complex (APC), which might couple cell division to cell differentiation by regulating cell cycle progression in the meristem or by restricting the response to differentiation cues, such as auxin, to dividing cells (Blilou et al, 2002). HBT might act in a different pathway than the proposed CLV-pathway because *hbt* mutants show primary defects in the QC region, and do not express SCR in the QC post-embryonically, suggesting that QC identity and stem cell maintenance may be affected prior to cell differentiation. However, preliminary analysis of post-embryonic *hbt* loss-of-function clones suggests that cell differentiation may be affected prior to QC identity and stem cell maintenance (Olivier Serralbo, unpublished results). Thus, it is possible that a root CLV-like pathway in differentiation is connected to HBT activity. Double mutant combinations of *hbt,sol1* and *hbt,sol2* should clarify whether *sol1* and/or *sol2* are able to suppress the root differentiation phenotype observed in *hbt*. In addition, introduction of a HBT:GFP protein fusion in RCH1-LLP1 plants would uncover whether HBT is a downstream target of the presumed CLV-like pathway. It will be also interesting to test whether downstream targets of HBT, as AXR3 (and may be other AXR/IAA proteins) are downregulated in RCH1-LLP1 plants.

Once the components of a CLV-like root pathway are known, the evolutionary relationships between signaling pathways involved in shoot and root meristem homeostasis can be investigated. The earliest unequivocal evidence for roots in the fossil record comes from Early Devonian vascular plants, which



have modified prostrate stems bearing rhizoids resembling those of living bryophytes (Kenrick and Crane, 1997). Regardless of their origin, the fossil record shows that many vascular-plant organs can be interpreted in terms of modification of basic structural units such as the spore-bearing tissues and the stems (Kenrick and Crane, 1997). Thus, possibly roots are derived from ancestral shoot structures like bifurcating leafless stems. Our preliminary data support a model in which shoot and root meristem maintenance through a CLV-like pathway could work differently. In shoots, the CLV pathway acts by restricting the expression of *WUS* in the organizing center, which in turn controls stem cell fate in the overlaying layer. In roots, the putative CLV-like pathway may promote differentiation or restrict cell division directly, without initially interfering with QC specification and stem cell maintenance. An interesting question that arises is which of these mechanisms resembles most the ancestral use of CLV signals in meristem development. Expanding knowledge of the molecular components acting in shoots and roots in different plant species should further our understanding of meristem evolution in vascular plants.



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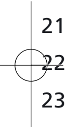
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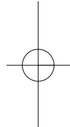
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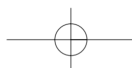

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Samenvatting in het Nederlands

De ontwikkeling van een plant stopt niet na de embryogenese maar duurt zolang de plant leeft mogelijk gemaakt door de aanwezigheid en activiteit van kleine groepen continu delende cellen, de meristemen. Vanuit de meristemen worden de organen gevormd door een process van gebalanceerde celdelingen en differentiatie. In het hart van het wortelmeristeem van Arabidopsis thaliana bevinden zich 4 mitotisch inactieve cellen, genaamd het "quiescent center (QC)", welke zijn omgeven door de stamcellen die verantwoordelijk zijn voor het genereren van alle cellen die samen de wortel vormen. Een functie van het QC is het behouden van de stam cel status van deze omliggende cellen.

Het doel van dit proefschrift was om signaalcomponenten te identificeren die nodig zijn voor het behoud van het wortelmeristeem. In hoofdstuk 2 heb ik uiteengezet dat overexpressie van een CLV3-homoloog gen (*LLP1*) onder een wortel-meristeem-specifieke promoter differentiatie veroorzaakt van het wortelmeristeem. Dit doet vermoeden dat een CLV-homologe signaaltransductie cascade verantwoordelijk is voor het behoud van het wortelmeristeem. Daarnaast werkt dit CLV-homologe signalering in de wortel onafhankelijk van SHR en SCR activiteit en zonder in eerste instantie de specificatie van het QC of de stamcellen te beïnvloeden.

Om de genen te identificeren die betrokken zijn bij deze signaal transductie heb ik een "suppressor"-mutagenese uitgevoerd op een lijn waarin *LLP1* ectopisch tot expressie is gebracht specifiek in het wortelmeristeem.

In twee verschillende loci, *sol1* en *sol2*, zijn mutaties geïdentificeerd als suppressors van ectopische *LLP1* expressie fenotypes.

Ik heb *SOL1* gekloneerd welke voor een op basis van de sequentie voorspelde Zn2+-carboxypeptidase codeert en daarom mogelijk een rol heeft in het processen van eiwitliganden. *sol2* mutanten laten bloemfenotypes zien die lijken op die van zwakke *clv* mutanten wat kan betekenen dat deze komponent zowel in een CLV signaaltransductie in de wortel als in de scheut een rol speelt.

In hoofdstuk 3 beschrijf ik een "reverse genetics" aanpak om te onderzoeken of CLV1-homologe receptoren betrokken zijn bij het behouden van

een actief wortelmeristeem. Hiervoor is een "clade" van 5 receptoren, waarvan 2 specifiek in het wortelmeristeem tot expressie komen, onderzocht op functionaliteit tijdens wortelontwikkeling. Er zijn onder andere expressie studies en enkele en dubbel mutant analyses gedaan. Het feit dat tot nu toe geen fenotype ondekt in deze mutanten wijst op de hoge mate van redundantie van deze receptoren.

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And finally, today that Aznar seems a re-incarnation of an obscure figure who died 27 years ago, and Bush tries to teach us good and evil, bombs are falling over Iraq. I just want to scream loud "Nunca Máis" and "No a la Guerra".

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Curriculum Vitae

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