The Roles of *WOL* and *APL* in Phloem Development in *Arabidopsis thaliana* Roots

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"Some days even my lucky rocketship underpants won't help."

Calvin, of Calvin and Hobbes

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List of original publications

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals.

I.   Mähönen AP, Bonke M, Kauppinen L, Riikonen M, Benfey PN, Helariutta Y. (2000) A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root. *Genes Dev*. 14: 23: 2938-2943.

II.   Bonke M, Thitamadee S, Mähönen AP, Hauser MT, Helariutta Y. (2003) APL regulates vascular tissue identity in Arabidopsis. *Nature*. 426: 6963: 181-186.

III.   Bonke M, Thitamadee S and Helariutta Y. Molecular characterization of the *APL* locus identifies a sequential process of phloem identity and differentiation. (*Manuscript*)

Contribution of the authors

Paper I: **Ari Pekka Mähönen**: Experimental designs, cloning of *WOODEN LEG* gene, complementation of the mutant, sequencing, part of the Northern analysis, writing the publication.
Martin Bonke: Experimental designs, histological studies, cloning of the cDNA, confocal microscopy, crossing of marker lines, writing a part of the publication, editing the publication.
Leila Kauppinen: Experimental designs, Northern analysis, part of *in situ* hybridization, editing the publication.
Marjukka Riikonen: Experimental designs, *in situ* hybridization, editing the publication.
Philip N. Benfey: Providing the *wol* mutant and information concerning the rough mapping location of the *WOL* gene, editing the publication.
Yrjö Helariutta: Experimental designs, editing the publication, supervised the work.

Paper II: **Martin Bonke**: Experimental designs, part of the cloning of the *APL* gene, complementation of mutant, crossing of marker lines, part of confocal microscopy, *in situ* hybridization, northern analysis, part of histological analysis, embryonic analysis, writing a part of the publication, editing the publication.
Siripong Thitamadee: Experimental designs, part of cloning of the *APL* gene, transgenic *APL* constructs, part of confocal microscopy, part of histological analysis, ultra structural analysis, writing a part of the publication, editing the publication.
Ari Pekka Mähönen: *WOL* promoter construct, editing the publication.
Marie-Theres Hauser: Providing the *apl* mutant, editing the publication.
Ykä Helariutta: Experimental designs, editing the publication, supervised the work.

Paper III: **Martin Bonke**: Experimental designs, RNAi construct, histological analysis, confocal microscopy, crossing of marker lines, embryonic analysis, writing of the publication.
Siripong Thitamadee: Experimental designs, transgenic *APL* constructs.
Ykä Helariutta: Experimental designs, editing the publication, supervised the work.

Abbreviations

apl	*altered phloem development* mutant
ARF	*AUXIN-RESPONSE FACTOR* gene family
BAC	Bacterial artificial chromosome
bdl	*bodenlos* mutant
BFA	Brefeldin A
bHLH	Basic/helix-loop-helix
CC	Companion cell
CLV1	*CLAVATA1* gene
CLV3	*CLAVATA3* gene
cpc	*caprice* mutant
CSN	COP9 signalosome
CUL1	*CULLIN1* gene
DhkA	*Dictyostelium discoideum* Histidine kinase A
E3	Ubiquitin ligase
EGL3	*ENHANCER OF GLABRA3* gene
ER	Endoplasmatic reticulum
EST	Expressed sequence tag
fs	*fass* mutant
gl2	*glabra2* mutant
GFP	*GREEN FLUORESCENT PROTEIN* gene
GL3	*GLABRA3* gene
glm	*gollum* mutant
gn	*gnom* mutant
GUS	*β-GLUCURONIDASE* gene
H-cell	Hair cell in epidermis
IAA	Indole-3-acetic acid
mp	*monopteros* mutant
MYB	M̲y̲e̲loblastosis, DNA binding domain
N-cell	Non-hair cell in epidermis
NPA	Naphthylphthalamic acid (auxin transport inhibitor)
OC	Organizing Center of shoot meristem
QC	Quiescent Center
PIN	*PIN-FORMED* gene family
RUB1	*RELATED TO UBIQUITIN1* gene
SCF	Skp1-Cullin-F-box
scr	*scarecrow* mutant
SE	Sieve element
shr	*shortroot* mutant
TAC	Transformation-competent artificial chromosome
TE	Tracheary element
TIR1	*TRANSPORT INHIBITOR RESISTANT1* gene
TRY	*TRYPTICHON* gene

ttg	*transparent testa glabra* mutant
wer	*werewolf* mutant
wol	*wooden leg* mutant
WUS	*WUSCHEL* gene

Abstract

The plant vascular tissue is necessary for transport of water, nutrients and macromolecules, and functions as a supporting structure. The network of xylem and phloem is expanded throughout plant life, both in the radial direction, due to cambial activity and in the apical-basal directions due to meristem activity. Although plant hormones, such as auxin and cytokinins, have been shown to be important for the general establishment of the vascular tissue, the genetic regulation of the patterning process is yet poorly understood.

In this study, the structural analysis of the provascular meristem of the *Arabidopsis* root and the identification of two genes that are involved in the regulation of this patterning process are presented. The *Arabidopsis* phloem poles are established after a series of asymmetric cell divisions. The first divisions, of the provascular cells adjoining the quiescent center (QC), appear random. The phloem specific cell divisions that form the companion cells (CC) and sieve element (SE) cell files are highly reproducible, indicative of a tightly regulated process.

The *wooden leg* (*wol*) mutation results in a vascular defect, in which cell proliferation of the vascular initials is restrained, resulting in fewer vascular cells. The remaining vascular cells all differentiate as protoxylem and therefore the primary root lacks phloem. Molecular cloning of the mutation identified the gene as a two-component histidine kinase receptor, which is specifically expressed within the vascular bundle, indicating that a phosphorelay signaling pathway regulates cell proliferation within the vascular tissue.

A second mutant, named *altered phloem development* (*apl*), has a more specific defect. Whereas the outer cell layers of the *apl* roots have a normal radial pattern, the vascular system is abnormal. The phloem specific tangential and periclinal cell divisions occur less frequent and subsequently the prospective SEs and CCs start to differentiate as xylem elements. The *APL* gene encodes a MYB (myeloblastosis, DNA binding domain) coiled-coil-type transcription factor, and from late embryogenesis onwards it is specifically expressed in developing phloem, consistent with a key role in phloem development. Ectopic *APL* expression in the vascular bundle inhibits xylem development. The presented results suggest that APL has a dual role both in promoting phloem differentiation and in repressing xylem differentiation during vascular development. A model of vascular development is proposed in which both WOL and APL are critical steps for vascular patterning.

1. Introduction

1.1 *Arabidopsis* as a model species for studying vascular development

Finland is the world's sixth-largest producer of paper and board and its paper exports make up a 15 per cent share of the world market. In order to make paper and board, a lot of wood is needed, in 2003 in Finland alone, a total of 50 million cubic meters of domestic roundwood was used as raw material (source: Finnish Forrest Industries, 2003; http://english.forestindustries.fi/files/ images/tilastot_en/raakaak.pdf). The amount of raw materials required for the different industries is steadily increasing, and although forest growth currently exceeds rates of harvest, environmental concerns are raising more and more objections to forest harvesting. One possible approach to solve this problem is knowledge based tree breeding to increase for example growth speed; this involves extensive studies on the genetic regulation of wood development. This knowledge can then be used to search for natural mutants with altered gene expression, or to genetically alter economically important trees that can be used in a breeding program for enhanced growth.

The major component of a tree trunk is the woody tissue, also known as xylem. The xylem is the transporting tissue that brings water and nutrients from the roots to the shoot. Each year a new ring of fresh xylem tissue grows around the layer of the previous year, steadily increasing the thickness of the trunk. This growth is dependent on the activity of the (pro)cambium, a layer of cells that are basically the stem cells of wood. (Pro)Cambial cells themselves are dependent on phloem, the transporting tissue on the outside of the tree trunk, which transports photosynthates from the shoot to the root, and to other sink tissues, such as (pro)cambium. Therefore, in order to fully understand wood formation, one needs to understand the genetic regulation of the cambial cell divisions, as well as the phloem function.

Studying vascular development in trees is challenging, due to their size and because it generally takes years for them to reach the fertile stage. Instead, the weed *Arabidopsis thaliana* (from now on called *Arabidopsis*) is used as a model organism. *Arabidopsis* has many properties that make it near perfect for research purposes and is widely used as a model organism. Some of its advantrages are: a small genome that has been sequenced, extensive genetic and physical maps of all 5 chromosomes, a rapid life cycle (about 6 weeks) with high seed production, easily cultivated in restricted space, efficiently transformed by *Agrobacterium tumefaciens*, and a large number of mutant lines and genomic resources are available. Furthermore, it has all the same tissues in its vasculature as a tree, making it the organism of choice for dissecting the mysteries of vascular development. New vascular tissue is continuously generated at the meristems of both shoot and root; however, the simple anatomical structure of the *Arabidopsis* root makes it especially suitable for studying the establishment of the vascular pattern.

1.2 Organization of the *Arabidopsis* root

1.2.1 General anatomy of the growing root tip

The main advantage of the *Arabidopsis* root is its simplicity. The root can be divided into

three distinct developmental zones: 1) the meristematic zone, where cell division occurs, 2) the elongation zone, where cells elongate before differentiating, and 3) the differentiation zone, where cells differentiate and reach maturity. A cross section through a mature *Arabidopsis* root can be seen as a series of concentrically layered tissues with the vascular bundle in the center, surrounded by the ground tissue layers of endodermis and cortex, and the epidermal layer on the outside (Fig. 1) (Dolan *et al*., 1993; Scheres *et al*., 1994). The root structure formation continuously takes place in the root tip at the root meristem, which includes the initial cells of all tissues and the mitotically inactive QC (Clowes, 1956; Dolan *et al*., 1993). The initials surround the QC on all sides: the vascular initials are located on the proximal-

or top-side, the columella and lateral rootcap initials on the distal- or lower side, and the cortex and endodermis initials on the radial flanks (Dolan *et al*., 1993) (Fig. 1). When the initial cells divide, two cells with different fates are formed. The cells connected to the QC maintain their undifferentiated state, whereas the other cell continues with the formation of the specific tissue. All tissues, except columella, will still undergo cell divisions before actual differentiation occurs (Dolan *et al*., 1993). These divisions can be anticlinal (in the horizontal plane) and result in more cells within the same cell file, or periclinal (in the radial plane) and result in two cell files that can differentiate into separate tissues, as happens in for example the formation of cortex and endodermis (Scheres *et al.*, 1994) and also in the vascular tissue.
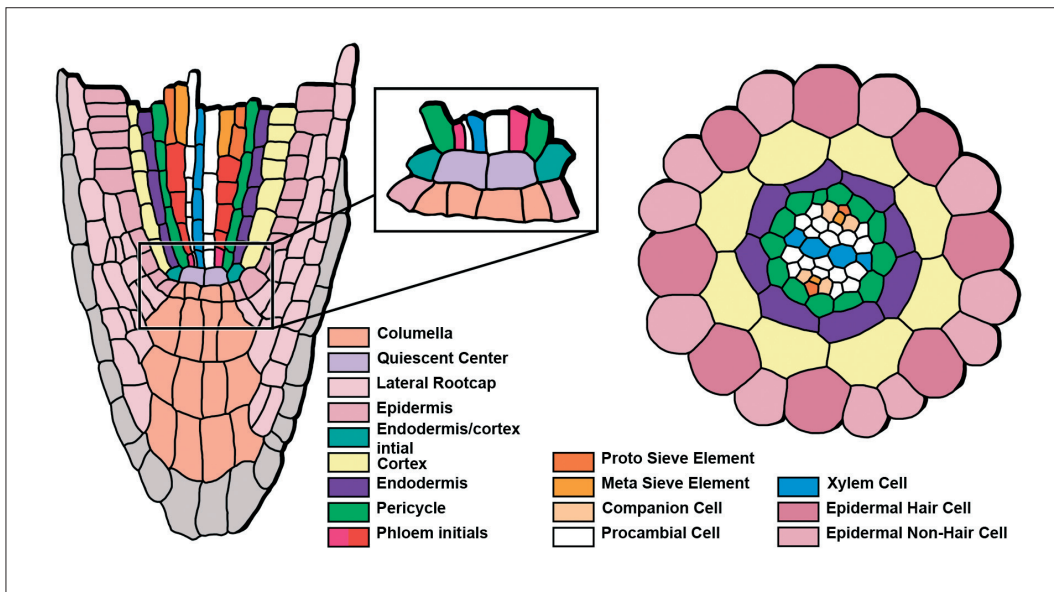

Figure 1. Cell lineages in the *Arabidopsis* root. Schematic; colors refer to cell lineages only, the differentiation state is unknown.

1.2.2 The establishment of the root structure during embryogenesis

In order to have proper root growth after germination, the plant has to form a functional pro-meristem (QC and initials) already during embryogenesis. *Arabidopsis* embryo formation has been closely monitored; the sequence of cell division after fertilization is known almost cell by cell. The zygote divides after it has elongated approximately three-fold, this is an asymmetrical division, resulting in a large basal cell and a smaller apical cell (Mansfield and Briarty, 1991). The basal cell will divide anticlinally several times and thus form the suspensor structure. The top most cell of the suspensor, which is connected to the smaller apical cell, is known as the hypophyseal cell. This cell will be part of the embryo and form the QC and columella initials of the root. The smaller apical cell will divide in a predictable manner and form most of the embryo. First it undergoes three rounds of cell division, leading to two layers of four cells on top of each other, also known as the octant stage. These two layers are the upper and lower tiers of the embryo, and will form the apical and most of the basal parts of the embryo and seedling, respectively (Scheres *et al.*, 1994). The cells subsequently undergo tangential cell divisions, which separate the epidermal or (protoderm) layer from the rest of the embryo; this is known as the dermatogen stage. The following cell divisions in the apical-basal plane indicate the axis formation of the embryo, which by then has a globular shape. From this stage onwards, the embryo starts taking on a more triangular shape. The cotyledons are initiated in the upper tier and the procambium is visible in the lower tier. The pericycle is separated from the other vascular tissue during the late globular stage. At late heart stage the prospective root meristem organization is first identified. The lowest of the protoderm cells undergoes the characteristic periclinal division and defines the lateral root cap initial (Scheres *et al.*, 1994; Dolan *et al.*, 1993). This allows for the identification of the neighboring cells as initials for cortex-endodermis, pericycle, and vascular tissue. Like the epidermis initials, at this stage the root endodermis-cortex initials already produce daughter cells that perform the periclinal divisions defining endodermis and cortex as separate layers (Scheres *et al.*, 1994).

Cell divisions now lead the embryo from "heart" stage through "torpedo" and "bent-cotyledon" until finally the "mature embryo". During these stages the shoot and root meristems are finalized and all tissues are set in place, but do not yet differentiate (Scheres *et al.*, 1994). Embryonic maturation starts when the final cell number of the embryo has been established: at that point seed storages are filled and dormancy and desiccation tolerance are established (Goldberg *et al.*, 1994).

1.2.3 Embryonic cell organization as functional basis for the seedling

The fate of embryonic meristem cells in germinated seedlings was determined by using the "clonal analysis" technique. Scheres *et al.* (1994) used a transgenic line that had an *EN-1* transposon inserted in the *β-glucuronidase* (*GUS*) gene. *EN-1* is an actively jumping transposon; when the *EN-1* is located in a gene, excision generally leads to repaired gene function, in this case, a functional *GUS* gene. When the *EN-1* transposon excises in an embryonic cell, the *GUS* gene in this cell and all its descendents

would be restored. By doing a GUS staining it was possible to analyze which cells were related to one another based on the size of the blue sector: a larger sector indicated that the excision had occurred in an earlier stage. Using this technique Scheres *et al*. (1994) established that the boundary between root and hypocotyl is generated at the early heart stage. However, this boundary is somewhat flexible, and cells mapped as "root-fate" can change into "hypocotyl-fate" cells until the end of embryogenesis and *vise versa*. Another result was that the QC and all initials are formed during the heart stage and maintained as such throughout the rest of embryonic development. The meristem is then activated into cell division during germination.

1.2.4 Plasticity of cell fate

The strict cell division patterns in wild type *Arabidopsis* embryos with the subsequent clonal relationship of cells within one tissue in the root suggests this clonal relationship to be important for proper pattern formation of the root. However, several independent experiments have shown that cells of the different tissues are capable to adapt a different identity under the right circumstances. For example, van den Berg *et al*. (1995) used the laser ablation technique to destroy the complete QC of a primary root; it was then observed that the vascular initials changed identity to reform a QC. These and other studies also showed the importance of the QC and tissue initials for the proper root patterning (van den Berg *et al*., 1995, van den Berg *et al*., 1997, Berger *et al*., 1998, Kidner *et al*., 2000).

Already during the 1950s, the QC was identified as being mitotically inactive (Clowes, 1956), but its function was not

deducted until the late 1990s. In a laser ablation experiment individual cells of the QC were destroyed (van den Berg *et al*., 1997). As a result, the columella initial that used to be in contact with the ablated cell no longer divided but started accumulating starch, a sign of differentiation (van den Berg *et al*., 1997). This indicated a role for the QC as a provider of a signal that keeps the contacting initial cells in an undifferentiated state in a cell-to-cell contact-dependent manner. The QC is not completely mitotically inactive, though rarely, its cells do divide. However, these divisions seem to occur randomly and the daughter cells can contribute to each tissue, further suggesting that position is important for cell fate (Kidner *et al*., 2000).

Also in radial orientation, cells can undergo changes in cell fate. Pericycle cells are capable of acquiring cortical cell identity, and cortical cells can take on epidermal cell identity when the original cells have died (van den Berg *et al*., 1995, Berger *et al*., 1998). These results strongly indicate that cells receive positional information from their neighboring cells, which decides their fate (Berger *et al*., 1998).

The laser ablation technique was also used to identify from which direction the positional information was coming during the formation of the endodermal and cortex layers. By ablation of the cortex-endodermis-initial daughter-cell, a separation was established of the initial cell from the mature cortex and endodermis layers. As a result, the newly generated daughter cells were unable to divide periclinally to form the cortical and endodermal layers. This suggested that either the daughter cells need information from the mature layers in order to divide in an oriented manner, or that the daughter

cells are accumulating stem-cell promoting factors from the QC (van den Berg *et al.*, 1995).

Altogether, from these results it can be concluded that root patterning involves cell-to-cell communication in which positional information is relayed between cells to establish cell identity. This was recently also shown in the formation of tracheary elements (TEs) of xylem, where the proteoglycan xylogen is secreted in a polar manner and mediates an inductive cell to cell interaction, leading to differentiation (Motose *et al.*, 2004; see also 1.3.5).

1.3 Signals and genes regulating root development

As *Arabidopsis* root formation is the result of a series of characteristic asymmetric cell divisions leading to the establishment of the different tissues found in the root, analysis of many informative *Arabidopsis* mutants and molecular cloning of the corresponding genes has led to a relatively good understanding of the genetics that control root development. In the following sections, the patterning processes of the different tissues are divided into separate units to clarify their mechanisms. Although they are not all directly linked to vascular patterning, their genetic regulation may serve as an example for how the patterning process takes place within the vascular bundle.

1.3.1 Distal pattern regulation by polar auxin transport

The plant hormone auxin is the most intensively studied signal molecules of the different hormones that have been identified in plants. Although the other plant hormones, such as gibberellic acid, abscisic acid, brassinosteroids have been shown to

be important for the formation and differentiation of the different tissues (for a review: Thomas and Sun, 2004; Gazzarrini and McCourt, 2001; Wang and He, 2004), only of auxin and cytokinin (see discussion) a clear function in vascular development has been established, therefore only these hormones will be discussed in this thesis.

Auxins such as indole-3-acetic acid (IAA) have been known as growth promoting factors for decades and recent research has led to a deeper understanding of their function in cell division, cell elongation, cell differentiation and the initiation of organ formation (for a review: Davies, 1995). Auxin is mainly produced in the shoot and then transported to locations where growth and differentiation occurs. Transport from the site of production to the site of action is thought to take place both in a polar and non-polar manner. Auxin is first loaded into the phloem and then travels in the phloem stream to the active sites where it is unloaded from the phloem. This phloem transport of auxin is non-polar and directed purely by the flow of the phloem stream. However, the auxin permease AUX1 is required for loading and unloading the auxin into and out of the phloem (Marchant *et al.*, 2002; Swarup *et al.*, 2001).

At the active sites, such as the root meristem, polar transport takes over. This involves auxin efflux carrier proteins of the PIN (PIN-FORMED) family (Galweiler *et al.*, 1998). Polar auxin transport already plays an important role during the earliest stages of embryogenesis. After division of the zygote, auxin transporter PIN7 is specifically localized in the apical membrane of the basal cell, directing auxin flow into the apical cell (Friml *et al.*, 2003). Interference with PIN7 function disrupts this auxin flow and affects apical cell fate, thus indicating that auxin is required for

apical cell fate specification. During the subsequent octant stage of embryogenesis, polar auxin transport is essential for the formation of the hypophyseal cell and apical-basal polarity of the embryo. The role of polar auxin transport in these processes was deducted from work on the *GNOM* (*GN*) gene. In *gn* mutants, neither hypophyseal cell nor apical-basal polarization were established (Mayer *et al.*, 1991, 1993). The *GN* gene encodes a Brefeldin A (BFA)-sensitive ARF GDP/GTP exchange factor that is essential for the polar localization of the PIN1 auxin efflux carrier. Evidence to support the role of PIN1, and thus polar auxin transport, was provided by treating wild type embryos with BFA. This phenocopied the mutant phenotype, while an engineered BFA-resistant *GNOM* line was unaffected by BFA (Geldner *et al.*, 2001, 2003).

When the globular stage (32 cells) has been reached, auxin production starts in the embryo itself. Auxin supply from the mother plant is then turned off by changes in the localization of the PIN7 auxin efflux carrier, which now is directed to the basal membrane of the suspensor cells. Furthermore, PIN1 localization shifts and is targeted to the basal membranes of the provascular tissue. This leads to an auxin maximum in the uppermost cell of the suspensor, which takes on the hypophysis identity (Friml *et al.*, 2003).

Polar auxin transport plays an important role in cell fate and patterning of the root meristem also after germination. In wild type *Arabidopsis* roots, an auxin maximum exists in the columella initials directly under the QC (Sabatini *et al.*, 1999). This auxin maximum is maintained by polar auxin transport, most likely driven by the PIN4 auxin efflux carrier, which is localized at the basal membranes of vascular cells in the

root tip (Friml *et al.*, 2002). In wild type, both naphthylphthalamic acid (NPA) treatment and *PIN4* disruption result in a shift of the auxin maximum to the location where *PIN4* is expressed. Furthermore, *pin4* mutants lacked or misexpressed QC marker lines and showed irregular cell divisions at the location where normally the QC, columella initials, and endodermis would be located, while above and at the location of the new auxin maximum the cells were taking on characteristics of QC and columella initials respectively (Friml *et al.*, 2002).

How exactly does auxin regulate the patterning process(es)? A receptor that binds auxin has yet to be identified, but the transcription pathway that is activated by auxin is understood for the greater part. At low auxin concentrations, the short-lived Aux/IAA repressor proteins are repressing the early auxin response genes by dimerizing with Auxin Response Factors (ARFs) (Ulmasov *et al.*, 1997; Tiwari *et al.*, 2004; for a review: Guilfoyle *et al.*, 1998). At higher auxin concentrations, such as available in the active zones of the meristem, turnover of the Aux/IAA proteins increases. This speedier turnover of Aux/IAA is established by a faster degradation in the 26S proteasome pathway through regulation of the E3 ubiquitin (U)-ligase function of SCF[TIR1] (Skp1-Cullin-F-box). The SCF[TIR1] complex consists of four subunits (ASK1/2, CUL1, RBX1 and TIR1), of which TIR1 (TRANSPORT INHIBITOR RESISTANT1) binds to the conserved degradation domain II of Aux/IAA proteins (Gray *et al.*, 2001; Dharmasiri *et al.*, 2003; Tian *et al.*, 2003). SCF[TIR] function is further regulated by modifications of the CUL1 (CULLIN) subunit by RUB1 (RELATED TO UBIQUITIN1) and COP9 signalosome

(CSN). This leaves free ARFs to activate the early auxin response genes either alone or as dimers (for a review: Berleth *et al*., 2004; Hellmann and Estelle, 2002).

Genetic evidence for the importance of these gene families has been around for quite some time. One of the first identified ARFs (ARF5) was linked to the *monopteros* (*mp*) mutant. In wild type, *MP* expression shifts from throughout the embryo during early stages to the basal domain and vascular tissue during the later stages. In *mp*, the apical-basal organization is disrupted during embryogenesis. After germination vascular strands are discontinuous, suggesting a vital role for *MP* in the establishment of the basal domain and vascular tissue (Berleth and Jürgens, 1993; Hardtke and Berleth, 1998). A similar phenotype is observed in the *bodenlos* (*bdl*) mutant, which codes for *IAA12* (Hamann *et al*., 1999). The mutation, localized in the conserved binding domain II, inhibits binding to TIR1 and thus results in stabilization of the protein. Both *BDL* and *MP* have similar expression patterns, and because the mutant phenotypes are alike, it is thought that they form a repressor-activator module that regulates basal- and vascular-patterning (Hamann *et al*., 2002). A third mutation, called *axr6*, is located in the CUL1 subunit of the SCF^{TIR1} complex. It causes a similar phenotype as *mp* and *bdl*, probably by the SCF^{TIR1} complex being unable to interact with the Aux/IAA proteins, which, as a result, are not degraded (Hellmann *et al*., 2003).

In all three mutants the hypophyseal cell fails to acquire the hypophysis identity, and, as a consequence, the QC and columella initials do not differentiate properly, this suggests that auxin is a positional cue that specifies their position (Friml *et al*., 2003). It remains to be seen whether the differentiation defect is the result of a disruption in perception of the auxin signal, lack of auxin signal transduction in the hypophyseal cell, or maybe due to a combination of both problems. Also, so far it has yet to be established whether the same pathway works both during embryogenesis and after germination, or if multiple pathways exist that separate these developmental stages.

1.3.2 Maintenance of the root meristem

As mentioned in 1.2.4, the QC is thought to maintain the surrounding initial cells in an undifferentiated state that enables them to continue dividing (van den Berg *et al*., 1997; Umeda *et al*., 2000). How the QC achieves this remarkable feat is still largely unknown, but recently some clues indicated that in the root meristem a similar pathway may exist to the one present in the shoot meristem. In the shoot apical meristem, the CLAVATA signaling complex (Clark *et al*., 1993) has been identified as a regulatory unit that maintains a set of stem cells within the meristem. This pathway includes several units that interact in a non-cell-autonomous manner. The CLAVATA3 (CLV3) protein is produced and secreted by the stem cells in the shoot meristem, and some of it can move to neighboring cells. CLV3 function is to repress the *WUSCHEL* (*WUS*) promoter, which normally drives the differentiation program of cells. The Organizing Center (OC) and initials strongly express *CLAVATA1* (*CLV1*) to avoid differentiation. The CLV1 receptor binds to CLV3 before it can get to the *WUS* promoter, and thus both OC and initials are maintained in their undifferentiated state (Lenhard and Laux, 2003).

A couple of genes which suggest that a similar mechanism of stem cell maintenance

exists in roots have been identified in recent years. *CLE19* is a homologue of *CLV3* that is weakly expressed in roots. When overexpressed in roots, it was shown to result in loss of the meristematic zone and shortening of the elongation zone, while the QC and initials remained intact. Overexpression of *CLV3* in the root had a similar effect (Casamitjana-Martinez *et al*., 2003). A putative Zn^{2+}-carboxypeptidase has been shown to be able to suppress the overexpression phenotype, suggesting a role in the processing of the ligand, but other members of a CLAVATA-like signaling complex in the root are yet to be identified. Moreover, a *CLE19* homologue from *Brassica napus* (*BnCLE19*) was recently associated with cotyledon development during embryogenesis. Although it is expressed in some pericycle cells of the root, and misexpression leads to strong phenotypic alterations in both root and shoot, knockouts did not show any changed phenotype, indicating that either it does not play an important role in normal development or that redundancy exists between the different *CLE* genes (Fiers *et al*., 2004). Recently also a *WUSCHEL*-like gene has been cloned that is expressed in the QC of Rice root meristems, and which seems to function in a similar fashion as WUS in specifying and maintaining the QC (Kamiya *et al*., 2003). Furthermore, the transcription factor *SCARECROW* (*SCR*), which is required for ground tissue formation, is necessary for the proper maintenance of the QC (Sabatini *et al*., 2003). Further research is needed in order to understand how and if all these components work together to maintain a functional root meristem.

1.3.3 Genetic regulation of epidermal patterning

In the radial pattern of the *Arabidopsis* root, the epidermal layer is the outermost. In this layer, two types of cells can be identified: 1) the trichoplasts or Hair cells (H-cells), which will develop root hairs, and 2) atrichoplasts or Non-hair cells (N-cells), which do not make hair cells. In wild type plants under normal conditions, the H-cell is located at the junction of two cortex cells and thus connected to both cells, whereas N-cells are in contact with only one cortical cell (Dolan *et al*., 1994; Galway *et al*., 1994). The genetic mechanisms that regulate epidermal cell fate have been widely studied, and individual components of the signaling pathway have been identified with mutant analysis. In mutant screenings, three mutants, *glabra2* (*gl2*), *transparent testa glabra* (*ttg*) and *werewolf* (*wer*), showed increased amounts of root hairs and therefore the encoding proteins are thought to be repressors of root hair formation. One mutant (*caprice* (*cpc*)) had less root hairs, and thus CPC probably is a stimulator of root hair formation (Galway *et al*., 1994; Di Cristina *et al*., 1996; Masucci *et al*., 1996; Lee and Schiefelbein, 1999; Wada *et al*., 1997). All four mutants were cloned and encoded putative transcription factors. The *GL2* gene contained a homeo-domain, and was expressed already during embryogenesis, where it is first found in all epidermal cells during the heart stage of embryogenesis. Its expression becomes specific for the N-cells during the bent-cotyledon stage (Rerie *et al*., 1994; Costa and Dolan, 2003). The *TTG* gene encodes a protein that contains four WD40 protein

binding repeats (Walker *et al*., 1999). Complementation of the *ttg* mutant with the Maize R cDNA, which encodes a basic helix-loop-helix transcriptional activator, indicated that it functions as a transcription factor by activating an *Arabidopsis* R homologue (Lloyd *et al*., 1992; Galway *et al*., 1994). In a yeast two-hybrid assay it was shown that TTG can interact with GLABRA3 (GL3), a basic/helix-loop-helix (bHLH) protein, indicating that TTG probably works by protein-protein interactions to specify N-cell fate (Payne et al., 2000).

WER codes for a putative MYB-related transcription factor that has also been shown to interact with a bHLH protein. It is expressed in the N-cells, where it positively regulates *GL2* and CPC expression (Lee and Schiefelbein 1999). The CPC protein promotes H-cell fate. The protein contains a MYB-like domain for DNA binding, but lacks the transcriptional activation domain that MYB transcription factors usually have. It is expressed in differentiating N-cells, where it either functions in concert with TTG, or in a separate pathway in order to down regulate *GL2*, *WER* and its own
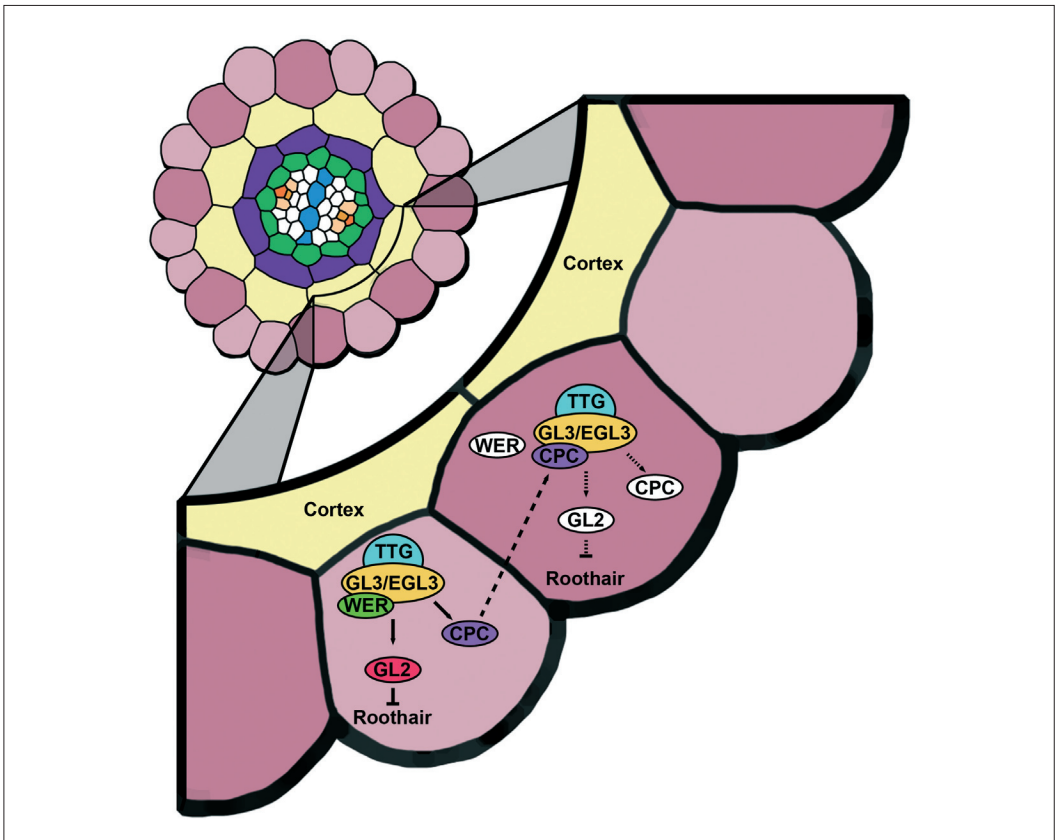
Figure 2. Root hair formation in the *Arabidopsis* root. Schematic; The current model of epidermal cell specification. In the N cell, a high level of WER relative to CPC enables the formation of WER-GL3/EGL3 and promotes GL2 and CPC transcription. CPC moves to the H position, where the relatively high level of CPC forms the inactive CPC-GL3/EGL3 complex. Proteins shown in white ovals are at low concentrations. Cell colors refer to cell lineages as in figure 1.

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expression (Wada *et al*., 1997, 2002; Schellmann *et al*., 2002; Lee and Schiefelbein, 2002). It also has the capability of moving into the adjacent H-cells where it can repress gene expression, suggesting that root hair formation is the result of transcriptional feedback loops (Wada *et al.*, 2002; Lee and Schiefelbein, 2002, Costa and Dolan, 2003). The bHLH proteins *GL3* and *ENHANCER OF GLABRA3* (*EGL3*) promote the N-cell fate by activation of *GL2* and are necessary for proper expression of CPC. This makes them part of both the N- and H-cell fate pathways (Bernhardt *et al*., 2003). Over the years, a model that puts all these components into a relatively simple competition model has emerged, explaining the epidermal patterning process (Fig. 2; Bernhardt *et al*., 2003).

The *GL3* and *EGL3* genes are expressed in both H and N position. The proteins interact with TTG, which is at least partially required for their activity. At the N position the interaction of the GL3-EGL3-TTG-complex with WER drives GL2 and CPC. The GL2 protein then inhibits root hair formation, while CPC moves into the adjacent epidermal cell where it out-competes WER in binding to the GL3-EGL3-TTG-complex (probably in combination with TRYPTICHON (TRY)), resulting in an inactive complex unable to activate GL2. Thus, a default pathway of root hair formation is followed (Bernhardt *et al*., 2003). How exactly the differential concentrations of WER and CPC accumulate at N and H position respectively is not known, but they possibly result from positional cues and genetic regulation. Whether GL3 and EGL3 have a similar function during embryogenesis, when epidermal patterning is first occurring, is yet to be established (Lin and Schiefelbein, 2001; Costa and Dolan, 2003).

1.3.4 Ground tissue patterning

The ground tissue consists of the two concentrically organized layers of cortex (outside) and endodermis (inside), which are already first established during embryogenesis. Both layers are descendents of a single layer of initial cells that are in contact with the QC. These initials divide anticlinally: the cell in contact with the QC maintains stem cell identity while the other continues with an asymmetrical periclinal cell division. These daughter cells will form the cortical and endodermal cell layers. The divisions are under genetic regulation by two members of the GRAS family of putative transcription factors, SHORTROOT (SHR) and SCR, which are both essential for the cell division of the daughter-initial cells (Benfey *et al*., 1993; Scheres *et al*., 1995; Di Laurenzio *et al*., 1996; Pysh *et al*., 1999; Helariutta *et al*., 2000). A mutation in the *SCR* gene suggests that its function is to drive the cell division, because it results in a single layer of ground tissue that has both endodermal and cortical characteristics (Scheres *et al*., 1995; Di Laurenzio *et al*., 1996). The *shr* mutation on the other hand, lacks endodermal markers and has only one layer of ground tissue, linking it to both cell division and cell differentiation (Benfey *et al*., 1993).

The expression patterns of both genes and protein localization studies led to a model of ground tissue patterning. The *SHR* gene is expressed in the vascular tissue (Helariutta *et al*., 2000). SHR protein then relocates from the stele into the adjacent ground tissue layer and the QC (Nakajima *et al*., 2001; Heidstra *et al*., 2004). In the QC and ground tissue initial cells SHR activates *SCR*, which then maintains its own expression. At this stage *SCR* expression is no longer inducible by SHR. Factors

originating from the QC restrain the ground tissue initials from dividing in the periclinal plane, allowing only anticlinal cell divisions to take place. The apical daughter cells from these anticlinal cell divisions are induced to divide periclinally by SCR and other factors, which also regulates the separation of the endodermal and cortical cell fate. Any left over SHR and SCR protein in the cortical cell file is degraded (Heidstra *et al.*, 2004). Furthermore, recent results showed that SHR movement is not passive, but requires specific factors in order to move from the stele into the adjacent cell layer (Sena *et al.*, 2004). Movement of SHR to the cortex layer, or to the extra layers of endodermis in the *SCRpro::SHR* line (Nakajima *et al.*, 2001), did not take place and is possibly prevented by SCR (Heidstra *et al.*, 2004). This regulation of SHR movement seems highly important, as misexpression of *SHR-GFP* (*GREEN FLUORESCENT PROTEIN*) resulted in endodermal cell fate in all cell layers outside of the vascular bundle where SHR-GFP was expressed (Sena *et al.*, 2004).

1.3.5 Vascular patterning

The vascular tissue of a plant functions as transport and support tissue and is one of the more complex structures in the plant. It is a continuous network that is expanded during plant life both in length as in width. In shoot and root, new vascular tissue is formed at the meristematic zones, and meanwhile new vascular cells are developing as the result of cambial cell division in the older tissues. The vascular tissue has a highly predictable structure, yet it is able to quickly adapt to local or environmental stimuli.

Within the vascular network three different tissue types can be identified: 1) xylem, 2) phloem, and 3) (pro)cambium. The xylem and phloem poles each consist of several cell types. Xylem tissues contain xylem parenchyma cells, xylem fibers, and TEs that form the actual transport vessels for water and nutrients transportation. During the differentiation of TEs, the secondary cell wall is formed, followed by programmed cell death. In angiosperms, the phloem poles consist of SEs, CCs, phloem fibers and phloem parenchyma cells. The SEs are the cells that form the actual conductive network: they undergo a partial autolysis, during which the nucleus and most of the ER and cytoplasm is lost, and callose is deposited in some areas. Between SE cells, special perforated sieve plates are formed that allow the mass flow of photosynthates. The SEs are maintained by the CCs, which supply them with macromolecules (Esau, 1977; Oparka *et al.*, 1999; Kuhn *et al.*, 1997).

The cambial zone lies between the xylem and phloem poles. Cambial cells can be seen as stem cells during radial growth of the vascular tissue, which occurs in many dicots. Cambium is formed from parenchymatous cells, two types can be identified: 1) primary cambium (or fascicular cambium), which lies between the primary xylem and phloem, and 2) secondary cambium (or interfascicular cambium) that is located between the vascular bundles only in stems. After a cambial cell division, the daughter cells differentiate into either phloem or xylem tissue, depending on which side of the cambial zone they are located. If they are on the phloem side, they will differentiate as phloem, and *vise versa* when they are located on the xylem side.

There are several distinct patterns in which xylem and phloem can be organized in the different plant species: both xylem

surrounding phloem (amphivasal) and phloem surrounding xylem (amphicribral) has been seen in stems. In leaves, collateral (external phloem) or bicollateral (internal and external phloem) has been observed (for a review: Ye, 2002). In roots on the other hand, the vascular morphology has a central symmetry.

Although these differences between root and shoot give the impression of separate vascular structures within the organs, the vascular bundle is one axial unit. During the early heart stage the first pro-vascular tissue is formed within the area that will be part of the upper hypocotyl (epicotyl). The vascular tissue within this epicotyl will elongate into the direction of both shoot and root, effectively joining them together and allowing for a continuous vascular bundle. When the first vascular bundles have been formed, procambial cell divisions throughout the root and hypocotyl establish the primary xylem and phloem poles. After germination, during secondary develop- ment, another phase of cell proliferation takes place when the cambium actively divides. It is likely that both the early procambial cell divisions and the later cambial cell divisions share some common developmental regulation.

Meanwhile, new vascular tissue is then also added at the meristems in root and shoot. One fundamental difference between root and shoot formation is the establishment of lateral organs. During shoot development, new leaves and their connecting vascular tissue are developing at the meristem. Lateral root formation is a separate process; they form from activated pericycle cells, and attachment of the lateral vascular tissues to the primary vascular tissue is an independent process.

Vascular initiation and adaptation is thought to involve both auxin transport and auxin signaling (For a review: Aloni, 1987; Ye, 2002; Sachs, 1991). Auxin was first implicated in vascular differentiation in the 1950s (Jacobs, 1952), when it was shown that xylem regeneration in damaged areas of *Coleus* stems was under the control of IAA, which was produced in newly forming leaves. Later studies with tissue cultures of *Syringa*, *Daucus*, and *Glycine*, indicated that auxin concentrations may regulate pattern formation within a vascular bundle, with low auxin concentrations inducing only SE formation and high auxin concentrations resulting in both TE and SE (Aloni, 1980). This lead to the hypothesis that auxin controls both xylem and phloem differentiation in plants, but that phloem differentiation is sensitive to lower auxin concentrations compared to xylem. Thus, xylem formation would always occur in the presence of phloem (For a review: Aloni, 1987). Other research demonstrated that in the lateral meristem of *Pinus sylvestris* a gradient of auxin concentrations exists, which peaks in the cambial zone (Uggla *et al.*, 1996). Furthermore, it was shown that ablation of the QC results in an auxin maximum in the vascular initials. Subsequently, these initials change cell fate and form a new QC and columella initials (Sabatini *et al.*, 1999). Taken together, all these results clearly indicate the importance of auxin in vascular patterning, but do not establish exactly how vascular pattern formation occurs. Although the auxin signaling response is now fairly well understood (see chapter 1.3.1), the genetic effectors of vascular patterning are yet to be identified.

For most root tissues, mutant analyses have resulted in a reasonably good understanding on how their formation takes place. As a result, an anatomical map, describing the cellular origin of these

tissues from their initial cells, has been available for some time (Dolan *et al*., 1993; Scheres *et al*., 1994). However, the formation of the root vascular pattern, from the approximately ten to twelve initial cells to the eventual differentiated tissue consisting of about 25 cells, has not yet been described. From several mutant screens a few mutants were isolated that showed radial pattern defects, of these, the *glm* (*gollum*), *wol* and *fass* (*fs*) mutants also showed changes in the radial pattern of the vascular tissue (Scheres *et al*., 1995). However, in *glm* and *fs*, the vascular tissue would eventually establish the pattern with "normal" xylem and phloem poles. In *wol* the vasculature did not recover in the same way. Both root and hypocotyl contained fewer cells in the vascular bundle and all these cells differentiated into xylem cells, hence the name "wooden leg". Higher up in the hypocotyl, the vascular system develops more cells and phloem is present. Eventually, adventitious roots, which sustain the seedling, will form from the hypocotyl. In all three mutants the vascular alterations were already present during embryogenesis in both root and hypocotyl, indicating that the vascular tissue patterning of both organs is regulated by the same genes during embryogenesis and post-germination (Scheres *et al*., 1995). As the *wol* mutant was the only mutation that resulted in a significant alteration of the vascular pattern of a mature root/hypocotyl, it was tested whether this change was the result of a modification in cell division or cell specification. The *fs* mutation could be used as a tool, because it caused an increase in cell division in all tissues. Therefore, *fs* and *wol* were crossed to examine whether the vascular tissue in the double mutant would be like *wol* and consist solely of xylem, in which case the *wol* mutation

influences cell specification, or like *fs*, which would indicate that *wol* only affects cell division. The latter phenotype was observed, indicating that in *wol*, the cell specification change is an indirect result of changes in local cell divisions. The number of xylem and phloem poles has been shown to increase with larger vascular size (Torrey, 1954; Feldman and Torrey, 1976; Torres-Ruiz and Jurgens, 1994). Based on this given, Scheres *et al*. (1995) hypothesized that if xylem is specified before phloem, then in *wol* the xylem takes over all available space and no room is left for phloem formation. It was assumed that the increased cell divisions, caused by the *fs* mutation, take place before cell specification within a tissue is established. The increased number of cells in the double mutant is then available for specification of both phloem and xylem in the double mutant. As *WOL* was the first putatively informative gene in vascular patterning, it was deemed worth the effort of cloning the gene, this work is described in section I.

Very recently another molecule was identified, which seems to be involved in the formation of vascular xylem strands (Motose *et al*., 2004). This proteoglycan-like factor, called xylogen for its capability of inducing xylem formation in plant tissue cultures, accumulates in the meristem, procambium and xylem. In differentiating TE cells, xylogen is localized in a polar manner at the apical side of the cell. As double knockouts for the two xylogen genes in *Arabidopsis* showed discontinuous xylem strands in both shoot and root, it is thought that the polar secretion of xylogen functions as a signal to induce the connecting cells to differentiate into xylem strands to form a continuous network. However, in the double knockout, TEs still differentiate in the vascular tissue, and the hypocotyl and

inflorescence stem did not show any clear defects. This indicates that xylogen is more likely to be a coordinator rather than a determining factor in maintaining the integrity of the vascular tissue and the patterning of minor veins. As xylogen has sterol binding capabilities, it is hypothesized that it possibly interacts in a complex with a sterol or sterol derived molecule (Motose *et al*., 2004). One candidate is COTYLEDON VASCULAR PATTERN (CVP), an enzyme in the sterol biosynthesis pathway, as mutations in the encoding gene result in a similar phenotype as the double knockout for both xylogen genes (Carland *et al*., 2002)

2. Aims of the Study

The general aim of this research was to examine how the vascular tissue of *Arabidopsis thaliana* develops from a set of initial cells to the final cylinder with phloem and xylem poles. Furthermore, this study focused on the identification of genes that are involved in the establishment of the vascular pattern. Therefore, two mutants, named *wol* and *apl* (*altered phloem development*), were selected based on their interesting defects in the patterning processes of the vascular tissue. They were extensively characterized and subsequently their encoding genes were cloned.

The specific aims were:

(1)   To study the cell division pattern of the procambial tissue. To characterize the *wol* mutant in which these divisions fail to take place and to identify the disrupted gene.

(2)   To characterize the *apl* mutant in which phloem pole formation is disturbed, and subsequently molecularly clone the corresponding gene.

(3)   To determine in greater detail the role of APL in the establishment and differentiation of phloem pole formation.

3. Materials and Methods

3.1 Plant material

The following *Arabidopsis* accessions were used; in **I** and **II**: Colombia (Col), Landsberg *erecta* (Ler), and C24. In **III**: Col. The various enhancer trap lines are described at http://www.plantsci.cam.ac.uk/ H a s e l o f f / C A T A L O G U E S / J L i n e s / index.html and were obtained through the Nottingham *Arabidopsis* Stock Centre (NASC, Nottingham, UK). The *wol* mutant was isolated as described (Scheres *et al.*, 1995). The *apl* mutant was isolated from a collection of EN-I mutagenized plants (Wisman *et al.*, 1998).

3.2 Genetic material

In **I**: The GenBank accession numbers for the genetic material are: AC007069 (T23K3 BAC (Bacterial artificial chromosome)); AA586219, AI992824, T20648 (ESTs (expressed sequence tags) representing the *WOL* gene); AJ278528, AJ278529, AJ278530 (three different splice variants representing *WOL*); U42597 (DhkA (*Dictyostelium discoideum* Histidine kinase A)); AC004557 (BAC containing F17L21.11); AB011485 (BAC containing MXH1.16); and AJ234550 (*WOL*-like genomic fragment in *H. vulgare*).

In **II**: AC007202 (BAC T8K14 containing At1g79430); 88N24 and 49O23 (TACs {Transformation-competent artificial chromosome} that complement the *apl* mutant phenotype, GeTCID, UK; Liu *et al.*, 1999); AY143841 (EST representing the *APL* gene).

3.3 Growth conditions

Seeds were sterilized in 5% sodium hypochloride, imbibed at 4° Celsius in the dark in sterile water for 2–5 days, and germinated on plates containing 0.5× Murashige and Skoog (MS) salt mixture, 4.5% (**I**) or 1% (**II** and **III**) sucrose, and 0.5 g/l 2-(N-morpholino) ethanesulfonic acid (MES) pH 5.8, in 0.8% agar. Plates were incubated in a near vertical position at 23° Celsius and a 12 hours light/dark cycle. Mature plants were grown at 23° Celsius in potting medium consisting of a 1:1 mixture of vermiculite and medium peat (type B2, Kekkilä, Espoo, Finland). The plants were sub-irrigated twice a week with tap water.

3.4 Fixation, imbedding and sectioning

Fixation of seedlings and embryos was performed overnight at 4° Celsius, in a solution containing 1% glutaraldehyde, 1.5% formaldehyde, and 50 mM sodium phosphate, pH 7.2. Triton X-100 was added in the fixative to a final concentration of 0.01% for embryo and stem fixation. Subsequently the plant material was dehydrated in a gradient of ethanol solutions, each step 20 minutes.

Leica Historesin was used for imbedding the plant material in plastic. First the material was incubated for four hours in a 1:1 mixture of 100% Ethanol : imbedding solution (10 ml basic resin, 0.1 g activator powder, 200 µl polyethylene glycol 400). Then a second incubation was done for two hours with only imbedding solution. Finally,

the material was imbedded in a mixture of 15:1 of imbedding solution : hardener liquid as described at http://www.bio.uu.nl/~mcbroots/orient.htm.

Sections (2-3 μm) were made on a Leica RM2165 rotary microtome, using a Leica microtome knife. Sections were stained with 42 ,6-diamidino-2-phenylindole (DAPI, **II**) or in a 0.05% toluidine blue solution in water and photographed on an Olympus Provis microscope using a Sensicam 12-bit cooled camera (PCO, ccd imaging). Images were processed with Adobe Photoshop 4.0.1 (**I**) and 6.0.1 (**II** and **III**).

3.5 Ultra-structural analysis (II)

Seedlings were first fixed for four hours at room temperature in 2% glutaraldehyde, 2% paraformaldehyde, 0.1% (w/v) tannic acid and 1% sucrose in 70 mM sodium phosphate buffer (pH 5.8). Then the material was washed in 70 mM phosphate buffer (pH 5.8) and stored overnight at 4° Celsius. Next, the samples were post-fixed for one hour in 1% OsO_4 in sodium phosphate buffer (pH 5.8) and washed again, first in 70 mM phosphate buffer (pH 5.8), and then in Tris-HCl (pH 5.8). Following this the material was stained for 30 minutes with 2% (w/v) uranyl acetate and dehydrated in an ethanol series at 4° Celsius. Finally the samples were imbedded in Spurr resin and ultra thin sections were made. The sections were analyzed and photographed on Jeol 1200 EX Transmission electron microscope (Jeol Ltd., Tokyo, Japan)

3.6 GUS staining

Gus staining (**II** and **III**) was performed overnight at 37º Celsius in staining solution

(10 ml) containing 29 mg NaCl, 6.6 mg $K_3Fe(CN)_6$, 0.01 M Tris-HCl (pH 7.2), 0.75 mg ml^{-1} X-gluc (Ducheva), and 20% methanol. Following this, the plant material was cleared, first by incubation with acidified methanol (10 ml of methanol, 2 ml of concentrated HCl (37%), 38 ml of H_2O) and incubated at 55°-57° Celsius for 15 min. Then the acidified methanol was replaced with basic solution (7% NaOH in 60% ethanol) and the samples incubated for 15 min at room temperature. The seedlings were then re-hydrated in a reverse ethanol gradient (40%, 20%, and finally in 10% ethanol) and mounted in 50% glycerol.

3.7 Fuchsin staining

Seedlings were first cleared using the same protocol as used after GUS staining. Then the material was stained for 5 min in 0.01% basic fuchsin solution in water, and then destained for 10 minutes in 70% ethanol. The samples were then re-hydrated in a reverse ethanol gradient (40%, 20%, and 10% ethanol) and mounted in 50% glycerol. Confocal microscopy images were taken on an Axiovert 135M confocal microscope with an argon ion laser (568 + 488-nm emission). Image processing consisted of Kalman filtering (n=3) during image acquisition (Bio-Rad software). Optical sections were projected together to form composite images of the vascular bundle.

3.8 Callose staining

Callose staining was performed as in Carland *et al*. (1999). Whole seedlings were fixed for 1 hr in acetic acid:95% ethanol (1:3), incubated in 2 M NaOH for 1 hour, neutralized briefly in 50 mM $NaPO_4$ (pH 6.8), and stained overnight in 0.005% aniline blue in 50 mM $NaPO_4$ (pH 6.8).

Fluorescence under UV light was visualized with a Olympus Provis microscope using a Sensicam 12-bit cooled camera (PCO, ccd imaging) equipped with a broad-band filter for detection of 49,6-diamidino-2-phenylindole.

3.9 Molecular techniques

The *WOL* and *APL* loci (**I** and **II**) were cloned using chromosome walking techniques. CAPS (Konieczny and Ausubel., 1993), markers were created by detecting polymorphisms between Ler and Col ecotypes. Binary vectors used for the different constructs were: pRD400 (**I**; pBIN19 derivative, Datla *et al*., 1992); pBI101 (**II**; Datla *et al*., 1992); and pHM3 (**III**; Mizuguchi and Kay, 1998). The constructs were transformed into *Agrobacterium tumefaciens* strains C58C1 (**I**; pGV2260; essentially as in Bevan, 1984) and C58C1 GV3101 (**II** and **III**) and then transformed into plants using the floral dip method (Clough and Bent, 1998). Transgenic seedlings (T1) were selected by resistance to kanamycin (50 µg ml^{-1}) on Petri plates. Transgene presence was confirmed by PCR, stereo microscopy and GUS staining. Segregation of the transgene in T2 generation was confirmed.

cDNA cloning (**I**) was performed with gene-specific primers by using the RobusT RT-PCR Kit (Finnzymes Oy) according to the manufacturer's instructions. To determine the 5'-UTR of the *WOL* mRNA, the 5'RACE system for rapid amplifications of cDNA ends version 2.0 (Life Technologies) was used according to the manufacturer's instructions (**I**).

The *APL* specific RNAi construct was generated from a 341 bp PCR fragment of the coding region of the APL gene as described in **III**.

3.10 Northern and Southern analysis

RNA was isolated according to Carpenter and Simon (1998). Northern blot analysis was carried out with 15 µg of total RNA. The membranes were hybridized with ^{32}P-labeled gene-specific probes: (in **I**: a 256-bp PCR fragment representing nucleotides 10143-10398 of the BAC clone T23K3; in **II**: a 768 bp NcoI – NotI fragment of AY143841). Genomic DNA was isolated from 17-d-old seedlings according to Doyle and Doyle (1990). Southern blot analysis (**I**) was performed as in Maniatis *et al*. (1982) with 15 µg of DNA and ^{32}P-labeled probes (256-bp PCR fragment (described above) of *WOL* and a 1067 bp cDNA fragment corresponding to nucleotides 10055-11204 of the BAC clone T23K3).

3.11 In situ hybridization

Seedlings were germinated and grown for five days. The *in situ* hybridization analyses were performed according to Di Laurenzio *et al*. (1996), with some minor modifications: tissue samples were cleared in xylene and embedded in Histoplast (Shandon); proteinase K was used at 10 µg ml^{-1}. The sense and anti-sense hydrolysed probes (in **I**: a 256-bp PCR fragment or a 1067-bp cDNA fragment as in Northern blot analysis; in **II**: a 757 bp, 5'-CTTCTGCCATGGATATTCAGCG-3', 5'-CACCCAAATGGCGAGTTTCTTCC-3') were labeled using the DIG RNA labeling kit (Boehringer) according to the manufacturer's protocol. The probes were used in the hybridization at a concentration of 50 ng ml^{-1} kb^{-1}. Processed sections (7 µm) were mounted in 50% glycerol or Immu-Mount (Shandon). Images were taken using differential interference contrast (DIC) settings.

4. Results

4.1 Vascular asymmetric cell divisions require WOL

Wild type and *wol* primary root meristematic regions were carefully analyzed to identify the cell divisions that require WOL. The roots were embedded in plastic and then serial sectioned. The sections were then stained with toluidine blue and examined under a microscope. By photographing every root section, the vascular cell lineages could be established and a 3D image of the root tip reconstructed (I, figure 1a-j, schematic)

In the wild type vascular bundle, a set of four to five initial cells in the middle form the axis that later will differentiate as xylem cells. These cells seem to divide almost exclusively in the anticlinal plane. The cells on the left and right of this axis will undergo some periclinal divisions to eventually form the two phloem poles and the undifferentiated cells that will later be part of the (pro)cambium (I, figure 1a-j). It is these cell divisions that are missing in the *wol* mutant (I, figure 1k). Although the final phloem specific cell divisions, which result in the cell files that will form the SEs and CCs, are quite regular and easily identified in wild type, the asymmetric cell divisions that lead up to this stage are somewhat variable (I, figure 1a-j).

As mentioned in the introduction, in *wol*, all vascular cells differentiate into xylem cells. Previous research has shown that this xylem differentiation is probably an indirect effect and that *WOL* is not required for phloem development *per se*. A cross between *wol* and *fs*, a mutation that increases cell division in all tissues, resulted in double mutants that had a *fs* phenotype, and thus contained phloem poles in the root

(Scheres *et al*., 1995). This result led to a model in which the specification of xylem temporally precedes that of phloem. The few cells in the *wol* vascular cylinder are specified as xylem, and no space is left for phloem specification. In wild type, the two outermost cells of the xylem axis differentiate as protoxylem; the other cells of the axis will become metaxylem. Cano-Delgado *et al*. (2000) showed that all cells in the *wol* primary root differentiate as protoxylem. We showed that in the *wol fs* double mutant both proto- and metaxylem was observed (I, figure 2c), indicating that WOL is not required for the formation of metaxylem, but indirectly influences the status of phloem and metaxylem by controlling vascular cell division.

The phenotype of the *wol* mutant indicated that the mutated gene could be an important regulator of vascular cell division and worthy of cloning. We identified *WOL* (I, figure 3a, b) as a previously annotated two-component histidine kinase receptor (Lin *et al*., 1999). The WOL protein structure is very similar to the two-component receptors that can be found in bacteria. It contains all the characteristic domains: a receptor area, a histidine kinase phosphorelay domain, and two receiver domains (I, figure 3d). In *wol*, a mutation was located in the predicted receiver domain (I, figure 3e), which suggested that either ligand binding or the transmission of information after ligand binding was probably restricted in *wol*. The mutation was complemented with a genomic fragment confirming the identity of the two-component receptor (I, figure 3c). The *WOL* gene was also identified by other groups, which named it *CRE1/AHK4* (Inoue *et al.*, 2001; Suzuki *et al.*, 2001). They established

that WOL functions as a cytokinin receptor. These results will be covered in the discussion.

4.2 Developmental expression of WOL

The reduced cell number in the *wol* vascular cylinder is visible already during embryogenesis in both root and hypocotyl. After germination cell divisions within the hypocotyl increase the vascular cell number and adventitious roots that rescue the seedling are formed. These cell divisions do not occur in the primary root of *wol*. Consistent with this phenotype, an *in situ* hybridization experiment showed that *WOL* expression is initiated during embryogenesis (I, figure 4d-g): first at the globular stage in the four vascular precursor cells, then throughout the subsequent stages within the provascular tissue of root, hypocotyl and cotyledon shoulders. After germination *WOL* is expressed in the vascular tissue, including pericycle, of the primary root (I, figure 4a, b). This expression is strongest in the meristematic region. As *WOL* expression could be detected in the *wol* vasculature (I, figure 4c), it was concluded that WOL is not required for its own expression.

4.3 Phloem pole formation requires APL

From a pool of seeds that was transformed with the EN-1 transposon from *Zea mays* (Wisman *et al.*, 1998), a second mutant, named *apl*, was selected (II). The phenotype of *apl* seedlings was very similar to *wol*, with short determinate primary roots that branch rarely, and eventually arrested shoot development (II, figure 1b). However, *apl* seedlings were unable to form the adventitious roots that rescue *wol* seedlings, and eventually died. Histological analysis of

apl primary roots by transverse sectioning showed that all tissues outside the vascular bundle existed as in wild type, but that the vascular tissue was somewhat abnormal in *apl*. Although a normal xylem axis could be identified in *apl* sections, SEs and CCs appeared absent in the areas that normally make up the phloem poles (II, figure 1c). This was confirmed by aniline blue staining of whole mounted seedlings, which stains the callose depositions in the phloem of wild type seedlings. In *apl* seedlings the callose depositions was lacking (II, figure 1d). Instead, after some 7 days the cells in the phloem pole areas often take on features of protoxylem TEs, with annular lignified cell wall structures (II, figure 1e). This phloem pole defect is evident in the vascular tissue throughout the seedling (II, supplementary information S1a, b).

4.4 Detailed phenotype analysis

In wild type, the SEs and CCs are formed after a set of highly reproducible cell divisions, whereas in *apl* the phloem poles are abnormal. Therefore a detailed analysis of the meristematic region was performed in order to investigate the status of these cell divisions. In *apl*, 23 per cent of the tangential SE divisions and 50 per cent of the periclinal CC divisions were delayed or absent (II, figure 2a). Also, the characteristic phloem differentiation, which can be visualized in plastic sections with toluidine blue staining, was lacking in *apl* mutants (II, supplementary information S2a). Furthermore, the protophloem specific green fluorescent protein (GFP) marker line from the Haseloff collection (J0701, http://www.plantsci.cam.ac.uk/Haseloff/CATALOGUES/Jlines/index.html) and the CC-specific *AtSUC2pro::GFP* (Imlau *et al.*, 1999) are

not expressed in the *apl* mutant (II, figure 2b, c). In contrast, marker lines specific for xylem (J1721) and xylem associated cells in the pericycle (J0121) were normally expressed, indicating that only phloem pole organization was disrupted (II, supplementary information S2c, d). The *apl* mutant was then crossed into the *fs* background in order to establish whether increased cell division could reverse the *apl* phenotype. The *apl fs* double mutant phenotype came out additive. The seedlings had very short roots and arrested shoot development similar to *apl*. Overall cell number, and thus also vascular cell number, was increased, but cells in the phloem pole areas still took on the xylem identity (II, figure 2d). Taken together, these results indicate that APL is required for both the phloem specific cell divisions and the subsequent differentiation of SE and CC. Disruption of APL function leads to a change of cell fate in the phloem poles, which take on xylem identity.

4.5 Identification of APL as a putative MYB-like transcription factor

The characterization of the *apl* mutant indicated that the *APL* gene encodes a key regulator of phloem development. This validated the effort of cloning the gene. A combined effort of positional cloning and PCR-based screening for the EN-1 transposon resulted in the identification of At1g79430 as the *APL* gene (II). The coding sequence had been annotated as a putative MYB-like transcription factor and was disrupted by an EN-1 transposon in the fifth exon (II, figure 3a). The 358 amino acids long predicted APL protein contains both a conserved MYB DNA-binding domain and a coiled-coil protein-protein dimeri-zation site (Rubio *et al*., 2001). Subsequent

complementation of the *apl* mutant with a genomic DNA fragment confirmed that the correct gene was identified (II, figure 1c). In *apl*, the EN-1 transposon is inserted at the end of the coiled-coil domain, and thus will either result in a truncated form of APL (159 amino acids), with reduced or altered function, or in a fully inactive *APL*. Loss of function is the most likely, because no *APL* RNA was found by Northern blot analysis with an *APL* specific probe (see materials and methods) in *apl* background (II, figure 3b).

4.6 The apl mutation resembles a severe loss-of-function

Only a single allele of the *apl* mutation was available, and although the northern hybridization indicated that *apl* was most likely a loss of function mutation, it was not possible to state this with absolute certainty. Therefore new alleles of *apl* were generated by introducing an *APL* specific RNAi construct into *Colombia* ecotype. The T1 generation seedlings showed a range of phenotypic alterations, varying from wild type to *apl*-like. Histological section analysis showed that the strongest phenotypic alterations phenocopied *apl*, indicating that *apl* is a loss of function (III, figure 1a-c). Of the T1 generation some plants survived on soil to the flowering stage, also in these plants an array of phenotypic changes was observed. The phenotypes of these flowering plants were somewhat variable, but in general they were small, had short inflorescence stems, infertile flowers and accumulated anthocyanin (III, figure 1d). The inflorescence stems of the flowering plants were sectioned and analyzed, but no significant differences from wild type were observed (III, figure 1e-h). The defects in

the phloem poles are probably too subtle to see with plastic sections. Phloem transport assays might possibly establish what is wrong with these plants.

4.7 APL expression in post embryonic roots

The histological analysis of the mutant and the Northern blot analysis indicated that APL's area of influence is limited to the vascular bundle in both shoot and root. This was confirmed by an *in situ* hybridization of root sections, which showed that in newly formed tissue *APL* is first expressed in the protophloem cell file and then, as the tissue matures, the CC and metaphloem SE also initiate *APL* expression (II, figure 3c). Consistent with the *in situ* hybridization, in a transgenic *APLpro::GUS* line, GUS staining was first detected in the protophloem cell file of the root meristem, and then, a little higher, in a slightly broader domain throughout the root (II, figure 3d). Being a putative transcription factor, it was likely that APL would be located in the nucleus. To validate this hypothesis, a fusion construct was generated between GFP and APL, driven by the APL promoter. Confocal microscopy showed nuclear localization of GFP-APL, it also confirmed the expected expression pattern as seen in the *in situ* hybridization experiment and *APLpro::GUS* line (II, figure 3f, g). Furthermore, the expression pattern reflected the developmental stages of phloem pole differentiation: loss of GFP signal could be observed when the nucleus of the protophloem sieve element disintegrated (II, figure 3g).

4.8 APL expression during embryogenesis

Although phloem only differentiates after germination, sectioning of *Arabidopsis* embryos showed that the phloem specific cell divisions are already observed between the early "bent-cotyledon" and "mature" stages of embryogenesis (II, figure 3h, i). This makes phloem patterning a relatively late process in the development of the embryo, because the outer cell layers are already established at the torpedo stage of embryogenesis. The phloem specific cell divisions coincided with the onset of *APLpro::GUS* expression in the phloem poles of late "bent cotyledon" stage embryos (II, figure 3e; III, figure 2a-k). However, unlike the phloem pattern in post-embryonic roots, in the embryonic hypocotyl these cell divisions did not seem to follow as exact a pattern (Data not shown). Therefore, *apl* embryos could not be identified unambiguously and thus their proper analysis was forestalled. Although vascular *GUS* expression is only present during the bent cotyledon stage, during the earlier stages GUS activity was observed in the cotyledons as early as the triangular stage (III, figure 2a-k). This expression is first present throughout the entire cotyledons during the heart stage, and then in the successive stages it becomes more and more limited to the cotyledon tips, until finally in the bent cotyledon stage it appears in the vascular tissue (III, figure 2a-k). The vascular expression pattern of *APL*, both during late embryogenesis, and after germination, indicates that APL functions as a phloem regulator throughout the life of the plant.

4.9 APL represses xylem formation

In *apl* mutants, the phloem pole areas developed lignified cells that appear like protoxylem TEs. This feature suggested that APL may have a secondary function besides promoting phloem development, namely

inhibiting xylem development. This possibility was studied by overexpressing *APL* throughout the vascular tissue near the root meristem under the *WOL* promoter. The transgenic *WOLpro::APL* seedlings grew normally and had, at first glance, a wild type cellular layout. However, closer analysis of xylem formation in *WOLpro::APL* roots showed that the cells at the protoxylem position stayed undifferentiated throughout the root (II, figure 4a, b). Furthermore, the central cells of the xylem axis only differentiated much higher up in the root, where the *WOL* promoter is less active (II, supplementary information S4e). Why the protoxylem cell files fail to differentiate at this higher level has yet to be established.

To investigate whether APL can repress the early specification of xylem, the same *WOLpro::APL* construct was transformed into the *wol* background. This resulted in a series of phloem – xylem patterns, ranging from *wol* phenotype to multiple phloem poles (Data not shown). It was concluded that *WOLpro::APL* is sufficient to repress TE differentiation. Furthermore, the undifferentiated cells at the protoxylem location maintained a nucleus (II, figure 4c-f; supplementary information S4f-i), suggesting that APL by itself is not sufficient to initiate phloem development at an ectopic location.

4.10 APL expression versus localization

The *apl* mutants generally show a disturbance in the phloem specific cell divisions. As these divisions take place some distance from where *APL* is expressed, it was thought that APL protein might move down from the cells where it is expressed to induce cell division in the area where the phloem pole lineages are established. To assess this hypothesis, *APLpro::GFP-APL* and *APLpro::GFP-ER* were crossed. In *APLpro::GFP-APL*, the GFP-APL fusion protein is localized in the nucleus, while in *APLpro::GFP-ER*, the GFP has an Endoplasmatic reticulum (ER) retention signal and a more global expression throughout the cell is observed. Using confocal microscopy it was determined that the marker lines have a matching expression pattern (III, figure 3a-c), indicating that APL protein does not move and thus the cell division defect can be addressed to (a) secondary factor(s).

4.11 Phloem specific APL expression domain is established independently from APL function

All results indicated that APL's function is to organize proper phloem differentiation and then maintain this differentiation state of the cells. In *apl* mutants this function is disrupted and phloem poles are disorganized. To investigate whether the cells at the phloem pole position in *apl* maintain the early phloem characteristics, the *APLpro::GFP-ER* marker line was crossed into the *apl* background. Confocal microscopy showed that the *APL* promoter is still active at the phloem pole location in *apl* (III, figure 3d). This indicates that the patterning process of phloem is not disrupted in the mutant; also, APL is not required for its own expression.

In *WOLpro::APL*, the cells in the protoxylem position remain undifferentiated and it remained unclear what their exact differentiation status was. To determine how much influence APL has on phloem patterning, the *APLpro::GFP-ER* line was crossed into the *WOLpro::APL* background. If APL can initiate phloem formation, then these cells should express

GFP. However, the undifferentiated cells did not express GFP (Data not shown), indicating that expressing *APL* at an ectopic location does not result in the specification of these cells into phloem cells. Furthermore, this also showed that APL does not drive its own expression, but that other factors initiate *APL* expression.

5. Discussion

All the three studies forming this thesis are discussed in their scientific context in detail in the corresponding published articles and manuscript. Therefore only a synopsis, relevant later published work and selected conclusions are presented in this chapter.

5.1 Phloem fate map

The anatomical fate map of the root meristem initials, which describes the cellular origin of most tissues, has been available for some time (Dolan *et al*., 1993; Scheres *et al*., 1994). In this model of root patterning it is thought that the QC maintains the surrounding initial cells in an undifferentiated state that allows them to keep dividing. However, unlike the establishment of the other tissues, phloem pole formation is the result of not one, but a series of asymmetrical cell divisions. The first divisions, of the cells near the QC, seem somewhat random, slightly higher up a series of easily identifiable periclinal and tangential cell divisions take place and establish the SE and CC cell files. This sets the cell divisions in the vascular bundle apart from the almost invariant cell divisions of the initials that establish the endodermis and outer layers of the root (Scheres *et al*., 1994). Therefore, just based on anatomy it is difficult to understand the status of phloem initials.

The variable nature of the first divisions versus the reliable cell divisions that establish the phloem lineages, indicates a second level of control, in which positional information, possibly coming from above, specifies the cells that will become phloem initial cells that establish the SEs and CCs. This is conform the expression pattern of the protophloem marker J0701, which is expressed only some cells above the QC.

5.2 Procambial cell divisions in roots require WOL

The vascular expression of *WOL* throughout embryogenesis and post-germination when procambial asymmetric cell divisions occur, the reduced cell number in the *wol* vascular bundle, and WOL's predicted protein structure as a two-component receptor, allow for the conclusion that WOL functions as a receptor protein in a signaling pathway that regulates the vascular cell proliferation. Furthermore, *WOL* expression remains high up into the differentiation zone, well beyond the area where the asymmetric cell divisions occur. The exact reason for this persistent expression is currently unknown, but possibly reflects a role in the differentiation of the vascular tissue. The presence of *WOL* expression in the pericycle could be related to the separation of the pericycle from the rest of the vascular cells, as *WOL* is detected before this modification takes place during embryogenesis. However, in *wol* the pericycle appears anatomically correct, and pericycle marker lines J0121 and J0272 are present. The slightly altered expression pattern of the J0121 marker line is the only indication that *wol* affects pericycle cell fate. In stead of the normal J0121 pattern, in which only the pericycle cells associated with the protoxylem cell files are expressing GFP, in *wol* all pericycle cells express GFP (data not shown). Whether this is a direct effect of the mutation, or an indirect effect of all pericycle cells now being in contact with protoxylem, remains unclear.

5.3 WOL encodes a cytokinin receptor

The structure of the WOL protein as a two-component receptor resembles that of the other two-component receptors found in the *Arabidopsis* genome. These other receptors have been shown to be involved in ethylene sensing and osmosensing (Chang *et al*, 1993; Urao *et al*., 1999), suggesting that WOL might possibly also function as a receptor for one of the phytohormones that can be found in the plant. One of the best candidates was cytokinin, which is produced in roots and has been shown to be involved in xylem differentiation already back in 1969 (Fosket and Torrey, 1969). Indeed, two groups working on the *WOL* alleles, *CRE1* and *AHK4*, showed that WOL and the two closely related *AHK2* and *AHK3* genes function as cytokinin receptors (Inoue *et al.*, 2001; Suzuki *et al.*, 2001; Higuchi *et al.*, 2004).

Combined later work led to a general model of cytokinin action, in which cytokinin receptors form dimers at the plasma membranes where they receive the cytokinin signal. Upon cytokinin binding a phosphorelay is activated in which a phosphate group is transferred from the receptor, through the receiver domain, onto a histidine phosphotransfer protein (AHP). This AHP then relocates to the nucleus where it can either interact with type-A response regulators (ARRs) or ARR complexes, or transfer the phosphate to its related type-B ARR receiver domain, which releases them from putative repressors in the nucleus. The dephosphorylated AHP then moves back to the cytosol, where it can re-enter the signaling pathway (Hwang *et al.*, 2002).

Since the published work of article I, full knockouts of the three cytokinin receptors (AHK2, 3 and 4) have been isolated (Higuchi *et al.*, 2004, Nishimura *et al.*, 2004). Analysis of these knockout lines showed that if either a functional *AHK2* or *AHK3* gene is present while the other two are missing, a wild type seedling growth phenotype is observed; if only a functional *AHK4* gene was present a reduced growth was observed (Nishimura *et al.*, 2004). However, all three single mutants show reduced callus formation and, furthermore, in all double mutant combinations the response to cytokinin was lost in callus and shoot induction assays, indicating that no single cytokinin receptor is sufficient for organ formation in these assays (Higuchi *et al.*, 2004). In the single mutants, the responses were lowered the most in the *ahk4* mutant; in double mutants the combinations including the *ahk4* mutant were most affected. *AHK4* has a tissue specific localization and inducible transcript regulation, and has been shown to be adaptive to external factors, such as phosphate starvation (Mähönen *et al*., 2000; Ueguchi *et al*., 2001; Che *et al*., 2002; Rashotte *et al*., 2003, Franco-Zorrilla *et al*., 2002). WOL/AHK4 protein reacts to external stimuli and possibly functions as a modulator of cytokinin sensitivity, allowing the plant to adapt to changes in the environment and growth conditions (Nishimura *et al*., 2004).

The three knockout lines were crossed to generate a triple knockout seedling that would lack all cytokinin receptors known to date. The striking result from this experiment was that although the resulting triple knockouts seemed to be completely insensitive to cytokinin signaling, viable, if severely retarded, seedlings were formed, suggesting that cytokinins may not be as essential as was previously believed. However, the reduction in size of both shoot and root meristems in the triple mutant

clearly shows that cytokinins are very important growth regulators (Higuchi *et al.*, 2004, Nishimura *et al.*, 2004). As cytokinins have been shown to be involved in advancing the G1/S and G2/M phases (Zhang *et al.*, 1996; Laureys *et al.*, 1998; Riou-Khamlichi *et al.*, 1999), the reduced meristem size may be the result from a decrease in cell cycle progression (Higuchi *et al.*, 2004).

A second interesting result from these knockout lines was that the root phenotype of the *WOL* knockout line was structurally wild type, while the *wol* root phenotype closely resembles that of the triple knockout, indicating that the *wol* receptor is not a loss-of-function allele, but has a negative activity on vascular proliferation.

The WOL-dependent asymmetric cell divisions in the primary root vascular tissue, located next to the xylem axis, are reminiscent of the cambial cell divisions that take place during the formation of wood and storage root development (Esau, 1977). Furthermore, all three cytokinin receptors show expression patterns within the vascular tissue of the *Arabidopsis* stem that seem to match the location of the primary cambium (Nishimura *et al.*, 2004). Therefore one can hypothesize that their role as growth regulator in the meristematic regions is extending to regulating the rate of cell division in the vascular cambium. Currently this involvement has yet to be proven, but if true, then finding out the targets of the cytokinin signaling pathway will be the next step in understanding vascular cell proliferation and differentiation.

5.4 APL regulates phloem differentiation

Of all the genes that have been shown to be involved in the formation of the vascular tissue, the *APL* gene is the first to be identified for having a specific function in one of the patterning processes of vascular development. Its expression pattern is initiated during the globular stage of embryogenesis and then maintained throughout the life of the plant. In the early stages of embryogenesis the *APL* expression pattern is somewhat surprising and localized to the cotyledons of the embryo. Only during the "bent cotyledon" stage the signal becomes specific for the vascular tissue. Currently, it is unknown what function APL has during these early stages of embryogenesis. In *apl* mutants the cotyledons appear normal, indicating that redundant factors probably take over APL function during these stages.

During the bent cotyledon stage, the asymmetric phloem cell divisions first occur, this is when the *APL* expression pattern becomes localized to the vasculature. However, although these cell divisions are delayed in *apl* post germination, the variable nature of these cell divisions during embryogenesis precluded the unambiguous identification of *apl* embryos from wild type embryos. An *apl* segregating line did not show embryos with significant differences in cell number and/or cell organization, therefore it is likely that APL is not involved in these cell divisions during embryogenesis. Possibly, APL function during embryogenesis is to prepare the cells for germination. Alternatively, as APL was shown to keep xylem cells in an undifferentiated state when overexpressed at an ectopic location, it is possible that it functions in a similar way in phloem poles during embryogenesis. After germination, the APL protein localization does not match with the area of cell division either. This strongly indicates that the observed defect in *apl* mutants is

the result of either secondary factors that drive these divisions do not reach their target area, or due to general metabolic problems because of dysfunctional phloem.

5.5 Phloem development is a multi step process

Overexpression of *APL* in the vascular cylinder under the *WOL* promoter demonstrated APL's ability to suppress xylem differentiation in the xylem axis. However, the *APLpro::GFP-ER* line showed that APL by itself was not enough to induce phloem formation at these ectopic locations, expression analyses with earlier markers may define the specification of these cells. One explanation for APL's xylem repressing abilities could be related to auxin transport. The phloem transports auxin throughout the plant and thus contains relatively high concentrations of auxin. Classical research has shown that high auxin concentrations can induce xylem formation (Aloni, 1980).

Therefore, one of APL's functions during phloem pole development may be to repress xylem differentiation in the phloem poles. This would then also explain why *APL* expression is maintained after the phloem is fully differentiated. Further research, such as with the weaker alleles of the APL RNAi series, is needed in order to find out whether this hypothesis is true or not.

The current results have established the APL function as one that regulates proper phloem differentiation. The earlier factors that regulate the patterning of the phloem poles are yet to be identified, but, as the patterning process is disturbed in *wol* and the triple knockout of the cytokinin receptors, they are likely to be regulated by cytokinin signaling. One set of candidate regulators are the transcription factors of the class III *HD-ZIP* and *KANADI* families, which have been shown to determine how xylem and phloem are organized in shoot tissue of *Arabidopsis* (Emery *et al.*, 2003; for a review: Bowman *et al.*, 2002).
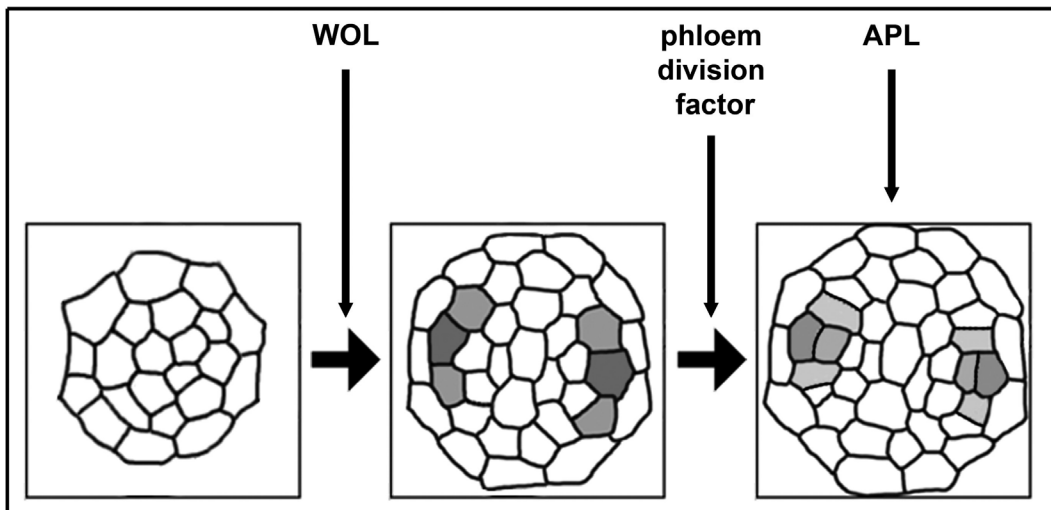

Figure 3. Model of vascular development. The initial vascular cell number is increased by cytokinin induced cell proliferation. Next a phloem specifying factor induces the phloem specific asymmetric cell divisions. In the final phase APL regulates phloem differentiation and maintenance. Cell colors refer to phloem cell lineages as in figure 1.

However, disruptions in these genes only change the layout of vascular tissue; the phloem tissues themselves are not affected. Also was recently shown that these genes do not affect the radial organization of the primary root vasculature (Hawker and Bowman, 2004). Whether these genes regulate APL, or are under regulation by APL, has yet to be established. Another candidate gene is *ZWILLE* (Moussian *et al*., 1998, 2003), which was recently shown to be specifically localized in the phloem poles already during the torpedo stage. This indicates a temporally earlier process upstream of APL, because *APL* expression has not yet become localized to the vasculature at this stage. ZWILLE is thought to regulate cell division and therefore possibly drives the early phloem related divisions. As the phloem specific cell divisions are disturbed in *apl* it will be interesting to analyze *ZWILLE*'s expression pattern and function in the *apl* background and the status of phloem in *zwille* mutants.

Taken together the results in this thesis hints to a temporal model (Fig. 3) in which auxin drives the establishment of the first provascular tissue, cytokinin is then required for the additional asymmetric cell divisions of which the phloem poles are formed. Finally, APL is required for the last phase of phloem development.

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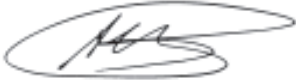
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