Cytokin Regulate Vascular Morphogenesis in the *Arabidopsis thaliana* root

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Academic dissertation

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Cover: Localisation of *ARR15* (left) and *AHP6* (right) transcripts on transverse sections of *Arabidopsis thaliana* root vascular bundle (*in situ* hybridisation carried out by Mikko Herpola)

*Success is the ability to go from one failure
to the next with great enthusiasm.*

Winston Churchill

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following two articles and two manuscripts. In the text, they are referred to by their Roman numerals.

I
Ari Pekka Mähönen, Martin Bonke, Leila Kauppinen, Marjukka Riikonen, Philip N.Benfey, Ykä Helariutta: **A novel two-component hybrid molecule regulates vascular morphogenesis of the Arabidopsis root**
Genes & Development. 2000, **14**: 2938-2943

II
Masayuki Higuchi, Melissa S. Pischke, **Ari Pekka Mähönen**, Kaori Miyawaki, Yukari Hashimoto, Motoaki Seki, Masatomo Kobayashi, Kazuo Shinozaki, Tomohiko Kato, Satoshi Tabata, Ykä Helariutta, Michael R. Sussman, and Tatsuo Kakimoto: *In planta* **functions of the** *Arabidopsis* **cytokinin receptor family**
Proceedings of the National Academy of Sciences of the United States of America. 2004, **101**: 8821-8826

III
Ari Pekka Mähönen, Anthony Bishopp, Masayuki Higuchi, Kaisa Nieminen, Kaori Kinoshita, Kirsi Törmäkangas, Yoshihisa Ikeda, Atsuhiro Oka, Tatsuo Kakimoto, Ykä Helariutta: **Cytokinin signaling and its inhibitor AHP6 regulate cell fate during vascular development**
Manuscript under revision for *Science*

IV
Ari Pekka Mähönen*, Masayuki Higuchi*, Kirsi Törmäkangas, Kaori Miyawaki, Melissa S. Pischke, Michael R. Sussman, Yka Helariutta, Tatsuo Kakimoto: **Cytokinins regulate a bidirectional phosphorelay network**
Manuscript

*equal contribution

ABBREVIATIONS

AHK	ARABIDOPSIS HISTIDINE KINASE
AHP	ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN
ANT	AINTEGUMENTA
APL	ALTERED PHLOEM DEVELOPMENT
APRR	ARABIDOPSIS PSEUDO RESPONSE REGULATOR
ARF	AUXIN RESPONSE FACTOR
ARR	ARABIDOPSIS RESPONSE REGULATOR
Asn, N	Asparagine
Asp, D	Aspartate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AXR	AUXIN-RESISTANT
BA	N^6-benzyladenine
BDL	BODENLOS
BR	brassinosteroid
CAPS	cleaved amplified polymorphic sequences
CDK	CYCLIN-DEPENDENT PROTEIN KINASE
CHASE	Cyclase/Histidine kinase-Associated Sensing Extracellular
CKI1	CYTOKININ-INDEPENDENT1
CLV1	CLAVATA1
CRE1	CYTOKININ RESPONSE1
CRE1(T278I)	CRE1 containing the *wol* mutation, Thr_{278} to Ile_{278}
CVP	COTYLEDON VASCULAR PATTERN
DMAPP	dimethylallyl pyrophosphate
DNA	deoxyribonucleic acid
GFP	GREEN FLUORESCENT PROTEIN
Gln, Q	Glutamine
GUS	beta-glucuronidase
HD-ZIPIII	homeodomain/leucine zipper class III
His, H	Histidine
HMBDP	1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate
HPt	HISTIDINE PHOSPHOTRANSFER PROTEIN
IAA	indole-3-acetic acid
Ile, I	Isoleucine
IP_3	inositol (1,4,5) triphosphate
iPDP	isopentenyladenosine-5'-diphosphate
iPMP	isopentenyladenosine-5'-monophosphate
ipt	isopentenyltransferase
iPTP	isopentenyladenosine-5'-triphosphate
KAN	KANADI
Leu, L	Leucine
LOP1	LOPPED
LUC	LUCIFERASE
MP	MONOPTEROS
mRNA	messenger RNA
PHAN	PHANTASTICA
PHB	PHABULOSA

Phe, F	Phenylalanine
PIN	PIN-FORMED
QC	quiescent center
REV/IFL	REVOLUTA/INTERFASCICULAR FIBERLESS
RNA	ribonucleic acid
RT	reverse transcription
SCF	<u>S</u>KP1, <u>C</u>ullin, <u>F</u>-box
SCR	SCARECROW
Ser, S	Serine
SFC	SCARFACE
T-DNA	transfer DNA
TE	tracheary element
Thr, T	Threonine
TIR	TRANSPORT INHIBITOR RESPONSE
VAN	VASCULAR NETWORK DEFECTIVE
VND	VASCULAR-RELATED NAC-DOMAIN
WOL	WOODEN LEG

SUMMARY

The plant vascular system is composed of a continuous network of strands called vascular bundles. These structures extend through each organ and throughout the entire plant. The vascular bundles contain two conducting tissue types: the xylem and the phloem. The xylem dually functions as a supporter of the plant body and as a conduit of water and minerals from the roots to the sites of photosynthesis in the leaves. From the leaves, photosynthesised carbohydrates are transported *via* the phloem to nourish other organs of the plant. During primary development vascular tissues differentiate from procambium, and during secondary development they differentiate from cambium. The mechanisms underlying the xylem and the phloem development are largely unknown.

The *Arabidopsis thaliana* mutant, *wooden leg* (*wol*) has a reduced number of vascular cell files in the root, and all these files differentiate into the xylem. Thus, *wol* root lacks the phloem. Through positional cloning, we determined that the *WOL* locus encodes a two-component sensor histidine kinase molecule, CRE1. It is now known that the *WOL/CRE1* receptor perceives cytokinin phytohormones. We found that cytokinin signalling through WOL/CRE1, and two other cytokinin receptors, regulate procambial cell proliferation and vascular cell identities. In order to identify new regulatory components of vascular morphogenesis, we carried out a suppressor screen for *wol*. Several recessive intragenic suppressor mutations were found, which led to identification of the bifunctional kinase/phosphatase activity of the WOL/CRE1 receptor. The role of the phosphatase activity is to negatively regulate cytokinin signalling and thereby normal procambial development. Two extragenic mutations were also identified in the screen. The mutations were allelic and by positional cloning, we ascertained that they inactivated a gene encoding a previously uncharacterized downstream component of cytokinin signalling, AHP6. With various genetic approaches, we demonstrated that cytokinin inhibits protoxylem differentiation. AHP6 counteracts cytokinin signalling locally, allowing protoxylem specification. Conversely, cytokinin signalling negatively regulates the spatial domain of *AHP6* expression. In conclusion, this study demonstrates a mechanism by which cytokinin, its receptor WOL/CRE1, and AHP6, a spatial inhibitor of cytokinin signalling, form a complex genetic network that regulates vascular morphogenesis.

INTRODUCTION

1. VASCULAR DEVELOPMENT

More than 400 million years ago, certain green algae began to adapt to living on land. These ancestors of the first terrestrial plants were able to cope with occasional drying, when growing on streams or mudflats, but they were still tightly depended on living on water. To successfully accomplish the transition from aquatic to terrestrial life, several structural and functional changes had to occur in plants. Adaptation was necessary, especially for efficient transport of nutrients, photoassimilates and water throughout the plant body. Mechanical support was also beneficial, allowing stem to grow upwards, away from the shade of other plants. The development of vascular tissue allowed plants to solve both problems. The vascular tissue extends throughout the entire plant as a network, and it consists of two transporting tissue types: the xylem and the phloem **(Fig. 1)**. The xylem has a dual function; it supports the plant body, and it is the main conduit of water and mineral nutrients that are transported, from the root, to the sites of photosynthesis in leaves. From leaves, photoassimilates, the end products of photosynthesis, are transported through the phloem to nourish other parts of the plant, such as the root and the reproductive organ.

During primary development, both the xylem and the phloem differentiate from a single meristematic tissue, procambium (Esau, 1977). In plants that later undergo radial thickening, secondary vascular tissues are formed as the result of periclinal cell divisions in a lateral meristem called cambium. In
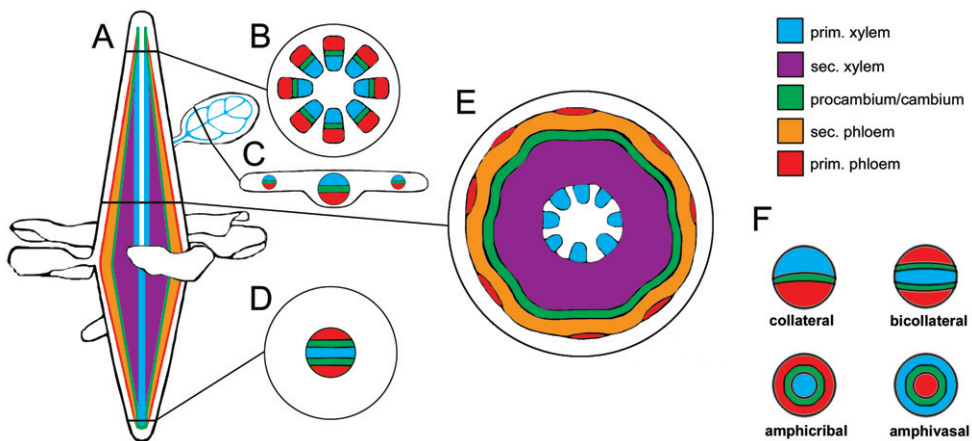
Figure 1. *The organisation of the primary and the secondary vascular tissues in Arabidopsis thaliana.* **(A)** Schematic presentation of a whole plant. **(B)** Transverse section of a stem close to the shoot apex. **(C)** Transverse section of a leaf. **(D)** Transverse section of a root close to the root apex. **(E)** Transverse section of the basal part of an inflorescence stem during secondary development. **(F)** Radial patterning of the vascular tissue. The xylem and the phloem are organised in a specific radial pattern, depending on species, organ or mutant. Modified from Nieminen *et al.*, 2004.

tree species, especially, the extent of secondary vascular tissue, wood, is evident.

In the following sections, the organization and the establishment of the vascular tissue are discussed, with the emphasis on early events in primary development of dicots, especially in the model species, *Arabidopsis thaliana*.

1.1 The vascular system

The vascular system of seed plants forms a continuous network of strands, called vascular bundles, which functionally connect the organs of a plant. In dicotyledonous species, vascular bundles are composed of several types of tissues: the meristematic tissues, procambium or vascular cambium, and the transporting tissues the xylem and the phloem. Monocotyledonous plants and ferns lack cambium.

1.1.1 Procambium and cambium

Procambium cells contain vascular stem cells that originate from the apical meristems, and they give rise to phloem and xylem precursor cells. Anatomically, procambial cells are cytoplasmically dense, and they appear as continuous strands of narrow, elongated cells. Characteristically, these cells and their initials, provascular cells, undergo cell divisions that are oriented parallel to the developing vascular bundle. As the bundle typically elongate in the same direction in which the organ is growing, the plane of cell division of the surrounding cells is usually different: they preferentially divide perpendicular to the direction of growth (Esau, 1977; Smith, 2001). Provascular cells are hard to visualise because they are polygonal and isodiametric, as are the surrounding cells (Turner, Sieburth, 2002). However, since provascular cell specific reporter lines, such as *ATHB8promoter::GUS* are available, it has become easier to distinguish these from the neighbouring cells (Baima, et al., 1995; Scarpella, et al., 2004).

Vascular cambium originates mainly from the procambium within the vascular bundle, and to some extent from the parenchyma between the vascular bundles. Fusiform initials and their derivatives are elongated cambial cells that give rise to the axial system, the vascular tissue that can be seen as annual rings. Ray initials are nearly isodiametric cambial cells that constitute the radial system. The fusiform initials are difficult to distinguish from their daughter cells, as these derivatives also divide periclinally before they begin to differentiate to xylem or phloem. Therefore, instead of the term cambium, many researchers refer to the cambial zone.

1.1.2 Transporting tissues

Xylem

The xylem is the principal water-conducting tissue in a vascular plant. In addition, it transports minerals and phytohormones, such as cytokinin and abscisic acid (Hartung, et al., 2002; Takei, et al., 2001b). It is composed of conducting tracheary elements, and nonconducting elements, such as xylem parenchyma and xylem fibers. Tracheary elements are elongated cells that when mature, lose all cell contents through programmed cell death, forming hollow tubes through which water and minerals flow. They have lignified secondary cell walls, which add strength and rigidity to the wall and prevent these elements from collapsing under the high pressure that is exerted upon water uptake from the roots.

Xylem fibers usually have thicker cell walls than tracheary elements, and they are specialised to function as support cells. Primary xylem differentiates from procambium during the formation of the primary plant body. Secondary xylem, or wood, results from the activity of the vascular cambium during the secondary development **(Fig. 1)**. Even though the xylem tissues are not organized in the same way, all the three major xylem cell types are found both in the primary and the secondary xylem.

Developmentally, primary xylem can be divided into earlier protoxylem and the later metaxylem. Differentiation to protoxylem cells takes place already within the actively elongating tissues. Their secondary cell walls appear ring-like (annular) or helical (spiral) thickenings, and can therefore be stretched when already differenti-ated, making it possible for them to elongate together with the surrounding developing tissue. However, later during primary development, the protoxylem cells are further stretched and eventually destroyed. The metaxylem cells differentiate later in development and may stay functional until primary growth is completed. The secondary cell wall is more uniform in metaxylem than in protoxylem cells, because metaxylem cells differentiate either as netlike (reticulate) thickenings or as pitted elements. Since the secondary cell walls of metaxylem are more uniform and thicker than those of protoxylem, metaxylem gives more support than does protoxylem. However, unlike protoxylem, it does not stretch after the formation of the secondary cell wall.

Phloem

The phloem serves as the conductor of the photosynthetic product sucrose, and as a translocator of proteins, phytohormones or mRNAs that are involved in plant development or viral infection (Baker, 2000; Citovsky, Zambryski, 2000; Oparka, Cruz, 2000). It is composed of conducting sieve elements, and of nonconducting cells such as fibers and parenchyma cells. The sieve elements are interconnected by sieve plates penetrated with pores, forming a continuous network through which photoassimilates are transported. Their metabolic activity is restricted, as the nucleus and the endoplasmic reticulum are usually degenerated. However, unlike the tracheary elements, mature sieve elements are living cells that contain cytoplasm. The sieve elements are supported by metabolically active companion cells, which provide them with nutrients. Photosynthesised sucrose is transported (loaded) to the sieve elements from the adjacent companion cells in source organs, such as leaves. Then, sucrose is transported *via* the phloem network and unloaded through companion cells to sink organs, such as roots and storage tissues. Similar to the xylem cells, phloem cells originate from procambium and vascular cambium during primary and secondary development, respectively **(Fig. 1)**. However, unlike wood, secondary phloem is eventually cut off from periderm due to its usual position near the periphery of the stem and the root.

1.2 Establishment of a vascular pattern

During primary development, vascular bundles appear as central cylinders in the embryonic root and hypocotyl, and as veins in the cotyledons. After

germination, vascular cylinders are propagated by the apical meristem of the primary root, and recapitulated by the lateral root meristems and leaf primordia that emerge from the flanks of the shoot apical meristems. The vascular system of the stem is formed as a result of the shoot apical meristem activity, and it is arranged as a ring of several vascular bundles **(Fig. 1)**.

The vascular bundles are first established as strands of procambial tissue, which differentiate to primary xylem and phloem. The spatial arrangement of the xylem and the phloem determines the radial pattern of the vascular bundle.

1.2.1 Specification of vascular bundles

Classical studies by Jacobs and Sachs demonstrated that the polar transport of the phytohormone auxin has a crucial role in specifying the vascular bundle (Jacobs, 1952; Sachs, 1981). Jacobs demonstrated that a signal from young leaf primordia is required for the generation of bypass vascular strands around a wound. Excision of leaves below a wound had no effect on the ability to bypass the wound. However, removal of leaf primordia above the wound markedly reduced vascular differentiation, suggesting that the signal is moving basipetally, *i.e.* from primordia down to the stem. Indole-3-acetic acid (IAA), the major auxin in plants, is predominantly synthesised in young apical regions, such as leaf primordia (Jacobs, 1952; Ljung, et al., 2001). Moreover, applied auxin is able to replace the induction of primordia on vascular regeneration. These findings suggested that polar auxin transport, from young leaf primordia through the stem, is required for vascular differentiation (Jacobs, 1952; Mcarthur, Steeves, 1972; Young, 1954). However, vascular morphogenesis in

response to auxin application does not readily occur in all plant species; monocots especially are known for their resistance for this process (Aloni, Plotkin, 1985).

Sachs demonstrated that a local application of auxin (source) to the side of a hypocotyl segment induces the transdifferentiation of parenchymatic cells within ground tissue, thereby forming continuous vascular strands into the existing vascular bundle (sink) **(Fig. 2)** (Sachs, 1981). Furthermore, he demonstrated the importance of polar auxin flux from the source to the sink, by establishing that when existing vascular cylinder was saturated, by an additional application of auxin on top of the segment (a change from sink to source) transdifferentiation did not take place **(Fig. 2)**. Based on these results, he concluded that canalized auxin flux during vascular specification would increase the ability of cells to transport auxin, and subsequently induce differentiation. Through this positive feedback mechanism, transport would drain the neighbouring cells from auxin and thus, inhibit vascular differentiation of these cells. Due to this mechanism, canalization would concentrate the flux on narrow strands resembling those seen in vascular bundles **(Fig. 2)**. An analogy for the canalization hypothesis would be the way erosion causes the formation of discrete channels for the flow of water (Sachs, 1981).

Discovery of auxin signalling and the transport components operating in vascular specification, combined with pharmacological studies of the auxin transport inhibitors, have further supported the fundamental role for auxin in the induction of vascular bundles. Components in auxin signalling required for vascular specification, and other factors needed for vascular

continuity, will be discussed in the following sections.

Mechanism of polar auxin transport

Polar auxin transport can be inhibited with a number of chemically heterogeneous compounds, such as 1-N-naphthylphthalamic acid (NPA) or 2,3,5-triiodobenzoic acid (TIBA) (Cande, Ray, 1976; Thomson, et al., 1973). The application of these inhibitors to *Arabidopsis* seedlings have been employed to investigate development of the vascular system under reduced polar auxin transport (Mattsson, et al., 1999; Sieburth, 1999). Under inhibitor treatment, vascular cells differentiated as local aggregates, rather than narrow continuous strands, indicating that polar auxin flux is required for continuous vascular pattern formation. However, inhibitors were less effective in inhibiting vascular patterning from established procambial tissues, such as embryonic cotyledons and leaf veins, at the time of their emergence as procambial strands. These data indicate that polar auxin inhibitors affect vascular development through disrupting the development of procambial patterns (Mattsson, et al., 1999; Sieburth, 1999).

The recent characterization of potential auxin influx and efflux carrier genes operating in the polar auxin transport, in *Arabidopsis*, has provided further support for the canalisation hypothesis. Although auxin is able to enter cells by diffusion, auxin influx carriers (AUX1 family) may be used where rapid influx is needed (Swarup, et al., 2001). Auxin is not able to diffuse out of cells, but it exits the cell through an efflux carrier apparatus that requires activity of at least two different groups of proteins: PIN-FORMED (PIN) family proteins, and proteins that bind auxin transport inhibitors, such as MULTIDRUG-RESISTANCE–like protein (for review, see Muday, Murphy, 2002). PIN1, an *Arabidopsis* auxin efflux
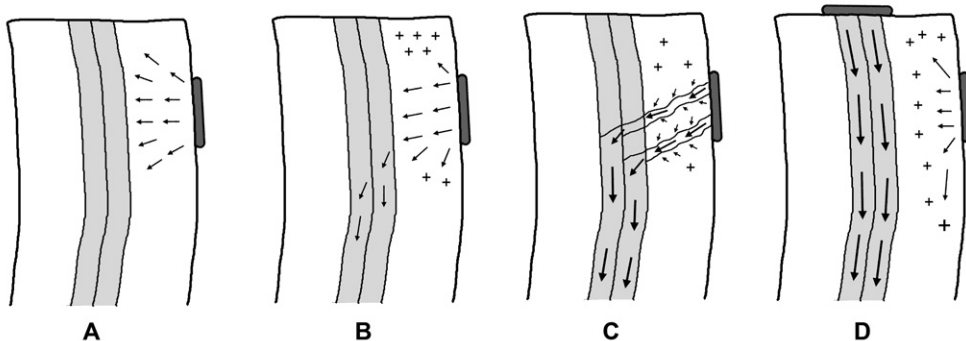
Figure 2. *Canalisation of auxin flux determines vascular pattern.* **(A)** Applied auxin on the side (dark grey spot) of excised hypocotyl diffuses away from the application source. **(B)** A proportion of applied auxin flows to an existing vascular bundle (light grey stripes), which leads to an enhancement of auxin flow from the source. Auxin accumulation (plus signs) inhibits auxin flux. **(C)** The preferred channels inhibit further canalisation in the surroundings and therefore differentiate as narrow vascular strands. **(D)** If the existing vascular bundle is saturated with an additional auxin application on top of the segment, the auxin flux from the lateral source is inhibited and the vascular strands fail to form. Modified from Sachs, 1981.

facilitator, is localised at the basal end of the embryonic provascular cells and postembryonic xylem parenchyma cells (Galweiler, et al., 1998; Steinmann, et al., 1999). The direction of the auxin flux, predicted by the polar localisation of PIN1, is consistent with the distribution of auxin maxima both in embryos, and in root apical meristems (Friml, et al., 2003; Sabatini, et al., 1999). Auxin transport from the young leaf primordia specifies the vascular tissue of the leaf, and connects it to the existing vascular network. Owing to poor auxin transport from primordia, auxin is accumulated in the *pin1* mutant, which leads to an over-proliferation of the xylem tissue in the vascular bundles right below the first cauline leaf (Galweiler, et al., 1998). As mentioned earlier, similar vascular tissue aggregates are observed when seedlings are treated with auxin transport inhibitors (Mattsson, et al., 1999; Sieburth, 1999).

PIN1, and several other members of the PIN family, are rapidly recycled between the plasma membrane and an intracellular compartment (Geldner, et al., 2001). Recessive mutations in *gnom* show defects in specifying apical-basal axis and, therefore, also in establishing the provascular tissue during early embryogenesis in *Arabidopsis* (Mayer, et al., 1993). A weaker allele of *gnom, van7,* displays discontinuous vascular bundles in leaves and cotyledons (Koizumi, et al., 2000). Furthermore, coordinated polar localisation of PIN1, and possibly other PINs, is defective in *gnom* embryos, indicating that PIN recycling is important for proper auxin canalisation (Steinmann, et al., 1999). Supporting this view, seedlings carrying mutations in multiple *PIN* genes show phenotypes reminiscent of *gnom* (Friml, et al., 2003). *GNOM* encodes a specific guanine-nucleotide exchange factor of the ADP ribosylation factor G

protein, which promotes the exocytic step in PIN1 cycling (Busch, et al., 1996; Geldner, et al., 2003; Steinmann, et al., 1999). Very recently, it was established that auxin inhibits endocytosis of PIN proteins and therefore, by maintaining the PINs localised to plasma membrane, auxin promotes its own efflux from the cells by a vesicle-trafficking-dependent mechanism (Paciorek, et al., 2005). These molecular data give a mechanistic view on how auxin canalises its own flux to establish a vascular bundle.

Auxin perception in vascular specification

Not all cells that are subjected to polar auxin flux differentiate as vascular cells. Therefore, proper auxin perception and triggering of the specific downstream components is essential for formation of continuous vascular strands. Several mutations related to auxin perception have been isolated in *Arabidopsis*, and many show vascular abnormalities (for review, see Berleth, et al., 2000; Scarpella, Meijer, 2004). The most severe mutations show seedling lethality, accompanied by reduced auxin response, vascular differentiation, and embryo axis formation. This complex phenotype suggests that there is a primary defect affecting cell alignment and specification along the axis of auxin flux, at different developmental stages.

The *MONOPTEROS* (*MP*) gene has an essential role in apical-basal pattern formation in the *Arabidopsis* embryo (Mayer, et al., 1991). *mp* seedlings lack basal structures, such as the embryonic root, hypocotyl and root meristem. Furthermore, mutant embryos lack the central provascular cylinder that can be seen as a set of narrow cells in heart-stage embryos in the wild-type (Berleth, Jurgens, 1993). Even though

mp seedlings are not viable, they can be rescued by generating adventitious roots in tissue culture, enabling studies in postembryonic stages. Throughout postembryonic development, vascular strands of *mp* are discontinuous and incompletely differentiated (Przemeck, et al., 1996). MP encodes the AUXIN RESPONSE FACTOR 5 (ARF5), a transcription factor that activates auxin responsive genes **(Fig. 3)** (Hardtke, Berleth, 1998). *MP* expression is restricted to provascular/procambial tissue, further supporting the role for MP in vascular specification. Since the initial expression domain of *MP* is broader, it is possible that auxin (and MP, as an auxin signalling component) acts primarily in apical-basal patterning and

secondarily in vascular development (Hardtke, Berleth, 1998).

AUX/IAA proteins, which are short-lived and rapidly upregulated by auxin, bind to specific ARFs (such as MP) and repress their transcriptional activities **(Fig. 3)**. The SCF^{TIR1} (SKP1, Cullin, F-box protein TIR1) complex catalyses the covalent addition of an ubiquitin molecule to AUX/IAA, which targets them to degradation *via* proteasomes **(Fig. 3)** (for review, see Callis, 2005; Hellmann, Estelle, 2002). Very recently, two laboratories demonstrated that the TIR1 protein of the SCF^{TIR1} complex is an auxin receptor. Auxin promotes SCF^{TIR1} – AUX/IAA interaction, by binding directly to TIR1, which leads to AUX/IAA degradation and consequently, to ARF-dependent auxin responses
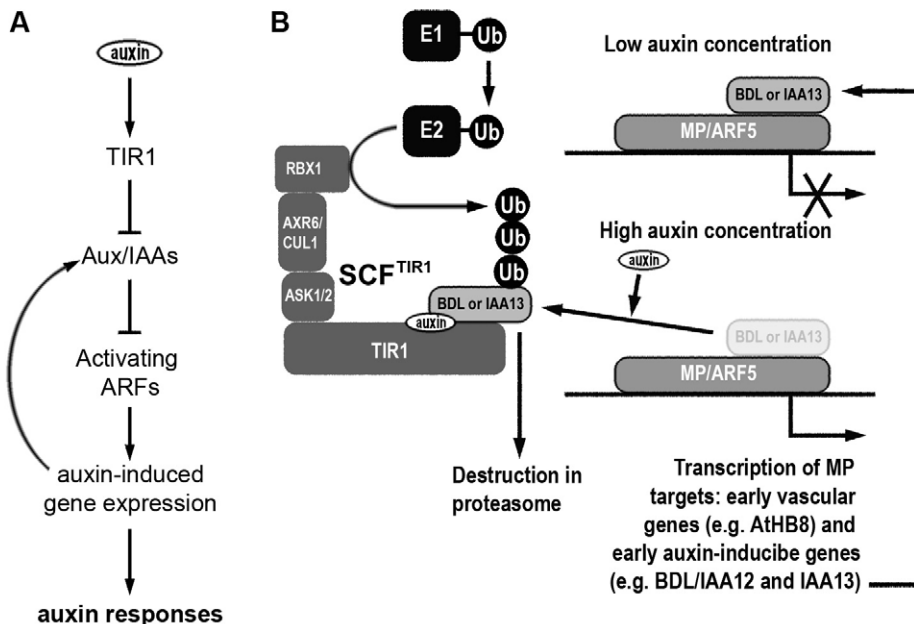
Figure 3. *Auxin signalling regulates vascular specification.* **(A)** A general illustration of auxin signalling. **(B)** The mechanisms of auxin signalling that regulates vascular specification. SCF^{TIR1} is composed of four subunits: RBX1, AXR6/CUL1, ASK1/2 and TIR1. E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; Ub, ubiquitin. Modified from Scarpella, 2004.

(Fig. 3) (Dharmasiri, et al., 2005; Kepinski, Leyser, 2005).

In addition to *mp*, mutations in several other components of auxin signalling pathway lead to vascular abnormalities. Dominant mutations in the *BODENLOS* (*BDL*) gene results in failure to establish basal body structures, thus *bdl* mutants resemble *mp* seedlings. However, the defects in *bdl* are weaker and do not result in loss of seedling viability (Hamann, et al., 1999). Additionally, the vascular system is reduced and mutant seedlings display reduced auxin responses (Hamann, et al., 1999; Hamann, et al., 2002). The *bdl* phenotype results from an amino acid change in the conserved degradation domain of IAA12, which leads to resistance to the SCF^{TIR1} mediated degradation of the protein. Consequently, BDL/IAA12 protein accumulates and constitutively represses the transcription of MP/ARF5, and perhaps also other ARFs **(Fig. 3)** (Hamann, et al., 2002; Hardtke, et al., 2004). When the homologous mutation was engineered to a sister gene, *IAA13*, the transgenic seedling displayed similar phenotypes to *bdl*. As the expression pattern of *IAA13* is similar to *BDL*, it is likely that both BDL/IAA12 and IAA13 need to be degraded in early embryogenesis for MP to promote root initiation and specification of provascular tissue **(Fig. 3)** (Weijers, et al., 2005). Homozygous *axr6* (*auxin-resistant6*) mutant seedlings show defects very similar to those of *mp* mutants; the mutant seedlings fail to produce hypocotyl and primary root and form severely reduced vascular system (Hobbie, et al., 2000). AXR6 is located upstream from MP and BDL, since it encodes CUL1, a component of SCF^{TIR1} complex (Hellmann, et al., 2003).

Taken together, these data suggest a model for auxin-mediated regulation of vascular specification **(Fig. 3)**. In low concentration of auxin, a set of AUX/IAA proteins, such as BDL/IAA12 and IAA13, accumulate and repress MP transcription. In high concentration of auxin, BDL/IAA12 and IAA13 are targeted to SCF^{TIR1}-mediated degradation, which would release MP from the inhibitory interaction to activate the transcription of genes involved in vascular specification. Simultaneously, MP activates expression of *AUX/IAA*, which leads to repression of *MP* transcription, ensuring a rapid modulation of auxin responses dependent on changes in auxin levels **(Fig. 3)**.

Vascular development in shoot apical meristem

Formation of the vascular tissue within a shoot is closely linked to the activity of the apical meristem. However, studies with two mutants, *pin* and *mp*, showing 'pin-like' structures suggest that vascular development in the stem may be independent of the formation of lateral organs (Okada, et al., 1991; Przemeck, et al., 1996). It was shown that even though the mutants failed to initiate the lateral organs from their inflorescence meristems, the vascular bundles under the meristems appeared relatively normal.

The role of vascular development in regulating leaf initiation and the pattern of phyllotaxis has been debated for many years (for review, see Turner, Sieburth, 2002). Leaf primordia and the vascular tissue beneath it seem to emerge almost simultaneously during leaf initiation. *PINHEAD/ZWILLE* was first identified by an analysis of mutants showing defects in shoot apical meristem establishment (Lynn, et al., 1999; Moussian, et al., 1998). Interestingly, *PINHEAD/ZWILLE* expression marks the site of vascular

development, below the site of future primordia, indicating that vascular specification for the primordia is initiated before any signs of primordia formation can be seen (Lynn, et al., 1999).

Continuity of vascular bundles in leaves

A number of leaf vascular (venation) pattern mutants have been identified in *Arabidopsis*, and many of them exhibit discontinuity in venation patterning, producing isolated, short stretches of veins, *i.e.* vascular bundles (for review, see Scarpella, Meijer, 2004). Many of these mutants, such as *gnom*/*van7*, *mp*, *bdl*, *axr6*, *scarface* (*sfc*), and *lopped1* (*lop1*), show reduced auxin transport or response (Carland, McHale, 1996; Deyholos, et al., 2000; Hamann, et al., 1999; Hobbie, et al., 2000; Koizumi, et al., 2000; Przemeck, et al., 1996; Steinmann, et al., 1999). However, there are also several venation pattern mutants that are perhaps not be related to auxin. In *cotyledon vascular pattern 1* (*cvp1*) mutant, vascular cells of cotyledons are not arranged in parallel files and are malformed. *CVP1* gene encodes STEROL METHYLTRANSFERASE-2, an enzyme in the sterol biosynthetic pathway, suggesting that sterols, such as brassinosteroids, may have a role in venation patterning or a general role in the membrane composition that is essential for the axialisation of procambial cells (Carland, et al., 2002). Even though *cvp1* has a normal auxin response and polar transport, it may still have some connection to auxin, as a related protein, STEROL METHYLTRANSFERASE-1, is required for the correct subcellular localisation of PIN, auxin efflux facilitators (Willemsen, et al., 2003). *cvp2* mutants exhibit an increase in free vein endings

in cotyledons and leaves (Carland, et al., 1999). *CVP2* encodes an inositol polyphosphate 5' phosphatase (5PTase), indicating an involvement of the inositol (1,4,5) triphosphate (IP$_3$) signal transduction pathway in connecting the free vein endings (Carland, Nelson, 2004). Discontinuity of procambial strands in *van3* leaves is caused by a mutation in ADENOSINE DIPHOSPHATE (ADP)-RIBOSYLATION FACTOR-GUANOSINE TRIPHOSPHATASE (GTPASE) ACTIVATING PROTEIN (Koizumi, et al., 2000; Koizumi, et al., 2005). Double mutant analyses, with auxin transport and signalling mutants, revealed that VAN3 may be involved in the auxin signal transduction, and not in the polar transport of auxin. (Koizumi, et al., 2005). These data indicate that polar auxin transport, *i.e.* canalisation, has a central role in specifying the venation pattern. However, other signalling pathways are also required for fine-tuning the pattern or for processes, like connecting the free vein endings, that cannot be explained by the canalisation hypothesis.

1.2.2 Radial patterning

Once vascular bundles are specified, they undergo procambial cell proliferation, prior to differentiation, to distinct patterns of the xylem and the phloem. A proportion of cells between the xylem and the phloem remains undifferentiated. These intervening procambial cells become mitotically active during the secondary development and form the lateral meristem, cambium. Different species of vascular plants form distinct radial patterns in each organ. The xylem and the phloem are arranged in these organs as collateral, bicollateral (bilateral), amphicribal or amphivasal patterns **(Fig. 1F)**.

Procambial and cambial cell maintenance and proliferation

Classical physiological studies have shown the involvement of the phytohormone auxin in initiating and promoting vascular cambium growth. Experiments with various species have demonstrated that auxin supply from the shoot apical meristem is required for cambial cell proliferation (Aloni, 1987; Shininger, 1979). Uggla and colleagues showed that there is an auxin gradient across the vascular cambium of *Pinus sylvestris*. Auxin maximum coincides with the cambial cells, suggesting that auxin may act as a positional signal to regulate the area of cell divisions in the cambial zone (Uggla, et al., 1996). Moreover, expression of *Populus tremula* homologues of the *Arabidopsis PIN* auxin efflux facilitator genes, and *AUX1*-family auxin influx carrier genes, correlate with the auxin gradient, indicating that the gradient may be maintained through the differential positioning of different auxin transporters (Schrader, et al., 2003). Also, a set of *AUX/IAA* auxin response factor homologues show gradient distribution in the cambium of *Populus tremula* (Moyle, et al., 2002). Proper auxin transport is required for normal procambial and/or cambial cell proliferation in *Arabidopsis* stem, since *pin1* loss-of-function mutant exhibits over-proliferation of the xylem tissue as a response to auxin accumulation (Galweiler, et al., 1998). Similarly, overexpression of auxin inducible HDZIPIII transcription factor, *AtHB8*, leads to over-proliferation of the xylem tissue in *Arabidopsis* stem (Baima, et al., 1995; Baima, et al., 2001). Together, these data suggest that auxin has a role in the vascular cambium cell proliferation. However, many aspects of auxin regulation on cambial cell proliferation need to be verified with genetic experiments. This may prove difficult, due to key role of auxin in plant development, from embryogenesis onwards; potential cambial phenotypes caused by disruptions of auxin signalling or transport, may be masked by earlier primary defects.

It seems probable that there are common mechanisms in controlling cell proliferation and maintenance during procambial (primary) and cambial (secondary) development. Many genes, such as *Arabidopsis* apical meristem regulators *CLAVATA1* (*CLV1*) and *AINTEGUMENTA* (*ANT*), and their *Populus* homologues, that are expressed in the primary meristem, are also expressed in the secondary meristems (cambium) of *Arabidopsis* and *Populus*, respectively (Schrader, et al., 2004; Zhao, et al., 2005). Typically, many mutants that show defects in vascular cambium proliferation, such as those with mutations affecting auxin transport or adaxial-abaxial polarity, have also defects in shoot apical meristem, supporting further the view that these meristems use common mechanisms. Moreover, cambium is formed from the procambial cells, products of the primary meristems, which may explain the absence of cambium specific mutants.

A recessive mutation *wooden leg* (*wol*) was isolated from a screen for reduced root growth (Scheres, et al., 1995). Seedlings homozygous for *wol* fail to produce lateral roots. However, the mutant is rescued by an adventitious root that emerges from the hypocotyl. Similar to the other mutants isolated from the screen, such as *scarecrow* (*scr*) lacking a ground tissue layer, *wol* also shows a defect in the radial patterning of the root. Unlike *scr, wol* has a defect in the vascular tissue. The vascular bundle of the *wol*

primary root shows a reduced number of cell files, which all differentiate into protoxylem elements (Cano-Delgado, et al., 2000; Scheres, et al., 1995). The lower part of the hypocotyl shows a similar vascular phenotype. However, the number of cell files increases and the phloem tissue appears in the upper part of the hypocotyl, close to the cotyledons. The reduced number of cell files is apparent already in mature embryos. The *wol* phenotype could be explained in two ways; *WOL* gene product may be required for the specification of the other vascular tissue types than protoxylem, or it may be required for vascular specific cell divisions (Scheres, et al., 1995). The *fass* mutant shows an increased number of cell layers in the root (Torres-Ruiz, Jurgens, 1994). *wol fass* double mutant shows all vascular tissue types, suggesting that the *WOL* gene is required for procambial cell divisions, not for cell specification. Scheres and colleagues suggested a hypothesis, in which other tissue types, such as phloem, are absent from the *wol* vascular bundle possibly because the xylem is specified earlier than the other vascular tissue types. Consequently, due to the reduced cell number, xylem takes over the restricted space in the *wol* vascular bundle. The occurrence of phloem in the upper part of the hypocotyl is consistent with this hypothesis, as the vascular bundle is wider near the shoot apical meristem (Scheres, et al., 1995). So far *wol* appears to be the only mutation related to procambial or cambial cell proliferation that exhibits a vascular specific defect.

Establishment of the abaxial-adaxial axis

Lateral organs of the aerial part of plants, such as leaves and floral organs,

are formed from the flanks of apical meristems. Consequently, they have intrinsic positional information: organ primordia have an adaxial (dorsal) side close to the meristem, and an abaxial (ventral) side away from the meristem. Once the axis of polarity is established in primordia, it serves as a reference for proper lamina (*e.g.* leaf blade) growth and asymmetric development, such as trichome formation on the adaxial side and stomata formation on the abaxial side of a leaf. Also, vascular patterning follows abaxial-adaxial polarity in these organs and in the stem: the xylem is localized on the adaxial side (internally in the stem) and the phloem on the abaxial side (peripherally in the stem) forming a collateral pattern for the vascular bundle (**Figure 1F**).

Studies of several *Antirrhinum majus* and *Arabidopsis thaliana* mutants have given insight on how polarity is established in plant organs (for review, see Bowman, et al., 2002). An *Antirrhinum PHANTASTICA* (*PHAN*) gene codes for a MYB-like transcription factor, and is expressed in organ primordia before organ initiation (Waites, et al., 1998). After organ initiation, *PHAN* expression becomes restricted to the adaxial side of developing leaves. Loss-of-function and conditional mutations of *PHAN* fail to maintain the apical meristem, and adaxial tissues of lateral organs are replaced by abaxial tissues, indicating that the *PHAN* gene product is required for adaxial identity of leaves (Waites, Hudson, 1995; Waites, et al., 1998). Interestingly, the vascular pattern of *phan* mutants changes from a collateral to an amphicribal symmetry, in which the xylem tissue is surrounded by the phloem **(Figure 1F)** (Waites, Hudson, 1995).

In contrast, gain-of-function mutations in *Arabidopsis* homeodomain/leucine zipper class III

genes (*HD-ZIPIII*) *PHABULOSA* (*PHB/ AtHB14*) or *REVOLUTA/ INTERFASCICULAR FIBERLESS 1* (*REV/ IFL1*) result in transformation of abaxial cell types of leaf to adaxial ones, and collateral vascular pattern to amphivasal, in which the phloem is encircled by the xylem **(Figure 1F)** (Emery, et al., 2003; McConnell, Barton, 1998; McConnell, et al., 2001). Furthermore, the most radialized *phb* leaves lack vascular tissue entirely, or only some xylem elements are present (McConnell, Barton, 1998). Recently, it was shown that a gain-of-function mutation of *PHB* occasionally results in lack of root, indicating that *HD-ZIPIII* genes also have a role in root patterning (Hawker, Bowman, 2004). Simultaneous loss-of-function of *PHB*, *REV* and another member of the family, *PHAVOLUTA* (*PHV/AtHB9*), results in abaxialization of cotyledons and lack of the formation of the shoot apical meristem. In the most severe cases, entire seedlings no longer have a bilateral symmetry which results in a single abaxialized radial cotyledon, demonstrating a vital role for PHB, REV, and PHV in radial patterning throughout plant development. Furthermore, vascular bundles show amphicribal symmetry in these radialized cotyledons (Emery, et al., 2003). A second class of genes in *Arabidopsis* needed for the proper radial patterning consists of three *KANADI* (*KAN1*, *KAN2* and *KAN3*) genes, which encode members of the GARP family of transcription factors (Eshed, et al., 2001). Simultaneous loss-of-function of the three *KANADI* genes results in adaxialization of lateral organs and amphivasal vascular symmetry, phenotypes similar to gain-of-function alleles of *PHB* and *REV* (Emery, et al., 2003). Moreover, abaxialized cotyledons and hypocotyl lack vascular tissue, when any of the three *KAN*

genes is ectopically expressed (Eshed, et al., 2001; Kerstetter, et al., 2001).

PHB, REV and PHV genes are expressed in overlapping domains of the adaxial regions of lateral organs, in vascular bundles and apical meristems, whereas *KANADI* genes are expressed in the developing phloem and abaxial regions of young lateral organs (Emery, et al., 2003; Kerstetter, et al., 2001; McConnell, et al., 2001). The complementary nature of both the phenotypes, and the expression patterns of the three HD-ZIPIII and the *KANADI* genes have given raise to a model, in which a juxtapositional expression of the two protein families is required for the lamina outgrowth, and for the establishment of collateral vascular bundles in all lateral organs **(Figure 4)** (Emery, et al., 2003). Also, the data imply that adaxial- and abaxial identity genes interact to specify xylem and phloem formation, respectively. This model, implicating an antagonistic interaction between the two families, is supported by loss-of-function studies. Simultaneous loss-of-function of *KAN1* and *KAN2* results in adaxialization of lateral organs and an expansion of the *REV* and *PHV* expression on the abaxial side of the organs (Eshed, et al., 2001). Recent studies have suggested an involvement of microRNAs in abaxial-adaxial axis formation. A gain-of-function phenotype of *REV*, and possibly also that of *PHB* and *PHV*, can be obtained by changing *REV* mRNA sequence in the microRNA165/166 target site, without manipulating the amino acid sequence (Emery, et al., 2003). Therefore, microRNA mediated negative regulation may be another mechanism through which *REV*, *PHB* and *PHV* expression is down-regulated from the abaxial domain to allow the proper radial patterning **(Figure 4)**.

Xylem specification and differentiation

In addition to the role in radial vascular pattern formation, HD-ZIPIII genes *REV*, *PHB* and *PHV* may have a role in vascular cell proliferation. Loss-of-function of *REV/IFL1* results in reduction of interfascicular xylem fibers, the tissue between vascular bundles, in the inflorescence stems (Zhong, Ye, 1999). The phenotype may be due to reduced auxin transport, because the auxin flow, as well as expression of auxin transporters are reduced in *rev/ifl1* mutants (Zhong, Ye, 2001). Furthermore, double mutants *rev phb/PHB* and *rev phv* demonstrate a more severe vascular defect than the *rev/ifl1* mutant (Prigge, et al., 2005). AtHB8 and AtHB15 are the other two members of the HD-ZIPIII family in *Arabidopsis*. They are specifically expressed in the procambium and the xylem precursor cells (Baima, et al., 1995; Ohashi-Ito, Fukuda, 2003). Loss-of-function of *AtHB8* does not show an obvious vascular phenotype. However, overexpression of the gene, or a *Zinnia elegans AtHB8* homologue, cause an increase in xylem tissue formation (Baima, et al., 2001; Ohashi-Ito, et al., 2005). Vascular bundles in *athb15* loss-of-function mutants are poorly distributed around the stem periphery and they show an increased number of the xylem cell files. Furthermore, overexpression of *AtHB15* leads to decreased xylem tissue formation, indicating that *AtHB15* is a negative regulator of vascular development (Kim, et al., 2005; Prigge, et al., 2005). These results suggest that each of the HD-ZIPIII genes has an important, in some cases even contrasting role in vascular morphogenesis, especially in proliferation of the xylem tissue.

Recently, it was proposed that two NAC-domain transcription factors act as transcriptional switches for protoxylem and metaxylem differentiation (Kubo, et al., 2005). When ectopically expressed in *Arabidopsis* or in poplar, *VASCULAR-RELATED NAC-DOMAIN6 (VND6)* and *VND7* induced transdifferentiation of various cell types into metaxylem and protoxylem, respectively. Furthermore, dominant negative versions of the transcription factors caused delay in xylem differentiation in the root vascular bundle (Kubo, et al., 2005). Since loss-of-function alleles of these two genes failed to show vascular phenotypes, there must be other factors required for xylem differentiation, that may act independently, or together with *VND6* and *VND7*.

Early physiological studies using cultured *Zinnia elegans* cells have shown that phytohormone brassinosteroids (BRs) have an important role in vascular development. They initiate tracheary element differentiation from the xylem precursors (for review, see Fukuda, 1997; Fukuda, 2004). Also, two brassinosteroid receptors show specific expression in the vascular
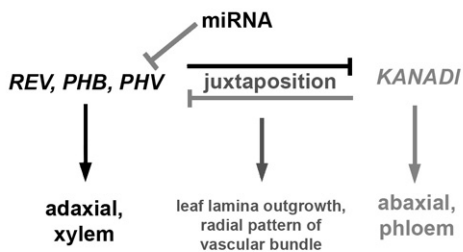
Figure 4. *Juxtaposition of HD-ZIPIII and KANADI activities pattern lateral organs and vascular bundle.* MicroRNA165/166 (miRNA) negatively regulates the expression of the three *HD-ZIPIII* genes, *REV*, *PHB* and *PHV*. Modified from Emery et al., 2003.

bundle (Cano-Delgado, et al., 2004). Loss-of-function mutation in one of the receptors, *BRL1*, or mutations in a brassinosteroid biosynthetic gene result in an increased phloem and a decreased xylem differentiation, compared to wild-type (Cano-Delgado, et al., 2004; Szekeres, et al., 1996). These data support further the role of BRs in promoting xylem differentiation, and it suggests also that BRs may have another role as inhibitors of phloem differentiation. HD-ZIPIII transcription factors REV, PHB and PHV share a putative steroid binding domain, raising the possibility that these factors act as receptors of steroids, such as BRs (Emery, et al., 2003). Furthermore, BRs induce expression of *Zinnia* HD-ZIPIII genes during xylem differentiation, suggesting that a link may exist between these two xylem promoting factors (Ohashi-Ito, Fukuda, 2003; Ohashi-Ito, et al., 2002).

Vascular cells interacting with each other differentiate to form a continuous strand. Therefore, inductive signals between differentiating vascular cells may guide the formation of a continuous network. Motose and colleagues showed that local intercellular communication in a gel-embedding cell culture promotes differentiation of *Zinnia* mesophyll cells into tracheary elements (TE), *i.e.* xylem cells (Motose, et al., 2001). Statistical analysis of the two dimensional distribution of the TEs showed that they were aggregated rather than randomly distributed, indicating that premature TEs draw neighbouring cells into the pathway of TE differentiation (Motose, et al., 2001). This inductive cell-to-cell interaction is mediated by a locally acting secreted factor, which was named xylogen, for it promotes xylogenesis. The activity of xylogen in

a bioassay, based on *Zinnia* cell culture, was recovered in the fraction of arabinogalactan proteins, a group of plant proteoglycans (Motose, et al., 2001). The expression of xylogen coincides with xylem differentiation in *Zinnia* transdifferentiation system, and in *Arabidopsis* vascular bundles. Furthermore, xylogen shows polar localisation on the apical cell walls of the immature xylem elements, implying that these cells secrete xylogen, in a polar manner, to their neighbouring cells. Double loss-of-function mutation of both of the *Arabidopsis* genes encoding xylogen results in a discontinuous vascular network similar to those seen in leaf venation pattern mutants (see 1.2.1), and in improperly interconnected xylem elements. However, xylogen seems not to be an essential component for xylem differentiation, because the double mutation resulted in a partial loss of xylem tissue, and it had only a limited influence on vascular development. Therefore, the role of xylogen is to coordinate, rather than to direct the xylem differentiation. Taken together, the polar secretion of xylogen stimulates neighbouring cells to differentiate into xylem, and therefore drives formation of a continuous vascular network (Motose, et al., 2004).

After specification, xylem cells undergo differentiation to various xylem cell types, *i.e.* to tracheary elements. The first step in differentiation of a TE is formation of a patterned secondary cell wall (see 1.1.2). Then, the developing tracheary elements undergo programmed cell death to form hollow tubes, through which water and water solutes flow. For more information related to these developmental processes, see reviews (Fukuda, 2004; Nieminen, et al., 2004).

Phloem specification and differentiation

It is probable that genes encoding HD-ZIPIII and KANADI transcription factors are also involved in the phloem specification. However, when the radial pattern is set up by these factors, there must be another set of genes needed for phloem differentiation. A recessive mutation in the *ALTERED PHLOEM DEVELOPMENT* (*APL*) locus results in an ectopic formation of xylem in place of phloem tissue (Bonke, et al., 2003). *APL* encodes a MYB-coiled coil transcription factor and it shows expression in developing phloem throughout the vascular system. Moreover, ectopic expression of *APL* in the whole vascular bundle inhibits xylem differentiation. Taken together, *APL* appears to have a dual role in promoting phloem differentiation and in inhibiting xylem differentiation. As discussed earlier, brassinosteroids may play a part also in the phloem development. Contrasting to the APL function, brassinosteroids appear to inhibit phloem differentiation.

2. CYTOKININS

From the 1940s, plant scientists made advances in developing plant tissue culture. Especially, addition of complex materials, such as coconut milk, to an otherwise chemically defined culture medium containing minerals, sugar and phytohormone auxin, results in the sustained growth of plant tissues. Coconut milk seemed to contain a substance or substances that could stimulate cell proliferation (Caplin, Steward, 1948). In the 1950s, Skoog and Miller found that autoclaved herring sperm DNA is a good activator of tobacco pith cell culture (Miller, et al., 1955b). They identified an adenine derivative, 6-furfurylaminopurine from herring sperm DNA as the potential active compound (Miller, et al., 1955a). When tobacco stem pieces were cultured together with a synthetical 6-furfurylaminopurine and auxin, the tissue began to proliferate, demonstrating that 6-furfuryl-aminopurine, which they named kinetin, is indeed the active compound **(Figure 5)** (Miller, et al., 1955a; Miller, et al., 1956). Ten years later, zeatin was isolated and identified from maize immature endosperm as the first naturally occurring member of this new group of phytohormones, cytokinins **(Figure 5)** (Letham, Miller, 1965; Skoog, et al., 1965). Moreover, zeatin turned out to be the most abundant cytokinin in coconut milk (Letham, 1974). Since the discovery of cytokinins, they have been implicated in many aspects of plant development, including cell division, shoot formation, leaf senescence, vascular development and activation of dormant lateral buds (Mok, Mok, 1994).

In the following sections, metabolism, transport and signalling of cytokinins, as well as their role in plant development are discussed, with the emphasis on cytokinin perception and signalling in *Arabidopsis thaliana* and its role in vascular development.

2.1 Structure and biosynthesis of cytokinins

Naturally occurring cytokinins are adenine derivatives and can be classified by the configuration of their N^6-side chain as isoprenoid or aromatic cytokinins (for review, see Mok, Mok, 2001). The most widespread, natural cytokinins are isopentyladenine and especially *trans*-zeatin, both containing an isoprenoid side chain **(Figure 5)**. Kinetin and N^6-benzyladenine (BA) are the best known cytokinins with ring substitutions at the N^6 atom, but only BA and its derivatives have been identified as natural cytokinins **(Figure 5)**. Ribose or ribose-5'-phosphate may be attached to the N^9 position of adenine backbone to form cytokinin ribosides or ribotides, respectively **(Figure 5)**. All of these compounds induce cytokinin responses when applied on a plant tissue. However, it is possible that many of these compounds undergo an interconversion to the actual active form *in planta*, and therefore the active forms may remain unknown. Recently, after identification of the cytokinin receptors, a few receptor binding assays have been carried out to identify the active forms. Binding assays indicate that the most active
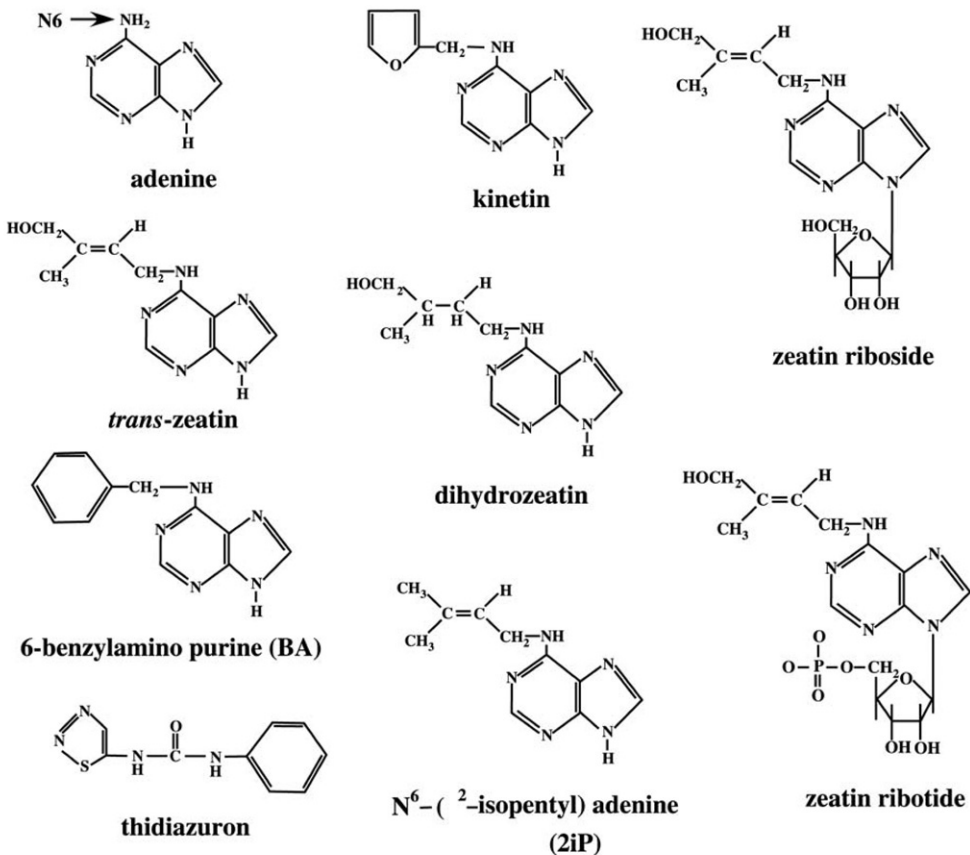

Figure 5. *Structures of cytokinins.*

cytokinins are isopentenyladenine and *trans*-zeatin, whereas only few ribosides, such as *trans*-zeatin riboside, and some synthetic cytokinins, such as urea-type cytokinin, thidiazuron **(Figure 5)**, show moderate, variable activity depending on the receptor (Spichal, et al., 2004; Yamada, et al., 2001). BA activity appeared to be poor in the binding assay, whereas a reporter gene assay in *Arabidopsis* suggested the activity to be strong, possibly because BA is more stable or it is modified in plant tissues (Spichal, et al., 2004). Also, a subset of *Zea mays* and *Arabidopsis* receptors bind *cis*-zeatin (Spichal, et al., 2004; Yonekura-Sakakibara, et al., 2004). Taken together, a subset of cytokinins has distinct affinities for different receptors, suggesting a fine-tuning mechanism at the perception level of cytokinin signalling.

The key enzyme in cytokinin biosynthesis was identified first from the slime mold, *Dictyostelium discoideum* (Taya, et al., 1978). A few years later, the *isopentenyltransferase* (*ipt*) gene was cloned from a transfer DNA (T-DNA) of *Agrobacterium tumefaciens* and it was shown to encode an enzyme with similar activity, *i.e.* it converts AMP and dimethylallyl pyrophosphate (DMAPP) to the active cytokinin isopentenyladenosine-5'-monophosphate (iPMP), **(Figure 6)** (Akiyoshi, et al., 1984; Barry, et al., 1984). ipt activity was found also in crude extracts of plant tissues, but it was only after sequencing of the *Arabidopsis* genome that homologous plant ipt genes were identified (Kakimoto, 2001; Takei, et al., 2001a). The *Arabidopsis* genome contains nine *AtIPT* genes, and when expressed in *E. coli*, seven of them yield secreted isopentenyladenine and *trans*-zeatin, indicating that these gene products posses ipt activity (Takei, et al., 2001a).

Many *AtIPT* genes show distinct, tissue-specific expression patterns that probably correspond to sites of cytokinin production (Miyawaki, et al., 2004). It is known that applied cytokinins together with auxin induce cell division and shoot formation in calli (Skoog, Miller, 1957). When *AtIPT4* was overexpressed in calli under the control of the CaMV 35S promoter, shoots were regenerated even in the absence of cytokinin, confirming that AtIPTs are the rate limiting enzymes in cytokinin biosynthesis (Kakimoto, 2001). Similar results were obtained when *AtIPT1, AtIPT3, AtIPT5, AtIPT7* or *AtIPT8* were overexpressed in calli (Miyawaki, et al., 2004). Unlike the agrobacterial ipt enzymes, the purified AtIPT4 utilized ATP and ADP rather than AMP as substrates **(Figure 6)** (Kakimoto, 2001). The product of the AtIPT-catalysed reaction are isopentenyladenosine-5'-triphosphate (iPTP) and isopentenyladenosine-5'-diphosphate (iPDP), both of which can be converted to zeatin by the cytokinin *trans*-hydroxylase activity of cytochrome P450 monooxygenase (CYP735A) **(Figure 6)** (Takei, et al., 2004). Recent studies suggest an alternative, bypass pathway for zeatin synthesis, in which a hydroxylated derivative of DMAPP, most probably 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (HMBDP), is directly transferred to the adenine moiety **(Figure 6)** (Astot, et al., 2000; Sakakibara, et al., 2005). However, DMAPP seems to be the major isoprenoid precursor for zeatin biosynthesis in *Arabidopsis*, while HMBDP is utilized mainly by *Agrobacterium* ipt in plastids of infected plant cells (Sakakibara, et al., 2005). A fraction of *trans*-zeatin may also be produced through the isomerisation of *cis*-zeatin, which is thought to be formed by the degradation of tRNA containing *cis*-

zeatin-type prenylation, but it is unknown whether this pathway has a biological relevance in *Arabidopsis* **(Figure 6)** (Mok, Mok, 1994). The biosynthetic pathway of aromatic cytokinins, such as BA, is entirely unknown. It is apparent that biosynthesis of these cytokinins utilizes a distinct pathway from the isoprenoid cytokinins, and may be related to the metabolism of phenolics (Mok, Mok, 2001).

2.2 Metabolism of cytokinins

O-glucosylation is an important step in the metabolism of *trans*-zeatin. The resulting *O*-glucosides serve as storage compounds of cytokinins and are resistant to degradation by cytokinin oxidases (see below). *O*-glucosides can easily be converted to active cytokinins by β-glucosidases **(Figure 6)** (Mok, Mok, 2001). Cytokinins can also be *N*-glycosylated in the adenine ring.
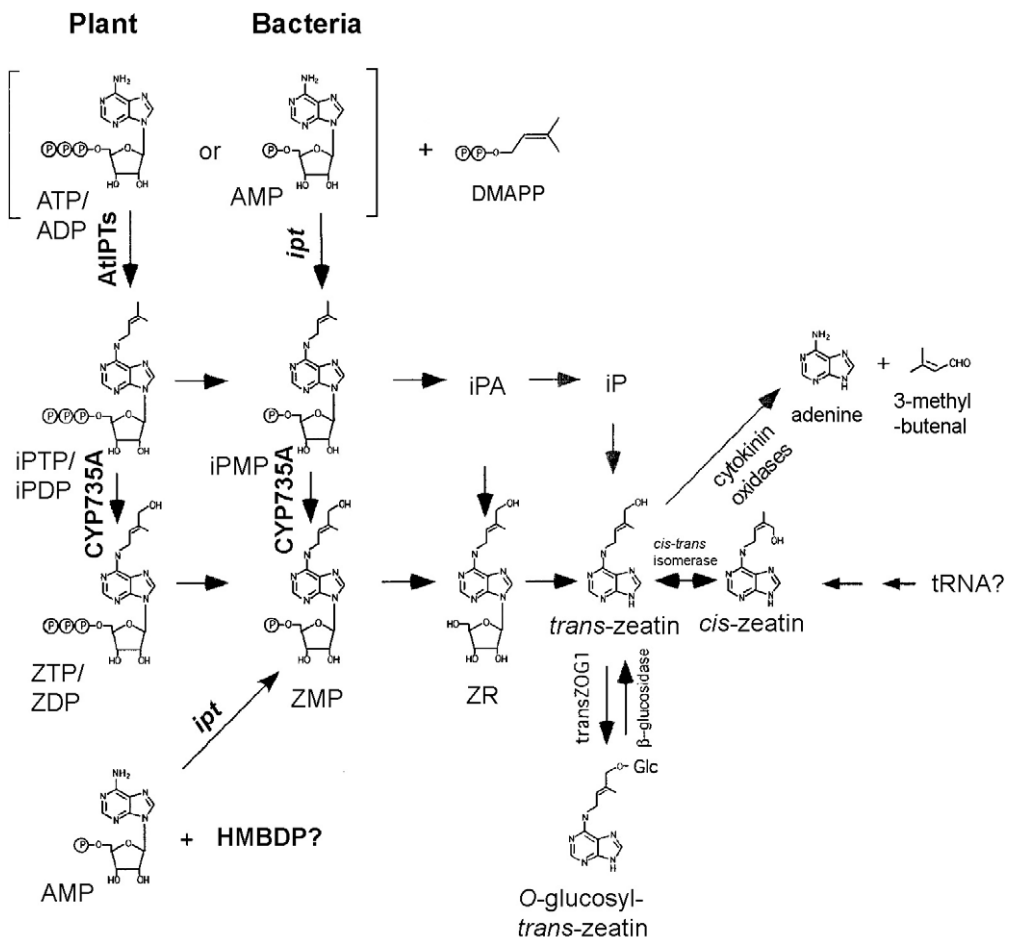
Figure 6. *Proposed biosynthetic and metabolic pathway for cytokinins.* HMBDP, 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate; trans-ZOG, *trans*-zeatin-*O*-glucosyl-transferase. Shown here are the major reactions in cytokinin biosynthesis and metabolism. For a more complete view of the pathways related to cytokinin metabolism, see Mok, 2001.

Presumably, most *N*-glycosylations cause irreversible inactivation of cytokinin, but the precise *in planta* role of these conjugations is unknown (for review, see Mok, Mok, 2001).

Many plants contain cytokinin oxidases, enzymes that irreversibly cleave the N^6-side chain from a subset of cytokinins. *Trans*-zeatin and isopentenyladenine have unsaturated N^6-side chains, and are therefore degraded by the oxidases **(Figure 6)**, while dihydrozeatin and BA are not (for review, see Mok, Mok, 2001). Recently, the first plant cytokinin oxidase (*CKX*) gene was isolated from *Zea mays*. The enzyme turned out to be a FAD-containing oxidoreductase, and it showed cytokinin oxidase activity when expressed in *Pichia pastoris* or in *Physcomitrella patens* (Houba-Herin, et al., 1999; Morris, et al., 1999). *Arabidopsis* genome contains seven cytokinin oxidase genes, *AtCKX1* to *AtCKX7* (Werner, et al., 2003). Overexpression of six of these genes under the control of the CaMV 35S promoter results in reduction of cytokinin content, and therefore in distinct phenotypes both in *Nicotiana tabacum* and in *Arabidosis* (Werner, et al., 2001; Werner, et al., 2003). Individual members of the *AtCKX* gene family show different expression patterns and subcellular localisations, suggesting that they may have different roles in cytokinin metabolism (Werner, et al., 2003). Recently, a quantitative trait locus (QTL) that increases grain production in *Oryza sativa,* rice, was shown to be a gene encoding cytokinin oxidase (OsCKX2). Reduced *OsCKX2* expression causes cytokinin accumulation in the inflorescence meristems that leads to an increased grain production, demonstrating the biological and agricultural importance of cytokinins and cytokinin oxidases (Ashikari, et al., 2005).

2.3 Transport of cytokinins

Cytokinins are adenine derivatives. Therefore, potential cytokinin transporters may be related to nucleotide transporters. Both purine and nucleoside transporters have been cloned from *Arabidopsis*, and they have shown to be capable of transporting free cytokinin bases and nucleoside type cytokinins in heterologous systems, respectively. However, whether nucleotide transporters have any biological relevance in cytokinin transport, remains to be assessed (Burkle, et al., 2003; Gillissen, et al., 2000; Hirose, et al., 2005).

Cytokinins have been found from virtually every organ of plants, and are probably present in every living cell. However, cytokinins can move within tissues or even from organ to organ and accumulate in specific regions, which make the detection of the actual biosynthesis sites difficult. It has long been believed that cytokinins are synthesised mainly in the root tips, and that shoot tissues have only a limited capacity for cytokinin biosynthesis. In several plant species, excision of roots results in a reduction of cytokinin levels and in slower growth, in the shoot. This process may be rescued by cytokinin application, suggesting that shoot tissues largely are dependent on the root-produced cytokinin, that is transported to the shoot *via* the xylem (for review, see Mok, Mok, 1994).

Contradictory results with respect to the biological significance of the cytokinin transport were obtained from reciprocal grafting experiments (Faiss, et al., 1997). The phenotypic effects of increased cytokinin were restricted to the part of the plant that was derived from a transgenic plant overexpressing *Agrobacterium ipt*. Hence, elevated levels of cytokinin in roots caused by the transgene had no phenotypic

consequence in the shoot, suggesting that cytokinins may act as a local rather than a systemic signal (Faiss, et al., 1997). Also, when systemic expression of *Agrobacterium ipt* was repressed locally in buds, using a dual, inducible-repressible transgenic system, cytokinin-induced bud outgrowth did not take place, supporting the view that cytokinins act locally (Bohner, Gatz, 2001). Moreover, by using a sensitive *in vivo* deuterium labelling and mass spectrometry analysis, cytokinins were shown to be synthesized in roots, but also equally in shoots, and preferentially in tissues rich in dividing cells, such as very young leaves and root tips (Nordstrom, et al., 2004). However, several recent studies have again suggested that cytokinins may act as a systemic signal, or at least, they may be transported from root to shoot. Cytokinin has been suggested to be a signalling component communicating nitrogen availability from the root to the shoot (Takei, et al., 2001b), it has been proposed to be passively transported by a transpiration stream, *via* the xylem, to the leaf primordia (Aloni, et al., 2005), and in contrast to the results from the grafting experiments carried out by Faiss and colleagues, *Agrobacterium ipt* induction in root results in bud outgrowth in the shoot (McKenzie, et al., 1998). Similarly, basally, but not apically applied cytokinins induced bud outgrowth in excised nodal sections of *Arabidopsis*, suggesting that cytokinin transport from root to shoot has a physiological significance (Chatfield, et al., 2000). One possible rationale for this contradiction is that cytokinin transport may require certain prerequisite conditions, such as low global cytokinin levels due to nutrient-starvation. Additionally, the expression of the *Agrobacterium ipt* may need to be above a certain threshold level

allowing a transport efficient enough to have an effect on the wild-type part of the plant. (Kakimoto, 2003). Also, grafting experiments should be repeated by overexpressing plant *IPTs* instead of *Agrobacterium ipt*, or cytokinin production-deficient mutants should be examined, in order to unravel the putative role of endogenous cytokinin in the cytokinin transport.

2.4 Cytokinin perception and signal transduction

Cytokinins are perceived by histidine kinases and the resulting signal is transduced by a phosphorelay pathway, similar to the prokaryotic two-component signalling system.

2.4.1 The two-component system

Reversible protein phosphorylation is the main mechanism of signal transduction in both prokaryotes and eukaryotes. In animal cells, phosphorylation typically occurs on a hydroxyl group of serine, threonine or tyrosine residues. In prokaryotes, it is common that a nitrogen atom of a histidine (His) residue or an acyl group of on aspartate (Asp) is phosphorylated (Klumpp, Krieglstein, 2002). The signalling *via* a phosphotransfer from His to Asp is called the two-component system. It was long believed that only prokaryotes have two-component systems, but then two histidine kinases, the ethylene receptor ETR1 of *Arabidopsis* (Chang, et al., 1993), and the osmosensor SLN1 from yeast *Saccharomyces cerevisiae* were identified (Maeda, et al., 1994; Ota, Varshavsky, 1993). It now seems probable that only animal cells lack the two-component system.

Prototypical two-component systems consist of two proteins, the

histidine kinase and the response regulator **(Figure 7a)**. Most histidine kinases function as transmembrane receptors. The input domain in the extracellular space senses the input signal, such as ligand binding. The binding of the ligand triggers the homodimerization of the two receptors monomers. Subsequently, within the intracellular transmitter domain of the two monomers, the histidine kinase subdomain of one monomer utilizes its ATPase activity to phosphorylate a conserved His residue within the other monomer. The phosphoryl group is subsequently transferred to a conserved aspartate residue within the receiver domain of a response regulator **(Figure 7a)**, which leads to a conformational change and modulation of the output domain activity. The receiver domain of a response regulator is conserved, whereas the output domain is more variable. Most output domains in prokaryotes are DNA-binding transcriptional regulators, while some of them have other functions, such as enzyme activity (for review, see Saito, 2001). Only a small proportion of the histidine kinase population exists in a phosphorylated state. Thus, the flux of phosphoryl groups rather than just the degree of phosphorylation is relevant for the function of the histidine kinases (Stock, et al., 2000).

The two-component system is modular, and it may be composed of various combinations of conserved and variable domains. Some prokaryotic and all eukaryotic systems consist of more than two components, and therefore the signalling *via* this multistep system is referred to as phosphorelay signalling **(Figure 7b)**. Histidine kinases of these systems are typically hybrids in which a receiver domain is fused to the carboxyl-terminus. Thus, following the
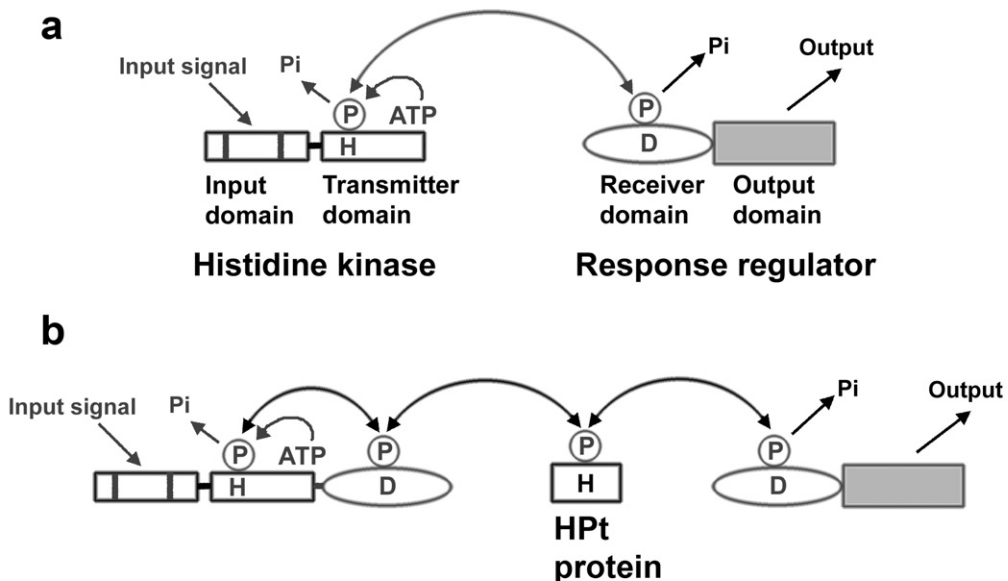
Figure 7. *General model of two-component systems.* **(A)** The prototypical two-component system. **(B)** Multistep phosphorelay system (His-Asp-His-Asp). Black bars, transmembrane domains; H, Histidine residue; D, Aspartate residue; P, phosphoryl group; Pi, inorganic phosphate. Modified from Kakimoto, 2003.

receptor activation, an intramolecular His-Asp phosphotransfer takes place, after which the phosphate is transferred *via* a His residue of another component, histidine phosphotransfer protein (HPt), to an Asp residue of a response regulator (Saito, 2001). The three-dimensional structure of the HPt is similar to that of the His residue-containing part of the transmitter domain (Kato, et al., 1997). However, HPt lacks the histidine kinase subdomain and acts as a monomeric signalling intermediate in the His-Asp-His-Asp phosphorelay **(Figure 7b)**. Many bacterial two-component histidine kinases are bifunctional. They have the above-mentioned kinase activity on His residues and also a phosphatase activity on a phospho-Asp residue **(Figure 7,** arrows pointing both directions**)**. Either of these activities may be regulated by the input signal, depending on the histidine kinase. In most cases, the His residue is not required for the phosphatase activity, but absolutely required for the kinase activity. Furthermore, some response regulators have autophosphatase activity, which affects the half-life of the phosphorylated state of the Asp residue. This bidirectional phosphotransfer is common in pathways that must be shut down rapidly (Stock, et al., 2000).

2.4.2 Cytokinin perception by histidine kinase receptors

The first indication that cytokinin signalling is linked to the two-component system came from a mutant screen of *Arabidopsis* genes whose overexpression resulted in rapid cell proliferation and greening in tissue culture, in the absence of applied cytokinin (Kakimoto, 1996). Hypocotyl segments were transformed with a T-DNA containing a strong transcriptional enhancer of CaMV 35S promoter, and the subsequent transgenic calli were screened for mutants exhibiting constitutive cytokinin responses. A gene named *CYTOKININ-INDEPENDENT1* (*CKI1*) that was tagged by the enhancer in four independent mutant lines, encodes a protein with sequence similarity to histidine kinases. *CKI1* is a candidate gene for a cytokinin receptor, because it is a putative sensor histidine kinase, and because its overexpression leads to cytokinin independent growth (Kakimoto, 1996). However, the additional data have not provided evidence that CKI1 is a cytokinin receptor. In fact, CKI1 constitutively activates phosphorelay when expressed in *Escherichia coli*, and the activity seems not to be regulated by cytokinins. Furthermore, isolated membranes of *Schizosaccharomyces pombe* containing CKI1 fails to bind cytokinins, suggesting that CKI1 is not a cytokinin receptor (Yamada, et al., 2001). *CKI1* has been found to be expressed in the female gametophytes and the endosperm of immature seeds. Since loss-of-function of *CKI1* leads to lethality already during female gametophyte development, a role for CKI1 after fertilization remains possible (Pischke, et al., 2002). Taken together, it seems reasonable to assume that overexpression of the constitutively active CKI1 results in an unexpected crosstalk with the endogenous cytokinin signalling pathway, with which it normally might not interact.

Recently, genuine cytokinin receptors were identified from *Arabidopsis*. The *cytokinin response1-1* (*cre1-1*) mutant was isolated from a screen for mutants impaired in the response for cytokinin in callus tissue culture (Inoue, et al., 2001). Seedlings homozygous for *cre1-1,* or for a T-DNA insertion allele, *cre1-2* showed reduced response to cytokinin, also in a root

elongation assay. Mapping and complementation analysis revealed that *CRE1* encodes a putative histidine kinase (Inoue, et al., 2001), which is identical to ARABIDOPSIS HISTIDINE KINASE4 (AHK4) (Ueguchi, et al., 2001b), a member of a protein family containing three highly homologous hybrid sensor histidine kinases; AHK2, AHK3 and AHK4 (Ueguchi, et al., 2001a). Each of these CRE-family histidine kinases contains a highly homologous extracellular domain in the N-terminal region (Ueguchi, et al., 2001a). This region resembles the ligand binding domain which is found in diverse receptors of prokaryotes, plants and the amoeba *Dictyostelium discoideum*, and was commonly named as <u>C</u>yclase/ <u>H</u>istidine kinase-<u>A</u>ssociated <u>S</u>ensing <u>E</u>xtracellular (CHASE) domain. The CHASE domain is bound by a diverse set of low molecular weight ligands (Anantharaman, Aravind, 2001; Mougel, Zhulin, 2001). Three laboratories independently demonstrated that CRE1/AHK4 is a cytokinin receptor by carrying out assays in which yeast and bacterial histidine kinase mutants were complemented by CRE1/AHK4 in a cytokinin-dependent manner (Inoue, et al., 2001; Suzuki, et al., 2001a; Ueguchi, et al., 2001b). Disruption of the SLN1 osmosensor, the only histidine kinase in the yeast *Saccharomyces cerevisiae*, is lethal, due to the inability to execute phosphotransfer (Maeda, et al., 1994; Posas, et al., 1996). Inoue and colleagues showed, and Ueguchi and colleagues confirmed, that expression of CRE1/AHK4 in the *sln1* mutant rescued the lethal phenotype, but only in the presence of cytokinins (Inoue, et al., 2001; Ueguchi, et al., 2001b). Similarly, Suzuki and colleagues demonstrated that CRE1/AHK4 can replace the function of histidine kinases in *Schizosaccharomyces pombe*,

and in *Escherichia coli*, only when cytokinins were applied to the cells (Suzuki, et al., 2001a). In the *S. cerevisiae* system, the conserved His and Asp residues of CRE1/AHK4, as well as Ypd1, a yeast HPt protein, were shown to be indispensable for complementation, suggesting that following cytokinin binding, CRE1/AHK4 initiates a phosphorelay in yeast (Inoue, et al., 2001). Additionally, the two other family members, AHK2 and AHK3 exhibited similar cytokinin-dependent activity (M. Higuchi & T. Kakimoto, personal communication) (Yamada, et al., 2001). In *S. pombe*, an analysis of isolated membranes of CRE1/AHK4-expressing cells was performed to demonstrate that CRE1/ AHK4 binds cytokinin. The membrane fraction was found to bind isopentenyladenine and other cytokinin bases in a highly specific manner (K_d = 4.6 nM for isopentenyladenine). Furthermore, a mutation in the CHASE domain, a receptor domain, abolished the binding of cytokinins, indicating that the CHASE domain senses cytokinins (Yamada, et al., 2001). Taken together, the CRE-family histidine kinases, AHK2, AHK3 and CRE1/AHK4, are the cytokinin receptors in *Arabidopsis*.

In addition to the CRE-family cytokinin receptors and *CKI1*, there are several other genes in *Arabidopsis* that show similarity to histidine kinases. There are five ethylene receptors (ETR1, ERS1, ETR2, EIN4 and ERS2), five phytochromes (PHYA to PHYE), one putative osmosensor (AtHK1) and a histidine kinase AHK5/CKI2 of unknown molecular function. However, not all ethylene receptors and none of the phytochromes contain all the conserved residues required for the canonical histidine kinase activity (for review, see Hwang, et al., 2002).

2.4.3 Response regulators

Arabidopsis genome contains 23 response regulator genes (*ARR*) that are divided into two main groups, type-A and type-B *ARR*s, depending on the sequence homology, domain structure and transcriptional response to cytokinin (D'Agostino, et al., 2000; Imamura, et al., 1999; Mason, et al., 2004). As revealed by *ARRpromoter*::*GUS* reporter analysis, *ARR* genes are expressed in distinct, yet often overlapping patterns in *Arabidopsis*, suggesting a functional redundancy among the group members (D'Agostino, et al., 2000; Kiba, et al., 2002; Mason, et al., 2004; Tajima, et al., 2004; To, et al., 2004).

The type-A ARRs have a receiver domain fused to a short variable carboxy-terminal extension, and most characteristically, their transcription is upregulated rapidly following cytokinin application without *de novo* protein synthesis. Thus, the type-A *ARR* genes are considered to be cytokinin primary response genes (Brandstatter, Kieber, 1998; D'Agostino, et al., 2000; Kiba, et al., 2002; Rashotte, et al., 2003). Numerous investigations indicate that type-A ARRs are negative regulators of cytokinin signalling. Overexpression of several type-A *ARR*s leads to decreased sensitivity for cytokinin (Hwang, Sheen, 2001; Kiba, et al., 2003). Furthermore, analysis of multiple loss-of-function type-A *ARR* mutants showed hypersensitivity for cytokinin in various assays. The severity of the hypersensitive phenotype generally correlated with the number of disrupted type-A *ARR*s, demonstrating highly overlapping function for members of this gene family in *Arabidopsis* (To, et al., 2004). Unlike in *Arabidopsis*, loss-of-function mutation in only one of the *Zea mays* type-A response regulators, *ABPH1*/*ZmRR3*

strikingly alters leaf phyllotaxy due to an increase of the meristem size (Giulini, et al., 2004). Thus, the degree of functional redundancy of these factors may differ between monocots and dicots, or *Arabidopsis* type-A *ARR*s mutants have not yet been analysed in enough detail.

Type-B ARRs are transcription factors that localize to the nucleus (Hwang, Sheen, 2001; Imamura, et al., 2001; Mason, et al., 2004; Sakai, et al., 2000). These factors have a receiver domain in the amino-terminus and a carboxy-terminal output domain containing a GARP, DNA-binding motif together with a transcriptional activation domain. Homologous GARP motifs occur widely in plant specific transcription factors, and the name GARP refers to the founding members of this family: GOLDEN2 of *Zea mays*, ARRs of *Arabidopsis* and Psr1 *Chlamydomonas* (Hosoda, et al., 2002; Riechmann, et al., 2000). Type-B ARRs, ARR1 and ARR2 bind to a consensus sequence GATCTT, which is found in promoters of the early response genes, including type-A *ARR*s (Rashotte, et al., 2003; Sakai, et al., 2000). In contrast to type-A *ARR*s, transcription of the type-B *ARR*s is not induced by cytokinin application (Imamura, et al., 1999). A number of studies suggest that type-B ARRs are positive regulators for cytokinin signalling. Overexpression of various type-B *ARR*s can induce cytokinin primary response genes in the absence of applied cytokinin in *Arabidopsis* protoplasts (Hwang, Sheen, 2001). Moreover, overexpression of various type-B *ARRs* containing a dominant gain-of-function mutation result in cytokinin hypersensitivity and abnormal cell proliferation in the shoot apex of *Arabidopsis* seedlings. This gain-of-function mutation can be engineered by deleting the receiver domain from

the ARRs, suggesting that the receiver domain is a repressor domain (Imamura, et al., 2003; Sakai, et al., 2001; Tajima, et al., 2004). A putative loss-of-function allele of *ARR1* showed reduced cytokinin sensitivity in the root elongation and in the callus growth assays, further supporting the role for type-B ARR*s* as positive elements in cytokinin signalling (Sakai, et al., 2001).

In addition to the true *ARR*s, there are also several *ARABIDOPSIS PSEUDO RESPONSE REGULATORS* (*APRR*s) in the *Arabidopsis* genome that encode proteins that contain a receiver domain, but lack the conserved Asp residue for phosphorylation. Many of the APRRs have been implicated in regulating normal circadian rhythm in plants (for review, see Mizuno, Nakamichi, 2005).

2.4.4 Histidine phosphotransfer proteins

A number of studies indicate that the five *Arabidopsis* histidine phosphotransfer proteins, AHP1 – AHP5 act as signalling intermediates in the phosphorelay operating cytokinin signalling. AHP1, AHP2 and AHP3 complement yeast *S. cerevisiae* HPt protein knock-out, *ypd1*, indicating that they can act as phosphorelay intermediates in yeast (Miyata, et al., 1998; Suzuki, et al., 1998). Yeast two-hybrid assays suggest that every AHP can interact with the type-B ARRs, such as ARR1 and ARR2, but not with the type-A ARRs, ARR3 and ARR4 (Imamura, et al., 1999; Lohrmann, et al., 2001; Suzuki, et al., 2001b; Tanaka, et al., 2004; Urao, et al., 2000). AHPs accept phosphoryl groups from *E. coli* membrane fraction and subsequently can transfer them to the type–B and unexpectedly to the type-A ARRs *in vitro* (Imamura, et al., 2001; Imamura, et al., 2003; Suzuki, et al., 1998; Tanaka,

et al., 2004). Furthermore, AHPs can compete for phosphotransfer with an *E. coli* HPt protein *in vivo* (Suzuki, et al., 2001a; Suzuki, et al., 2002). However, no *in vitro* phosphotransfer from cytokinin receptors to AHPs has been reported. Arabidopsis seedlings overexpressing *AHP2* were slightly hypersensitive specifically to cytokinins, further supporting the role of AHPs as positive mediator in cytokinin signalling (Suzuki, et al., 2002).

2.4.5 Cytokinin signal transduction

A model for the cytokinin signalling pathway, and especially the different roles of type-A and type-B ARRs in cytokinin signalling, have been suggested by two laboratories **(Figure 8)** (Hwang, Sheen, 2001; Sakai, et al., 2001). Hwang and Sheen used a transient expression system of *Arabidopsis* mesophyll protoplasts to study the roles of the above mentioned components in cytokinin signalling (Hwang, Sheen, 2001). First, they confirmed that the CRE-family receptors mediated cytokinin responses. Overexpression of the receptors in the protoplast system induced the expression of *ARR6promoter::LUC*, a reporter for cytokinin primary responses, in a cytokinin-dependent manner. Next, they showed with an AHP-GFP protein fusion that AHP1 and AHP2, but not AHP5, translocate to nucleus following cytokinin application, suggesting that AHPs function as shuttles that carry the phosphoryl groups from the cytoplasm to the nucleus **(Figure 8)**. Furthermore, they showed that type-A and type-B ARRs may have different roles in the cytokinin signalling. Overexpression of type-B *ARR*s (*ARR1, ARR2* and *ARR10*) resulted in induction of *ARR6promoter::LUC* expression, a

type-A ARR reporter. This induction was further induced by cytokinin application. Conversely, the overexpression of type-A ARRs (*ARR4* and *ARR7*) inhibited cytokinin-mediated induction of *ARR6promoter::LUC* (Hwang, Sheen, 2001). These results combined with the data discussed in chapter 2.4.3 demonstrate that type-B ARRs are transcriptional activators and type-A ARRs are transcriptional repressors of cytokinin signalling. Moreover, these data imply that these two types of response regulators form a negative feedback mechanism **(Figure 8)**. Sakai and colleagues demonstrated using a glucocorticoid inducible system that
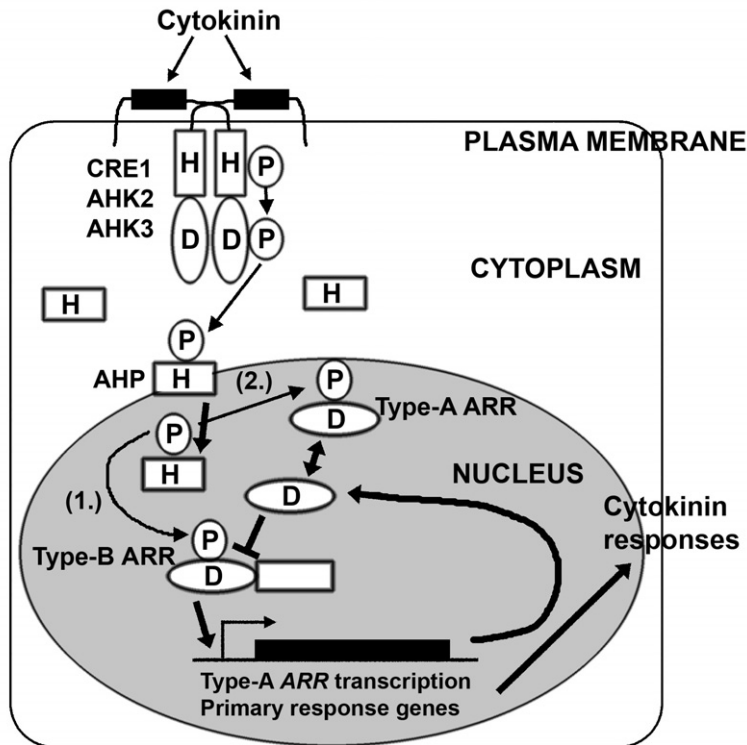
Figure 8. *A model of cytokinin signal transduction in Arabidopsis.* Cytokinin is perceived by the CHASE domain of the three CRE-family cytokinin receptors. Cytokinin binding leads to autophosphorylation of a His residue within the transmitter domain of a cytokinin receptor upon which the phosphoryl group is transferred intramolecularly to an Asp residue in the receiver domain of the sensor. From a cytokinin receptor the phosphoryl group is transferred to a His residue on an AHP protein. Phosphorylated AHP then translocates to the nucleus and transfers the phosphate to an Asp residue in a type-A and/or type-B response regulator. The type-B ARR receiver domain negatively regulates its own transactivation domain. It is possible that following phosphorylation (1.), this repression is relieved and type-B ARRs are able to induce expression of type-A and other primary response genes. Type-A ARRs negatively regulate cytokinin signalling and therefore their own expression. Another possibility is that AHP transfers the phosphate to a type-A ARR (2.), which would then relieve type-B ARR to activate transcription. Modified from Kakimoto, 2003.

type-B ARRs can directly activate type-A ARRs (Sakai, et al., 2001). *Arabidopsis* was transformed with a construct containing a glucocorticoid-binding domain fused to the gain-of-function version of ARR1, a type-B ARR, which lacks the receiver domain. When ARR1 fusion protein is activated by glucocorticoids, it induces *ARR6* transcription. Glucocorticoid application induces *ARR6* expression even in the presence of protein synthesis inhibitors, confirming that ARR1 can bind to the binding sites present on the *ARR6* promoter, and activate *ARR6* transcription (Sakai, et al., 2001). Taken together, cytokinins activate type-B ARRs, which then activate the expression of the primary response genes **(Figure 8)**. In addition to type-A *ARR*s, several other primary response and other downstream genes have been identified through microarray and massive parallel signature sequencing analyses (Hoth, et al., 2003; Kiba, et al., 2005; Rashotte, et al., 2003). The future challenge is to investigate how these components mediate cytokinin responses to regulate various developmental and physiological processes.

The cytokinin signalling resembles the auxin signalling pathway **(Figure 3)**, even though the signalling components are completely different. In both signalling pathways, a subset of primary response genes serves as negative regulators of the pathway; type-A ARR in cytokinin and AUX/IAA in auxin signalling pathway. Both negative regulators have domains homologous to the transcription factors that activate their transcription; type-A and type-B ARRs have similar receiver domains, and AUX/IAAs and ARFs similar domains III and IV. However, hormone perception differs significantly between these two pathways. Auxin directly promotes SCF^{TIR1} – AUX/IAA interaction in nucleus,

which leads to AUX/IAA destruction by the 26S proteasome, and subsequent dimerization and activation of ARFs **(Figure 3)**. Conversely, cytokinin is perceived most probably at the cell surface by receptor histidine kinases, which activates type-B ARRs through a phosphorelay. Bacterial response regulators have been shown to be dimers as the result of phosphorylation (Da Re, et al., 1999). Therefore, it is possible that phosphorylation activates dimerization also in cytokinin signalling pathway. Phosphorylation of type-A ARRs could release type-Bs to homodimerize and activate transcription **(Figure 8)**. However, no evidence indicating that ARRs could dimerize has emerged. It is interesting to notice that in addition to auxin signalling and many other phytohormone signalling pathways, cytokinin signalling might also utilize 26S proteasome mediated degradation in its signalling (Smalle, et al., 2002). However, the exact role of proteasome mediated degradation related to cytokinin signalling is currently unknown.

2.5 Roles of cytokinins in development

During the last 50 years, cytokinins have been implicated in many plant growth and developmental processes. Cytokinins induce cell division, chloroplast development and *de novo* shoot formation, promote seed germination, release buds from apical dominance, stimulate leaf expansion, delay senescence, increase sink strength and regulate vascular development (for review, see Mok, Mok, 1994; Mok, Mok, 2001). In most of these early studies exogenously applied cytokinin has been the experimental approach to understand the role of the phytohormone in plants. Since cytokinin application can cause

secondary defects, the endogenous role of cytokinins in various biological processes remains to be verified. During the last two decades researchers have taken advantage of the modern genetic and transgenic techniques in combination with the classical techniques to unravel the exact roles of the phytohormone.

2.5.1 Cytokinins in cell proliferation

Skoog and Miller described the hormonal control of *de novo* organ generation in plants (Skoog, Miller, 1957). They showed that the concentration ratio of two phytohormones, auxin and cytokinin, determines the type of organ generated from the undifferentiated *in vitro* callus tissue. High cytokinin to auxin ratio usually favours shoot formation, while low cytokinin to auxin ratio favours root generation. Equal concentrations of the phytohormones result in the proliferation of undifferentiated callus. These regeneration techniques have been crucial for the development of plant transformation systems.

Numerous studies indicate that the key mechanism through which cytokinins regulate development, is promoting cell proliferation. The first visible change in the *de novo* shoot regeneration from root explants is that periclinal cell divisions occur in the pericycle layer (Cary, et al., 2002). This is followed by further cell proliferation, development of chloroplasts and upregulation of several genes required for shoot apical meristem development, such as *CUP SHAPED COTYLEDON* and *Class1 Knotted1-like homeobox (KNOX)* genes, *SHOOTMERISTEMLESS* and *KNAT1*, suggesting that cytokinins act upstream of these genes in shoot development (Cary, et al., 2002).

Supporting these results, overexpression of *KNAT1* leads to such phenotypes as reduced apical dominance and ectopic shoot formation, which are reminiscent of the transgenic plants overproducing cytokinins (Kerstetter, Hake, 1997; Rupp, et al., 1999). Furthermore, *KNOX* genes are sufficient to elevate cytokinin levels and to induce the expression of cytokinin biosynthesis genes, *AtIPT5* and *AtIPT7*, as well as the cytokinin primary response gene, *ARR5* (Jasinski, et al., 2005; Yanai, et al., 2005). Taken together these data demonstrate the intimate interplay between the phytohormone cytokinin and KNOX transcription factors in promoting shoot meristem activity and maintenance. In addition, since the cytokinin receptor CRE1 was identified from a mutant screen for inability to proliferate callus tissue, cytokinins clearly have a central role in cell proliferation (Inoue, et al., 2001). However, the role of cytokinin in organ regeneration could also be to promote meristematic competence, after which many factors, including cytokinins, are able to promote cell division. Since it is difficult to distinguish between the direct promotion and the competence for cell division, the question concerning the possible dual role of cytokinins remains to be answered.

In order to understand the role of cytokinins in whole plants, cytokinins were depleted globally from *Nicotiana tabacum* and *Arabidosis* by overexpressing *CKX* genes under the control of the CaMV 35S promoter (Werner, et al., 2001; Werner, et al., 2003). Transgenic plants showed retarded growth in the shoot apical and floral meristems. These phenotypes were caused mainly by reduced rate of cell division. Root growth, however, was significantly increased in these lines due to an increased number of

meristematic cells in the root apical meristem, suggesting that cytokinins may have an opposite role in the root growth. Contrasting the previous, well-established findings (Gan, Amasino, 1995; Mok, Mok, 1994), the transgenic plants showed decreased apical dominance and they senescenced later. Even though the transgenic plants had less than half of the total cytokinin content, the local concentrations of cytokinins related to the phenotypes could be different. In addition, the balance between different types of cytokinins must be altered, because some cytokinins, such as BA, are resistant for cytokinins oxidases (see 2.2). Therefore, detailed analysis of mutants lacking cytokinin signalling or synthesis is required to solve the discrepancy.

The cell cycle consists of a round of DNA replication (S phase) followed by mitosis and cytokinesis (M phase). These two phases are separated by two gap phases (G_1 and G_2). Current data suggest that cytokinins are promoting G_1–S and G_2–M transitions (for review, see De Veylder, et al., 2003; Inze, 2005). Cytokinins have been shown to induce transcription of a cyclin D3, whose accumulation initiates G_1–S entry in the cell cycle (Soni, et al., 1995). The induction was not inhibited by the addition of cycloheximide, indicating that the cytokinin signalling induces cyclin D3 transcription directly, without further protein synthesis (Riou-Khamlichi, et al., 1999). However, inhibitors of protein phosphorylation inhibited cytokinin meditated cyclin D3 induction. Constitutive overexpression of cyclin D3 results in cytokinin independent proliferation of calli in an *Arabidopsis* tissue culture (Riou-Khamlichi, et al., 1999). In addition, the transgenic tissue turned green similarly to a cytokinin treated tissue, indicating that cytokinins induce chloroplast development through cyclin D3. However, the tissue failed to develop shoots both in the presence and absence of cytokinins. Interestingly, the cyclin D3 overexpressors showed delayed senescence, similarly to the transgenic plants overproducing cytokinins. These transgenic lines did not show elevated cytokinin levels, demonstrating that cyclin D3 can independently promote tissue proliferation (Riou-Khamlichi, et al., 1999). Taken together, cytokinins promote cell division directly by inducing the expression of cyclin D3, the promoter of G_1–S transition.

Various data indicates that cytokinins regulate also G_2–M transition. In the cytokinin-autonomous tobacco cell line, BY2, an endogenous cytokinin peak around S and M phases (Redig, et al., 1996). When cytokinin accumulation is inhibited, BY2 cells can not enter mitosis. However, inhibited BY2 cells are able to resume mitotic progress if zeatin is added, indicating that the cytokinin is indispensable for G_2–M transition (Laureys, et al., 1998). G_2–M transition is mediated by activation of CYCLIN-DEPENDENT PROTEIN KINASE (CDK). Cultured cells of *Nicotiana plumbaginifolia* are unable to enter mitosis without cytokinin application, because CDK is phosphorylated to its tyrosine residue and therefore it remains inactive. However, cytokinin action can be replaced by expressing a tyrosine phosphatase, *cdc25*, in the cell culture, indicating that cytokinins promote G_2–M transition through cdc25 (Zhang, et al., 2005).

2.5.2 Cytokinins in vascular development

When isolated *Zinnia elegans* mesophyll cells are cultured *in vitro* in the presence of auxin and cytokinin, they differentiate at high frequency into tracheary elements (TEs) (Fukuda,

1997). It is believed that cytokinins and auxin work together in this transdifferentiation process first to maintain procambial activities and subsequently to promote TE differentiation (Church, Galston, 1988; Fukuda, 2004). Cytokinin is also a limiting and controlling factor in the formation of TEs and phloem sieve elements around a wound in excised internodes of *Coleus blumei* (Aloni, et al., 1990; Baum, et al., 1991). Auxin application alone exhibited a small number of TE and sieve element formation. However, auxin application together with cytokinin markedly increased vascular differentiation around a wound. Later stages of the vascular differentiation seemed to occur even without applied cytokinin (Aloni, 1982).

AIMS OF THE STUDY

1) To identify the gene underlying the *wooden leg* (*wol*) phenotype

2) To perform a suppressor screen for the determinate growth habit of *wol*, and find new factors regulating vascular morphogenesis

3) To have a deeper understanding of the vascular development in *Arabidopsis* root by characterising suppressor mutations

MATERIALS AND METHODS

The materials and methods are described in detail in the respective publications. **Table1** summarises the mutants employed in this study; the types of mutations, the ecotypes to which mutations were introduced, and the references to the publications in which the mutants have been studied. The methods employed in this study are summarised in **Table2** with references to the publications in which they have been applied.

Table1. The *Arabidopsis thaliana* mutants used in this study

Relevant mutant genotype	Type of mutation	Ecotype	Publication
wol	a point mutation in the receptor domain of CRE1	Columbia	I, III, IV
cre1-2	T-DNA insertion*	Columbia	IV
cre1-10	T-DNA insertion*	Wassilewskija	II
cre1-11	T-DNA insertion*	Wassilewskija	II, IV
cre1-12	T-DNA insertion*	Columbia	II, III, IV
ahk2-1ms	T-DNA insertion*	Wassilewskija	II, IV
ahk2-2tk	T-DNA insertion*	Columbia	II, III, IV
ahk3-1ms	T-DNA insertion*	Wassilewskija	II, IV
ahk3-2	T-DNA insertion*	Columbia	II, III, IV
ahk3-3	T-DNA insertion*	Columbia	II
ahp6-1	a point mutation resulting in a premature stop codon in frame	Columbia	III
ahp6-2	a point mutation, affects splicing	Columbia	III
ahp6-3	T-DNA insertion*	Columbia	III
fass	a point mutation in a gene (At5G18580) with unknown function	Columbia	I

* T-DNA insertion leads to partial or complete loss-of-function

Table 2. The methods used in this study

Methods	Publication
Confocal light microscopy	(I), (II), III, IV
Ethylmethane sulfonate (EMS) mutagenesis and mutant screen	III, IV
Fuchsin staining	(I), III, IV
Gene identification through positional cloning	I, III
Genetic crossing of *Arabidopsis*	I, II, III, IV
Histological staining for GUS activity	(II), III, IV
In situ RNA hybridisation	(I), III, IV
In vitro phosphotransfer assay	(III), (IV)
Northern blot analysis	I, (II)
Phase contrast microscopy	(I), III, IV
Plasmid construction	I, (II), III, IV
Polymerase chain reaction (PCR) analysis	I, II, III, IV
Quantitative real-time PCR analysis	II, (III), IV
Sectioning of plastic embedded samples	I, II, III, IV
Site-directed mutagenesis	III, IV
Tissue culture assays for cytokinin response	(II), (III), (IV)
Transformation of *Arabidopsis* using floral dipping method	I, (II), III, IV
Yeast assay for kinase and phosphatase activities of CRE1	(IV)
Western blot analysis	IV

Methods in brackets were performed by co-authors in the particular publications

RESULTS AND DISCUSSION

1. CYTOKININS REGULATE VASCULAR CELL IDENTITIES, AND PROLIFERATION OF PROCAMBIAL CELL FILES, THROUGH CRE-FAMILY CYTOKININ RECEPTORS

The vascular system of *Arabidopsis thaliana* root has a central axis of the xylem cell files, consisting of the marginally located protoxylem and centrally located metaxylem. The xylem axis is flanked by the phloem and the intervening procambial cell files. All the vascular cell files originate from the procambial provascular stem cells located above the QC cells **(Figure 9A)**.

1.1 *WOL* regulates vascular cell identities and periclinal cell divisions in the procambium

The recessive *wooden leg* (*wol*) mutation results in a reduced number of vascular cell files in the root, and all of these vascular cell files differentiate as xylem **(Figure 10)**, which in turn results in determinate root growth (Scheres, et al., 1995). The cell division defect is evident already in mature embryos, and it appears also in adventitious roots, suggesting that the *WOL* gene product regulates vascular cell divisions throughout plant development (Scheres, et al., 1995). In order to fully understand the cell division defect in *wol*, and the subsequent loss of phloem and intervening procambium, the vascular cell lineage relationships were determined in the primary root of wild-
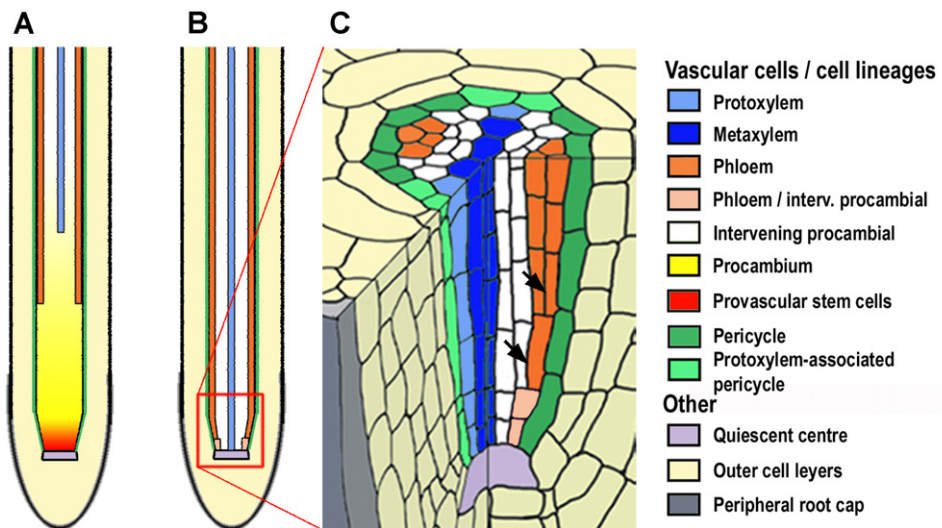
Figure 9. *Schematic presentations of the cell types and cell lineages in Arabidopsis root vascular bundle.* **(A)** Cell types in vascular bundle. Vascular cells are originated from the provascular stem cells. A proportion of the procambial cell files remain undifferentiated between the xylem and the phloem to form the intervening procambium, which is later activated to develop the lateral meristem, cambium. Phloem is differentiated before protoxylem. **(B, C)** Vascular cell lineages. Protoxylem cell files can be traced down to QC, whereas phloem cell files and a proportion of intervening procambial cell files originate as a result of asymmetric cell divisions (C, arrows).

type and *wol* (**I, Fig. 1; Table 1**). Serial transverse section analysis of the primary root meristem revealed that the xylem cell lineages form an axis composed of 4-5 cell files adjacent to the underlying quiescent center (QC) (**Figure 9C**) (**I, Fig. 1C,** schematic). Phloem and a proportion of intervening procambial cell lineages, however, appeared to originate as a result of asymmetric, periclinal cell divisions in the procambium (**Figure 9C**) (**I, Fig. 1C-G, J,** schematic). Consistent with the previous findings (Esau, 1977), the phloem sieve elements differentiated earlier than the xylem elements (**I, Fig. 1H, I,** schematic). However, xylem was specified earlier than phloem, as the xylem cell lineages were traced right above QC, while the phloem cell lineages were established later as a result of asymmetric cell divisions (**Figure 9**) (**I, Fig. 1,** schematic).

The periclinal cell divisions in procambium were largely absent in *wol* (**I, Fig. 1J, K** schematic; **Table 1**). In addition, the number of provascular stem cells above the QC were slightly lower in *wol* compared to wild-type, supporting the earlier finding that some of the embryonic divisions required for the formation of the normal number of provascular stem cells do not occur in *wol* (**Table 1**) (Scheres, et al., 1995). Taken together, the *WOL* gene product regulates the procambial periclinal cell divisions that are required for proper intervening procambium proliferation and for phloem specification.

1.2 *WOL* encodes a putative sensor histidine kinase

In order to understand the molecular nature of the *wol* phenotype, the *WOL* gene was identified through positional cloning (**I, Fig. 3A**). Analysis of 416 individuals of F2 mapping population restricted the *WOL* locus to an 11 kb region situated between two CAPS (cleaved amplified polymorphic sequences) molecular markers, nga1145 and RNS1, located in the top of chromosome two. This region contains only one annotated open reading frame, T23K3.2 (At2g01830), encoding a putative sensor histidine kinase. Sequencing of the entire 11 kb region revealed only one point mutation, conversion of Thr_{278} to Ile_{278} in the predicted open reading frame. The *wol* mutant phenotypes were fully complemented by a wild-type genomic fragment that contained the entire *WOL* gene, indicating that the Thr_{278} to Ile_{278} mutation in T23K3.2 underlies the *wol* phenotype (**I, Fig. 3C**).

Next, the coding region of *WOL* was identified using RT-PCR and 5'RACE-PCR techniques. Three transcripts were found, that corresponded to three different transcription start sites (**I, Fig. 3B**). Two of them had the same translational start site resulting in an identical open reading frame of 1057 amino acids. Later, it was shown that the third transcript had a start codon slightly earlier, resulting in a 23 amino acid extension in the N-terminus (Inoue, et al., 2001). The predicted WOL protein has an apparent sequence similarity to hybrid histidine kinases, involved in two-component signalling, suggesting that *WOL* may be a signal transducer. The predicted WOL consists of a short cytoplasmic N-terminal region, followed by an extracellular putative receptor domain flanked by two transmembrane domains, and a C-terminal cytoplasmic histidine kinase domain followed by two receiver domains (**I, Fig. 3D**). Interestingly, the *Arabidopsis* genome contains two other hybrid histidine kinases, MXH1.16 (At5g35750) and F17L21.11 (At1g27320), with high similarity to *WOL* throughout the protein sequence.

Most importantly, these WOL protein family members have a very similar predicted extracellular receptor domain, the CHASE domain, possibly involved in ligand binding (**I, Fig. 3E**) (Anantharaman, Aravind, 2001; Mougel, Zhulin, 2001). The receptor domain of a *Dictyostelium discoideum* sensor histidine kinase DhkA is 24% identical to the putative *WOL* receptor domain (Wang, et al., 1996), further supporting the idea that the WOL protein family members encode receptor molecules. The Thr_{278} residue mutated in *wol* is located in the putative receptor domain and is conserved among the WOL protein family (**I, Fig. 3E**), indicating that the recessive *wol* mutation may reduce the ability of the receptor to bind a ligand.

1.3 *WOL* is expressed in procambium

Organ and tissue specificity of *WOL* expression was determined using a gene specific probe (**I, Fig. 3B**). The RNA blot analysis revealed a single major band of ~3.7 kb, which is in accordance with the sizes of the *WOL* transcripts. The expression appeared to be more abundant in the root than in the shoot (**I, Fig. 3F**). The tissue specificity of *WOL* was determined by *in situ* localisation of the mRNA in wild-type embryonic and root sections (**I, Fig. 4**). In the globular stage embryo the expression of *WOL* first appeared in the four innermost cells, the provascular cells (**I, Fig. 4D**). During the heart, torpedo and nearly mature stages of embryogenesis, *WOL* mRNA was detected consistently from the procambium of cotyledon shoulders, future hypocotyl, and embryonic root (**I, Fig. 4E-G**). Post-embryonically, *WOL* expression was restricted to the procambial and the pericycle cells of the root meristem, consistent with the *wol* phenotype apparent in these cells

(**I, Fig. 4A, B**). *WOL* was expressed also in *wol* background suggesting that the gene product does not regulate its own expression. Taken together, *WOL* expression coincides, both spatially and temporally, with the divisions of the procambial cells of the embryonic and primary root that are defective in *wol* mutant. Additionally, *WOL* expression coincides with the vascular cell specification defect as well, as a faint *WOL* expression extends to the region where vascular cells begin to differentiate. Therefore, WOL may be a receptor molecule that controls vascular cell identities and asymmetric cell divisions in procambium through a specific signal transduction pathway involving a phosphotransfer reaction, characteristic of two-component molecules.

1.4 WOL is allelic to CRE1, a cytokinin receptor

Three groups reported independently that they have identified a cytokinin receptor called CRE1 or AHK4 (Inoue, et al., 2001; Suzuki, et al., 2001a; Ueguchi, et al., 2001b) (For more details, see section 2.4.2 of Introduction). *CRE1/AHK4* encodes a histidine kinase which turned out to be identical to *WOL*. Moreover, the two other WOL family members, MXH1.16 and F17L21.11 are identical to AHK2 and AHK3, respectively, which also seem act as cytokinin receptors (M. Higuchi & T. Kakimoto, personal communication) (Ueguchi, et al., 2001a; Yamada, et al., 2001). Hereafter, these three histidine kinases are called the CRE-family cytokinin receptors. It was shown that the putative receptor domain, the CHASE domain, of CRE1/WOL/AHK4 (hereafter CRE1/WOL or just CRE1) is indeed a receptor domain, because it binds cytokinins. Interestingly, introduction of the *wol* mutation, Thr_{278}

to Ile$_{278}$, to the CHASE domain abolished cytokinins binding (Yamada, et al., 2001). Recent 3-D modelling data of the CHASE domain suggest that the Thr$_{278}$ residue is located in the binding pocket of the CHASE domain and may directly interact with the ligand (Pas, et al., 2004). Taken together, these data suggest indirectly that vascular cell identities and the periclinal cell divisions in the procambium are regulated by cytokinins through the CRE1 cytokinin receptor. Furthermore, CRE1(T278I) (Thr$_{278}$ to Ile$_{278}$) mutation in *wol* results in the inability to sense cytokinins, and may therefore be the reason for the impaired procambial development.

1.5 Cytokinins promote procambial cell maintenance and proliferation, and inhibit protoxylem identity

In order to directly determine whether cytokinins regulate vascular morphogenesis, cytokinins were depleted from wild-type procambium by overexpressing *CYTOKININ OXIDASE2* (*CKX2*) under the control of the strong procambium specific promoter, *CRE1*. The resultant transgenic lines exhibited a similar phenotype to *wol*; determined root growth, reduced vascular cell file number and differentiation of all the root vascular cells to protoxylem (**IV, Fig. 1G**). This demonstrates that cytokinins regulate procambial cell proliferation and vascular cell identity.

As mentioned earlier, in addition to the cell division defect, *wol* mutants also show a defect in cell specification, since all the vascular cell files differentiate as xylem. Scheres and colleagues analyzed the role of CRE1/WOL in phloem specification by crossing *wol* to *fass*: a mutation resulting in supernumerary cell layers (Scheres, et al., 1995). The double

mutant showed increased number of cell files, and phloem markers were observed in the vascular bundle, suggesting that the primary role of CRE1/WOL is to regulate procambial cell divisions. The vascular bundle of wild-type roots contains two types of xylem: the protoxylem, which differentiates early, and the metaxylem, which differentiates later on **(Figure 9)**. Conversely, the *wol* vascular bundle contains only protoxylem (**I, Fig. 2B**) (Cano-Delgado, et al., 2000), raising the possibility that *wol* promotes metaxylem specification. However, a closer histological analysis revealed that both the protoxylem and the metaxylem were present in the *wol* x *fass* double mutant (**I, Fig. 2C**). Collectively, the analysis of the *wol* x *fass* double mutant suggests that the primary role of CRE1/WOL, and therefore cytokinins, is to promote periclinal cell divisions in the procambium. A proportion of these cell divisions is asymmetric, and gives rise to the intervening procambium and phloem cell lineages. As the *wol* mutation results in lack of these asymmetric cell divisions, phloem cell lineages fail to be established. However, like the protoxylem, the metaxylem cell files are also specified early, and yet *wol* contains only protoxylem. This may be explained by the fact that metaxylem differentiates later, and therefore protoxylem may use up the restricted space. However, despite of the restricted space, *wol* mutant contains even more protoxylem cell files than wild-type (wild-type, 2; *wol*, ~9), leaving open the possibility that cytokinins may have other roles in vascular morphogenesis in addition to their role in promoting procambial cell divisions.

In order to circumvent the problem of restricted space in the vascular cylinder of *wol* and seedlings expressing

CRE1promoter::*CKX2*, cytokinins were depleted post-embryonically using an inducible system (Zuo, et al., 2000). Oestrogen receptor fused to a DNA binding domain was expressed under the control of the *CRE1* promoter (**III, Fig. S1A**). Introduction of oestrogen led to induction of *CKX1-YFP* expression, and subsequently cytokinin degradation. When transgenic seedlings were germinated on media containing oestrogen, all the vascular cell files differentiated as protoxylem (**III, Fig. 1B, S1**). Importantly, the number of vascular cell files remained similar to the wild-type, demonstrating that the reduced cell number is not a prerequisite for exclusively protoxylem differentiation. Furthermore, when wild-type seedlings were germinated on media containing cytokinins, protoxylem differentiation was severely impaired, or even completely missing from the root (**III, Fig. 1B, S2A, S4B**). Reinforcing this result, an enhancer trap line J0121 (Laplaze, et al., 2005), that highlights the protoxylem-associated pericycle cells, was also absent as a result of cytokinin treatment (**III, Fig. 2E, S2C**) (**Figure 10**). These results indicate that cytokinins are required to promote and maintain cell identities other than protoxylem; in the absence or in low concentration of cytokinin protoxylem is the default identity.

In contrast to the above, analysis of the *wol* x *fass* double mutant suggests that the primary role of CRE1/ WOL and therefore cytokinins is to promote cell proliferation in root procambium. However, *fass* is a pleiotropic mutant, and it exhibits typically even more cell layers than does wild-type (Torres-Ruiz, Jurgens, 1994). Thus, the effect of the *wol* mutation in *fass* may be diluted, and functional redundancy may take over the role of CRE1/WOL. The absence of

cell divisions in *wol* may be explained by the specification of all the cell files, at an early stage, as protoxylem. Consequently, the protoxylem identity would lead to the absence of the periclinal cell divisions. Supporting this hypothesis, serial transverse section data imply that the wild-type root protoxylem cell files undergo periclinal cell divisions less often than other cell files in the procambium (A.P. Mähönen, unpublished data). As a result of the *wol* mutation there are less vascular cell files already during embryogenesis (Scheres, et al., 1995). Since the protoxylem differentiates post-embryonically, the embryonic cell division defect of *wol* may be independent of the vascular cell identity defect. Therefore, cytokinins may play an additional role in promoting cell proliferation in root procambium through CRE1/WOL after all. However, the protoxylem cells may be specified already during embryogenesis, and thus restrict cell proliferation already at this point.

1.6 *wol* negatively regulates normal procambial development

In order to discover new factors regulating vascular morphogenesis in *Arabidopsis*, a suppressor screen for the determinate root growth habit of *wol* was carried out. Several suppressor mutations were identified. Genetic experiments revealed that among sixteen suppressors, thirteen appeared to be intragenic suppressors, *i.e.* second site mutations in *CRE1(T278I)* (**IV, Fig. 1A**). Interestingly, five of these mutations were due to an introduction of either a new splice site or a stop codon in frame within the *CRE1(T278I)*. Furthermore, backcrosses to *wol* revealed that the five mutations disrupting *CRE1(T278I)* were recessive over *wol* (**IV, Table S1**). Transverse

sections demonstrated that the vascular bundles of the disruptants were similar to wild-type, with all the vascular cell types present (**IV, Fig. 1D**). These results indicate that a disruption of the *CRE1(T278I)* gene leads to the reversion of the all-protoxylem phenotype of *wol* to wild-type. Moreover, two T-DNA insertion alleles of *CRE1, cre1-2 and cre1-11* (**IV, Fig. 1E, S1**) exhibited wild-type vascular bundles, and they both were recessive over *wol* (**IV, Table 1**). Taken together, these results indicate that *wol* is not a loss-of function mutation as was first assumed by the recessive nature of the mutation. In fact, *wol* possesses a dose-dependent activity that negatively affects proliferation of procambial cell files. The various disruptive intragenic mutations act to eliminate this negative activity.

1.7 *CRE1/WOL*, *AHK2* and *AHK3* are mutually necessary for normal vascular morphogenesis

1.7.1 CRE-family triple mutant phenocopies *wol* in root vascular bundle

cre1-2 and *cre1-11*, the putative null alleles of *CRE1*, display wild-type vascular bundles, suggesting that CRE1 acts redundantly with other factors to regulate vascular morphogenesis. Alternatively, CRE1 function may be unrelated to vascular development, and the negative activity exerted by *CRE1(T278I)* could be due to unexpected interactions with downstream components involved in vascular morphogenesis. As discussed earlier, there are two other CRE-family cytokinin receptors, AHK2 and AHK3, in the *Arabidopsis* genome (**I, Fig. 3E**)

(Ueguchi, et al., 2001a). In order to understand the role of CRE1 in vascular morphogenesis, single, double, and triple mutant combinations of the three receptors were made. Plants with a single T-DNA insertion mutation grew normally and had normal vascular bundles in the root (**II, Fig. 11; IV, Fig. S1**). *cre1-12 ahk2-2tk*, *cre1-12 ahk3-3*, *cre1-10 ahk2-1ms*, and *cre1-10 ahk3-1ms* mutations displayed no obvious seedling phenotypes (**II, Fig. 11**), whereas the *ahk2-2tk ahk3-3* and *ahk2-1ms ahk3-1ms* double mutants had smaller leaves and shorter stems than the wild-type plants (**II, Fig. 11**). Vascular bundles of a few double mutants, such as *cre1-12 ahk3-3*, frequently exhibited ectopic protoxylem formation in the vicinity of the existing protoxylem file (**III, Fig. 1B**). Unexpectedly, triple mutants lacking every CRE-family cytokinin receptors, both in the Wassilewskija (Ws) background (*cre1-11 ahk2-1ms ahk3-1ms*, and *cre1-10 ahk2-1ms ahk3-1ms*) and in the Columbia (Col) background (*cre1-12 ahk2-2tk ahk3-3*), germinated and possessed basic organs: root, shoot and stem (**II, Fig. 3D, 11**) (Nishimura, et al., 2004). Primary roots of *cre1-12 ahk2-2tk ahk3-3* and *cre1-11 ahk2-1ms ahk3-1ms* exhibited determinate growth, and the transverse sections of the root tip revealed severely reduced cell number in the vascular bundle (**IV, Fig. 1F, S1**). Similar to *wol*, all the cells in the vasculature of the triple mutants differentiated into protoxylem. These results indicate that CRE1, AHK2 and AHK3 together control vascular cell identities and normal proliferation of procambial cell files. The dose-dependent negative activity conferred by the *wol* allele of CRE1 acts against signalling initiated by AHK2 and AHK3.

1.7.2 CRE-family genes show distinct, yet overlapping expression patterns

In order to understand the functional redundancy of the CRE-family cytokinin receptors, the expression patterns of the three receptors were studied. Expression analysis of the *GUS* reporter gene driven under the control of *CRE1, AHK2 and AHK3* promoters, as well as RNA blot analysis, revealed distinct, yet overlapping expression patterns for the three genes throughout the plant (**II, Fig. 1**). *CRE1promoter::GUS* activity in leaves was much weaker than the activity of *AHK2promoter::GUS* and *AHK3promoter::GUS*, illuminating why only the *ahk2 ahk3* combination of the double mutants showed reduced growth in leaves. In the root tips, *CRE1promoter::GUS* was expressed strongly in procambium, *AHK2promoter::GUS* expression maximum was in the QC cells and the surrounding stem cells, while *AHK3promoter::GUS* was expressed in broader regions of root meristem and vascular cylinder (**IV, Fig. S2**). GUS expression patterns of *CRE1* and *AHK3* were consistent with their mRNA localisation shown by *in situ* RNA hybridisation (**I, Fig 4; IV, Fig. S2**). The *AHK2* transcript was below the detection limit.

1.8 Vascular phenotypes of the CRE-family mutants correlate with their cytokinin responses

1.8.1 CRE-family triple mutant is completely resistant to cytokinins

Cytokinin sensitivity of single, double and triple mutants of the cytokinin receptors were measured with various assays. Normally, applied cytokinins inhibit root elongation. However, *cre1* mutants showed reduced sensitivity to cytokinin in this assay, whereas the *ahk2* and *ahk3* mutants exhibited slightly reduced sensitivity (Inoue, et al., 2001) (**II, Fig. 4**). Additive effects were seen in the double mutants, whereas the triple mutants were completely insensitive to cytokinins (**II, Fig. 4**). Next, cytokinin response was tested in the adventitious root formation assay (Kuroha, et al., 2002); When seedling is cut in half from the root-hypocotyl junction, and the upper part is transferred onto media, adventitious roots begin to emerge from the cut end. In wild-type this process is inhibited by exogenous application of cytokinin (**II, Fig. 5**). In this assay, *cre1-12* exhibited strong insensitivity and *ahk3-3* moderate insensitivity for cytokinin, whereas *ahk2-2tk* had a similar response to wild-type. The *cre1-12 ahk3-3* double mutant was completely resistant to cytokinin, indicating that *CRE1* and *AHK3* jointly regulate cytokinin induced inhibition of adventitious root formation (**II, Fig. 5**). In the presence of auxin, cytokinins stimulate cell proliferation and greening of calli from the excised hypocotyl segments (Miller, et al., 1956). In accordance with previous studies, *cre1* mutants also exhibited reduced sensitivity for cytokinin with this assay (Inoue, et al., 2001) (**II, Fig. 6**). In contrast, cytokinin response in *ahk2* and *ahk3* mutants were similar to wild-type. Double mutant combinations showed additive effects for cytokinin sensitivity, and *cre1-12 ahk2-2tk ahk3-3* was completely resistant to cytokinin (**II, Fig. 6**). A high ratio of cytokinin to auxin concentrations in media favours shoot formation from wild-type plant segments (Skoog, Miller, 1957). All the single mutants responded like wild-type in this assay, whereas double mutants showed reduced sensitivity for cytokinin (**II, Fig. 12**). The triple mutants were not tested with this assay.

Next, cytokinin response was tested with molecular markers. Following cytokinin application, transcription of type-A response regulators, such as *ARR5* and *ARR15*, is upregulated rapidly, without *de novo* protein synthesis (see 2.4.3 for details). If the CRE-family cytokinin receptor triple mutant is completely resistant to cytokinins, as the aforementioned assays suggest, expression of the primary response genes *ARR5* and *ARR15* should not be changed following cytokinin induction. Therefore, following 30 min benzyladenine induction, double RNA samples and reverse transcription (RT) reactions were prepared both from the wild-type and from *cre1-12 ahk2-2tk ahk3-3*. Subsequently, quantitative real-time PCR analysis was performed from the cDNA samples. As expected, in wild-type cytokinin treatment induced *ARR5*

and *ARR15* expression ~14 and ~13 fold, respectively (**II, Fig. 7**). Conversely, cytokinin treatment caused no change to the expression of the primary response genes in *cre1-12 ahk2-2tk ahk3-3*, confirming that the CRE-family triple mutant is completely resistant to cytokinins. In a nearly simultaneous publication with (**II**), another set of single, double and triple mutants of CRE-family genes were generated and analysed, and essentially the same conclusions were made (Nishimura, et al., 2004).

Root vascular bundle of the CRE-family triple mutant, as well as *CRE1promoter::CKX2* and *wol*, exhibit the same phenotype: a reduced number of cell files, from which all differentiate as protoxylem (**Figure 10**). Since CRE-family triple mutant is completely resistant to cytokinins, the above mentioned phenotype describes
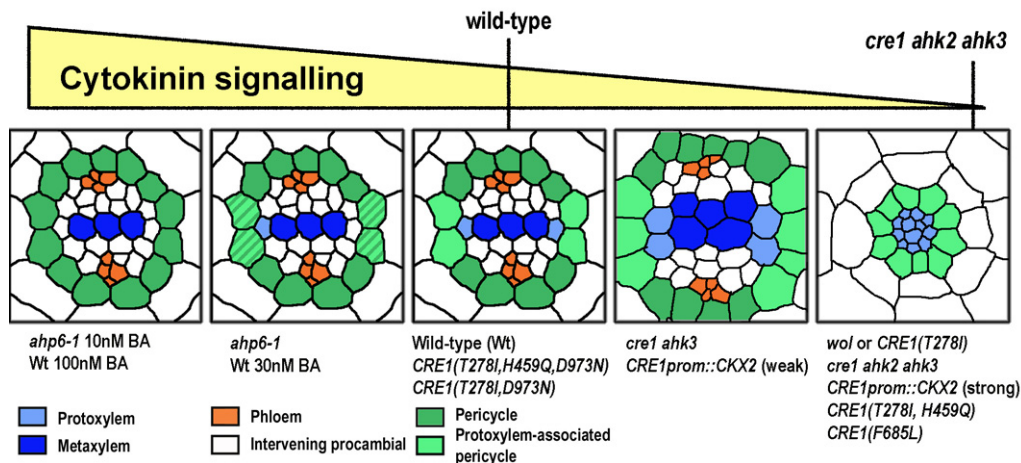
Figure 10. *The number of the protoxylem cell files present in various roots correlate with their level of cytokinin signalling and expression of AHP6 and J0121.* Schematic presentations for the transverse sections of the primary root tip. *AHP6* expression coincides with the protoxylem cells (light blue) and with the protoxylem-associated pericycle cells (light green), while the expression of enhancer trap line, J0121, coincides with the protoxylem-associated pericycle cells. Note, the expression of these two markers have been examined at least in one representative root of each five phenotypes, but not every listed genotype has been examined. Only J0121 marker expression in *cre1-12 ahk3-3* phenotype has not been examined. BA, benzyladenine (a cytokinin).

a situation when there is no cytokinin signalling in the root procambium. Taken together, cytokinin signalling through CRE-family cytokinin receptors is required for regulating vascular cell identities and for normal proliferation of procambial cell files.

1.8.2 *wol* shows reduced sensitivity to cytokinins

As discussed before, *wol* has a negative activity for proliferation of procambial cell files. A recent report suggests that the negative activity exerted by the *wol* allele of *CRE1* is independent from the canonical cytokinin signalling (de Leon, et al., 2004). Global cytokinin response of *wol* alleles were compared with that of *CRE1* putative null alleles using various assays. For example, *wol* seemed to be slightly more sensitive to cytokinins than putative null alleles, when type-A ARR inducibility for whole seedlings were assayed (de Leon, et al., 2004). As described earlier, *wol* phenotype is observed in the procambium of the root tip where the *CRE1* expression dominates. In order to study the cytokinin response where the vascular defect of *wol* is observed, RNA was isolated from root tips for quantitative RT-PCR analysis. *ARR15* was selected as a marker, because its cytokinin induced expression is dependent on *CRE1* (Kiba, et al., 2002). As expected, 30 min cytokinin treatment induced *ARR15* expression in wild-type ~40 fold, and in *cre1-2*, an insertion allele of *CRE1*, ~25 fold (**IV, Fig.**

1H). In *wol*, *ARR15* expression was induced only ~5 fold, and in *cre1-12 ahk2-2tk ahk3-3* there was no change in *ARR15* expression following cytokinin treatment. Furthermore, intragenic suppressors, *wol-sup9* and *wol-sup10*, partially restored their cytokinin response, demonstrating that cytokinin insensitivity in procambium correlates with the vascular phenotype (**IV, Fig. 1H**). Similar results were obtained from adventitious root formation assay: *wol* were more insensitive to cytokinin than an intragenic suppressor, *wol-sup10*, and an insertion allele *cre1-2* (**IV, Fig. S4**). The observation that *wol* is insensitive to cytokinin in some assays is puzzling. A possible explanation is that in many tissues the negative activity of *wol* may cause a complex feedback mechanism, which would lead to an enhancement of cytokinin signalling, and subsequently to an increase in cytokinin response. In root procambium, in which *CRE1* expression dominates, the negative activity of *wol* may be too strong for the feedback mechanism to overcome. These results combined with the observations that cytokinin depletion from procambium, and disruption of the CRE-family genes lead to the *wol* phenotype, demonstrates that *wol* has a negative activity on cytokinin signalling initiated by AHK2 and AHK3. This negative activity mediates inhibition of vascular cell file proliferation and of other vascular cell identities than protoxylem.

2. AHP6 COUNTERACTS CYTOKININ SIGNALLING ALLOWING PROTOXYLEM SPECIFICATION

As discussed earlier, cytokinins inhibit protoxylem specification in root procambium. Thus, it seems contradictory that the protoxylem

differentiates early on as two cell files in the vascular bundle, while the root tip is the major site of cytokinin biosynthesis. The following

experiments demonstrate that AHP6 locally inhibits cytokinin signalling, therefore, enabling protoxylem differentiation.

2.1 *AHP6* promotes the specification of protoxylem

In addition to the thirteen intragenic suppressor mutations, three recessive extragenic suppressor mutations were isolated from the suppressor screen for the determinate root growth of *wol*. Two mutations were mapped to the bottom of chromosome 1 and they failed to complement each other indicating that they were allelic. These two were named *ahp6-1* and *ahp6-2* (the nomenclature will be explained below). The analysis of the third mutant is under progress (A.P. Mähönen and A. Bishopp, unpublished results). *ahp6* mutations resulted in a partial suppression of the *wol* phenotype. Primary roots of *wol ahp6* mutants underwent elongation, although slower than in wild-type, and several roots emerged from their root-hypocotyl junction (**III, Fig. 2A**). Both *ahp6* mutations in *wol* resulted in an increased number of vascular cell files in the hypocotyl and the root with undifferentiated files present, as opposed to the exclusively protoxylem cell files present in *wol* (**III, Fig. 1B, S3C**). This proliferation allowed the phloem network to first reach the upper part of the primary root, and later in development, the root tip, which could be seen by the expression of the phloem companion cell specific *AtSUC2promoter::GFP* reporter and in the transverse sections (**III, Fig. 1B, S3A, S3C**). These observations suggest that the *AHP6* locus has a role in regulating the balance of cell proliferation and differentiation during vascular development.

The *ahp6* mutants displayed no obvious seedling phenotypes. However, a detailed histological analysis revealed a distinct phenotype in the root vascular bundle, in which protoxylem differentiation occurred sporadically along the root, but proceeded normally in the hypocotyl (**III, Fig. 1B, S3D, S4A**). Statistical analysis revealed that this phenotype is more dramatic in *ahp6-1* than in *ahp6-2*, indicating that *ahp6-1* is a stronger allele (**III, Fig. 1B, S3D, S4B**). Expression of the molecular marker *ZCP4promoter::GUS*, indicated the location of the immature tracheary elements *i.e.* immature xylem cells in the vascular bundle (Pyo, et al., 2004). In wild-type GUS expression was restricted to the immature protoxylem cells in the root tip, and it occurred almost simultaneously in both protoxylem files. In *ahp6-1*, GUS expression typically appeared sporadically along the root at least in one of the two protoxylem files, therefore, correlating well with the visible xylem differentiation (**III, Fig. 2D**). Additionally, expression of an enhancer trap line, J0121, which highlights the protoxylem-associated pericycle cells (Laplaze, et al., 2005), was slightly down-regulated in *ahp6-1*, when compared to wild-type (**III, Fig. 2E**, data not shown) **(Figure 10)**. Interestingly, lateral roots seemed to emerge from the protoxylem-associated pericycle cells in *ahp6-1*, even when the underlying protoxylem was missing, suggesting that the associated protoxylem was not required for lateral root initiation. During secondary development, a proportion of the intervening procambial cells start to divide periclinally, and thus act as cambium (**III, Fig. 1A**). Similar cell proliferation in intervening procambial cells could also be seen in *ahp6-1*. However, the undifferentiated cells in *ahp6-1*, that

appear as stretches of several cells in the protoxylem cell file position, underwent simultaneous periclinal cell divisions with the surrounding cambial cells (**III, Fig. 1B, S5**). In wild-type the protoxylem cells are invariably differentiated as protoxylem and were not therefore able to divide. Taken together, the phenotypes of *ahp6*, both in *wol* and in wild-type background, indicate that *AHP6* has a role in promoting protoxylem specification in the root vascular bundle.

2.2 AHP6 is a pseudo phosphotransfer protein

To elucidate the molecular nature of the *AHP6* locus, the *AHP6* gene was identified through positional cloning. Analysis of 987 individuals of the F2 mapping population delimited the *AHP6* gene to a 68.5 kb window between two markers in the bottom of chromosome one. Sequencing of the candidate gene *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (At1g80100) revealed that both *ahp6-1* and *ahp6-2* had a mutation in the gene (**III, Fig. 3A**). A genomic DNA fragment containing the wild-type *AHP6* gene and 1.6 kb of the 5' regulatory sequences complemented *ahp6-1*, demonstrating that the mutation in At1g80100 underlies the *ahp6-1* phenotype (**III, Fig. 2A, S3C**). As discussed earlier, cytokinins activate phosphotransfer from the three CRE-family receptors, *via* the conserved histidine residue of the AHP proteins (AHP1-AHP5), to response regulators, which lead to physiological responses. AHP6 lacks the conserved histidine residue (**III, Fig. S6**) (Asn83 in AHP6b, see below), which is required for phosphotransfer, and is present in the other *Arabidopsis* HPt proteins, AHP1-5 (Hwang, et al., 2002; Stock, et al.,

2000). Therefore, AHP6 was designated a pseudo phosphotransfer protein. Next, the coding region of the *AHP6* gene was identified using RT-PCR. Two transcripts, *AHP6a* (DQ093642) and *AHP6b* (DQ093643), were identified. The 3'-end of the first exon of *AHP6b* was 66 bp longer than in *AHP6a* (**III, Fig. 3A, C**). The mutation in *ahp6-1* introduced a premature stop codon in the first exon (CAG to TAG, at Gln35), while the mutation in *ahp6-2* (G to A) was located in the first intron, 5 bp from the 5'-border of the *AHP6b* splice variant. Presumably, due to the mutation close to the intron-exon border of *AHP6b*, only the *AHP6a* transcript is present in *ahp6-2* seedlings (**III, Fig. 3C**). Since *ahp6-2* is the weaker allele of *ahp6* mutations, it is therefore probable that both transcripts are functional. In addition to the two *ahp6* alleles isolated from the mutant screen, a T-DNA insertion allele, *ahp6-3*, was identified from the SALK T-DNA insertion mutant collection (Alonso, et al., 2003). In *ahp6-3* the T-DNA insertion disrupts the protein sequence at Ser111 of AHP6b (**III, Fig. 3A**). *ahp6-3* phenocopies *ahp6-1* both in *wol* and in wild-type background (**III, Fig. S3D, S4B**, data not shown). In conclusion, *ahp6-1* and *ahp6-3* correspond probably to null alleles, because the open reading frame is terminated early in *ahp6-1*, and there is only reduced level of *AHP6* message upstream with no message downstream of the T-DNA insertion site in *ahp6-3* (**III, Fig. 3C**).

2.3 AHP6 inhibits phosphotransfer *in vitro*

The conserved histidine residue required for the phosphotransfer in the five AHP proteins is replaced by an asparagine residue in AHP6. Therefore, in order to understand the biochemical role of AHP6, its *in vitro*

phosphotransfer activity with the yeast SLN1 histidine kinase receptor was tested. In this assay, ³²P-labelled ATP was incubated in the presence of the SLN1-transmitter domain, the SLN1-receiver domain and an HPt protein. This was expected to lead to phosphotransfer from the His residue within the SLN1-transmitter domain, to a conserved Asp residue within the SLN1-receiver domain, and subsequently onto a conserved His residue within an HPt protein. The yeast HPt YPD, or a mutant version of AHP6b, in which Asn83 is replaced with the phosphoacceptor His, were able to accept a phosphoryl group from SLN1. In contrast, native AHP6 was unable to accept a phosphoryl group (**III, Fig. 3B**). These results suggest that AHP6 is unable to function as phosphotransfer protein, due to the replacement of the conserved His residue of AHP6 with an Asn residue. Furthermore, AHP6 appeared not to contain any other sites that could be phosphorylated by the phosphorelay system, as AHP6 failed to accept ³²P label in the *in vitro* phosphotransfer assay. In contrast to the five AHP proteins, the positive mediators of phosphorelay, AHP6 seemed to inhibit phosphotransfer from the SLN1-transmitter domain to the SLN1-receiver domain (**III, Fig. 3B**). In conclusion, the molecular and biochemical data suggest, that AHP6 acts as a non-specific inhibitor of phosphorelay in relation to cytokinin signalling. A systematic investigation of DNA sequence databases revealed that there are homologous pseudo HPt proteins present also in other plant species (**III, Fig. S6**). Perhaps, the pseudo HPt mediated negative regulation of the phosphorelay signalling is a common mechanism throughout the plant kingdom.

2.4 *AHP6* is expressed in the protoxylem and the associated pericycle cells

Organ and tissue specificity of *AHP6* expression was determined using various molecular techniques. RT-PCR analysis with *AHP6* specific primers revealed that the gene is expressed both in the root and in the shoot (**III, Fig. 3C**). Tissue specificity of *AHP6* was studied with *in situ* hybridisation, as well as *AHP6promoter::GUS* and *AHP6promoter::GFP* reporter constructs. In wild-type embryos, GFP expression was first detected in the cotyledons of heart-shaped embryos, and by the early torpedo stage the expression was gradually restricted to two strands extending from both cotyledons towards the embryonic root tip (**III, Fig. 3G, S7B**). In the mature embryos, GFP signal was specified further to an intense signal at the cotyledon apices, and to two strands in the embryonic root, which are presumably protoxylem lineages. All the three techniques demonstrated that *AHP6* is expressed in the protoxylem and in the protoxylem-associated pericycle cell files in the root, which is consistent with the mutant phenotype (**Figure 10**) (**III, Fig. 3D, E, 4A, S7A**). In the aerial part of the plant, *AHP6* is expressed in the shoot apex and young leaves (**III, Fig. S7A**). In order to determine the subcellular localization of AHP6, a translational fusion between *AHP6* and the reporter gene *GFP* was constructed. The resultant *AHP6promoter::AHP6-GFP* was functional, as it was capable of complementing the *ahp6-1* mutation. Confocal microscopy analysis revealed that AHP6-GFP localised predominantly to the nucleus (**III, Fig. 3F**).

2.5 AHP6 is a negative regulator of cytokinin signalling

Since AHP6 inhibits phosphorelay *in vitro,* and a loss-of-function of *AHP6* leads to suppression of a cytokinin receptor mutation, AHP6 may play a role as a negative regulator of cytokinin signalling. First, it was examined, whether cytokinin signalling is required for AHP6 function. As discussed earlier, *wol* and *cre1-12 ahk2-2tk ahk3-3* phenocopy each other in the primary root vasculature **(Figure 10)**. When *ahp6-1* was crossed to *cre1-12 ahk2-2tk ahk3-3*, which lacks cytokinin responses, the subsequent quadruple mutant failed to suppress the triple mutant phenotypes. However, since *ahp6-*1 can suppress *wol* (*i.e. wol ahp6-1,* identified from the mutant screen), it indicates that the residual cytokinin signalling present in the *wol* background is required for the suppression by *ahp6-1* (**IV, Fig. 1H**, data not shown). Next, the cytokinin responsiveness of the *ahp6* mutants was examined in the adventitious root formation assay. Both *ahp6-1* and *ahp6-2* were able to suppress the reduced cytokinin sensitivity of *wol*, further supporting the role of AHP6 as a negative regulator of cytokinin signalling (**III, Fig. 2B**). Since protoxylem differentiation, both in *ahp6-1* mutant and wild-type seedlings treated with cytokinins, was severely impaired, enhanced cytokinin signalling in procambium may lead to the *ahp6* phenotype. Therefore, cytokinin response was studied in the procambium of cytokinin-induced wild-type and *ahp6-1* root samples, using *ARR15* as a marker. *In situ* hybridisation analysis revealed that in wild-type, the *ARR15* signal is localised to the intervening procambial tissue (**III, Fig. 2C**) **(Figure 9C)**. In *ahp6-1* the expression domain expanded to include

the positions normally occupied by protoxylem files (**III, Fig. 2C**). These results indicate that AHP6 has a role in locally inhibiting cytokinin signalling in the protoxylem position, thus facilitating protoxylem specification. In order to confirm the *in vivo* role of AHP6 as a local inhibitor of cytokinin signalling, cytokinins were depleted locally by introducing *AHP6promoter*::*CKX2* into *ahp6-1*. *AHP6promoter*::*CKX2* was able to suppress the *ahp6-1* phenotype, indicating that *CKX2* can replace the role of *AHP6* (**III, Fig. S3D**). Furthermore, when *ahp6-1* seedlings were germinated on media containing BA, a cytokinin, the inhibitory effects of the *ahp6-1* mutation and exogenously applied cytokinins on protoxylem differentiation were synergistic; in 10 nM BA concentration *ahp6-1* seedlings typically lacked both protoxylem files, whereas wild-type exhibited the normal two-protoxylem-file phenotype in the same condition (**III Fig. S2A, S4B**). These results demonstrate that AHP6 acts as a negative regulator of cytokinin signalling.

2.6 Cytokinin signalling negatively regulates the spatial domain of *AHP6* expression

As discussed earlier, cytokinin treatment of wild-type can phenocopy the *ahp6* phenotype. Applied cytokinin either enhances cytokinin signalling to levels that dilute the inhibitory effect of AHP6, or it down-regulates *AHP6* expression, which would then lead to enhanced cytokinin signalling in the protoxylem position. The latter hypothesis seemed to be more accurate, as quantitative RT-PCR analysis revealed that the *AHP6* transcript was down-regulated after a 6 hour treatment with cytokinin (**III, Fig.**

4B). Similarly, the expression of AHP6promoter::GFP was reduced in wild-type in response to cytokinin application (30 nM BA) (**III, Fig. 4C, S8**). In *ahp6-1* the GFP signal was decreased at lower concentration of cytokinins (10 nM BA). Interestingly, the decrease in GFP fluorescence along a root was not even but formed sporadic patterns. Without cytokinin application, the *AHP6* expression was slightly reduced in *ahp6-1* when compared to wild-type (data not shown).

Cytokinin application experiments suggest that cytokinin may intrinsically down-regulate *AHP6* expression. Therefore, *AHP6* expression was studied in mutant backgrounds that show reduced cytokinin response. As discussed earlier, *cre1-12 ahk3-3* exhibits intermediate cytokinin responsiveness (**II, Fig. 4,5,6,12**). *In situ* hybridisation analysis revealed that the *AHP6* expression domain in *cre1-12 ahk3-3* is broader than in wild-type, as *AHP6 expression* is typically found in three (as opposed to two) pericycle cell files and in two (as opposed to one) adjacent protoxylem cell files (**III, Fig. 4A**). Interestingly, the expanded expression domain coincides with the ectopic protoxylem cell files present in 91% of the *cre1-12 ahk3-3* roots (**III, Fig. 1B**) (**Figure 10**). Similar ectopic protoxylem file formation could be seen in the weakest *CRE1promoter::CKX2* lines (**III, Fig. S3B**), indicating that the appearance of ectopic protoxylem is the first sign of limited cytokinin signalling. Furthermore, the loss of *AHP6* function in *ahp6-1 cre1-12 ahk3-3* was able to suppress the formation of ectopic protoxylem present in *cre1-12 ahk3-3*, and the remaining protoxylem files often showed sporadic differentiation, similar to *ahp6-1* (**III, Fig. 1B**). These results indicate that the expanded expression domain of AHP6 is responsible for the formation of the ectopic protoxylem in *cre1-12 ahk3-3*. When cytokinin signalling was severely impaired in procambium, such as in *wol* or in *cre1-12 ahk2-2tk ahk3-3* (**IV, Fig. 1H**), *AHP6* expression expanded throughout the vascular bundle including the pericycle layer (**III, Fig. 4A**). The expanded expression corresponds to the exclusively protoxylem differentiation that could be observed in the vasculature of the mutant roots (**Figure 10**). Furthermore, the *AHP6promoter::GFP* expression was expanded throughout the *wol* procambium already during embryogenesis, as opposed to the two narrow strands of fluorescence in wild-type (**III, Fig. 3G, S7B**). Differentiation of protoxylem takes place during germination. Therefore, since the expanded expression observed in *wol* is set up during embryogenesis, well before protoxylem differentiation, it indicates that cytokinin signalling negatively regulates the spatial domain of *AHP6* expression upstream of protoxylem differentiation.

2.7 Cytokinin signalling, and its spatially specific modulation, control vascular cell identities

Arabidopsis root procambium propagates two files of protoxylem, even though cytokinins inhibit protoxylem specification and the root tip is the major site for cytokinin biosynthesis. In order to allow protoxylem specification, cytokinin signalling needs to be inhibited specifically in the protoxylem cell lineages. This spatial inhibition is mediated by AHP6 (**III, Fig. 4D**), perhaps together with other factors, as the variable *ahp6* phenotype can be further enhanced with cytokinin treatments. The *ahp6* phenotype can be phenocopied by degrading cytokinins

from *AHP6* expression domain, further supporting the idea that cytokinins primarily regulate vascular cell identities in root procambium, rather than cell proliferation. Cytokinin signalling and its spatially specific downstream inhibitor, AHP6, may also play a role in early vascular patterning, because the two-pole expression pattern of *AHP6* is established already during embryogenesis, when procambium is still anatomically radially symmetric. Furthermore, when *ahp6-1* seedlings were germinated in the presence of 100 nM BA, phloem files were often miss-positioned in the vascular bundle (**III, Fig. S2A**). AHP6 is the founder member of a new "pseudo" subclass of HPt proteins. Protein database searches suggested that AHP6-like proteins are found throughout the flowering plants. It remains to be seen whether AHP6-like proteins, which inhibit phosphorelay, exist also in other eukaryotes and prokaryotes.

AHP6 inhibits cytokinin signalling, and reciprocally, cytokinin inhibits the spatial domain of AHP6 expression. The expression of AHP6 may therefore form a positive feedback loop, in which AHP6 inhibits cytokinin signalling, and subsequently *AHP6* expression would be enhanced due to decreased inhibitory effect of cytokinin signalling. This would in turn lead to enhanced inhibition of cytokinin signalling by AHP6, and so on (**III, Fig. 4D**). The positive feedback loop could be required for the stabilisation of the less responsive domain of cytokinin, permitting protoxylem differentiation. Interruption of the loop by the *ahp6* mutation, or by cytokinin treatments, might destabilise the system, leading to the observed sporadic protoxylem formation and *AHP6* expression (**III, Fig. S4A, 4C**). Since cytokinin signalling inhibits *AHP6* expression, other factors

must be required to promote *AHP6* expression. One candidate for such a factor is the phytohormone auxin, which is believed to be transported through the xylem parenchyma cells (Booker, et al., 2003; Galweiler, et al., 1998; Gee, et al., 1991; Steinmann, et al., 1999). Auxin has been asserted to be the major factor for initiating lateral root primordia (for review, see Casson, Lindsey, 2003). Interestingly, lateral roots emerge from the protoxylem-associated pericycle cells, the same cells where *AHP6* is expressed. Perhaps auxin promotes *AHP6* expression in the vascular bundle from the protoxylem-associated pericycle cells, while cytokinin counteracts this promotion by restricting *AHP6* domain to the two strands (**III, Fig. 3D**).

In addition to the cytokinin - AHP6 feedback loop, regulating vascular cell fates, there are well established feedback loops regulating cell fates both in root and in shoot apical meristems. In shoot apical meristem, organising centre specific WUSCHEL promotes stem cell identity and induces expression of *CLAVATA3*, which in turn, spatially restricts *WUSCHEL* expression (Fletcher, et al., 1999; Schoof, et al., 2000). In root apical meristem, auxin accumulation in the root tip induces expression of the PLETHORA transcription factors, which specify root stem cells (Aida, et al., 2004). In turn, *PLETHORA* genes are required to maintain the auxin maximum in the root tip (Blilou, et al., 2005). All the above mentioned processes are related to meristematic or stem cell maintenance, and may therefore need robust auto-regulatory systems to optimally maintain the size of stem cell population. It is not therefore a surprise that they all employ regulatory feedback loops, which are inherently relatively stable as they are self-correcting (Doerner,

2003). Recently it was reported, that cytokinins induce expression of ABPHYL1, a *Zea mays* response regulator, which in turn negatively regulates the cytokinin-induced expansion of shoot apical meristem (Giulini, et al., 2004). Therefore, AHP6 mediated inhibition is not the only mechanism inhibiting cytokinin signalling and thereby, ensuring normal development. Perhaps, spatially specific down-regulation of cytokinin signalling serves as a general mechanism in tissue patterning and meristem regulation.

3. CYTOKININS PROMOTE CELL PROLIFERATION AND MERISTEMATIC COMPETENCE

In apical meristems and vascular tissue of shoot, cytokinins promote cell proliferation and meristematic competence.

3.1 Cytokinins promote cell proliferation and meristematic competence in apical meristems

Cytokinins have been shown to promote cell proliferation and formation of shoots from tissue culture (Skoog, Miller, 1957). Confirming earlier results, analysis of the CRE-family triple mutants revealed that cytokinins were required to promote meristematic competence and cell proliferation in root and shoot apical meristems (**II, Fig. 8,9**) (Nishimura, et al., 2004). Similar results were obtained in shoot apical meristem when cytokinins were degraded by overexpressing *CKX* genes under the control of the CaMV 35S promoter (Werner, et al., 2001; Werner, et al., 2003). In contrast to the triple mutant roots, *CKX* overexpressing lines resulted in an increased root growth, due to an increased number of meristematic cells in the root meristem. The contradicting results may be due to the incomplete degradation of cytokinins in the *CKX* overexpressing lines. A feedback mechanism may be activated in specific tissue as a response to global cytokinin degradation, which may result in a local increase in cytokinin signalling, and subsequently in an increase in root growth. Perhaps, a more plausible explanation is to assume that cytokinins have both inhibitory and stimulatory roles in root growth. It has been shown that cytokinins induce ethylene production, and subsequently ethylene inhibits root growth (Cary, et al., 1995). Therefore, the inhibitory effect on root elongation could be mediated by cytokinin-induced ethylene production at high cytokinin concentrations. When a proportion of the cytokinin pool is degraded, less ethylene is produced, and therefore root growth is enhanced. Only when cytokinin signalling is severely inhibited, as seen in the CRE-family triple mutant, cell proliferation and meristematic tissue maintenance defects are observed. CaMV 35S promoter is not a very strong promoter in the root procambium (A. P. Mähönen, unpublished results). When CKX2 was expressed under the control of a strong procambial promoter of *CRE1*, root growth was severely decreased, similar to the triple mutant (**IV, Fig. 1G**). This result supports the hypothesis that cytokinin signalling needs to be severely inhibited for the cell proliferation defect to be seen. Future studies with combinations of ethylene synthesis mutants and *CKX* overexpressors may help to elucidate the roles of these two hormones in root growth.

In response to auxin and cytokinin, cultured *Zinnia elegans* mesophyll cells differentiate to tracheary elements (Fukuda, 2004). Cytokinins are believed to promote procambial activities in this process. In this study a similar role for cytokinins was also found *in planta*. Lack of cytokinin signalling in root procambium results in differentiation of all vascular cell files into protoxylem, indicating that cytokinins are required to promote and maintain procambial cell identity in intact plants **(Figure 10)**. The additional role of cytokinin as an inhibitor of protoxylem identity has not been reported before. In this context, cytokinins are also required to maintain procambial cell identity, and if cytokinin signalling is absent, the default pathway, protoxylem specification, is activated **(III, Fig. 4D)**. It is probable that cytokinins may also have a similar role, as promoters of undifferentiated stage, in other developmental processes, such as proliferation of apical meristems. However, cytokinins are not completely required for the activity of meristems, since the CRE-family triple mutants had a group of undifferentiated cells in their meristems that proliferated and produced organs, albeit at a slow rate **(II, Fig. 8,9)** (Nishimura, et al., 2004). Since a subpopulation of meristematic cells contain stem cells, from which all the postembryonic cells originate, it is possible that the general role of cytokinins is to promote stem cell identity in all meristems, including root procambium. Now that we are beginning to understand the concept of stem cells in plants (Scheres, 2005), it is time to investigate the possible role of cytokinin in relation to stem cells.

3.2 Cytokinins promote procambial cell proliferation throughout the vascular system

All CRE-family cytokinin receptors are also expressed in the vascular bundles of the aerial part of the plant, *i.e.* vasculature of the petioles and stems **(IV, Fig. S2)**. Consistent with these expression patterns, the triple mutant *cre1-11 ahk2-1ms ahk3-1ms* had a reduced number of vascular cell files in the petioles of the first true leaves as well as in hypocotyl **(IV, Fig. S3**, data not shown). Interestingly, lack of cytokinin signalling in petiole vascular bundle did not lead to differentiation of all the vascular cell files as protoxylem. This data indicates that cytokinin mediated inhibition of protoxylem specification is needed only in the root or, alternatively, there is a redundant mechanism in petiole vascular bundles inhibiting protoxylem. Similarly, expression of *35Spromoter::CKX1* in *Arabidopsis* resulted in a decreased number of vascular cell files in leaf vascular bundle (Werner, et al., 2003). These results are in accordance with studies on increased cytokinin signalling in the vascular bundle; When *ARR11*, a type-B ARR, containing a gain-of-function mutation was overexpressed under the control of the 35S promoter, more vascular cells were observed in the leaf vascular bundle (Imamura, et al., 2003). Similarly, induction of *Agrobacterium ipt* expression resulted in over-proliferation of vascular tissue in *Arabidopsis* hypocotyl (Rupp, et al., 1999). Collectively, these results indicate that cytokinin signalling through CRE-family receptors is required for procambial cell maintenance and/or normal proliferation of procambial cells throughout the vascular system.

4. *CYTOKININS REGULATE A BIDIRECTIONAL PHOSPHORELAY IN ARABIDOPSIS*

Many bacterial histidine kinases are bifunctional, because they posses both kinase and phosphatase activities. These histidine kinases can both phosphorylate and dephosphorylate the same target, depending on the presence of an input signal **(Figure 7)**. The *wol* mutation (Thr$_{278}$ to Ile$_{278}$) located in the receptor domain of CRE1, results in an inability to bind cytokinins and in a negative activity on cytokinin signalling and procambial cell file proliferation. It is therefore possible that the negative activity conferred by CRE1(T278I) is a phosphatase activity leading to dephosphorylation of HPt proteins. CRE1(T278I) may mimic a cytokinin-unbound form of CRE1, raising the possibility that wild-type CRE1 might possess a phosphatase activity as well.

4.1 CRE1 possesses kinase and phosphatase activities

To test the hypothesis of a bifunctional CRE1, its kinase and phosphate activities were examined *in vitro*. CRE1 was first expressed in the yeast *Saccharomyces cerevisiae* and in *Escherichia coli*, but these experiments failed to produce enough protein, possibly on account of CRE1 being a membrane-spanning protein. Additionally, overexpression of a histidine kinase in systems that already contain two-component molecules may lead to toxicity. In order to avoid the toxicity problems, and possible contamination from the endogenous histidine kinases, CRE1 was expressed in an insect cell line derived from *Spodoptera frugiperda*, which does not contain two-component molecules. In the kinase assay, a membrane fraction of the insect cells containing CRE1, and an HPt protein (YPD1, AHP1, AHP2, AHP3, or AHP5), were incubated in the presence of ^{32}P-labelled ATP. The phosphoryl group was transferred to the HPt protein only in the presence of cytokinin, demonstrating for the first time a phosphotransfer reaction between CRE1 and an AHP protein *in vitro* (**IV, Fig. 2A, B**). Consistent with the idea that a phosphoryl group is transferred *via* the conserved phosphoaccepting Asp973, phosphorylation of the HPt protein did not occur when Asp973 was mutated. In the phosphatase *in vitro* assay, AHP1, carrying a ^{32}[P]-phosphoryl group, was incubated with CRE1. In the course of time, the level of AHP1 radioactivity decreased, indicating that CRE1 has a phosphatase activity *in vitro* (**IV, Fig. 2C**). Asp973 was required, and His459 was partially required, for the phosphatase activity (**IV, Fig. 2C, S5**). To summarise, CRE1 has both kinase and phosphatase activities for AHPs *in vitro*; The kinase activity seems to be regulated by cytokinins.

Next, we examined the kinase and phosphatase activities of CRE1 in a yeast system. Previously, it was shown that CRE1 can complement sln1, the disruption of the only histidine kinase in yeast, in a cytokinin dependent manner (Inoue, et al., 2001) (**IV, Fig. 2D**). CRE1 was expected to transfer the phosphoryl group to YPD1, an HPt, as the complementation by CRE1 failed in the yeast line lacking YPD1. It was also demonstrated that both His459 and D973N are required for the phosphorelay, whereas in this study only His459 was absolutely required (**IV, Fig. 2D**). The discrepancy may be explained by the weak promoter that was used previously. If yeast contain insufficient amounts of the slightly disrupted CRE1(D973N), the complementation may not occur. Wild-

type yeast, in which the SLN1 is present, was utilized to assay the phosphatase activity. Using this system CRE1 was demonstrated to exhibit a phosphatase activity on YPD1, since wild-type yeast expressing *CRE1* was nonviable in the absence of cytokinin; In the presence of cytokinin yeast grew normally (**IV, Fig. 2D**). In this assay Asp973 was absolutely required and His459 partially required for the phosphatase activity.

4.2 CRE1(T278I) possesses weak kinase activity, and constitutive phosphatase activity

In order to study the molecular nature of the negative activity conferred by the *wol* allele, CRE1(T278I) was examined in yeast and *in vitro* assays. CRE1(T278I) showed very weak kinase activity, *in vitro*, in the absence of cytokinin, and the activity was only slightly increased by cytokinin (**IV, Fig. 2A**). However, the CRE1(T278I) mutation did not affect the *in vitro* phosphatase activity of CRE1. The phosphatase activity of CRE1(T278I) was disrupted by the additional mutations H459Q and D973N (**IV, Fig. 2C, S6**). The *in vitro* data suggest that *wol* has a negative activity on cytokinin signalling; With almost complete loss of the kinase activity, CRE1(T278I) preferentially dephosphorylates AHPs.

In the yeast assay, CRE1(T278I) severely inhibited the growth of wild-type yeast regardless of the presence or absence of cytokinin (**IV, Fig. 2D**). This result is consistent with the interpretation that CRE1(T278I) resembles the cytokinin-free form of CRE1, and is therefore locked in a state characterised by little kinase activity, yet constitutive phosphatase activity. However, the negative activity exerted by CRE1(T278I) was weaker in comparison to wild type CRE1, in the

absence of cytokinins, possibly because Thr_{278} to Ile_{278} mutation results in conformational changes. Moreover, CRE1(T278I, H459Q, D973N) had no negative activity, indicating that it is dependent on the phosphorelay from AHP1 to CRE1(T278I) (**IV, Fig. 2D**).

Certain mutations in bacterial histidine kinases can independently destroy either the kinase or the phosphatase activity, therefore making it possible to study the role of these activities separately *in vivo*. One such mutation is the F390L mutation in the *E. coli* osmosensor EnvZ, which destroys kinase activity without affecting phosphatase activity (Hsing, et al., 1998). The corresponding residue (F685) is conserved in CRE1. When F685 was replaced with Leu, the kinase activity of CRE1 was destroyed, both in the yeast and *in vitro* assay (**IV, Fig. 2A, D**). In the absence of cytokinin, CRE1(F685L) retained phosphatase activity in both assays. However, the phosphatase activity was eliminated by cytokinin only in the yeast assay (**IV, Fig. 2C, D**). Therefore, it is possible that phosphatase activity is also regulated by cytokinins. The yeast system might be more sensitive than the *in vitro* assay in detecting subtle changes in phosphatase and kinase activities.

4.3 Constitutive phosphatase activity of CRE1 leads to reduced cytokinin response and *wol*-like phenotype *in planta*

To elucidate the role of kinase and phosphatase activities of *CRE1* in *planta*, various *CRE1* point mutants were expressed under the control of the *CRE1* promoter. The *CRE1promoter::CRE1* variants were transformed to a putative null allele, *cre1-2*, to minimize unwanted interactions with the endogenous

CRE1. As expected, *CRE1-HA* exhibited wild-type root vascular phenotype, whereas *CRE1(T278I)-HA* exhibited *wol*-like root vascular bundles (**IV, Fig. 3A**, data not shown). Even though plants carrying *CRE1(T278I, H459Q, N973N)-HA* expressed more CRE1 protein than plants carrying *CRE1(T278I)-HA*, only the former line exhibited wild-type vascular bundle (**IV, Fig. 3A-C**) (**Figure 10**). These results, similar to those in the yeast assay, indicate that both His459 and Asp973 are required for the negative activity conferred by CRE1(T278I). Similarly, expression of *CRE1(T278I, N973N)-HA* resulted in a wild-type phenotype, even when the protein expression level was higher than in a line carrying *CRE1(T278I)-HA* (**IV, Fig. 3C,E**). This result demonstrates the absolute requirement of Asp973 for the negative activity. However, *CRE1(T278I, H459Q)-HA* resulted in a *wol*-like phenotype with moderate protein expression levels (**IV, Fig. 3C,D**). Therefore, His459 seems not to be fully required for the negative activity, which is in accordance with the yeast and *in vitro* results, as well as with the notion that the conserved His residue is not required for the phosphatase activity in many other histidine kinases (**IV, Fig. 2D, S5**) (Stock, et al., 2000). Expression of CRE1(F685L), which lacks kinase activity but has phosphatase activity *in vitro*, resulted in *wol*-like roots in *cre1-2* background, indicating that a decreased kinase / phosphatase ratio of CRE1 leads to development of a *wol*-like vascular bundle (**IV, Fig. 3F**). These results demonstrate that the negative activity of *wol* relies on phosphotransfer *in planta*, and when combined with the yeast and *in vitro* results, consists of a constitutive phosphatase activity on the AHP proteins. The Thr_{278} to Ile_{278} mutation in *wol* results in the inability of the receptor to bind cytokinins, and therefore, to induce kinase activity.

Next, the effects of various mutant *CRE1* genes were tested for primary response to cytokinin. Following cytokinin treatment for 30 minutes, quantitative RT-PCR analysis for *ARR15* expression was performed. The cytokinin responses were consistent with the mutant phenotypes, since transgenic lines expressing *CRE1(T278I)* or *CRE1(F685L)* resulted in a reduced *ARR15* induction, while expression of *CRE1(T278I, H459Q, D973N)* had no effect on *ARR15* expression, when compared to the response in *cre1-2* background (**IV, Fig. 3G**). These results indicate that the constitutive phosphatase activity of CRE1(T278I) or CRE1(F685L) on AHPs results in reduced cytokinin response and *wol*-like root vascular bundle (**Figure 10**).

4.4 Overexpression of CRE1(T278I) results in a phenotype similar to the CRE-family triple mutant

The phosphatase activity of *wol* results in a dramatic phenotype in the root vascular bundle, while the shoot appears relatively normal. The cytokinin receptor triple mutant displays the same phenotype in root, but has additionally a strong growth defect in shoot. The phosphatase activity does not lead to a dominant negative effect. Rather, the phosphatase activity must dephosphorylate the phospho-AHP protein pool in a dose-dependent manner. The restriction of the *wol* phenotype to the root may then be explained by the dominance of *CRE1* expression in the root procambium (see 1.7). In order to study the effect of the constitutive phosphatase activity in the whole plant, *CRE1(T278I)* was overexpressed under the strong CaMV 35S promoter. Wild-type seedlings

expressing *35Spromoter*::*CRE1*(*T278I*) appeared normal, whereas *wol* seedlings expressing the same transgene exhibited severely retarded growth in the shoot, supporting the idea of dose-dependent phosphatase activity (**IV, Fig. S7**). *35Spromoter*::*CRE1*(*T278I*) in *wol* resulted in a reduced number of cell files in leaf petiole vasculature and the shoot apical meristem was diminished in size (**IV, Fig. S3,** data not shown). Moreover, in the callus formation assay, hypocotyl segments lacked cytokinin responses (**IV, Fig. 3H**). Therefore, ectopic expression of *CRE1*(*T278I*) in *wol* background phenocopied the *cre1-12 ahk2-2tk ahk3-3* triple mutant (**IV, Fig. S7**). Apparently, the ubiquitously expressed CRE1(T278I) enhanced dephosphorylation of phospho-AHPs in shoot, which resulted in reduction of forward phosphorelay to a similar level with the *cre1-12 ahk2-2tk ahk3-3* triple mutant.

4.5 Kinase and phosphatase activities of CRE1 imply a bidirectional phosphorelay network

The *Arabidopsis* genome contains 5 *AHP* genes, relatively few compared to the number of *ARRs* or histidine kinase genes. Yeast-two-hybrid and *in vitro* phosphotransfer assays indicate that AHP proteins do not exhibit strict specificity towards their signalling partners (**IV, Fig. 2B**) (see 2.4.4 in Introduction). Therefore, AHP proteins may be considered as signalling intermediates that serve as an integration point for the CRE-family phosphorelay, as well as for other phosphorelay signalling. CRE-family histidine kinases, and perhaps several other histidine kinases, such as CKI1, putative osmosensor AtHK1 and ethylene receptors, may also transmit their phosphorelay through AHP proteins. It is therefore possible that CRE1 can interact with other signalling pathways, by dephosphorylating phospho-AHPs that have been phosphorylated by other histidine kinases. Thus, the existence of cytokinin regulated kinase and phosphatase activities in CRE1 enable a bidirectional phosphorelay network downstream of the CRE-family of cytokinin receptors in plants (**IV, Fig. 4**).

5. CONCLUDING REMARKS

The present study demonstrates that cytokinin signalling, through the CRE-family cytokinin receptors, regulates vascular cell identities and proliferation of procambial cell files in *Arabidopsis*. When cytokinin signalling is absent from the root procambium, all the vascular cell files differentiate as protoxylem, indicating that cytokinins inhibit protoxylem specification and promote procambial cell identity. Subsequently, cells with procambial identity are able to have other identities and proliferate as phloem, intervening procambium and metaxylem tissues by yet unidentified cues. In wild-type root procambium, protoxylem specification is enabled by spatially specific inhibition of cytokinin signalling. This inhibition is mediated by AHP6, the first member of a novel "pseudo" subclass of HPt proteins. The double negative-feedback loop between cytokinin signalling and AHP6 presumably form a positive-feedback loop to stabilise *AHP6* expression, and thereby, to down-regulate cytokinin signalling and allow protoxylem specification. In the vascular tissue of aerial part of the plant, the role of cytokinin is to enhance proliferation of procambial cells and/or maintain intervening procambial tissue identity.

This study identifies two levels of negative regulation in cytokinin signalling that regulate vascular morphogenesis. In addition to AHP6, which inhibits signalling at an intermediate level, there is a negative activity for cytokinin signalling at the receptor level as well. When cytokinin is present, cytokinin receptor CRE1 phosphorylates the downstream AHP molecule, and subsequently activates cytokinin signalling. In low cytokinin concentrations CRE1 preferentially dephosphorylates phospho-AHP molecules, thereby negatively regulating cytokinin signalling, which is initiated by the other two cytokinin receptors. Additionally, CRE1 may dephosphorylate phospho-AHPs, which were phosphorylated by histidine kinases unrelated to cytokinin perception, thus enabling crosstalk with other signalling pathways.

The decision to specify as xylem, or remain as a procambial cell, is regulated by reciprocal interaction of cytokinin signalling and AHP6, in root procambium. Cambial cells have to decide between the same two options as they proliferate during secondary development. It is therefore tempting to speculate that cytokinin signalling and its spatially specific inhibition have a role in specifying cell fates during secondary development as well.

In the absence of cytokinins, all the vascular cells differentiate as protoxylem suggesting that protoxylem differentiation was the default pathway in root vascular morphogenesis of early plants, and that specification of other tissue types enabled by cytokinin appeared later in evolution. This is an interesting question that requires investigation of these components in early land plants.

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Helsinki, November 2005
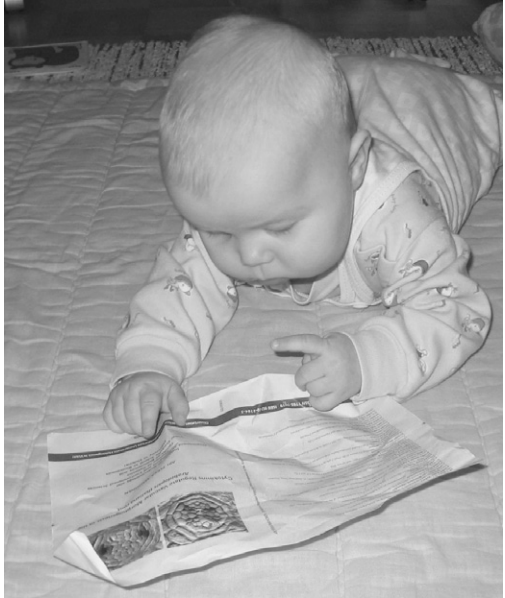
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