Symplastically transmitted signals regulate pattern formation during root development in *Arabidopsis thaliana*

Anne Vatén

Faculty of Biological and Environmental Sciences
Department of Biosciences
Division of Plant Biology
and
Institute of Biotechnology
and
Viikki Doctoral Programme in Molecular Biosciences
University of Helsinki

ACADEMIC DISSERTATION

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Supervised by

Professor Yrjö Helariutta
Institute of Biotechnology
Department of Biosciences
University of Helsinki

Members of the follow up group

Professor Teemu Teeri
Department of Applied Biology
University of Helsinki

Dr. Mikko Frilander
Institute of Biotechnology
University of Helsinki

Reviewed by

Professor Jari Valkonen
Department of Applied Biology
University of Helsinki

Research Professor Päivi Rinne
Department of Plant and Environmental Sciences
Norwegian University of Life Sciences, Norway

Opponent

Professor David Jackson
Cold Spring Harbor Laboratory
Cold Spring Harbor, NY, USA

Custos

Professor Jaakko Kangasjärvi
Department of Biosciences
University of Helsinki

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It is not the mountain we conquer but ourselves.

Edmund Hillary

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following original articles and a manuscript which are referred to in the text by their roman numerals, and some unpublished results. The articles are printed with a kind permission of their copyright holders.

I Carlsbecker A[1], Lee JY[1], Roberts CJ[2], Dettmer J[2], Lehesranta S[2], Zhou J[2], Lindgren O[3], Moreno-Risueno MA[3], **Vatén A**[3], Thitamadee S, Campilho A, Sebastian J, Bowman JL, Helariutta Y[4], Benfey PN[4]. (2010) Cell signalling by microRNA165/6 directs gene dose-dependent root cell fate. Nature 465, 316–321.

II Fitzgibbon J, **Vatén A.** (2011) Plasmodesmata 2010: plasmodesmata down under. Protoplasma 248, 3–7. Conference report.

III Bishopp A, Lehesranta S[1], **Vatén A**[1], Help H, El-Showk S, Scheres B, Helariutta K, Mähönen AP, Sakakibara H, Helariutta Y. (2011) Cytokinin transported through the phloem regulates polar auxin transport and maintains vascular pattern in the root meristem. Current Biology 21, 927–932.

IV **Vatén A**, Dettmer J, Wu S[1], Stierhof Y-D[1], Miyashima S[1], Yadav SR, Roberts CJ, Campilho A, Bulone V, Lichtenberger R, Lehesranta S, Mähönen AP, Kim J-Y, Jokitalo E, Sauer N, Scheres B, Nakajima K, Carlsbecker A[2], Gallagher KL[2], Helariutta Y. (2011) Callose biosynthesis regulates symplastic trafficking during root development. Developmental Cell 21, 1144–1155.

V Miyashima S[1], **Vatén A**[1], Helariutta Y. Symplastic connectivity is required for maintenance of the quiescent center. (Manuscript).

[1-4] These authors contributed equally to this work

AUTHOR'S CONTRIBUTION

I AV participated in positional cloning of *phb-7d*, established and analysed *shr-2, J0571, UAS::miR165a* line. AV had minor part in writing the manuscript.

II AV participated in designing and writing of the manuscript.

III AV designed experiments to follow effect of *icals3m* in the phloem, established all the transgenic lines containing *icals3m* and participated in their characterization. AV had minor part in writing the manuscript.

IV AV designed and performed experiments to screen the *cals3-d* mutations, identified and characterized them at the molecular level, including construction and analysis of the *icals3m* lines in various genetic backgrounds. AV identified and participated in the analysis of *CALS7* knock-out line. AV wrote the manuscript with KG and YH.

V AV designed and performed experiments to block symplastic signaling in *WOX5* and *CALS3* expression domains. AV followed auxin response in *cals3-d* mutants and auxin response and transport in *pCALS3::icals3m* line. AV wrote the manuscript with YH.

ABBREVIATIONS

ABA	abscisic acid
CC	companion cells
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
DT	desmotubule
EMS	ethylmethane sulfonate
ER	endoplasmic reticulum
GA	gibberellic acid
GUS	β-glucuronidase
miRNA/miR	microRNA
mRNA	messenger RNA
nt	nucleotide
PCR	polymerase chain reaction
PD	plasmodesmata
PM	plasmamembrane
QC	quiescent centre
RAM	root apical meristem
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
SA	salicylic acid
SAM	shoot apical meristem
SE	sieve elements
siRNA	small interfering RNA
ta-siRNA	trans-acting siRNA
TE	tracheary elements
TEM	transmission electron microscopy

ABSTRACT

The exchange of positional information largely determines the fate of plant cells during organ development. Hence, the mobility of the molecular signals that direct plant development is crucially important. Plasmodesmata (PD) are membrane-lined channels that connect the plant cells and allow symplastic cell-to-cell movement of molecules between neighbouring cells. Currently, various developmental regulators have been proposed to move symplastically through the PD. However, little is known about the role of symplastic signaling in plant morphogenesis. In this study, the *Arabidopsis* root was used as a model to investigate molecular control of the pattern formation and to elucidate how symplastic signaling regulates root development.

We identified four novel mutants, *phb-7d*, *cals3-1d*, *-2d*, and *-3d* (collectively referred as *cals3-d*) that display defective protoxylem development. Characterization of these mutants revealed that the protoxylem defects are a result of an altered regulatory pathway involving the transcription factor SHORT-ROOT (SHR). It has been shown that SHR moves from the stele into the endodermis and the quiescent centre (QC) where it regulates cell division of the endodermis/cortex initials and maintains the stem cell identity. Through a positional cloning we identified *phb-7d* as a novel gain-of-function allele of *PHABULOSA* (*PHB*). *PHB* is post-transcriptionally regulated by microRNA165/6 (miR165/6). *phb-7d* carries a point mutation in the miR165/6 recognition site of *PHB* which renders it insensitive to regulation through miR165/6 and results in a loss of protoxylem identity. We found that SHR is required in the endodermis to turn on the expression of *MIR165/6*, which in turn is essential for the formation of the proper concentration gradient of *PHB* in the stele. We demonstrated that the non-cell-autonomous actions of SHR and miR165/6 are essential for the regulation of *PHB*, and therefore, for the regulation of protoxylem development.

The movement of SHR from the stele is impeded in *cals3-d*. We defined that the *cals3-d* mutations are in the *CALLOSE SYNTHASE 3* (*CALS3)/ GLUCAN SYNTHASE-LIKE 12* (*GSL12*) gene, whose product participates in the biosynthesis of callose, β-1,3-glucan. The *cals3-1d*, *-2d* and *-3d* lead to amino acid changes in the predicted cytosolic chains of CALS3 resulting in an increased callose biosynthesis. In order to characterize the role of the *cals3-d* in cell-to-cell communication, we engineered a temporally and spatially controlled miss-expression system for *cals3-d*. Using this system, we demonstrated that the aperture of a PD is controlled by the callose biosynthesis at the surrounding cell wall domain. Moreover, we identified symplastically transmitted cell signaling events. First, our study reveals that SHR moves from the stele to the endodermis and to the QC via the PD. Second, we found that miR165 traffics from the endodermis to the stele via the PD. Third, we show that the phloem-borne symplastically transported signals are essential for the root development. In conclusion, the results of my thesis work demonstrate the fundamental importance of symplastic connectivity for the regulation of root morphogenesis.

1 INTRODUCTION

Once the position of a given plant cell has been established through cell division, it remains fixed through the life of the plant. This is because plant cells have rigid cell walls that anchor the cells in position and provide rigidity and support for plants. In contrast, animal cells do not have cell wall and hence, they are able to migrate from one position to another during embryogenesis and organogenesis; this creates three-dimensional forms seen in animals. A tremendous range of complex shapes and forms are evident also throughout the plant kingdom. How are plants able to generate such a structural complexity without the cell movement? In the 1990s a set of laser ablation studies demonstrated that cell identity is reversible in plants (van den Berg et al., 1995; van den Berg et al., 1997). Destruction of a cell in a particular layer resulted in a change of fate of cells in nearby layers. These studies revealed that the identity of plant cells is largely dependent upon positional information. Hence, intercellular signaling is a major force in the regulation of plant development. As a result, several signaling pathways have evolved and these display a wide array of signaling molecules to ensure the correct assignment of cell fate. One of the intercellular signaling pathways, a symplastic signaling route, operates directly from cell to cell via the membranous cell wall channels, plasmodesmata (PD). Several signals have been proposed to move via the PD to provide the positional cues required for the plant growth and development (eg. Lucas et al., 1995; Nakajima et al., 2001; Kim et al., 2002b; Chitwood et al., 2009; Schlereth et al., 2010; Meng et al., 2010). Intriguingly, plant pathogens are able to manipulate and use PD as a route to invade their host plant. This results in a loss of crop yields and affects food production as well as sources of energy and materials for industry. Despite the fundamental importance of symplastic trafficking, it has remained poorly characterized.

In the following sections, an overview of root development and intercellular signaling is presented. Subsequently, the non-cell-autonomous processes regulating root development are discussed and the symplastic signaling route and its regulation are summarized. Finally, a putative regulator of symplastic signaling, callose, is presented and its structure, biosynthesis, degradation, and the role in plant development are discussed.

1.1 ROOT DEVELOPMENT AND INTERCELLULAR SIGNALING

Formation of the *Arabidopsis* root begins as the developing embryo reaches the globular stage of embryogenesis. Here, a single extra embryonic suspensor cell is specified to form the hypophysis and subsequently undergoes an asymmetric cell division (for review see Jenik et al., 2007; De Smet et al., 2010). This division creates two cells; an upper lens shaped cell that will give rise to the quiescent center (QC) and a lower cell that will give rise to the upper cells of the columella. In the root meristem the QC is surrounded by stem cells, which will give rise to various tissue types according to their position; epidermis, ground tissue (containing cortex and endodermis), vasculature or stele (containing

pericycle, xylem, phloem, and procambium), columella, and root cap (Dolan et al., 1997) (**Figure 1**). The stem cell daughters divide several times and finally enter zones of cell elongation and differentiation which are processes driving root growth. The rates of cell division and cell differentiation reach a balance as the seedling reaches maturity five days after germination, resulting in a meristem with a fixed number of cells (Dello Ioio et al., 2007).

Root development is controlled by various non-cell-autonomous signals such as plant hormones, RNA molecules, transcription factors, membrane-bound proteins, and peptides (for review see Robert and Friml, 2009). The transport of intercellular signals can be classified based on the distance. Long-distance transport occurs through the vascular tissues (via the phloem and xylem) as well as the systemic auxin transport (through specific auxin transporters). Short-distance transport includes the local cell-to-cell transport events. Another way to classify signal movement is based on the route through which the signals move; this can be apoplastic, symplastic, or transcellular. Symplastic transport via the PD is a plant specific pathway and operates in a targeted or a non-targeted fashion in the cytoplasmic continuum via the endoplasmic reticulum (ER)-containing PD. The apoplastic transport occurs by a passive diffusion through the apoplastic space between the cell wall and the plasmamembranes (PMs). In transcellular transport a signal moves across the PM and the apoplastic space to the next cell with the help of a specific import-export mechanism. This could occur by secretion of a signal to the apoplast and by endocytosis to the next cell or through a channel-mediated transport to the apoplast and with the help of a carrier to the next cell. In a similar manner to short-distance signals, long-distance signals can also be communicated through the symplastic (the phloem transport), apoplastic (the xylem transport) or transcellular pathways (e.g. polar auxin transport).

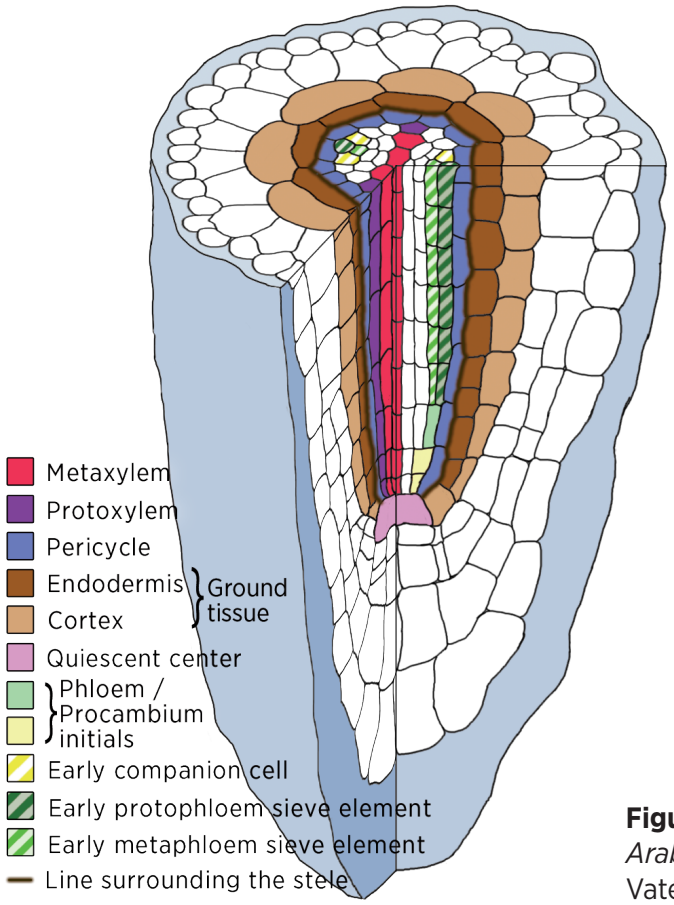

Figure 1 Schematic representation of the *Arabidopsis thaliana* root tip. Modified from Vatén et al., 2011.

1.1.1 LONG-DISTANCE TRANSPORT VIA VASCULAR TISSUES

The vascular tissues form a continuous network throughout the plant. They function as a route for long-distance transport, strengthen the plant body and provide support against changing environmental conditions. The vascular bundle contains tissues specialized for the transport, the xylem and phloem, and the pluripotent procambial cells (Dolan et al., 1997). Mature xylem consists of the water-conducting tracheary elements (TE), and non-conducting, supporting xylem fibers and xylem parenchyma cells. Phloem consists of conducting sieve elements (SE) connected to companion cells (CC), non-conducting phloem fibers and phloem parenchyma cells. The procambial cells are stem cells which remain in an undifferentiated state and retain an ability to divide. Division of the procambial cells produce the new cells potent to differentiate into the phloem or xylem cells, allowing the plant to produce a continuous network of vascular strands.

The xylem transports water, nutrients, and hormones, such as abscisic acid (ABA), cytokinin, and strigolactones from the root to the shoot (shootwards). The xylem stream is regulated by the opening and closure of the stomata in an ABA-dependent manner (for review see Jiang and Hartung, 2008). During a period of drought, ABA is transported to the shoot, which results in the closure of stomata. Strigolactones and cytokinin are both regulators of the shoot branching (Chatfield et al., 2000; Bennett et al., 2006; Umehara et al., 2008; Gomez-Roldan et al., 2008; for review see Domagalska and Leyser, 2011). Cytokinin promotes shoot branching whereas strigolactones inhibit it. The transport of these hormones is affected by the availability of nutrients, such as nitrogen, phosphorus, sulfur, and iron. Hence, xylem transport provides a mechanism to maintain the balance between growth of the root and shoot systems in relation to the available resources.

The phloem forms a symplastic continuum, and hence, transport through the phloem can be tightly regulated. The phloem sieve tubes are connected via sieve pores (type of modified PD). Phloem sap contains photosynthetic assimilates (for review see Gibson, 2004; Dinant and Lemoine, 2010) and signaling molecules, such as mRNAs, small RNAs (for review see Kehr and Buhzt, 2008), proteins, and hormones (ABA, auxin, and cytokinin). Certain plant viruses are able to invade plants via the phloem (for review see Vuorinen et al., 2011). A wide variety of proteins have been shown to be abundant in phloem sap (Lin et al., 2009). However, only a subset of these proteins have been shown to be transported via the phloem and furthermore, only in a few specific examples has the mobility of these proteins been shown to be essential for their function. The transcription factor regulating flowering time, FLOWERING LOCUS T (FT), is produced in the vascular tissues of leaves (Takada and Goto, 2003) and has been shown to move from the CC to the SE and finally to the shoot apical meristem (SAM) to control the meristem determinacy and the floral transition (Corbesier et al., 2007; Mathieu et al., 2007; Jaeger and Wigge, 2007). Also microRNA399 (miRNA399/miR399) has been shown to accumulate in the phloem during phosphorus starvation (Pant et al., 2008). Furthermore, when the shoots of plants overexpressing miR399 were grafted onto wild-type roots, an accumulation of miR399 and a decrease of the levels of miR399 target transcript, *PHOSPHATE 2* (*PHO2)*, were observed in the root.

Cytokinin is transported both via the phloem and the xylem. Specific cytokinin species seem to be enriched in the phloem (such as N^6- (Δ^2-isopentenyl) adenine (iP) and *cis*-zeatin) and other species are enriched in the xylem (for example, *trans*-zeatin) (Corbesier et al., 2003; for review see Hirose, 2008, Kudo et al., 2010). In addition, it has been proposed that the phloem-transported cytokinin is predominantly transported towards the root (rootwards) whereas the cytokinin in the xylem is transported shootwards. Tight compartmentalization of different cytokinin species in the vasculature suggests that they may display different biological roles. It is unknown whether the cell-to-cell transport of cytokinin requires specific transporters. PURINE PERMEASES (PUP) and EQUILIBRATIVE NUCLEOSIDE TRANSPORTERS (ENT) have been proposed to be such a transporters (Bürkle et al., 2003; Sun et al., 2005). Although auxin can move long distances via the phloem by mass flow (Cambridge and Morris, 1996), the biological function of such a bulk flow is unknown.

1.1.2 AUXIN TRANSPORT AND THE FORMATION OF VASCULATURE

The phytohormone auxin regulates plant growth and development through the establishment of asymmetries in the local concentration of auxin. The specific spatial distribution patterns of auxin are driven through local biosynthesis and a directional cell-to-cell auxin transport. It is well established that auxin moves through a specific transport system involving auxin efflux and influx transporters belonging to PIN-FORMED (PIN), p-glycoproteins of the ATP-binding cassette (ABC)-type transporter (ABCB/PGP), and AUXIN-RESISTANT 1/LIKE-AUX 1 (AUX1/LAX) families (Gälweiler et al., 1998; Swarup et al., 2001; Blakeslee et al., 2007; for review see Zažímalová et al., 2010). AUX1/LAX are responsible for the auxin influx whereas PINs and ABCBs/PGPs promote the auxin efflux. It has been shown that the polar localization of PINs determines the direction of auxin flow. In contrast, ABCB/PGPs are mostly apolarly localized and maintain stable auxin flow throughout the tissues. PIN1-4 and 7 are polarly localized at the PM and generate the polar auxin transport stream (Petrášek et al., 2006) whereas PIN5, 6, and 8 are localized at the endomembranes and are likely to regulate the intracellular auxin distribution (Mravec et al., 2009). PM-localized PINs are continuously recycled between the PM and the endosomal compartments (Geldner et al., 2001; Dhonukse et al., 2008).

Differentiation of the vascular bundles has long been suggested to occur via a process directed by the auxin flux, which further up-regulates and polarizes the transport of auxin (Sachs 1981). PIN1 is polarly localized on the surface layer of the leaves to create an auxin maximum (Scarpella et al., 2006). Next, the auxin flux is directed to inner tissues to define the site of the future procambium which is followed by the expression of an auxin-inducible pre-procambial marker gene *ARABIDOPSIS THALIANA HOMEOBOX 8 (ATHB8)* and auxin reporter *pDR5rev::GFP* at the site of the future venation (Scarpella et al., 2006; Bayer et al., 2009). AUXIN RESPONSE FACTOR 5 (ARF5)/MONOPTEROS (MP) is an important transcription factor required for the formation of the embryonic root and is a regulator of the auxin signaling in the procambium precursor cells. The *mp* mutant displays a distorted embryonic axis and vascular discontinuity (Przemeck et al.,

1996; Hardtke and Berleth, 1998). *ATHB8* has been shown to be a direct target of MP (Donner et al., 2009). ATHB8 functions to stabilize the specification of preprocambial cell fate against the auxin transport perturbation by reducing sensitivity to auxin in a *PIN1* mediated manner (Donner et al., 2009). In addition to *ATHB8*, at least two additional direct targets of MP, TARGET OF MP 5 (TMO5), a basic helix-loop-helix (bHLH) type transcription factor, and TMO6, a Dof-type transcription factor, are expressed in the vascular tissues and are probably involved in the regulation of vascular development (Schlereth et al., 2010). The specialized and strictly regulated auxin transport system is essential for patterning of the vascular tissues, as well as numerous other aspects of plant development. Since the mobility of auxin regulates the formation of the specialized transport tissues (the vasculature), auxin also has the ability to modify the mobility of other substances moving via the xylem and the phloem (for review see Leyser, 2011).

1.1.3 PEPTIDE SIGNALING

Peptide-receptor signaling is a common mechanism to regulate plant development (for review see Katsir et al., 2011): in such cases, a short peptide is produced, cleaved, and processed in the cytosol and then secreted to the apoplast where it diffuses until it binds to a receptor. One of the most commonly known peptide-receptor signaling scheme functions in the maintenance of the shoot apical meristem (SAM). In the SAM the homeobox protein WUSCHEL (WUS) functions in a non-cell-autonomous manner to forms a negative feedback loop with CLAVATA 3 (CLV3) peptide and CLV1 and CLV2/CORYNE (CRN) receptors (Schoof et al., 2000). The CLV3 peptide is produced by the stem cells and diffuses into the underlying tissues to prevent the WUS activity and in doing so regulates the size of the stem cell population. A gene homologous to *WUS* also operates in the root tip to maintain the stem cells. *WUSCHEL-RELATED HOMEOBOX 5, WOX5* is expressed in the QC cells at the root tip (Haecker et al., 2004; Sarkar et al., 2007). The CLV3 homolog, CLE40, which is a member of CLV3/ENDOSPERM SURROUNDING REGION (ESR)-related (CLE) clade, has been shown to control stem cell proliferation in the distal root meristem (Stahl et al., 2009). CLE40 is thought to regulate *WOX5* expression via its putative receptor, ARABIDOPSIS CRINKLY 4 (ACR4) (de Smet et al., 2008; Stahl et al., 2009). Recently, it has been shown that CLE-peptide signaling plays an additional key role in the vascular tissues where it regulates the balance between the differentiation of the xylem and the maintenance of the procambial cell fate. The CLE41/CLE44 peptide TDIF (tracheary element differentiation inhibitory factor) inhibits xylem cell differentiation and promotes the proliferation of the procambial cells in both hypocotyls and leaves (Ito et al., 2006; Hirakawa et al., 2008). The cambium and the procambium specific CLV-like receptor kinase PHLOEM INTERCALATED WITH XYLEM (PXY)/TDIF RECEPTOR (TDR) acts as the TDIF receptor (Fisher and Turner, 2007; Hirakawa et al., 2008). TDIF is secreted from the phloem, it then moves to the cambial cells where it is directly bound by PXY. A procambium/cambium specific gene, *WOX4* (Ji et al., 2009; Ji et al., 2010; Hirakawa et al., 2010), is a downstream target of the TDIF/PXY pathway and it promotes the procambial cell divisions in a cell-autonomous fashion. Interestingly, it does not affect the xylem

differentiation. Hence, after the TDIF/PXY signal-recognition step, at least two routes must diverge; one regulating the procambial cell divisions in a *WOX4*-dependent manner and another regulating the xylem cell differentiation in a *WOX4*-independent manner.

1.1.4 MOBILE PROTEINS

A maize mutant with a peculiar knotted leaf phenotype was identified in the 1980s (Freeling and Hake, 1985). Mosaic analysis revealed that in the leaves of *Knotted 1* (*Kn1*), a mutant sector was non-cell-autonomously regulating cell divisions in the epidermis (Hake and Freeling, 1986). This phenotype was shown to be the result of a dominant mutation in the *KN1* gene (Vollbrecht et al., 1991). *KN1* mRNA was detected in the shoot meristems but it was excluded from the epidermal (L1) layer whereas KN1 protein was detected also in the L1 (Jackson et al., 1994). These observations lead to hypothesis that KN1 could move from cell to cell. KN1 was subsequently shown to be mobile and hence it was the first plant protein found to move from cell to cell (Lucas et al., 1995; Kim et al., 2002b). KN1 is sufficient to increase the PD size exclusion limit (SEL) and promote the mobility of KN1 protein-RNA complex from cell to cell (Kragler et al., 2000; Lucas et al., 1995). The movement of KN1 is developmentally regulated; when GFP-KN1 was overexpressed in the SAM epidermal layer in *Arabidopsis*, it was capable to traffic to the L2 and L3 layers underneath the L1 (Kim et al., 2003). However, overexpression of the GFP-KN1 in the leaves revealed directional trafficking pattern; GFP-KN1 moved from the leaf mesophyll to the epidermis, but could not traffic to opposite direction. KN1 contains a KNOX homeodomain and this is required and sufficient for the movement of KN1 (Kim et al., 2005a). A corresponding *Arabidopsis* gene, SHOOTMERISTEMLESS 1 (STM1), also functions in the maintenance of the SAM in a cell-automous fashion (Long et al., 1996). Like KN1, STM1 moves from cell to cell. Recently, it was found that the non-cell-autonomous action of KN1 is mediated by chaperonins (Xu et al., 2011; See chapter 1.2.2.).

Since the discovery of the KN1 locus, a wide array of mobile proteins regulating plant development has been identified. Characterization of the mobility of transcription factors in the root suggests that as many as 25–29% of the transcription factors may be able to move from cell to cell (Lee et al., 2006; Rim et al., 2011). However, these must be studied case by case to determine whether or not their mobility is biologically relevant. The *Arabidopsis* root forms during the globular stage of the embryo development in which MP regulates the specification of the hypophysis (See chapter 1.1.2). *MP* is expressed in the cells adjacent to the hypophysis and it acts non-cell-autonomously. The auxin-mediated degradation of the MP interacting protein, BODENLOS (BDL) (Hamann et al., 1999; Hamann et al., 2002), is required for the differentiation of the embryonic root. This degradation releases MP from the auxin-mediated transcriptional inhibition and allows the activation of its target genes (Dharmasiri et al., 2005; Weijers et al., 2005; Weijers et al., 2006; Schlereth et al., 2010). Besides the mobility of auxin, also the movement of a bHLH transcription factor TMO7 is required for the *MP*-dependent formation of the embryonic root (Schlereth et al., 2010). In the globular embryos, *TMO7* mRNA is detected in the same domain as *MP,* next to the hypophysis, but TMO7 is also detected in the

hypophysis, indicating an additional non-cell-autonomous activity. A reduced level of *TMO7* results in altered division of cells in the hypophysis and occasionally the formation rootless seedlings. When *TMO7* is mis-expressed in the hypophysis, *TMO7* is sufficient to rescue the weak *mp* allele indicating that the hypophysis is the site of the TMO7 function. Hence, TMO7 and auxin are MP-mediated non-cell-autonomous signals required for the embryonic root formation.

SHORT-ROOT (SHR) is a GRAS transcription factor required for cell division of the endodermis-cortex initial cell, the specification of QC fate, and the maintenance of QC identity (Helariutta et al., 2000; Sabatini et al., 2003; Heidstra et al., 2004). A loss of *SHR* leads to dwarfed phenotype with a short root containing a single ground tissue layer with cortex identity. SHR and another GRAS-type transcription factor, SCARECROW (SCR), have been shown to regulate the formative cell division producing two ground tissue layers via direct transcriptional regulation of the cell-cycle gene *CYCD6;1* (Sozzani et al., 2010). *SHR* is transcribed in a subset of stele cells and SHR protein moves to the endodermal layer surrounding the stele and to the QC cells, where it enters the nucleus (Nakajima et al., 2001; Sabatini et al., 2003; Heidstra et al., 2004). When SHR is ectopically expressed in the epidermis or in the phloem CC it is not sufficient to move to the surrounding layers suggesting that the movement of SHR is controlled in a tissue specific fashion (Sena et al., 2004). Several regions in the GRAS domain of SHR are required both for the cell-to-cell movement and for the correct subcellular localization of SHR (Gallagher et al., 2009). Sequestering of SHR to the nucleus in the endodermal cells is dependent on cell-autonomously acting SCR, which is transcribed in the endodermis (Cui et al., 2007). Loss of *SCR* also leads to a phenotype with a single ground tissue layer, however, it displays characteristics of both of the cortex and endodermis cells. Interaction of SHR and SCR in the endodermis directs SHR to the nucleus and thus limits the mobility of SHR to the endodermis only. The outer cell layers have a capacity to respond to SHR and therefore the restriction of the SHR movement is crucial for the correct patterning of the ground tissues (Sena et al., 2004). In addition to SCR, zinc finger proteins such as JACKDAW (JKD) and MAGPIE (MGP) control the activity of SHR (Welch et al., 2007). The SHR-SCR regulated radial pattern formation has been postulated to be an evolutionary conserved mechanism to give a rise to the single endodermal layer observed among almost all land plants (Cui et al., 2007).

Recently, Koizumi et al. (2011) identified a novel protein interacting with SHR and promoting SHR movement. Expression of *SHORT ROOT INTERACTING EMRYONIC LETHAL (SIEL)* is regulated by *SCR* and *SHR*. In addition to SHR, SIEL interacts with other non-cell-autonomous proteins acting in the root such as CAPRICE (CPC) (Wada et al., 1997), TMO7, and the genes acting on the SHR pathway such as SCR, JKD, and MGP. In line with this, a complete loss of *SIEL* leads to an embryonic lethality whereas a milder allele displays a pleiotropic phenotype including altered patterning of the ground tissue and the root hairs (Koizumi et al., 2011). SIEL localizes to both the nucleus and the cytoplasm, where it associates to the endosomes in the latter. It was proposed that SIEL could promote the endosome-mediated transport of SHR to the PD.

CPC is a R3 type Myb-like protein promoting root hair differentiation (Wada et al., 1997). A loss of *CPC* leads to reduced root hair formation. *CPC* mRNA is observed in the root epidermal layer in the atrichoblasts (non-root hair cells) whereas *pCPC::CPC:GFP* is detected in the nuclei throughout the epidermal layer. This indicates that CPC acts non-cell-autonomously (Wada et al., 2002). An S1 region in the N-terminal domain and an S2 region in the Myb domain of CPC are both required for the movement from the atrichoblasts to the trichoblasts (root hair cells) and the S2 domain is also required for the nuclear localization of CPC (Kurata et al., 2005). When *CPC* was ectopically expressed in the stele, CPC was unable to move to the endodermis, however, when it was expressed in the trichoblasts CPC was detected throughout the epidermis. This indicates that the trafficking of CPC is under a tissue specific control. GLABRA3 (GL3) is expressed in the trichoblast and moves to the atrichoblast to promote the cell fate of non-hair cells (Savage et al., 2008). A study incorporating the use of mathematical models proposed that a mutual support mechanism based on the mobility of CPC and GL3 patterns the root epidermis. Recently, it was reported that the entire regulatory network specifying root hairs is controlled non-cell-autonomously by JKD, which is expressed in the QC and ground tissue (Hassan et al., 2010).

Two genes involved in reactive oxygen species (ROS) signaling have been shown to act non-cell-autonomously. A bHLH transcription factor UPBEAT1 (UPB1) was identified as a direct regulator of a set of peroxidases (Tsukagoshi et al., 2010). Loss of *UPB1* leads to an altered concentration of ROS (increase in O_2 and decrease in H_2O_2 levels) and to an increased size of the root apical meristem (RAM). ROS are messengers mediating plant stress responses by regulating the redox balance of the cells. The redox balance has been shown to play a role in the maintenance of root meristem activity (Vernoux et al., 2000) and in the regulation of cell-to-cell communication (Benitez-Alfonso et al., 2009; Rutschow et al., 2011; Stonebloom et al., 2011). UPB1 was proposed to maintain the balance between the cell proliferation and differentiation by regulating the production of ROS. *UPB1* mRNA is detected in the lateral root cap and in the vasculature whereas UPB1 is detected mostly in the nuclei of the transition and elongation zone cells (Tsukagoshi et al., 2010). Therefore, it is possible that UPB1 itself acts as a non-cell-autonomous signal. Additionally, thioredoxin H9 (TRX H9) has been shown to move from cell to cell (Meng et al., 2010). When *TRX H9-GFP* was expressed ectopically under the control of SCR promoter, GFP signal was detected throughout the root tip. TRX H9 is a PM-bound protein and thioredoxins regulate the ROS levels in the cells. Loss of *TRX H9* results in dwarfed plants with short roots, indicating that its function is necessary for plant development. Meng et al. (2010) proposed that TRX H9 could relay redox information from cell to cell.

1.1.5 SMALL RNAS

Small RNAs regulate gene expression in most eukaryotic organisms. They direct the cleavage of their target transcripts and/or modify their target genes by the translational inhibition and thereby cause a negative regulation (for review see Brosnan and Voinnet, 2009; Voinnet, 2009). In addition, small RNAs direct epigenetic modifications (for review

see Lejeune and Allshire, 2011). Small interfering RNAs (siRNAs) lead to RNA interference (RNAi), and this protects plants against harmful RNAs, such as viral, transposon and transgene RNAs. In this scheme a long double stranded RNA (dsRNA) is produced, the DICER-LIKE (DCL) enzymes further process this to form siRNA duplexes (Xie et al., 2004). Next, a single stranded siRNA-strand guides ARGONAUTE (AGO) to cleave the target transcript leading to a degradation of a complementary strand in the target RNA sequence (Baumberger and Baulcombe, 2005). It has been well established that RNAi operates in a non-cell-autonomous manner, as the silencing of the target RNA spreads from cell to cell and more widely through the vasculature (Voinnet and Baulcombe, 1997; Palauqui et al., 1997; Schwach et al., 2005). However, the identity of the mobile silencing signal has remained unknown. Dunoyer et al. (2010) expressed *SUC-SUL*, inverted repeat construct designed to raise the non-cell-autonomous silencing effect against ubiquitously expressed *SULFUR* (*SUL)* gene under the control of CC-specific SUCROSE TRANSPORTER 2 (SUC) promoter. This resulted in a chlorotic phenotype in the tissues where silencing occurs. In wild-type plants, genetic silencing spreads via the vasculature and from the vasculature to the surrounding cells. Dunoyer and colleagues were able to visualize this by the chlorotic lesions in and around the vascular tissues, whereas in *dcl4* mutants silencing does not spread and the chlorotic lesions do not form. When *DCL4* was expressed in the CC in the *dcl4* mutant, recovery of the silencing signal mobility was observed (Dunoyer et al., 2010). Furthermore, CC-specific expression of the cell-autonomous silencing suppressor p19, which binds specifically to the siRNA duplexes, decreased the mobility of the silencing. This suggests that the DCL4 depedent (21- nucleotide) siRNA duplex could be a mobile signal mediating the RNAi in this specific example. By grafting the *dcl2 dcl3 dcl4* mutant reciprocally with the wild-type and by directly sequencing all the siRNAs, Molnar et al. (2010) showed that also the DCL3 dependent (24-nucleotide) siRNAs could act as a mobile silencing signal. In addition, they found that siRNAs direct the epigenetic modification in their target cells.

A second group of small RNAs, miRNAs regulate the transcript levels of endogenous genes. Unlike siRNAs, miRNAs are encoded by the endogenous *MIR* genes, which are independent transcriptional units (Xie et al., 2005). miRNAs are processed by DCL1 and they contain a complementary region for the target transcript, which directs AGO1-mediated degradation of the target mRNA (Rhoades et al., 2002; Xie et al., 2003; Jones-Rhoades and Bartel, 2004; Baumberger and Baulcombe, 2005). miRNAs regulate the expression levels of key regulators of plant growth and development. For example, class III homeodomain leucine zipper (HD-ZIP III) genes (*PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *ATHB8* and *CORONA* (*CNA*)), which by themselves regulate the radial pattern of the organs, are post-transcriptionally controlled by miR165 and miR166 (miR165/6) (McConnel et al., 2001; Emery et al., 2003; Mallory et al., 2004; Prigge et al., 2005; Zhou et al., 2007). miR165/6 are encoded by nine *MIR* genes, which display distinct and tissue specific expression patterns throughout plant development indicating a diversified regulation for the *HD-ZIP III* gene expression patterns (Jung and Park, 2007). All the five *HD-ZIP III* genes contain a miR recognition sequence complementary

to miR165/6 (Mallory et al., 2004; Zhou et al., 2007). Binding of miR165/6 with the *HD-ZIP III* transcript directs the RNA to the degradation pathway. A mutation in the miRNA recognition sequence of the *HD-ZIP III* genes leads to the formation of a mRNA, that is resistant to miRNA-mediated degradation. The importance of miRNA-mediated control of plant development can be seen through the ectopic expression of HD-ZIP III genes that results in severe pleiotropic phenotypes (McConnel et al., 2001).

How is miRNA activity regulated? Some hints have come from studies on the promoter regions of the *MIR* genes; these contain a conserved TATA-box element, biotic and abiotic stress response elements and an overrepresentation of binding sites for the transcription factors LEAFY (LFY), ARFs, and MYC2 (Xie et al., 2005; Megraw et al., 2006). ARFs modulate the expression of the auxin response genes (for review see Guilfoyle and Hagen, 2007). LFY is a homeotic gene controlling flowering time and is itself controlled by gibberellic acid (GA) (Weigel et al., 1992; Eriksson et al., 2006). MYC2 acts as a repressor of blue-light mediated photomorphogenic growth and increases sensitivity to ABA leading to an enhanced responsiveness to drought (Abe et al., 2003; Yadav et al., 2005). Presence of these elements in the *MIR* promoters suggests that hormonal control among other factors may play an important role in the transcriptional regulation of miRNAs.

Many miRNAs and genes involved in their biogenesis display tissue-specific expression patterns. This has led to a hypothesis that miR function in a cell-autonomous manner. However, miRNA species have been detected in the phloem both by in situ hybridization and by cloning them from the phloem sap (Valoczi et al., 2006; Buhtz et al., 2008). Separate experiments were performed where plants were grown in the absence of sulphate, copper, or phosphate. Under such conditions, the levels of the mature miRNAs respond to starvation of these nutrients in the non-vascular-tissues, and the levels of these miRNAs were also strongly increased in the phloem (Buhtz et al., 2008). In addition, when shoots over-expressing miR399 were grafted with the wild-type roots, a downregulation of the miR399 target *PHO2* was observed in the root, demonstrating that miR399 is transported via the phloem (Pant et al., 2008)

Third small RNA group is composed of trans-acting siRNAs (tasiRNAs) (Vazquez et al., 2004; Peragine et al., 2004). tasiRNAs are processed from mRNAs encoded by the endogenous genes and, furthermore, cleaved by them in a miR-mediated manner. The processing of a *TAS3* encoded transcript is guided by miR390 to produce 21-nt siRNAs (tasiR-ARFs), which target the expression of *ARF3* (Allen et al., 2005). Chitwood et al. (2009) showed that the tasiR-ARF biogenesis localizes to the two upper (adaxial) layers of the leaf, whereas their target, *ARF3*, is expressed outside this region on the lower (abaxial) side of the leaf. TasiR-ARFs form a gradient in the leaf; a high level of tasiR-ARFs is observed at the upper surface of the leaf (the domain in which they are synthesized), and the levels gradually decrease towards the lower surface of the leaf. These observations suggest that tasiR-ARFs could move out of the cells in which they are synthesized. The resulting gradient of tasiR-ARFs could create the observed sharp expression boundary of *ARF3*. These results are consistent with a modelling study, which predicted that the non-cell-autonomous action of small RNAs have the potential to sharpen spatial expression patterns (Levine et al., 2007).

1.2 SYMPLASTIC SIGNALING OCCURS VIA PD

1.2.1 STRUCTURE, FUNCTION, AND EVOLUTIONARY ORIGIN OF PD

PD are cylindrical structures penetrating the cell wall with two coaxial membrane tubes (**Figure 2A–B**). An inner tube is called the desmotubule (DT) and it connects the ERs of bordering cells. The PM forms outer membrane and the space between these membranes is called the cytoplasmic sleeve. Ultrastructural studies on PD have revealed the presence of spoke-like linkers between the DT and the PM as well as between the PM and the cell wall (Ding et al., 1992; Overall and Blackman, 1996). The DT has been suggested to contain proteinaceous components in its lumen (Hepler, 1982; Ding et al., 1992). However, the identity of these structures is currently unclear. The cytoplasmic sleeve contains callose (Allison and Shalla, 1974), cytoskeletal components, such as actin (White et al., 1994) and myosin (Baluška et al., 2001). Furthermore, it is likely to contain numerous proteins reported to localize at the PD (Levy et al., 2007a; Thomas et al., 2008; Simpson et al., 2009; Jo et al., 2011; Fernandez-Calvino et al., 2011).

A molecular flux through the PD is thought to happen primarily via the cytoplasmic sleeve (**Figure 2A**). Consistent with this hypothesis, studies on the mobility of fluorescently labeled sphingolipids suggest that there may be a barrier preventing lipid-diffusion through the PM of the PD (Grabski et al., 1993). Recently, the PM-bound TRX H9 was shown to traffic from cell to cell and its binding to the PM was shown to be essential for its cell-to cell mobility indicating that the trafficking via the plasmolemma might be possible (Meng et al., 2009). Moreover, it has been reported that ER-bound (Guanoune-Gilbert et al., 2008) and small ER-lumen targeted molecules (Barton et al., 2011) move from cell to cell suggesting that also DT have the potential to mediate trafficking.

The exact mechanisms through which PD are formed are poorly understood and the molecular components involved in this process are currently not known. However, some PD are known to form during cytokinesis (primary PD), whereas the others form after cytokinesis (secondary PD). It is thought that during the formation of the primary PD, ER tubules are inserted across the phragmoplast of the dividing cell (Hepler, 1982). The Golgi

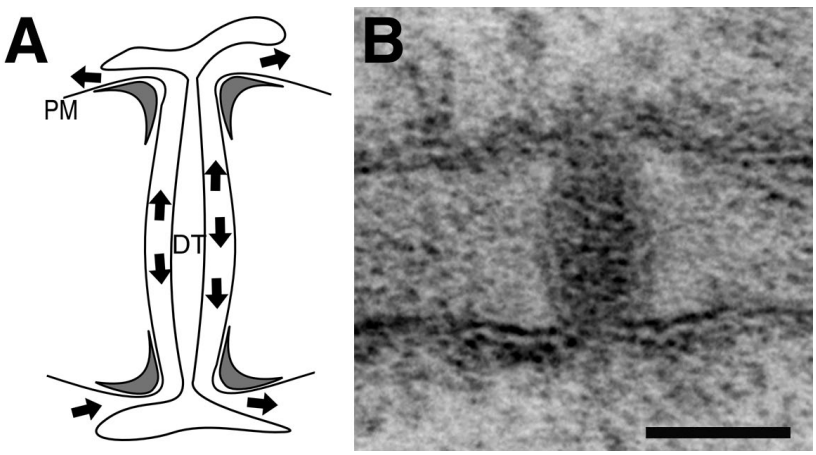

Figure 2 Structure of the plasmodesmata containing callose at the neck region (grey) (A). A transmission electron micrograph showing the PD ultrastructure in the wild-type stele (B). PM, plasmamembrane; DT, desmotubule; scalebar, 50 nm. Modified from Vatén et al., 2011.

vesicles transport cell wall material to the forming cell plates, then fuse together and push the ER tubules between them. A strong callose accumulation at the cell plate coincides with this process. Finally, the extensively remodeled and constricted ER forms the DT and the Golgi-derived membranes form the PM of the new PD. It has been hypothesized that the tightly constricted organization of the DT is achieved by a protein-scaffold stabilizing the structure (Tilsner et al., 2011). The PM is likely to undergo changes in its lipid composition to attract PD-proteins. Among the PD-localized proteins are PD callose binding protein 1 (PDCB1), β-1,3-glucanase, and remorin (Levy et al., 2007a; Raffaele et al., 2009; Simpson et al., 2009), which are suggested to localize to lipid rafts containing more saturated phopholipids in the tightly packed conformation than the surrounding PM (Tilsner et al., 2011) (see chapter 1.2.2).

The secondary PD exist either between clonally related or unrelated cells (Bergmans et al., 1997; van der Schoot and Rinne, 1999). However, the mechanism through which the secondary PD form is the subject of debate. One scenario explaining how the secondary PD may form is by a fission of the primary PD resulting in a twinned secondary PD (Faulkner et al., 2008). A second scheme suggests that the ER of the pre-existing PD may act as a template for the formation of the new PD nearby the pre-existing PD (Faulkner et al., 2008). In a third scheme, cell wall thinning initiates the formation of the PD followed by a modification of the cell wall leading to the formation of *de novo* secondary PD (for review see Ehlers and Kohlmann, 2001). Silencing of two RNA helicases, ISE1 and ISE2, leads to a formation of fewer secondary PD between the epidermis and the mesophyll cells in *Nicotiana benthamiana* (Burch-Smith and Zambryski, 2010). Also, either the transition between vegetative growth and competency to flower or exogenous applications of cytokinin has been shown to promote the formation of the secondary PD in the SAM in *Sinapis alba* (Ormenese et al., 2000; Ormenese et al., 2006). The PD between clonally unrelated cells form continuously and their formation must be tightly controlled to ensure correct cell signaling during plant development (Zhu et al., 1998a; Zhu et al., 1998b).

As the tissues mature the PD structures become more complex (Burch-Smith and Zambryski, 2010). The primary PD can be extensively modified and can display either a simple or a complex and branched morphology similar to the secondary PD (for review see Burch-Smith et al., 2011a). Therefore, their origin cannot be concluded based solely on the PD structure. It is possible that modification of the PD may also modify their function. Embryos have been shown to contain several symplastic domains (eg. unloading domains displaying different SEL compared to surrounding tissues). Consistently with this, embryonic tissues contain branched PD.

PD exist in all land plants (embryophytes). The evolution of multicellularity has created a need for extensive communication between cells. However, it is not clear when and where the PD first appeared in plants. Embryophytes are phylogenetically the closest clade of plants to the small and diverse group of green algae (streptophyta) (for review see Becker and Marin, 2009). PD are present in streptophyta algae in species where formation of the phragmoplast takes place during cell division (Pickett-Heaps, 1967; Marchant and Pickett-Heaps, 1973; Fowke and Pickett-Heaps, 1969). Therefore,

it has been suggested that the existence of the phragmoplast may have preceded the appearance of the PD (Graham et al., 2000). Based on phylogenetic analyses, one of the three streptophyta clades is likely to be the closest sister lineage for the embryophytes; the Charales, the Zygnematales, or the Coleochaetales. Until now, studies defining the evolution of the PD have focused on Charales, most likely because this lineage has been considered to be morphologically the most complex (Franceshi et al., 1994; Cook et al., 1997; Faulkner et al., 2005; Brecknock et al., 2011). Some studies have failed to detect primary PD in Charales (Franceshi et al., 1994), whereas other studies have shown that this group displays similar PD to embryophytes (Cook et al., 1997; Brecknock et al., 2011). Recent phylogenetic investigations suggest that Charales may not be, in fact, the closest group to the embryophytes (Finet et al., 2010; Wodniok et al., 2011). Instead, the Coleochaetales (Finet et al., 2010) or the Zygnematales, or a group consisting of both the Zygnematales and the Coleochaetales (Wodniok et al., 2011) are likely to be the sister group of embryophytes. As new imaging technologies (Fizgibbon et al., 2010; for review see Bell and Oparka, 2011), stable transformation methods for streptophyta species (Abe et al., 2011), and a list of the PD-components (Fernandez-Calvino et al., 2011) will come available, the appearance of the PD may be clarified.

1.2.2 REGULATION OF SYMPLASTIC TRANSPORT

The PD connect plant cells creating supra-cellular entities called the “symplasts” (Lucas et al., 1993). Symplastic trafficking refers to all trafficking via the symplast. Studies based on microinjections of small molecules have revealed that sugars and minerals are able to traffic passively by diffusing from cell to cell and have established the term size exclusion limit, SEL (Terry and Robards, 1987). The SEL indicates the size of the largest molecule, which may move passively via the PD. However, later studies have revealed that the nature of the PD SEL is very dynamic: it is sensitive to environmental conditions (Rinne and van der Schoot, 1998; Crawford and Zambryski, 2001) and different species, organs, and tissues display differences in the PD SEL (Howard et al., 1994; Oparka et al., 1999; Imlau et al., 1999; Kim et al., 2002a; Kim et al., 2005b). A decreased SEL leads to reduced movement, whereas an increased SEL results in an elevated transport capacity. It has been established that the mobility through the PD can happen via the targeted or non-targeted pathway (Crawford and Zambryski, 2001). Molecules moving via the targeted pathway, such as KN1, actively increase the PD SEL to enter the PD channel and the neighboring cell, whereas molecules moving in a non-targeted fashion diffuse via the PD. Plant viruses move from cell to cell and this movement has been shown to involve elaborate interaction with the PD (Wolf et al., 1989; Waigmann et al., 1994; for review see Niehl and Heinlein, 2011). Finally, several endogenous proteins regulating plant growth and development have been shown to act non-cell-autonomously and these are likely to move from cell to cell via the PD (see chapter 1.1.4. and for review see van Norman et al., 2011).

Genetic screens have been performed to identify factors regulating symplastic transport during embryogenesis (Kim et al., 2002a) and during post-embryogenic

growth (Benitez-Alfonso et al., 2009). A developmental transition involving the down-regulation of the PD SEL occurs at the mid-torpedo stage of embryogenesis in *Arabidopsis* (Kim et al., 2002a). Two embryonically lethal mutants *increased size exclusion limit of the plasmodesmata1* (*ise1*) and *ise2*, which failed to undergo this transition, have been identified. The *ise1* and *ise2* mutants display increased molecular trafficking and contain a higher proportion of twinned and branched PD compared to wild-type embryos (Kim et al., 2002a; Burch-Smith and Zambryski, 2010). Silencing of *ISE1* and *ISE2* in *Nicotiana benthamiana* results in a phenotype resembling the *Arabidopsis ise1* and *ise2* mutants with an increased molecular trafficking and an increased formation of the twinned and branched secondary PD (Kobayashi et al., 2007; Stonebloom et al., 2009; Burch-Smith and Zambryski, 2010). *ISE1* encodes a mitochondria-localized DEAD-box RNA helicase (Stonebloom et al., 2009) and *ISE2* encodes a choloroplast stroma localized DEVH-box RNA helicase (Kobayashi et al., 2007; Burch-Smith et al., 2011b). Whole genome expression analysis of *ise1* and *ise2* revealed that both display altered expression of cell wall, PD and plastid function related genes; this is consistent with the defective plastid development observed in these mutants (Burch-Smith et al., 2011b). Based on this work it was suggested that an organelle-nucleus cross talk regulates the PD mediated cell-to-cell signaling in plants. Furthermore, by using redox-sensitive organelle-specific fluorescent proteins, Stonebloom et al. (2011) showed that silencing of *ISE1* and *ISE2* leads to a decreased level of ROS in the plastids, whereas silencing of *ISE1* leads to an elevated level of ROS in the mitochondria. By using inhibitors of the mitochondrial or the plastid metabolism known to increase the ROS production, it was shown that the mitochondrial ROS production leads to an increased cell-to-cell transport, whereas the ROS production in the plastids leads to a decreased cell-to-cell transport (Stonebloom et al., 2011). This suggests that *ISE1* and *ISE2* regulate the redox-state of organelles and this may be one factor that affects the PD formation and function. Hence, the production of ROS may regulate loosening of the cell wall during the *de novo* secondary PD formation and/or promote the deposition of callose (a sugar molecule suggested to regulate the PD SEL, see chapter 1.3) at the PD. Stonebloom et al. (2011) hypothesized that under anoxic conditions a greater degree of cell-to-cell mobility might be promoted to enhance the transport of carbohydrates away from the anoxic zones, and during an oxidative stress the cell-to-cell connectivity could be reduced to minimize the harmful effects.

In parallel to these studies, Benitez-Alfonso et al. (2009) discovered that ROS are involved in the regulation of the PD permeability. The seedling lethal *gfp arrested trafficking1* (*gat1*) mutant displays reduced molecular trafficking, an altered PD structure, an elevated level of ROS and an increase in callose deposition throughout the root tip. *GAT1* encodes a plastid localized m-type thioredoxin expressed in the organ primordia and meristems. Phenotypic defects in *gat1* could be due to a loss of the redox balance, which induces callose deposition and results in altered PD structure or regulation, and as a consequence, lead to the decreased PD-mediated trafficking. Furthermore, Rutschow et al. (2011) analyzed the PD permeability in root tips during ROS treatment by combining an analysis of the fluorescence recovery following photobleaching with a mathematical

model on symplastic diffusion. Interestingly, they found that a two hour treatment with low doses of ROS leads to a strikingly increase in the permeability of PD, whereas high doses of ROS decreased PD permeability. These results show that the regulation of ROS production is involved in the modulation of cell-to-cell signaling. Furthermore, this mechanism seems to be sensitive at least to the subcellular localization of ROS production as well as to ROS dosage. The exact mechanisms as well as the molecular components acting in the ROS-mediated regulatory pathway must be identified to further clarify this complex scheme.

The deposition of callose is thought to regulate the PD aperture. The callose deposition in the neck regions of the PD has been observed in studies based on TEM (Allison and Shalla, 1974; Delmer et al., 1993). When callose deposition was chemically inhibited with 2-deoxy-D-glucose (DDG) (Jaffe and Leopold, 1984), the PD neck region did not show a constricted pattern as it shows without the inhibiting treatment (Radford et al., 1998; Radford and White, 2001). This raised a question regarding the presence of callose at the PD; is callose a natural component of the PD, or is the callose deposition induced by physical wounding during the fixation process? However, callose deposition at the PD has been shown to modify cell-to-cell signaling. Callose degradation enhances cell-to-cell movement (Bucher et al., 2001; Rinne et al., 2005) and consistent with these results, the ectopic expression of *GAT1*, a negative regulator of the ROS-activated callose synthesis, results in increased trafficking in leaves (Benite-Alfonso et al., 2009). In contrast, cell-to-cell trafficking is impaired as a result of conditions leading to an increase in callose accumulation at the PD, such as aluminium treatment (Sivaguru et al., 2000), over-expression of callose binding protein PDCB1 (Simpson et al., 2009), loss of *GAT1*-dependent negative regulation of callose synthesis (Benitez-Alfonso et al., 2009), or loss of callose degrading enzymes, β-1,3-glucanases (Iglesias and Meins, 2000; Levy et al., 2007a). These results suggest an important role for the callose biosynthesis in the regulation of the PD aperture.

How is callose deposited at the PD? *CALLOSE SYNTHASE* (*CALS*)/ *GLUCAN SYNTHASE-LIKE* (*GSL*) genes encode proteins, which are likely to function as callose synthesizing enzymes (see chapter 1.3.2). However, none of them have so far been shown to localize to the PD. Guseman et al. (2010) showed that a loss of *CALS10/GSL8* leads to an enhanced cell-to-cell connectivity in leaves suggesting that *CALS10* could be involved in the callose biosynthesis at the PD. However, *CALS10* encodes a protein required for the timely deposition of callose at the cell plate during the cell division and as a result, *cals10* displays severe cytokinesis defects involving incomplete cell walls (Chen et al., 2009; Thiele et al., 2009; Guseman et al., 2010). Hence, the altered cell-to-cell connectivity in *cals10* and the role of *CALS10* in the synthesis of the PD-localized callose has remained an open question. Interestingly, Simpson et al. (2009) identified PDCB1, which displays callose-binding activity and localizes mostly to the PD neck region. Overexpression of *PDCB1* caused an increase in callose accumulation accompanied by a decrease in cell-to-cell mobility, whereas a loss of *PDCB1* did not lead to any detectable phenotype (Simpson et al., 2009). The latter could be due to a redundant activity of the 11 PDCB1-like proteins.

It was postulated that PDCB1 could function as a scaffold between the ER and the cell wall to stabilize the PD structure. It will be interesting to learn what is the exact role of PDCB proteins in the deposition of PD-localized callose.

It has been hypothesized that trafficking via the PD may involve changes in the structure of mobile proteins. Kragler et al. (1998) noticed that mobility of KN1 was inhibited by an internal crosslinking of the protein, suggesting that the modification of the protein conformation could be involved in the trafficking of KN1 through the PD. It was proposed that this conformational change could be facilitated by the interaction with chaperons (Kragler et al., 1998). A family of heat shock proteins including (HSP70) contains a group of molecular chaperons conserved among eukaryotes, which are involved in multiple processes, such as protein transport, protein aggregation, and assistance in protein folding (Saibil, 2008). The yellow beet virus homolog of the HSP70 (HSP70h) was found to associate with the PD (Medina et al., 1999) and facilitate translocation of the virus from cell to cell (Peremyslov et al., 1999). Furthermore, two HSP70 resembling proteins were detected from PD enriched cell wall fractions. This was followed by the identification of two heat shock cognate 70 (HSC70) proteins from the phloem stream with the ability to move from cell to cell (Aoki et al., 2002). Recently, it was found that the non-cell-autonomous action of KN1 is mediated by chaperonins (Xu et al., 2011). A gene encoding chaperonin containing TCP1 (CCT8) was identified in a screen based on a trichome rescue system (Kim et al., 2005a). In this experiment, a trichome deficient *Arabidopsis* mutant *glabra1* (*gl1*) was rescued by a mobile GFP-GL1-KN1 protein fusion expressed in the mesophyll cell layer adjacent to the epidermal layer. In wild type GL1 acts cell-autonomously in the epidermis to promote trichome differentiation. The *cct8* mutation decreased the mobility of GFP-GL1-KN1 from the mesophyll to the epidermis and resulted in a loss of the trichome rescue in the *gl1* (Xu et al., 2011). CCT8 is a subunit of type II chaperonin complex, which forms oligomeric double-ring assemblies that assist in protein folding. When the levels of other members of the CCT family, CCT1-5, were reduced by artificial miRNAs, the rescue of the trichome development was lost suggesting that the whole type II chaperonin complex is likely to be involved in promoting KN1 mobility. The weak *cct8* mutant does not display any clear defects in meristem maintenance, but it enhances the severity of the weak *stm1* allele. Therefore, it is possible that CCT8 regulates also the STM1 protein trafficking or folding. Moreover, CCT8 interacted directly with KN1, STM1, and TRANSPARENT TESTA GLABRA (TTG). TTG is a WD40 repeat protein involved in the promotion of the trichome development in a non-cell-autonomous fashion (Larkin et al., 1994). CCT8 also modified the mobility of TTG, but not SHR movement, suggesting that trafficking of non-cell-autonomous proteins does not occur through a single pathway (Xu et al., 2011). Only when expressed in the epidermis was CCT8 sufficient to rescue trichome development (and hence the KN1 mobility) in *cct8* suggesting that CCT8 acts in the destination cell of KN1. It is therefore possible that the chaperonin complex promotes KN1 trafficking by assisting the refolding of KN1 after its entrance in destination cells. Further, it was recently reported that *cct8* mutation leads to reduced spread of tobamovirus infection suggesting that chaperonins may also function in their cell-to-cell translocation (Fichtenbauer et al., 2012).

A receptor-like kinase (RLK) CRINKLY4 (CR4) has been shown to localize to the PD in a tissue specific manner and to regulate the SEL of the PD in the maize endosperm (Tian et al., 2007). Recently, nine other RLKs have been shown to localize to the PD (Jo et al., 2011; Fernandez-Calvino et al., 2011). The corresponding ligands for these RLKs are currently unknown. A family of PD-localized proteins (PDLP) resembling type I membrane receptor-like proteins has been shown to regulate cell-to-cell trafficking (Thomas et al., 2008; Lee et al., 2011). PDLP1 localizes to the PD and contains a single transmembrane domain, which has been shown to be necessary for its localization to the PD. Overexpression of either *PDLP1* or *PDLP5* leads to a reduction in cell-to-cell trafficking, whereas when combined with a loss of function mutant of another member of PDLP family, loss of either *PDLP5* or *PDLP1* leads to an increase in cell-to-cell connectivity. Surprisingly, the *pdlp1 pdlp2 pdlp3* mutant displays a reduced viral trafficking (Amari et al., 2010). Further investigations revealed that PDLP1-3 proteins interact with viral proteins and promote the mobility of tubule-forming viruses. It has been hypothesized that endogenous PDLPs might display a receptor-like function for viruses and facilitate the assembly of proteins associated with viral movement at the PD (Amari et al., 2010). Decreased levels of *PDLP5* cause a reduced accumulation of callose at the PD, suggesting that PDLP5 promotes callose synthesis at the PD (Lee et al., 2011). *PDLP5* expression is strongly induced by salicylic acid (SA) treatment, bacterial invasion, and senescence, whereas *PDLP5* overexpression induces the hyperaccumulation of SA, deposition of callose at the PD, chlorosis, and ultimately, cell death. These results suggest that PDLP5 might function as a regulator of the plant innate immunity responses and decrease the symplastic connectivity during the pathogen attack to protect neighboring cells from the pathogen invasion (Lee et al., 2011). These findings show a clear connection between PD permeability and plant pathogen related signaling; in which the innate plant immunity strongly relies on cell-to-cell communication. On the other hand, pathogens benefit from this signaling system and this has been demonstrated by the receptor-like function of PDLP1-3.

Immunolocalization studies have shown that cytoskeletal components such as actin (White et al., 1994), myosin (Radford and White 1998; Reichelt et al., 1999), and proteins resembling tropomyosin (Faulkner et al., 2009) are localized at the PD. It has been suggested that they might play a role in the regulation of the PD function or act as a structural PD component (White et al., 1994; Radford and White, 1998; Radford and White, 2011). A function has been characterized for these cytoskeletal components through studies using chemicals with stabilizing or disrupting effects. However, the function of these particular chemical compounds varies depending on the tissue and the species used (for review see White and Barton, 2011). There has been a critical discussion on the physical constrains for the localization of the large cytoskeletal components at the PD (Tilsner et al., 2011). Although a contractile protein centrin has been shown to localize to the PD, its function in the PD is unknown (Blackman et al., 1999). Studies with plant viruses strongly indicate that the cytoskeleton might play a role in the trafficking via the PD. Amari et al. (2011) showed that targeting of PDLP1 to the PD occurs via a myosin-dependent

endomembrane pathway. A dominant negative myosin mutant displays a mislocalization of both PDLP1 and viral MP, and in addition hinders cell-to-cell trafficking during tubule formation by the *Grapevine fanleaf* virus. This suggests that these processes are myosin-dependent (Amari et al., 2011). An endogenous microtubule-associated protein MPB2C has been shown to have the ability to interact with both of *Tobacco mosaic virus* (TMV) -MP and KN1 (Kragler et al., 2003; Winter et al., 2007). Interestingly, MPB2C is sufficient to prevent cell-to-cell trafficking of both KN1 and viruses. It has been hypothesized that MPB2C could act in a regulatory pathway controlling passage of viruses and HD-proteins via PD (Winter et al., 2007).

Rinne et al. (2001) observed that when plants were subject to a period of cold treatment, this was followed by a restoration of symplastic connectivity in the SAM and an accumulation of spherosome-like vacuoles containing β-1,3-glucanase in the vicinity of the PD. This suggests that the secretory delivery may play a role in the PD targeting. Studies on synaptogamin (SYTA) showed that it binds to both the viral *Cabbage leaf curl virus* (CaLCuV) and TMV MPs and localizes to the endosomes (Lewis and Lazarowitz, 2010). Moreover, a dominant negative form of SYTA results in an inhibition of viral trafficking and prevents the endocytosis. These results suggest that the endocytic recycling pathway is involved in the CaLCuV and TMV trafficking to the PD.

In addition to various factors promoting and preventing cell-to-cell movement, also information contained within a mobile protein itself is likely to play a role in the ability of that protein to undergo trafficking. In a few cases a domain required for the protein trafficking has been identified, such as KN1 and SHR (Kim et al., 2005a; Gallagher et al., 2009). In other circumstances a post-translational modification, such as glycosylation and phosphorylation, has been shown to be important for the trafficking through the PD (Taoka et al., 2007). However, no common signal necessary for protein trafficking has been identified so far among the sequences and structures of the various mobile proteins. In conclusion, it is possible that PD mediated transport is not a single pathway; instead, it is likely to involve several parallel transport routes involving specific, elaborate regulation.

1.3 CALLOSE

1.3.1 CALLOSE FORM AND FUNCTION

β-1,3-glucans are polymers composed of glucose residues linked by (1,3)-β-glucosidic bonds (**Figure 3A**). Despite their simple basic structure they display a wide range of forms and biological functions (Bacic et al., 2009). This is mirrored by the common occurrence of β-1,3-glucans among living organisms; they are found in gram-positive and gram-negative eubacteria, fungi, plants (in green algae, red algae, and embryophytes), and chromalveolates. Common features for β-1,3-glucans are borderline solubility in aqueous systems (leading to a tendency to form molecular associations), stiffness of the polymer chain, and a stable triple-stranded helical conformation (that acts as a cross-link in gels).

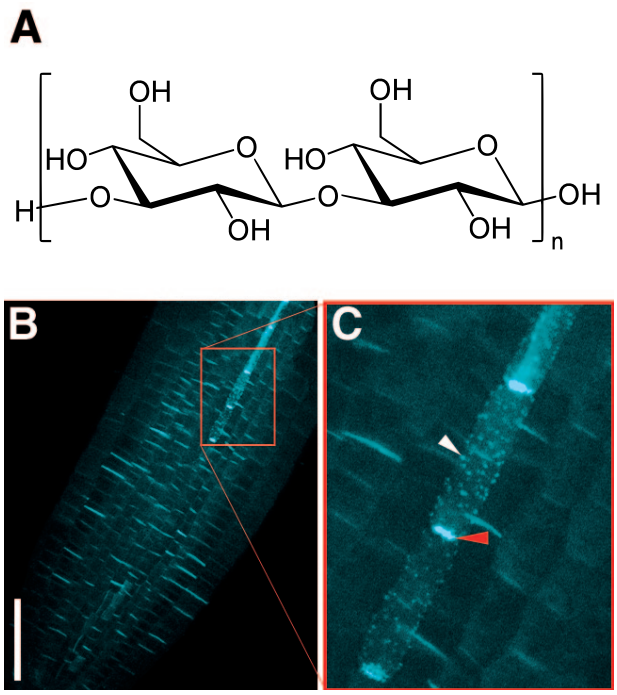

Figure 3 Structure of linear β-1,3-glucan (A). Callose deposition of the *Arabidopsis* root tip (B), and the phloem (C), visualized with aniline blue fluorochrome. Red arrowhead, sieve plate; white arrowhead, callose deposition in the lateral walls of the sieve elements; scalebar, 50 μm. Modified from Vatén et al., 2011.

Linear β-1,3-glucans are the least soluble forms and they form triple helices that can aggregate to form extended solid structures.

Callose is a linear β-1,3-glucan widely observed in the plant kingdom which occurs at many stages throughout plant development (Maltby, 1979; Currier, 1979). It is predominantly composed of glucose residues linked by (1,3)-β-glucosidic bonds, although (1,6)-β-glucosidic bonds have also been observed with low frequency (Aspinall and Kessler, 1957). Callose can be identified by its staining properties with aniline blue (Wilhelm, 1880) or aniline blue fluorochrome (Evans and Hoyne, 1982) (**Figure 3B**), or by labelling with callose specific antibody (Meikle et al., 1991). The functional role of callose in plant has been poorly determined in many cases. Nevertheless, it has been suggested to act as a scaffold for the addition of other cell wall components, as a reinforcing component, or as a permeability barrier (Bacic et al., 2009). The molecular conformation of callose *in vivo* is still unknown.

1.3.2 BIOSYNTHESIS OF CALLOSE

Enzymes for β-1,3-glucan biosynthesis in fungi (Douglas et al., 1994) and plants (Hong et al., 2001a; Cui et al., 2001) belong to the family of glucosyl transferase 48 (GT48). However, a catalytic activity of the GT48 proteins has not been defined since a catalytic site in their amino acid sequence has not been identified. Thus, it has remained an open question whether they act as catalytic subunits with an unknown catalytic site or alternatively, as pore-forming units in the callose synthase complex. However, both biochemical (Li et al., 2003; Brownfield et al., 2007) and genetic studies (which will be discussed later in this chapter) strongly support the role of CALS/GSL as catalytically active callose synthesizing enzymes.

The *CALS* genes were originally identified based on their sequence similarity to the yeast FK506 hypersensitivity locus (*FKS1*), which encodes an enzyme responsible for β-1,3-glucan biosynthesis (Douglas et al., 1994; Hong et al., 2001a; Cui et al., 2001). The number of *CALS* genes varies between species. *Arabidopsis* has 12 genes encoding large CALS enzymes, which are predicted to be membrane-bound proteins based on their sequence and on the localization of *GFP-CALS12* in the PM of onion cells (Østergaard et al., 2002). CALSs are predicted to contain a cytosolic NH_2-terminus, followed by 6 transmembrane helices, a large cytosolic central loop followed by 10 transmembrane helices, and a cytosolic COOH-terminus (Hong et al. 2001a). The function of the particular domains has not been defined. CALS may participate in callose synthesis as a part of a multi-subunit enzyme complex. Since *CALS*s display overlapping expression patterns, it is possible that they form the complexes consisting of heteromeric CALS subunits (Dong et al., 2008). In addition to CALSs, other proteins could function as a part of the callose synthesizing-complex. CALS1/GSL6 has been reported to interact with UDP-glucose transferase (UGT1) and phragmoplastin (Hong et al., 2001a; Hong et al., 2001b). Yeast FKS1 has been shown to interact with Rho-type GTPase (RHO1P), which regulates its glucan synthase activity (Qadota et al., 1996). *Arabidopsis* UGT1 was shown to interact with a Rho-like protein (ROP1) (Hong et al., 2001b) and thus, it was suggested that ROP1 could regulate CALS1 through an interaction with UGT1. The existence of multi-subunit callose synthase-complexes is further supported by biochemical studies showing co-purification of multiple polypeptides with CALSs (Li et al., 2003). Some of these peptides are likely to be contaminants due to difficulties in purifying membrane bound proteins, whereas some of them could be true interacting partners, such as calmodulin, sucrose synthase (SuSy), and annexin (Cui et al., 2001; Amor et al., 1995; Andrawis et al., 1993). Since callose has multiple functions in plant cell, its synthesis is likely to be regulated at least in a tissue and isoform specific manner.

1.3.3 DEGRADATION OF CALLOSE

Deposition of callose is a transient process. The enzymes responsible for the transient nature of callose deposition are β-1,3-glucanases (Levy et al., 2007b). These hydrolytic enzymes catalyze cleavage of (1,3)-β-glucosidic linkages. Similar to β-1,3-glucan synthases, β-1,3-glucanases also function in a wide variety of processes during plant development, pathogen attack, and stress responses. There are 50 members of the *Arabidopsis* β-1,3-glucanase family and this family is divided to 13 expression clusters based on their expression patterns and phylogenetic relationships (Doxey et al., 2007). Viral infection has been shown to induce the expression of β-1,3-glucanases (Whitham et al., 2003; Ascencio-Ibanez et al., 2008). This is likely to cause the hydrolysis of the PD-associated callose and therefore increase the passage of viruses through the PD. β-1,3-glucan hydrolysis during the cell division and the cell wall remodelling has been suggested as an ancestral function of β-1,3-glucanases and therefore their function in the pathogenesis would be a late evolutionary event (Doxey et al., 2007).

1.3.4 CALLOSE IN PLANT GROWTH AND DEVELOPMENT

Callose is detected at the cell plate of freshly divided cell (Northcote et al., 1989). In this position, it has been suggested that callose creates a fast spreading force that converts tubules into plate-like structures and provides mechanical support as well as flexibility for the new cell wall before the rigid cell wall is synthesized (Samuels et al., 1995). Degradation of callose also provides building blocks for cellulose synthesis, since both of these polymers consist of UDP-glucose units. The first *CALS* gene to be connected with cell division was *CALS1/GSL6* (Hong et al., 2001a). GFP-CALS1 is localized to the dividing cell plate in tobacco BY2 cells and caused an increased aniline blue signal indicating callose overaccumulation at this position. CALS1 interacts with phragmoplastin and UGT1 (Hong et al., 2001a). Additionally, UGT1 and phragmoplastin have been shown to interact at the forming cell plate (Hong et al., 2001b). However, loss of *CALS1* does not lead to cytokinesis-related defects suggesting a redundant function of CALSs in the cytokinesis. Surprisingly, expression of phragmoplastin also leads to the overaccumulation of callose at the cell plate (Geisler-Lee et al., 2002). More recently, several groups reported that another *CALS* gene, *CALS10*, function during cytokinesis (Chen et al., 2009; Thiele et al., 2009; Guseman et al., 2010). Loss of *CALS10* results in a cytokinesis-defective phenotype including bi- and multinucleate cells, cell-wall stubs, and incomplete cell walls. However, it does not lead to a loss of callose in the cell division plane; there is only a delay in callose accumulation and this suggests that CALS10 is essential for the timing of callose deposition during the cytokinesis. It has been hypothesized that CALS10 could be required for either the insertion of the new cell wall to the cross-wall at the cell division site or the direct stabilization of the nascent cell wall (Thiele et al., 2009).

Callose deposition in the phloem has been observed during both phloem development (**Figure 3B**) and in mature phloem that has entered the non-functional phase (Engleman and Esau, 1964; Hollis and Tepper, 1971; Evert, 1977). Callose is also detected in the developing sieve plate and therefore, it has been proposed that callose could be required for the process of sieve plate formation (**Figure 3C**). Recently, *CALS7/GSL7* was identified and this locus encodes an enzyme acting in phloem specific callose synthesis (Xie et al., 2011; Barrat et al., 2011). *CALS7* is expressed in the phloem cells and the *cals7* mutant displays reduced callose accumulation in the phloem, this is accompanied by an altered structure of the sieve plates in the hypocotyl. These results reveal that the callose deposition in the phloem is indeed important for the sieve plate formation.

Callose is often detected in the vicinity of the PD and it is thought to regulate the PD SEL (Allison and Shalla, 1974). The role of callose in formation of PD or maintenance of the PD ultrastructure has not been characterized. None of the *CALS* genes have been localized to the PD or showed to synthesize callose at the PD. However, loss of *CALS10* has been shown to lead to an increase in cell-to-cell trafficking (Guseman et al., 2010). Callose deposition at the PD has been shown to regulate plant developmental processes in at least in two independent cases. Ruan et al. (2001) showed that a transient gating of the PD facilitates elongation of cotton fibers. Gating of the PD creates a high turgor inside the cell and this is necessary for the elongation of the cell. Furthermore, it was

shown that closure of the PD was followed by a reduction in the activity of the callose-degrading enzyme, GhGluc1, and consistent with this, callose accumulation was observed at the PD (Ruan et al., 2004). Rinne and van der Schoot (1998) showed in the birch SAM symplastic fields co-ordinate morphogenesis. Treatment of birch plants with short photoperiod caused callose deposition at the PD and therefore resulted in a symplastic isolation of shoot meristem cells and dissipation of symplastic fields, preceding dormancy development. Furthermore, callose disappeared from the PD during a period of cold treatment; this caused the recovery of symplastic connectivity within the SAM fields, and is likely to be the result of the activation of β-1,3-glucanases (Rinne et al., 2001; Rinne et al., 2011). Therefore, regulation of connectivity in this symplastic field could control the passage of the morphogens that regulate morphogenesis (Ruonala et al., 2008; Rinne et al., 2011).

CALSs also play a role in microgametogenesis and microsporogenesis. Østergaard et al. (2002) noticed that *CALS12/GSL5* is expressed in pollen and suggested that it acts there as a callose synthesizing enzyme. Furthermore, Enns et al. (2005) showed that *CALS12* acts redundantly with *CALS11/GSL1* during pollen development and reported that both of these are required for the synthesis of the callose wall separating the microspores of the tetrad. In addition, *CALS11* and *CALS12* play a role in the pollen grain maturation. *CALS9/GSL10* has been shown to be involved in pollen development at the mitotic division stage and it has been suggested to act in this process redundantly with *CALS10* (Töller et al., 2008; Huang et al., 2009; Xie et al., 2010). *CALS5/GSL2* has been shown to act in the synthesis of callose localized at the wall surrounding the pollen mother cell (Dong et al., 2005) and callose plugs deposited in the growing pollen tube (Nishikawa et al., 2005). Based on phylogenetic analyses and analysis of callose deposition in *Physcomitrella patens* it has been suggested that a *CALS5* ortholog has been the ancient callose synthesizing enzyme acting in the spore germination (Schuette et al., 2009)

Besides its multiple roles in plant development, callose deposition is also an important component in plant stress responses. Callose deposition is induced during abiotic and biotic stresses (Siwaguru et al., 2000; Hofmann et al., 2010; Lee et al., 2011). Callose accumulation at the PD may function as a barrier to prevent the trafficking of harmful substances from cell to cell during stress (for review see Zavaliev et al., 2011; Lee and Lu, 2011). However, only *CALS1* and *CALS12* are clearly upregulated by a SA treatment or fungal infection (Østergaard et al., 2002; Jacobs et al., 2003; Dong et al., 2008) suggesting specific roles for these two CALS isoforms in stress response. Surprisingly, a loss of *CALS12* leads to an increased resistance against powdery mildew fungus (Nishimura et al., 2003; Jacobs et al., 2003). When the SA signaling pathway was genetically depleted from the *cals12* mutant, its disease resistance recovered, suggesting that *CALS12* or callose produced by CALS12 could negatively regulate the SA pathway (Nishimura et al., 2003).

2 AIMS OF THE STUDY

In order to gain an understanding of plant vascular development we performed genetic screens using the mobile phloem marker, *pSUC2::GFP* and a protoxylem-specific marker, *pAHP6::GFP*. Four mutants, *phb-7d*, *cals3-1d, -2d*, and *-3d* (referred collectively as *cals3-d*) displaying an altered xylem development, were identified from these screens. During the course of this work a spatiotemporally controlled expression system based on the *cals3-1d* and *cals3-2d* mutations (*icals3m*) was engineered and used as a tool to study PD-mediated trafficking during root development.

The specific aims of this study were:

1. To identify genes underlying the *phb-7d* and *cals3-d* phenotypes (I, IV).

2. To understand how *PHB* and *CALS3* are involved in the regulation of vascular development in general and more specifically in protoxylem development (I, IV).

3. To manipulate symplastic signalling in a targeted manner using the *icals3m* system, which was developed specifically for this study. (IV)

4. To identify symplastic signaling events which regulate key aspects of root development (III, IV, V).

3 MATERIALS AND METHODS

The materials and methods are described in detail in publications I, III, IV, and V and the publication(s) in which they appear are indicated in the **Table 1**.

Table 1 Methods used in this study. Those in parenthesis were performed by co-authors in the respective publications.

Method	Publication
Agrobacterium mediated transformation of *Arabidopsis*	(I), III, IV, V
Aniline blue staining	III, IV, V
Confocal light microscopy	(I), III, IV, V
Ethylmethane sulfonate (EMS) mutagenesis	(I), (IV)
Fuchsin staining	(I), IV
Gene identification through positional cloning	I, IV
Genetic crossing of *Arabidopsis*	I, IV, V
Histological staining for GUS activity	(I), (III), IV
Hormone transport assay	(III)
Immuno-transmission electron microscopy	(IV)
In situ RNA hybridisation	(I), (IV)
In vitro callose synthase activity measurement	(IV)
Light microscopy	I, III, IV, (V)
Mutant screen	I, IV
Plasmid construction	(I), III, IV, V
Polymerase chain reaction (PCR) analysis	I, IV, V
Quantitative real-time PCR analysis	(I), (III), (IV)
Histological sectioning of plastic embedded samples	(I), IV
DNA sequencing	I, III, IV
Site-directed mutagenesis	IV
Transmission electron microscopy (TEM)	(IV)
Lugol staining	(V)

4 RESULTS AND DISCUSSION

4.1 SIGNALING BETWEEN THE ENDODERMIS AND THE STELE IS REQUIRED FOR PROTOXYLEM DEVELOPMENT

The phloem is a tissue specialized for transport located in the inner tissues of the plant, in the vascular cylinder (**Figure 4**). Small proteins driven by a CC- specific *SUC2* promoter traffic from the CC to the SE (via the PD) and further through the phloem (via the sieve pores) and finally freely diffuse throughout the entire root tip (Imlau et al., 1999). In order to identify factors regulating phloem development we performed a genetic screen using the phloem marker *pSUC2::GFP*. We identified three short rooted mutants *phb-7d*, *cals3-1d*, and *cals3-2d*, which displayed an impairment in the formation of phloem, which we observed through an altered pattern of *pSUC2::GFP* (I, Fig S5; IV, Fig 1E). In these mutants *pSUC2::GFP* was detectable only in the upper part of the root and there was no *GFP* signal in the elongation and meristematic zones of the root tip. A test for allelic complementation revealed that *cals3-1d* and *cals3-2d* mutants are allelic. Subsequently, a third allele, *cals3-3d*, was identified in an independent screen based on the misexpression of the protoxylem marker *pAHP6::GFP* (Mähönen et al., 2006). The *cals3-1d, -2d*, and *-3d* mutants are collectively referred to hereafter as *cals3-d*.

In addition to the phenotype in which defects in phloem development were observed, *phb-7d* and *cals3-d* mutants also share defects in xylem development. In wild-type, protoxylem cells can be distinguished from metaxylem cells due to the deposition of lignin

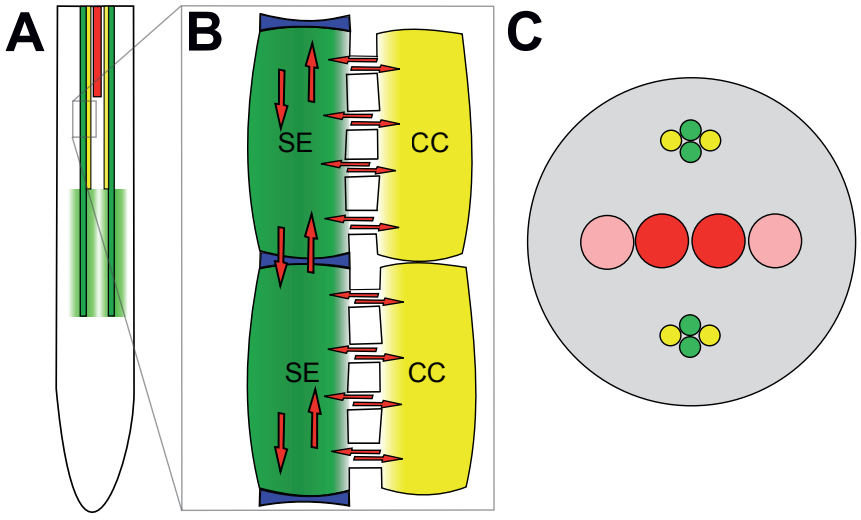

Figure 4 Schematic representation of the phloem (green-yellow) and the xylem (red) cell files in the root tip (A). CC and SE are symplastically connected by the PD, whereas SEs are connected by the sievepores (B). Proto- and metaxylem cells form the xylem axis across the vascular cylinder flanked by the phloem poles (C). Red, metaxylem; pink; protoxylem; yellow, CC; green, SE. Modified from Vatén et al., 2011.

in a helical or ring-like pattern and in addition these cells differentiate in a spatially and temporally distinct position. Metaxylem cells have more pronounced cell wall thickenings and differentiate later in the central position of the xylem axis (**Figure 4C**). The *phb-7d* and *cals3-d* mutants displayed defects in the formation of protoxylem, and this was accompanied by the ectopic differentiation of metaxylem cells in the protoxylem position (I, Fig 1H; IV, Fig 6B). In addition, cell files with characteristics of both of protoxylem and metaxylem were observed in *cals3-d* mutants (IV, Fig 6C; **Figure 5**). Consistent with these changes in the xylem identity, the expression of *pAHP6::GFP* was lost in the primary root tip of *phb-7d*, whereas it was expressed in a weak and fragmented pattern in *cals3-d* (I, Fig S6_2; IV, Fig S5). These results suggest that mutations in the *PHB* and *CALS3* loci modify a pathway required for protoxylem development.

In order to understand the molecular nature of the *phb-7d* we used a positional cloning approach to indentify the location of the mutation. We mapped the *phb-7d* mutation to the window containing HD-ZIP III gene *PHB* and through DNA sequencing defined that *phb-7d* carries a point mutation in a miR165/6 target site of *PHB* (I, Fig S5). We showed that in wild-type roots *PHB* mRNA localizes primarily to the center of the stele with a maximum at the metaxylem position, whereas mRNA levels in the protoxylem position are low (I, Fig 2A). In contrast, *phb-7d* displayed high levels of *PHB* mRNA throughout the stele and this expression pattern extended into the pericycle and ground tissues (I, Fig 2B). Through this work we revealed that the *phb-7d* mutation in the miR165/6 target site results in a failure of the miRNA165/6 species to target the *PHB* mRNA in the root. In contrast to earlier findings (Hawker and Bowman, 2004), these results revealed that the correct targeting of *PHB* is required for the development of vasculature in the *Arabidopsis* root.

In addition to *phb-7d* and *cals3-d* mutants, loss of *SHR* also leads to defects in protoxylem identity (I, Fig 1F). In order to define if the failure to form protoxylem in all these mutants is dependent on *PHB*, loss-of-function *phb-6* and *phb-13* alleles were introduced to *shr-2* and *cals3-1d* backgrounds, respectively. In both cases a suppression of the defective protoxylem formation was observed (I, Fig 1I; IV, Fig 6A–E; **Figure 5**). Furthermore, an expansion of the *PHB* domain was observed at *shr-2* (I, Fig 2G). These results suggest that the loss of protoxylem formation in *cals1-d* and *shr-2* is a result of the misregulation of *PHB*.

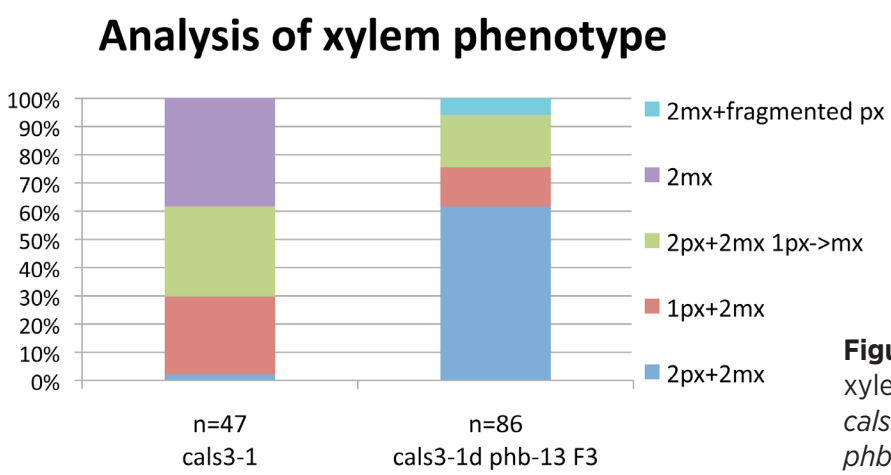

Figure 5 Quantification of xylem phenotypes in the *cals3-1d* and in the *cals3-1d phb-13.*

In order to understand the role of *SHR* in the targeting of *PHB* mRNA in the stele, the expression patterns of *MIR165/6* genes were defined. Analysis of the *MIR165/6* promoter::GFP fusions in wild-type revealed that only *pMIR165a::GFP* and *pMIR166b::GFP* were active in the root tip, however they were not active in the stele (I, Fig 3). *pMIR165a::GFP* was strongly expressed in the endodermis, whereas *pMIR166b::GFP* was strongly expressed in both the endodermis and QC, and at low levels in the epidermis and cortex of the RAM. In the *shr-2* background these drivers showed only very modest activity. This strongly suggests that SHR is required for the *MIR165a* and *MIR166b* expression in the root. Since *PHB* is under the post-transcriptional control of miR165/6, their absence in *shr-2* is likely to be the reason for the expanded *PHB* domain and the loss of protoxylem observed in *shr-2*.

Since a gradient of *PHB* mRNA is observed across the stele and *MIR165/6* are required for the regulation of cell identity in this domain, but are only transcribed outside the stele, we asked whether a non-cell-autonomous signal could move from the endodermis to the stele. We created transgenic *shr-2* lines where *MIR165a* was expressed under a ground tissue-specific enhancer, *J0571 (J0571;* www.plantsci.cam.ac.uk/Haseloff/Home.html) and the protoxylem cell identity was followed in the resulting lines (*shr-2, J0571; UAS::MIR165a*). Five independent lines showed a recovery in protoxylem specification and this was accompanied by the suppression of *PHB* levels (I, Fig 4C and Fig S16A). One of these lines was backcrossed and an analysis of the resulting F2 population confirmed that the protoxylem recovery co-segregated with the activator. These results indicate that miR165a produced in the ground tissue is sufficient to target the *PHB* transcript in the stele and therefore, it is highly likely that miR165a is a mobile signal.

We encompassed all this data into a single mechanism where we proposed that a novel bidirectional signaling pathway operated to determine xylem differentiation. In this pathway, SHR is produced in the stele, from where it moves into the endodermis to induce the transcription of miR165/6. The endodermally produced miR165/6 restrict the *PHB* mRNA domain non-cell-autonomously and this results in the downregulation of levels of *PHB* mRNA inside the stele. As a result of this process, a high level of the *PHB* mRNA can be observed at the metaxylem position whilst there is only a low level at the protoxylem position. Therefore, this bidirectional signaling mechanism is required to set up the post-transcriptional regulation of *PHB* and allow the proper differentiation of the protoxylem.

4.2 THE *CALS3-D* MUTATIONS RESULT IN REDUCED SYMPLASTIC SIGNALING

In addition to the defects in both xylem and phloem development, *cals3-1d* and *cals3-2d* mutants display abnormal cell divisions in the ground tissue resulting in sporadically missing cells or extra cells in the ground tissue. Koizumi et al. (2011) reported recently that also *shr-2 /+* heterozygotes display the latter phenotype. Since decreased levels of *SHR* can cause a ground tissue phenotype resembling that of *cals3-1d* and *cals3-2d* mutants, we asked whether or not SHR levels are affected in these mutants. We found

out that the *pSHR::SHR:GFP* signal was decreased in the endodermis of the *cals3-d* mutants (IV, Fig S5E). This suggests that the decreased movement of SHR from the stele to the endodermis could result in the ground tissue and protoxylem phenotypes observed in *cals3-d* mutants. Besides the severe developmental phenotype observed in the homozygous *cals3-d* mutants, the root development in the heterozygous *cals3-d* mutants was otherwise normal except that an altered pattern of unloading of *pSUC2::GFP* (27 kDa) was detected in the root tip. In the heterozygous plants, GFP did not diffuse throughout the root tip but moved only to the post-phloem domain of the root, which was similar to *pSUC2::GFP-sporamin* (47 kDa) in the wild-type root tips (IV, Fig 1H and 1I) (Stadler et al., 2005). Since large molecules, such as *pSUC2::GFP-sporamin*, are unable to travel through the post-phloem domain to the root tip, it is thought that PD SEL in this domain is reduced compared to PD SEL in the tissues above them. These results suggested that *cals3-d* mutations cause an altered cell-to-cell connectivity in the root tip.

In order to understand the molecular nature of *cals3-d,* we identified the site of the mutation in the *CALS3* through a positional cloning approach. We mapped the *cals3-d* mutations to *At5g13000* locus (IV, Fig 1L), which encodes a putative callose synthase (Hong et al., 2001), *CALS3/GSL12*. The *cals3-1d* and *-2d* phenotypes were partially suppressed with a genomic fragment containing the wild-type *CALS3* gene indicating that *cals3-d* mutations cause the defects seen in *cals3-d* mutants. Next we added *cals3-1d* and *cals3-2d* mutations to the aforementioned fragment (called *cals3m*) and studied its effect in the wild-type background (IV, Fig 1J). Transgenic plants containing this mutated fragment displayed a phenotype resembling the *cals3-d* (IV, Fig 1K). Taken together with the *cals3-d* heterozygous phenotype these results suggest that the more *CALS3-D* protein is present the stronger the phenotype will be. Therefore, it is likely that *cals3-d* acts in a dose dependent manner.

Furthermore, we cloned the *CALS3* cDNA and discovered that it encodes a putative protein with 1947 amino acid residues (IV, Fig 1J). We defined lesions that caused the *cals3-1d*, *cals3-2d*, and *cals3-3d* mutations, and the following amino acid changes in the deduced amino acid sequence: R84K, R1926K, and P189L respectively. Since all the three amino acid changes are in the predicted cytosolic faces of CALS3, it is possible that they share a mechanism in which they modulate a CALS3 function. Moreover, we analyzed sequence predictions of other proteins belonging to the GT48 family in *Arabidopsis* and in other plant species. We noticed that the amino acids corresponding to R84, R1926, and P189 and the domains surrounding them in CALS3 were highly conserved both among CALSs in *Arabidopsis* and throughout the genomes of diverse plant species, including poplar (*Populus trichocarpa*), tobacco (*Nicotiana alata*), grapevine (*Vitis Vinifera*), rice (*Oryza sativa*), barley (*Hordeum vulgare*), and the moss species (*Physcomitrella patens* and *Selaginella moellendorffii*) (**Appendix 1**). The strong conservation of the amino acids R84, R1926, and P189 suggests that they could be important for protein function of CALS.

Using RT-PCR, *In situ* hybridization, and a transcriptional reporter analysis we defined the expression level and domain of *CALS3*, (IV, Fig 2C, S2B-D). *CALS3* mRNA is strongly expressed throughout the seedling and the CALS3 promoter is active in

vasculature, especially in the phloem and root tip. Next, we studied the subcellular localization of CALS3 using a constitutively expressed GFP-CALS3 fusion protein, which partially suppressed *cals3-1d*. Interestingly, CALS3 was localized to cell wall punctae, (putatively at the PD) and the PM in the cotyledon of epidermal cells in *Arabidopsis* (IV, Fig 2A and 2B). In order to study whether or not *cals3-1d* mutations modify the subcellular localization of CALS3, we defined the localization of *35S::GFP-cals3-1d* (IV, Fig S2A). We were unable to detect any difference in the subcellular localization of the *GFP-CALS3-1D* protein compared with wild-type CALS3. These results suggest that CALS3 is a PD-localized protein and furthermore, *cals3-d* mutations are not sufficient to alter its subcellular localization. An independent study also recently identified CALS3 in a proteomic analysis of the PD (Fernandez-Calvino et al., 2011).

We studied the *CALS3* transcript levels in the roots of *cals3-d* mutants using RT-PCR and found that *cals3-d* mutants accumulate similar amounts of *CALS3* transcripts to wild-type (IV, Fig S2E). We then identified T-DNA insertion lines in the *CALS3* locus (*cals3-4*, *cals3-5*, and *cals3-6*), but did not detect any deviation in root development to wild-type (IV, Fig 1L). This suggested that the activity of CALSs is redundant during plant development (Brady et al., 2007). Taken together, our analysis indicates that *cals3-d* mutations lead to an altered activity of CALS3 at the PD and this activity is dose dependent. Based on our results *cals3-d* mutations neither modify the *CALS3* transcript level nor alter the subcellular localization of the CALS3 protein. Since each of the three mutations leads to a change in a conserved amino acid in the predicted cytosolic chains of CALS3, it is possible that *cals3-d* mutations result in altered regulation of CALS3.

In order to understand the effect of the *cals3-d* mutations on callose biosynthesis, we analyzed the callose deposition in the primary roots of the *cals3-d* mutants using the aniline blue staining technique (Ingram et al., 2011) We failed to observe any difference in the callose accumulation patterns during primary root development, however, we detected a slightly increased callose accumulation during early stages of lateral root development (IV, Fig 2E and 2F). Callose deposition during plant development is thought to be transient in nature and this transiency must be a result of the balanced action of the callose deposition and degradation. Therefore, to address whether *cals3-d* results in an elevation of callose biosynthesis, we analysed the PD-localized β-1,3-glucanase gene (*At5g42100*), which has been reported to function in callose degradation at the PD (Levy et al., 2007a). We expressed *At5g42100* in the *cals3-1d* background under the CALS3 promoter, and detected a partial rescue of the root elongation (IV, Fig S3A), suggesting that *cals3-1d* could cause an in increased callose deposition at the PD.

The transient pattern of callose accumulation complicates the analysis of callose deposition in both genetic mutants and constitutive transgenic lines. Furthermore, the viability of the cells is likely to suffer by an increase in callose accumulation. To overcome these difficulties, we engineered a series of inducible overexpression lines of the CALS3 based on the XVE-system created by Zuo et al. (2000). In these lines, CALS3, *cals3-1d,* or combination of *cals3-1d* and *cals3-2d* (called *icals3m)* are expressed under the control of tissue specific promoters, which are induced with an estradiol treatment. Using this

system we found that when the CALS3 promoter was used to driving wild-type CALS3 this caused only a very modest increase in the callose accumulation, whereas when either *cals3-1d* or *icals3m* where driven under the CALS3 promoter this resulted in a clear callose overproduction in the cell walls in cells in which the CALS3 promoter was active. After the induction of *icals3m*, callose accumulation was clearly stronger than after the induction of *cals3-1d* indicating that the *cals3-1d* and *cals3-2d* mutations caused an additive effect (IV, Fig S2F–K). In addition, we defined a callose synthase activity *in vitro*, from tissue extracts prepared from the roots of *pCALS3::icals3m* plants and found that activity was 10–50% higher after 12 hour induction compared to uninduced plants (IV, supplementary Table 1). Taken together, these results suggest that the *cals3-d* mutations disturb the regulation of callose synthesis, resulting in an increased accumulation of callose.

Since callose has been reported to have multiple roles and be present in several positions in the cell, we decided to characterize the effect of *icals3m* on EM level. We induced two transgenic lines which specifically expressed *icals3m* in either the vascular cylinder or ground tissue, using *CRE1* (Bonke et al., 2003) and *J0571*; *p6xUAS promoters*, respectively. Both of the lines showed a strong and rapid overaccumulation of callose in a domain corresponding to the specificity of the promoter driving expression of the transgene, this was indicated by observing the pattern of aniline blue staining and through callose immunofluorescence labelling (IV, Fig 3). In order to localize callose more specifically we performed an immunogold labelling against callose in the stele cells of *CRE1::icals3m* and in the negative control line *J0571*; *p6xUAS::icals3m* after 8 and 5 hours of induction, respectively. In both of these lines the majority of gold particles were localized in clusters in the cell wall, at the vicinity of the PD. Interestingly, three times more gold particles were detected in these PD-associated clusters in *CRE1::icals3m* than in *J0571*; *p6xUAS::icals3m*. In addition, 80.4 % of the PD were at swollen cell wall domains associated with an enhanced gold labelling in *CRE1::icals3m*. In contrast, the swollen cell wall domains were not observed in *J0571*; *p6xUAS::icals3m* (IV, Fig 4B, 4E, and S4). Next, we measured the PD aperture in the vascular cylinder following *pCRE1::icals3m* induction and found that the size of the aperture was significantly decreased upon induction (IV, Fig 4F). After a 24 hour induction the PD aperture was 24% narrower in *pCRE1::icals3m* line compared with wild-type. Taken together, these results suggest that the overexpression of *cals3-d* leads to an increased production of PD-localized callose, which leads to a decrease in the size of PD apertures.

4.3 SYMPLASTIC SIGNALING IS REQUIRED FOR PROTOXYLEM DEVELOPMENT

The *cals3-d* mutants display an overaccumulation of callose, decreased level of SHR at the endodermis and *PHB* dependent xylem defects (see chapter 4.1 and 4.2). It is therefore possible that the reciprocal signaling between the vascular tissues and the endodermis causes these defects. The signaling mechanism proposed earlier involves movement of both SHR and miR165/6 (Nakajima et al., 2001, Publication I, Miyashima et

al., 2011). However, the route of their movement has not been characterized. We asked whether or not the movement of SHR or *miR165/6,* or both could be regulated by the callose deposition at the PD. We analysed *icals3m* under the ground tissue-specific enhancer (*J0571; p6xUAS::icals3m*) and found that callose accumulation in the ground tissue results in an elevation of *PHB* transcript in 6 hours and that this is accompanied by an expansion of its expression domain (in 12 hours) inside the stele (IV, Fig 6F–H). This observation was proceeded by the ectopic formation of the metaxylem at the expense of the protoxylem specification (IV, Fig 6I–K). Hence, an increased amount of callose in the tissues surrounding the stele likely results in an inhibition of the bidirectional signaling mechanism, which directs protoxylem development. Next we analyzed the *pSHR::SHR:GFP* signal in a line expressing *icals3m* under the vascular cylinder-specific *CRE1* promoter and detected decreased SHR signal in the endodermis after 7 hours, whereas the strength of the SHR signal in the stele was not affected. After a 22 hour period of induction the SHR signal in the endodermis was hardly detectable (IV, Fig 6L–N). Since callose accumulation at the PD of the stele cells is sufficient to prevent SHR trafficking from the stele to the endodermis, SHR is likely to move via the symplastic pathway.

To study the mobility of miR165/6 we used the previously mentioned transgenic *shr-2* mutant which was rescued by the expression of miR165a in the ground tissue (*shr-2* with *J0571; p6xUAS:MIR165a*). This was originally created for study I, (see chapter 4.1 for more information). By using this line the mobility of miR165a could be studied independently of SHR movement and its effect on the expression of *MIR165* could be elucidated. We studied how the production of callose in the ground tissue in the line, *shr-2* with *J0571; p6xUAS:MIR165a*, *p6xUAS::icals3m*, affects the spatial distribution of the mature miR165 in the root tip using LNA-probes against miR165. We noticed that without the induction a strong signal (indicating high levels of miR165) was detected ubiquitously throughout the root cross-section. However, after 24 hours of induction the strong miR165 signal was limited to the ground tissue (IV, Fig 7A and 7B). Consistently we detected *PHB* signal throughout the stele after 24 hours of induction (IV, Fig 7C and 7D). These results suggest that synthesis of callose specifically in the ground tissue hinders the mobility of miR165 from the endodermis to the surrounding tissues in the root tip and as a consequence, is unable to maintain the correct post-transcriptional regulation of *PHB*. To further characterize the effect that *J0571; p6xUAS:MIR165a* had regarding the mobility of miRNA we introduced a `miRNA-sensor` system (Miyashima et al., 2011) to *J0571; p6xUAS::icals3m* line. Here, the action of a modified version of *MIR165* can be visualized by following the expression pattern of a reporter containing a target sequence for a modified miR165 fused to nls-YFP. In the resulting line (*pSPR1::nlsYFP_165mu_tgt/UAS::MIR165Amu/J0571; p6xUAS::icals3*) miR165Amu is constitutively transcribed in the ground tissue, whereas callose deposition could be induced in the same domain. Without induction, the nls-YFP signal was absent in the root tip due to the mobility of miR165Amu and its suppressive function. In contrast, after 24 hours of induction the nls-YFP signal was detected in the stele cells, suggesting that the miR165Amu movement from the ground tissue is inhibited by the accumulation of callose in the same tissue (IV, Fig 7E and 7F).

Therefore, callose production in the ground tissue is sufficient to limit the movement of miR165Amu from the ground tissue to the stele. Based on these results, both SHR and miR165 are likely to move between the endodermis and the stele via the PD to allow proper protoxylem development (**Figure 6**).

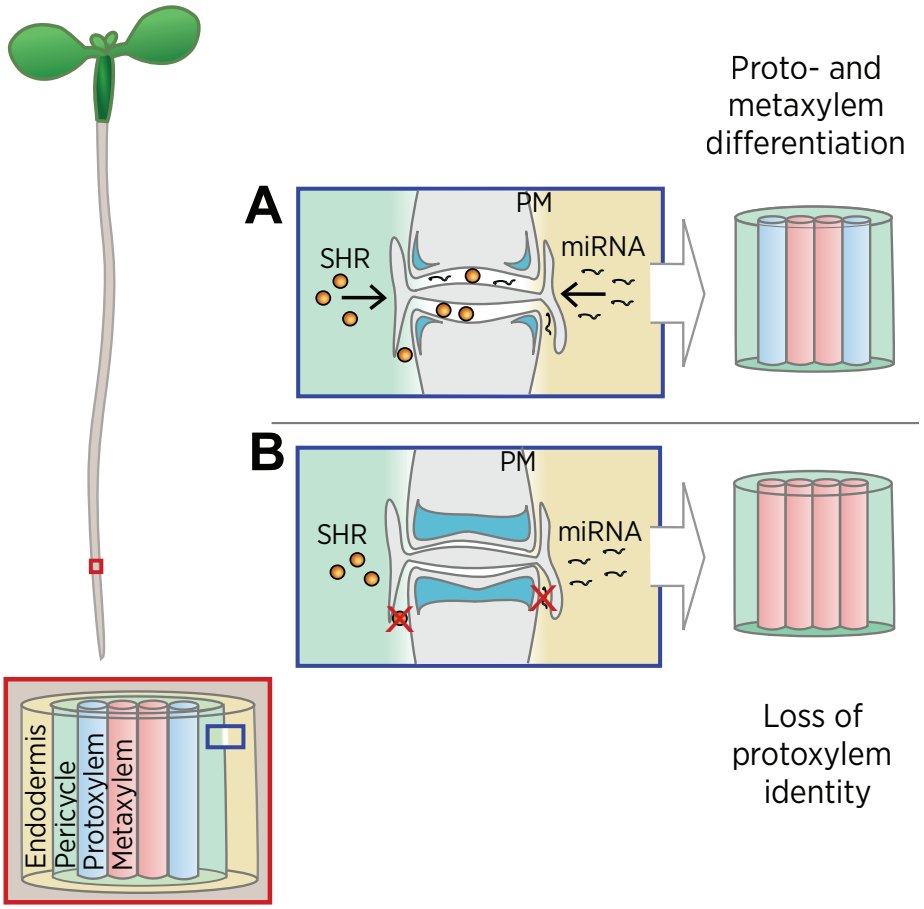

Figure 6 Schematic image showing the bidirectional signals that are trafficked through the PD to direct protoxylem development in the *Arabidopsis* root (A). Callose deposition at the PD prevents mobility of these signals and results in altered xylem development (B). Modified from Vatén et al. (2011).

4.4 SYMPLASTIC CONNECTIVITY IS NECESSARY FOR PHLOEM TRANSPORT AND MAINTENANCE OF QC

In order to further characterize the effect of callose biosynthesis during root development, we focused on the phloem and the QC, which were the subdomains of CALS3 activity with the strong expression. The trafficking from the CC to the SE occurs via the PD and the subsequent transport through the phloem occurs via the sieve pores (**Figure 4**). We noticed that wild-type phloem was strongly stained with aniline blue indicating high levels of callose (**Figure 3**; IV, Fig 2D). The sieve plates display a strong even staining pattern, whereas the lateral walls of the protophloem SEs showed a punctated staining. First, we expressed *icals3m* under the control of the phloem specific APL promoter (Bonke et al., 2003) and as a result, we observed dramatically more callose in the phloem cells and an impaired unloading of *pSUC2::GFP* to the root tip (IV, Fig S3B and S3C). This is consistent with the lack of unloading observed in the *cals3-d* heterozygous plants (IV, Fig 1H) and the ability of the overexpression of *icals3m* to reduce the PD aperture (IV, Fig 6F). Surprisingly, we noticed a rapid expansion in the size of the differentiation zone (at the expense of the cell division zone) towards the root tip when we induced *pAPL::icals3m* (IV, Fig S3B, S3D, and S3E). These results indicate that the symplastic connectivity in the phloem is essential for the maintenance of RAM. It is possible that the induction of *pAPL::icals3m* prevents the movement of an unknown phloem-borne signal (or signals) required for the meristem function. A similar decrease in the RAM has been observed in the *gat1* mutant, which also displays increased accumulation of callose, altered ROS levels, and reduced cell-to-cell trafficking of *pSUC2::GFP* in the root tip (Benitez-Alfonso et al., 2009). It remains to be seen if the *gat1* and *pAPL::icals3m* both modify the same non-cell-autonomous pathway required for the meristem maintenance.

In order to study the role of callose synthesis in the phloem in greater depth we searched a published list of phloem specific *CALS* genes (Brady et al., 2007) and noticed that one of them, *CALS7*, is predicted to show phloem specific expression; this observation had been recently reported by two other studies (Xie et al., 2011; Barratt et al., 2011). We studied the expression of *CALS7 in situ* and with a promoter-fusion and identified it as a protophloem SE specific gene throughout the root development (IV, Fig 5A and 5B). Consistent with this, we found that loss of *CALS7* caused a severely reduced callose deposition specifically at the protophloem SE (IV, Fig 5C). Next, we introduced *pAPL::icals3m* to *cals7-1*, and followed the callose deposition in this line. After the induction of *pAPL::icals3m,* a massive accumulation of callose in the phloem of *cals7-1* was observed (IV, Fig 5G) indicating that the presence of *icals3m* in the phloem is sufficient to overcome the absence of CALS7. To define the role of wild-type CALS3 as a phloem callose synthase we drove it under the control of the APL promoter in *cals7-1*. The induction of *pAPL::iCALS3* resulted in a partial restoration of the callose deposition in the *cals7-1* phloem (IV, Fig 5E–G), suggesting that CALS3 acts as a callose synthase capable of synthesizing callose in the phloem.

Both cytokinin and auxin have been detected in phloem sap (Cambridge and Morris,

1996; Corbesier et al., 2003). We used the line with phloem specific expression of *icals3m* to define whether or not the transport of these hormones occurs via the symplastic pathway. First we characterized the cytokinin response followed by the phloem specific callose accumulation using a cytokinin response marker, *pARR5::GUS*. After the induction of *pAPL::icals3m* the level of *pARR5::GUS* was decreased in the root tip indicating a reduced cytokinin signaling (III, Fig 3P). In order to test whether cytokinin is transported in the phloem, the mobility of a ^{14}C-labelled cytokinin was followed after its exogenous application to the hypocotyl before and after the induction of *pAPL::icals3m*. We observed that in wild-type plants, cytokinin was rapidly transported from the hypocotyl to the root tip. However, we detected a clear decrease in the amount of ^{14}C-labelled cytokinin in the root tip after the induction of phloem specific callose deposition (III; Fig 2A, 2C, and S2C). Therefore, a reduction in symplastic connectivity in the phloem prevents both cytokinin transport and limits the response to cytokinin in the root tip. This indicates that the basipetal transport of cytokinin through the phloem to the root tip is a significant source of cytokinin. Next, we characterized the auxin response by following the expression domain of auxin reporter *DR5rev::GFP* after the induction of *pAPL::icals3m*. We detected only modest changes in the pattern of *DR5rev::GFP* in the QC and columella (III, Fig 3N and S3A). A similar effect on auxin response had been previously observed when cytokinin level were depleted in transgenic lines (Pernisova et al., 2008); this suggests that the observed changes in *pAPL::icals3m* are likely to be caused by an alteration in cytokinin signaling. Furthermore, we analysed the transport of ^{14}C labelled IAA in wild-type and *pAPL::icals3m* and observed that the basipetal transport of auxin was reduced. However, unlike in the case of cytokinin transport, some ^{14}C labelled IAA was still able to accumulate in the root tip, (III, Fig 2B and 2D). Based on these results, auxin is capable to move basipetally via the phloem, however, it seems likely that it has a minor role for the auxin response in the root tip. Therefore, it is possible that there is an additional transport mechanism responsible to maintain a sufficient flow of auxin throughout the root tip. To further characterize this scheme, the direct and indirect cytokinin regulated marker genes, *pPIN7::PIN7:GFP*, *DR5rev::GFP,* and *pAHP6::GFP* (Bishopp et al., 2011) were analyzed in the *pAPL::icals3m* line. Without induction all of these markers were expressed sharply in the positions that determine the locations of distinct domains of high auxin and high cytokinin signaling; *DR5rev::GFP* and *pAHP6::GFP* were strongly expressed in the protoxylem position where there is high auxin signalling response, whereas *pPIN7::PIN7:GFP* was expressed in the procambium where there is high cytokinin signaling (III, Fig 3A–C) as was recently described by Bishopp et al. (2011). However, after the induction of *pAPL::icals3m,* we observed that the expression of these markers became destablized; the signals of *DR5rev::GFP* and *pAHP6::GFP* spread to extra cells next to the protoxylem position (III, Fig 3 N–O). In line with this, the *pPIN7::PIN7:GFP* signal was excluded from a few procambial cells flanking the xylem axis (III, Fig 3 M). Thus, the domain of the high auxin signaling expanded to neighbouring cells when the symplastic connectivity in the phloem was reduced. These results suggest that cytokinin moves symplastically from shoot to root and it is likely that this particular source of

cytokinin is essential for maintaining specific domains of high auxin and high cytokinin in the root tip.

The QC cells of the root meristem have been shown to promote the undifferentiated state of the surrounding stem cells in a contact-dependent manner (van den Berg et al., 1997). It has been suggested that SHR promotes the maintenance of the QC through its movement from the stele to the QC (Heidstra et al., 2004). In order to characterize the symplastic signaling events at the QC, we followed the effect of inducible *icals3m* under the QC-specific *WOX5* promoter (Xu et al., 2006; Sarkar et al., 2007). Induction of *pWOX5::icals3m* caused a QC-specific callose accumulation after only six hours (V, Fig 1A and 1B) and in 24 hours callose was also detected outside of the QC in the stem cells and in the vascular cells above the QC (V, Fig 1C–E). A dynamic behavior of the WOX5 promoter activity has been reported when the QC has been laser ablated (Xu et al., 2006). Since the described behavior resembles the pattern of callose accumulation in the *pWOX5::icals3m*, it is possible that the perturbation of the symplastic connectivity between the QC and the surrounding cells leads to the differentiation of the stem cells. This would cause an expansion of the *WOX5* expression domain from the QC cells to the neighboring cells. Therefore, the dynamic pattern of callose synthesis driven by the WOX5 promoter could reflect the nature of this particular promoter and possibly, the importance on symplastic connectivity for the QC maintenance. Next, we followed the localization of *pSHR::SHR:GFP* upon induction of *pWOX5::icals3m*. After 22 hours of induction we detected a decreased SHR signal at the QC suggesting that the callose synthesis at the PD is sufficient to hinder the trafficking of SHR from the stele to the QC (V, Fig 1F–H). In addition, we followed the differentiation of columella cells in the *pWOX5::icals3m* line by staining starch granules with lugol. Without induction the columella cells differentiated to the distal part of the root tip in an organized manner, which could be observed by the presence of starch granules in these cells. After 48 hours of induction we observed an ectopic accumulation of starch granules in the columella stem cells suggesting that their stem cell identity was lost (V, Fig 1I and 1J). Further analysis is required to determine whether all of these observations are due to reduced symplastic signaling in and around the QC.

In order to investigate whether the effect of inducing *icals3m* has more general effects on intercellular trafficking we focused on the auxin transport. First, we expressed *icals3m* under the control of the CALS promoter and compared the localization of *DR5rev::GFP* after the treatment with an auxin transport inhibitor NPA (Katekar and Geissler, 1980) and a β-17-estradiol, which induces the expression of *pCALS3::icals3m*. The *DR5rev::GFP* signal was completely abolished from the stele as a result of a 48 hour NPA treatment (Bishopp et al., 2011). In addition, expression of this marker extended laterally into the ground tissue stem cells (V, Fig 2C and 2D). In contrast, after a 48 hour induction of *pCALS3::icals3m* we detected no deviation from the wild-type *DR5rev::GFP* pattern with respect to the QC and the surrounding stem cells. However, in the vascular cylinder we occasionally observed a broader domain of GFP signal, and in the root cap the signal extended peripherally into the epidermis (V, Fig 2E) similar to our results

on the *pAPL::icals3m* line described earlier in this chapter. Furthermore, localization of *pPIN1::PIN1:GFP* was unaffected by a 12 hour induction of the *pCALS3::icals3m* (Fig 2F–H). To confirm that the strength of the symplastic blockage was sufficient in these conditions, we followed the protoxylem development in *pCALS3::icals3m* after a 48 hour induction and observed defects in protoxylem differentiation (V, Fig 2I). Finally, we analyzed the pattern of *pDR5rev::3xvenus-N7* (Heisler et al., 2005) in the root tip of the line displaying the QC-specific callose accumulation. We did not detect any deviation from the wild-type auxin response, whereas SHR mobility was severely hindered in *pWOX5::icals3m* (V, Fig 2A and 2B). These results are consistent with our previous observations where the expression of *DR5rev::GFP* in the *cals3-d* mutants is relatively normal (V, Fig 3). Taken together these results show that *icals3m* is sufficient to prevent symplastic signaling during root development, however, it only has a modest effect on polar auxin transport and auxin response in the root tip.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

In this study we have shown that symplastic trafficking is essential for root development. Moreover, we have demonstrated that callose deposition at the PD is sufficient to decrease the PD aperture and thus, reduce symplastic trafficking leading to altered root morphology. This work was based on the identification of the *cals3-1d, -2d,* and *-3d* mutations in the *CALS3* gene encoding a PD-localized protein synthesizing callose. The *cals3-d* leads to an overaccumulation of callose at the PD. Our studies show that the *cals3-d* mutations do not alter either the CALS3 localization or the mRNA levels of *CALS3*. Instead, it is possible that they alter the regulation of CALS3; the *cals3-d* mutation could release CALS3 from negative regulation and hence, lead to an increased callose production. This could occur, for example, if *cals3-d* alters the putative binding site(s) of the negative regulator(s). Further analysis of the *cals3-d* mutation sites is required to reveal whether these hypotheses are correct or not.

Despite the universal presence of callose in a wide variety of plant species and at various positions in the plant body, there is surprisingly little evidence of its function. Several studies have suggested that the presence of callose at the PD could regulate symplastic transport. Our results indicate that callose deposition controls the PD SEL and in doing so controls molecular trafficking via the PD. However, it is still not defined whether the presence of callose in the PD is purely regulatory or whether it has also a role in the PD formation. Analysis of the role of callose and the callose synthesis in a particular biological context is challenging, since *CALS*s belong to a multigene family and they are likely to display redundant functions. This was also observed in this study, as the loss of *CALS3* did not produce any detectable phenotypes. It is possible that the function of multiple *CALS*s synthesizing callose at the PD must be removed to obtain altered symplastic connectivity. Another way to approach function of the CALSs could be with the help of the *cals3-d* mutation. Since the amino acids corresponding to R84, R1926, and P189 and the domains surrounding them are conserved among the CALSs in *Arabidopsis*, it is possible that the function of the other CALS proteins could be controlled if these amino acids were manipulated. It is possible that this could help elucidate the role of callose synthesized by these proteins. However, it should be verified on a case by case basis whether the regulatory mechanisms related to *cals3-d* loci are diversified or shared among CALSs. Conservation of these domains even among relatively distant plant species suggests that they are important for the CALSs function. Further investigation is required to define the role of these conserved domains.

The majority of plant cells are symplastically connected and various signals have been postulated to move via the PD. The symplastic signaling route has been difficult to study largely due to challenges in the genetic analysis of the PD formation and function. By engineering a spatially and temporally controlled expression system using the CALS3

mutant form (*icals3m*), we were able to control the PD aperture in selected tissues and at specific time points and hence, to monitor the role of molecular trafficking in specific contexts. We suspected that the bidirectional signaling involving the mobility of SHR and miR165/6 could be disrupted in the *cals3-d* mutants, since they display an altered protoxylem differentiation. By modulating the PD SEL and through this the symplastic connectivity in both the stele and ground tissue using *icals3m,* we showed that the mobility of both SHR and miR165a could be restricted. Our results indicate that SHR and miR165a indeed move symplastically between the stele and the ground tissues. Various questions arise from these findings: How do these molecules move via the PD? Does their movement involve other assisting components? How general is the ability of miRs to move from cell to cell via the PD and how is this phenomenon regulated?

We also characterized the symplastic connectivity between the QC and surrounding cells and found out that it was essential for both the trafficking of SHR and for the assignment of correct cell identity. It still remains unclear if there are some other additional symplastic signals acting in the maintenance of the QC. We identified novel interactions between the phloem and surrounding tissues; the phloem specific callose deposition prevented the basipetal transport of cytokinin via the phloem and was accompanied by the expansion of the domain of high auxin signalling in the vascular cylinder. Furthermore, reduced symplastic connectivity in the phloem resulted in a rapid expansion of the differentiation zone and a reduction in the size of the cell division zone in the root tip. This indicates that symplastic connectivity of the phloem is important for the maintenance of the RAM. The molecular nature of this interaction remains to be identified. In contrast, we noticed that the effect of *icals3m* on polar auxin transport was modest. However, auxin also moves symplastically through the phloem and its mobility was reduced, but not completely prevented by the phloem specific *icals3m*. Since the auxin response in the root tip was unaffected, it remains an open question whether the symplastically transported auxin has a functional role in the root.

In this study we created the *icals3m* system, which allows the regulation of the PD aperture. This system could be beneficial for the characterization of various signals known to act non-cell-autonomously and also for analysis of the symplastic connectivity between tissues. This system could be used to modify any plant characteristic that involves symplastically moving regulatory signals, such as the flowering-time. Taken together, the use of *cals3-d* mutations in spatiotemporally controlled manner allows a wide range of novel studies in the plant research community, and has the potential to inspire new applications related to food, biomass, and biomaterial production.

SUMMARY IN FINNISH

TIIVISTELMÄ

Jokaisessa monisoluisessa eliössä soluista toiseen kulkee sekä tietoa, että solujen rakennusmateriaalia. Viestintä on kasvisoluille erityisen tärkeää, sillä ne erilaistuvat sijaintinsa perusteella; solun ympärillä olevat solut välittävät solulle tietoa siitä, millaiseksi sen tulee kehittyä. Nämä viesti voivat kulkea lyhyitä tai pitkiä matkoja. Viestit voivat kulkea solusta toiseen soluseinissä olevien aukkojen, plasmodesmien, kautta. Johtosolukot ovat pitkänmatkan kuljetukseen erikoistuneita solukoita, joiden avulla jopa useiden metrien päässä toisistaan sijaitsevat kasvisolut ovat yhteyksissä toisiinsa.

Tutkin väitöskirjassani solujen välistä viestintää lituruohossa. Löysimme sarjan mutanttikasveja, joiden johtosolukoiden kehitys on puutteellinen; tietyt johtosolukon solut kehittyvät väärään kohtaan ja toiset solutyypit jäävät kehittymättä kokonaan. Ensimmäinen mutantti, *phb-7d*, kantaa perimässään mutaatiota, joka estää sen koodaaman *PHB* mRNA:n rajoitusmekanismin toiminnan. Tämän seurauksena *PHB* mRNA leviää liian laajalle alueelle mutantin juuressa, mikä aiheuttaa sen johtosolukon solujen virheellisen erilaistumisen. Tämä osoittaa, että *PHB* mRNA:n oikea sijainti on tärkeää johtosolukon kehitykselle. Selvitimme, että *PHB* mRNA:n sijaintia juuressa säätelee johtosolukon ja sitä ympäröivän perussolukon välinen kaksisuuntainen viestintä; Ensimmäinen viestimolekyyli, transkriptiotekijä SHORT-ROOT (SHR), kulkee perussolukosta johtosolukkoon, jossa se aiheuttaa toisten viestimolekyylien, microRNA165/6 (miR165/6), ilmentymisen. Havaitsimme, että miR165/6 siirtyy takaisin perussolukkoon, jossa se saa aikaan *PHB* mRNA:n rajoittumisen oikealle alueelle.

Loput mutanteista, *cals3-1d*, *-2d* ja *-3d*, kantavat perimässään muutoksia *CALS3* geenissä, jonka koodaama proteiini tuottaa kalloosia, suurta sokeripolymeeria. Havaitsimme, että sekä CALS3 proteiini, että sen tuottama kalloosi sijaitsevat plasmodesmien läheisyydessä. *cals3-d* mutaatioiden seurauksena kalloosin tuotanto kasvissa lisääntyy ja solujen välinen viestintä vähenee. Kehitimme muuttuneen CALS3 proteiinin avulla kasvilinjoja, joissa kalloosia voidaan tuottaa tietyssä solukossa tiettynä ajankohtana altistamalla kasvit kemikaalille. Näissä kasvilinjoissa solujen välistä viestintää voidaan siis säädellä kohdennetusti. Tämän työkalun avulla tunnistimme, että SHR ja miR165/6 liikkuvat plasmodesmien läpi. Lisäksi havaitsimme, että tiettyjen solukoiden välisen viestinnän estäminen aiheutti kasvin ilmiasun muuttumisen. Tämä osoittaa, että näiden solukoiden välillä kulkee kehitystä säätteleviä viestejä, jotka ovat toistaiseksi tuntemattomia. Tunnistimme myös muita solujen välisiä viestejä, jotka kulkevat plasmodesmien läpi sekä toisaalta viestejä, jotka kulkevat toisia viestireittejä pitkin.

Kehitetyllä työkalulla on mahdollista kartoittaa mitkä kaikista solujen välisistä viesteistä kulkevat plasmodesmeja pitkin. Tällä työkalulla on myös mahdollista muokata kasvien kasvua kohdistetusti, esimerkiksi muuttaa kasvin rakennetta, kukkimisen ajankohtaa tai taudinkestävyyttä.

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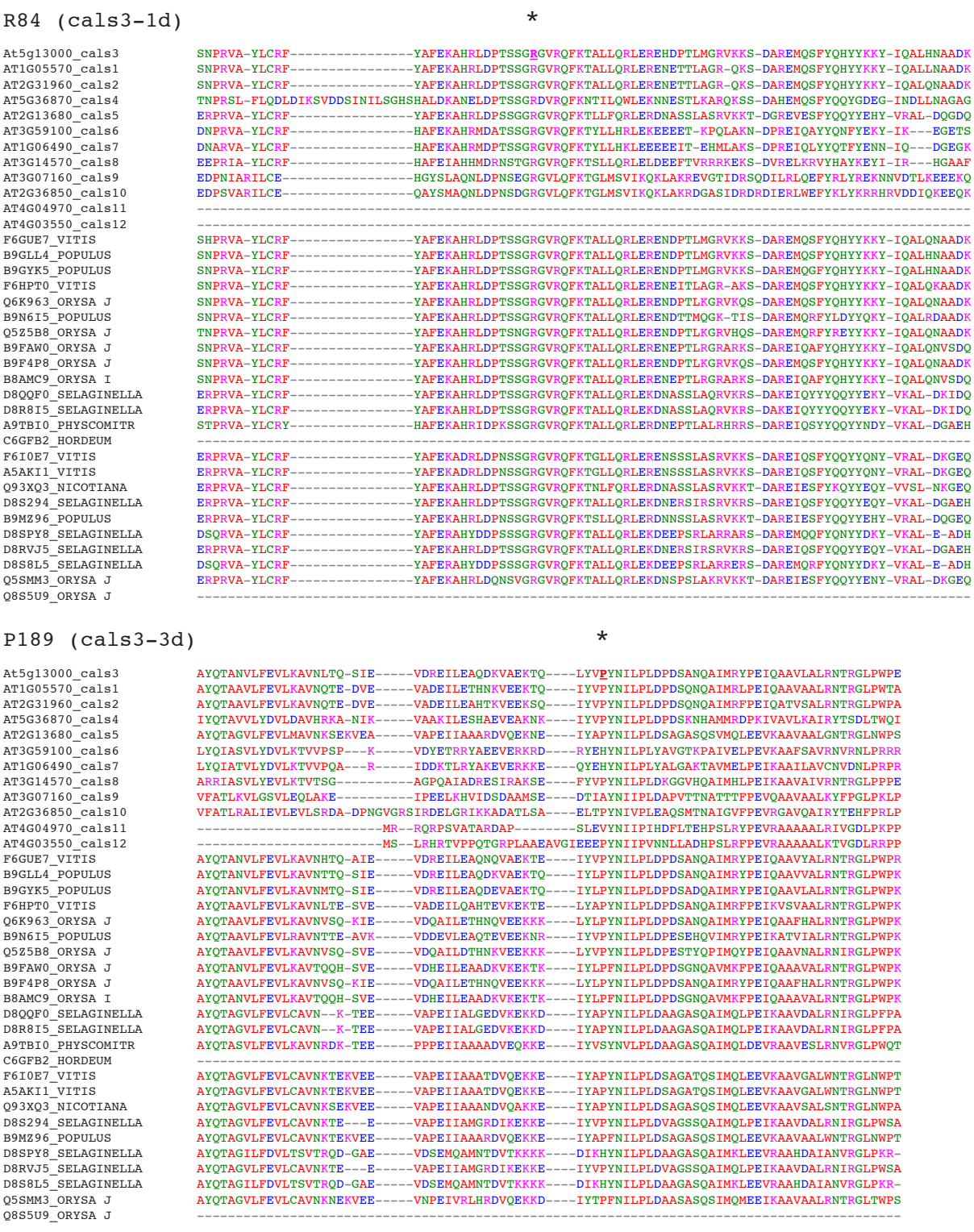
APPENDIX 1

Partial alignment of callose synthase sequences surrounding the conserved amino acid residues R84, P189, and R1926.



R1926 (Cals3-2d) *

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At5g13000_cals3          IAQACKPVVHR-AGFWGSVRTLARGY--EIVMGLLLFTPVAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGHRKDR----SSR--NKE
AT1G05570_cals1          IAQACKPLIQQ-LGIWSSVRTLARGY--EIVMGLLLFTPVAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGQRKDR----SSK--NKE
AT2G31960_cals2          IAQACKPLIQR-LGFWSSVRTLARGY--EILMGLLLFTPVAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGQRKDR----SSK--NKE
AT5G36870_cals4          IAQSCKPLIQQ-PGIWSWVMTLAWVY--DLVMGSLLFIPIAFMAWFPFISEFQTRMLFNQAFSRGLH-ISRILSGQRKHR----SSK--NKD
AT2G13680_cals5          ISQVARPLMKT-VGMWGSVKALARGY--EYIMGVVIFMPVTVLAWFPFVSEFQTRLLFNQAFSRGLQ-IQRILAGGKKQK------------
AT3G59100_cals6          IGQVLRSPIKA-LGVWDSVKELGRAY--ENIMGLVIFAPIAVLSWFPIVSEFQARLLFNQAFSRGLQ-ISMILAGRKDKA----TSS--HK-
AT1G06490_cals7          IGQALRSVFKG-LGFWDSVKELGRAY--EYIMGLVIFTPIAVLSWFPFVSEFQTRLLFNQAFSRGLQ-ISMILAGKKDKE----TPS--TK-
AT3G14570_cals8          IAQAVRPKIEG-TSLWEFTQVLARAY--DYGMGVVLFAPMAILAWLPIISAFQTRFLFNEAFNRRLQ-IQPILAGKKKNR------------
AT3G07160_cals9          IACAWKPVLKR-MGMWKSIRSLARLY--DALMGMLIFLPVALCSWFPFVSTFQTRMMFNQAFSRGLE-ISLILAGDNPNS-GL---------
AT2G36850_cals10         LAITWKQVLRV-LGLWETVREFGRIY--DAAMGMLIFSPIALLSWFPFISTFQSRLLFNQAFSRGLE-ISIILAGNRANV-ET---------
AT4G04970_cals11         IAQVLKPFLLS-TVVWDTVISVARFY--DLFFGLIVMAPVALLSWLPGFQNMQTRILFNEAFSRGLQ-ISIILAGKKST------------
AT4G03550_cals12         IAQTQRKWLKNYTIFWNAVVSVARMY--DILFGILIMVPVAFLSWMPGFQSMQTRILFNEAFSRGLR-IMQIVTGKKSKG-DV---------
F6GUE7_VITIS             IAQACKPVVER-AGFWASVRTLARGY--EIIMGLLLFTPVAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGHRKDR----SSR--NKD
B9GLL4_POPULUS           IAQACKPIVQR-AGFWGSVQTLARGY--EIVMGLLLFTPVAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGPRKDR----SSR--NKE
B9GYK5_POPULUS           IAQACKPVVQR-AGFWGSVRTLARGY--EIVMGLLLFTPVAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGHRKDR----SSR--NKE
F6HPT0_VITIS             IAQACKPLVVR-AGIWKSVRTLARSY--ELFMGLILFIPVAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGQRKDN----SSN--NKD
Q6K963_ORYSA J           VAQAIKPVIVR-IGLWGSIKALARGY--EIIMGLLLFTPIAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGHKKDR----ATR--NKE
B9N6I5_POPULUS           IAQACKPLIQH-AGFWGSVRTLARGY--EIVMGLLLFTPVAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGPRKDR----TSR--NKE
Q5Z5B8_ORYSA J           IAQAIKPAVQA-IGLWGSIKALARGY--EILMGLLLFTPIAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGHKKDR----STR--NKE
B9FAW0_ORYSA J           IAQACKPLARR-AGLWGSVRALARAY--EIIMGVLLFTPITILAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGQKKER-ER-SSR--NKD
B9F4P8_ORYSA J           VAQAIKPVIVR-IGLWGSIKALARGY--EIIMGLLLFTPIAFLAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGHKKDR----ATR--NKE
B8AMC9_ORYSA I           IAQACKPLARR-AGLWGSVRALARAY--EIIMGVLLFTPITILAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGQKKER-ER-SSR--NKD
D8QQF0_SELAGINELLA       IFMASRPVIVK-LGFWDSIRALARTY--EFVMGLVLFAPVAVLAWFPFVSEFQTRLLFNQAFSRGLQ-ISRILAGRKGKK----SNQDKSTH
D8R8I5_SELAGINELLA       IFMASRPVIVK-LGFWDSIRALARTY--EFVMGLVLFAPVAVLAWFPFVSEFQTRLLFNQAFSRGLQ-ISRILAGRKGKK----SNQDKSTH
A9TBI0_PHYSCOMITR        IAVACKPVVIN-LGFWKSVKSLARGY--EYMMGILLFTPIAVLSWFPFVSEFQTRLLFNQAFSRGLQ-ISRILAGRKKL------------
C6GFB2_HORDEUM           VAQALKPAIMR-VGLWGSIRALARGY--EIIMGLVLFTPYAFLAWFPFVFEFQTRMLFNQAFSRGLQ-ISRILGGH----------------
F6I0E7_VITIS             ISQALRPAVKA-LGMWGSVKALGRGY--EYMMGLSIFAPVAILAWFPFVSEFQTRLLFNQAFSRGLQ-IQRILAGGKKNK------------
A5AKI1_VITIS             ISQALRPAVKA-LGMWGSVKALGRGY--EYMMGLSIFAPVAILAWFPFVSEFQTRLLFNQAFSRGLQ-IQRILAGGKKNK------------
Q93XQ3_NICOTIANA         IAQACRPVVKG-IGMWGSVKALARGY--EYLMGLVIFAPVAVLAWFPFVSEFQTRLLFNQAFSRGLQ-IQRILAGGKKHK------------
D8S294_SELAGINELLA       IGMACRPLVES-MGFWGSVRALARSY--EFFMGLLIFTPVAILAWFPFVSEFQTRLLFNQAFSRGLQ-ISRILAGRKKLGE-----------
B9MZ96_POPULUS           IAQACRPVVKG-LKMWGSVKALARGY--EYMMALVIFAPVAVLAWFPFVSEFQTRLLFNQAFSRGLQ-IQRILAGGKKNK------------
D8SPY8_SELAGINELLA       IFQACRPVIVT-YGMWDSVQALARTY--EYVMGLLLFAPVAILAWFPFVSEFQTRLLFNQAFSRGLQ-ISRILAGKRKKV----ADD-----
D8RVJ5_SELAGINELLA       IGMACRPLVES-MGFWGSVRALARSY--EFFMGLLIFTPVAILAWFPFVSEFQTRLLFNQAFSRASRSLGSLRAGRNSVNETSTISP-----
D8S8L5_SELAGINELLA       IFQACRPVIVT-YGMWDSVQALARTY--EYVMGLLLFAPVAILAWFPFVSEFQTRLLFNQAFSRGLQ-ISRILAGKRKKV----ADD-----
Q5SMM3_ORYSA J           ISQASKPVVKA-FGLWGSVKALSRGY--EYLMGILIFVPVAVLAWFPFVSEFQTRLLFNQAFSRGLQ-ISRILAGGKKQS------------
Q8S5U9_ORYSA J           IAQACKPLARR-AGLWGSVRALARAY--EIIMGVLLFTPITILAWFPFVSEFQTRMLFNQAFSRGLQ-ISRILGGQKKER-ER-SSR--NKD
```

Alignment was created using Multiple Sequence Alignment program Kalign (http://www.ebi.ac.uk/Tools/msa/kalign/).

F6GUE7_VITI: Putative uncharacterized protein OS=Vitis vinifera GN=VIT_06s0004g01270 PE=4 SV=1
B9GLL4_POPULUS: Predicted protein OS=Populus trichocarpa GN=POPTRDRAFT_814785 PE=4 SV=1
B9GYK5_POPULUS: Predicted protein OS=Populus trichocarpa GN=POPTRDRAFT_817697 PE=4 SV=1
F6HPT0_VITIS: Putative uncharacterized protein OS=Vitis vinifera GN=VIT_13s0156g00210 PE=4 SV=1
Q6K963_ORYSA J: Putative callose synthase 1 catalytic subunit OS=Oryza sativa subsp. japonica GN=OJ1149_C12.24 PE=4 SV=1
B9N6I5_POPULUS: Predicted protein OS=Populus trichocarpa GN=POPTRDRAFT_828302 PE=4 SV=1
Q5Z5B8_ORYSA J: Putative callose synthase 1 catalytic subunit OS=Oryza sativa subsp. japonica GN=OSJNBa0069C14.2 PE=4 SV=1
B9FAW0_ORYSA J: Putative uncharacterized protein OS=Oryza sativa subsp. japonica GN=OsJ_09261 PE=4 SV=1
B9F4P8_ORYSA J: Putative uncharacterized protein OS=Oryza sativa subsp. japonica GN=OsJ_09005 PE=4 SV=1
B8AMC9_ORYSA I: Putative uncharacterized protein OS=Oryza sativa subsp. indica GN=OsI_09824 PE=4 SV=1
D8QQF0_SELAGINELLA: Glucan synthase like 3 OS=Selaginella moellendorffii GN=GSL3-1 PE=4 SV=1
D8R8I5_SELAGINELLA: Glucan synthase like 3 OS=Selaginella moellendorffii GN=Gsl3-2 PE=4 SV=1
A9TBI0_PHYSCOMITR: Predicted protein OS=Physcomitrella patens subsp. patens GN=PHYPADRAFT_143195 PE=4 SV=1
C6GFB2_HORDEUM: Glucan synthase-like 2 (Fragment) OS=Hordeum vulgare GN=GSL2 PE=2 SV=1
F6I0E7_VITIS: Putative uncharacterized protein OS=Vitis vinifera GN=VIT_04s0044g01280 PE=4 SV=1
A5AKI1_VITIS: Putative uncharacterized protein OS=Vitis vinifera GN=VITISV_008958 PE=4 SV=1
Q93XQ3_NICOTIANA: Putative beta-1,3-glucan synthase OS=Nicotiana alata GN=Gsl1 PE=2 SV=2
D8S294_SELAGINELLA: Glucan synthase like 4 OS=Selaginella moellendorffii GN=Gsl4-2 PE=4 SV=1
B9MZ96_POPULUS: Predicted protein OS=Populus trichocarpa GN=POPTRDRAFT_782672 PE=4 SV=1
D8SPY8_SELAGINELLA: Putative uncharacterized protein OS=Selaginella moellendorffii GN=SELMODRAFT_157296 PE=4 SV=1
D8RVJ5_SELAGINELLA: Glucan synthase like 4 OS=Selaginella moellendorffii GN=Gsl4-1 PE=4 SV=1
D8S8L5_SELAGINELLA: Glucan synthase like 2 OS=Selaginella moellendorffii GN=Gsl2 PE=4 SV=1
Q5SMM3_ORYSA J: Putative callose synthase 1 catalytic subunit OS=Oryza sativa subsp. japonica GN=OSJNBb0036B04.3 PE=4 SV=1
Q8S5U9_ORYSA J: 1,3-beta-glucan synthase component family protein, expressed OS=Oryza sativa subsp. japonica GN=OJ1015F07.18 PE=4 SV=1