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Cell-to-cell communication via plasmodesmata in vascular plants

Iris Sevilem, Shunsuke Miyashima and Ykä Helariutta*

Department of Bio and Environmental Sciences; Institute of Biotechnology; University of Helsinki; Helsinki, Finland

In plant development, cell-to-cell signaling is mediated by mobile signals, including transcription factors and small RNA molecules. This communication is essential for growth and patterning. Short-range movement of signals occurs in the extracellular space via the apoplastic pathway or directly from cell-to-cell via the symplastic pathway. Symplastic transport is mediated by plant specific structures called plasmodesmata, which are plasma membrane-lined pores that traverse the cell walls of adjacent cells thus connecting their cytoplasms. However, a thorough understanding of molecules moving via plasmodesmata and regulatory networks relying on symplastic signaling is lacking. Traffic via plasmodesmata is highly regulated, and callose turnover is known to be one mechanism. In Arabidopsis, plasmodesmata apertures can be regulated in a spatially and temporally specific manner with the *icals3m*, an inducible vector system expressing the mutated *CalS3* gene encoding a plasmodesmata localized callose synthase that increases callose deposition at plasmodesmata. We discuss strategies to use the *icals3m* system for global analyses on symplastic signaling in plants.

Keywords: symplastic communication, mobile signal, Arabidopsis, root development, plasmodesmata, callose

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*Correspondence to: Ykä Helariutta;
Email: yrjo.helariutta@helsinki.fi

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Positional Information Determines Cell Fate in Plants

Continuous growth and development is an important characteristic of plants. As plants are sessile, continuous plastic growth is a mechanism of adaptation to environmental conditions and maintaining fitness. The root system of Arabidopsis consists of a primary root formed during embryogenesis and several orders of post-embryonic lateral roots originating from the primary root. In the root apical meristem there is an area called the stem cell niche, where self-renewing stem cells called initials surround the quiescent center (QC), a small group of mitotically less active organizing cells necessary for the maintenance of the initials.[1] The initials divide asymmetrically to produce a new initial that remains next to the QC and another daughter cell that eventually differentiates. The daughters then undergo divisions in the meristematic zone located above the stem cell niche to increase their number. After the meristem, the cells enter the elongation zone, where divisions cease and strong expansion begins. Finally, in the differentiation zone, cells acquire their specific fates. Since plant cells do not move, the daughters of the initials are organized in cell files that form lineages where mature cells are located further from the tip. Thus, the age of a cell can be determined by its position along the longitudinal axis. The final radial organization of the root consists of concentric layers of distinct tissues along the radial axis (**Fig. 1**). The vasculature with its conductive tissues xylem and phloem and intervening procambium are located in the center of the root. The xylem axis develops bilaterally differentiating into central metaxylem with pitted cell walls and peripheral protoxylem with spiral cell walls. Surrounding the vasculature is the pericycle, the site for lateral root initiation. The vasculature and pericycle, together forming the stele, are surrounded by two layers of ground-tissue, a single layer of

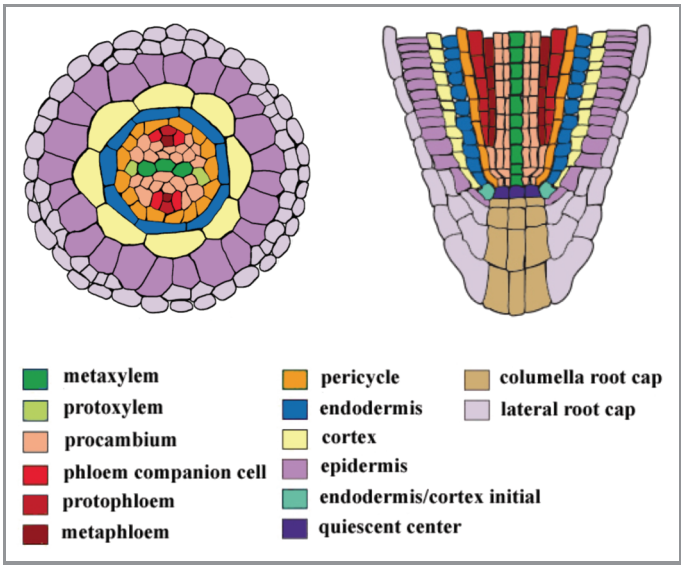

Figure 1. Cell types in the Arabidopsis root. Radial (left) and longitudinal (right) sections of the Arabidopsis root tip. Cell types are marked by different colors. Modified from Miyashima et al.[9] and Carlsbecker et al.[8]

endodermis and cortex. The outermost layer is the epidermis with alternating hair cells and non-hair cells.[2]

In plants, the fate of cells is largely determined by their spatial context rather than by their cell lineages. Genetic studies and the removal of QC cells by laser ablation first showed that the maintenance of the initials in an undifferentiated state depends on signals from the QC cells.[3,4] Furthermore, ablation of cortex/endodermal initials has shown that adjacent cells can replace their function.[5] Thus, positional information that is exchanged between cells predominantly coordinates pattern formation during development and underlies the flexibility of plant growth. Cell-to-cell communication is mediated by moving of regulatory molecules. Mobile signals include phytohormones, transcription factors and small RNAs, which vary in their mechanisms of action and the developmental outcomes they produce.

Bidirectional Signaling Coordinates Radial Development

In Arabidopsis root, a bidirectional signaling pathway was recently identified operating between the stele and endodermis that directs their patterning (**Fig. 2**). A GRAS family transcription factor SHORTROOT (SHR) is transcribed in the stele and moves from the stele to the endodermis to activate the transcription of SCARECROW (SCR).[6,7] Carlsbecker et al.[8] demonstrated that SHR and SCR located in the endodermis are needed for correct specification of protoxylem and metaxylem. SHR, together with SCR, activates transcription of *MIR165A* and *MIR166B* in the endodermis, and the miRNAs move radially to the stele periphery to cleave the mRNAs of *PHABULOSA* (*PHB*), a Class III homeodomain leucine zipper (*HD-ZIP III*) TF, and other members of the family, resulting in protoxylem differentiation. *shr* and *scr* mutants have abnormal xylem patterning in which metaxylem is formed in the protoxylem position. *phb-7d* gain of function mutant with a point mutation in the miR165/166 target site forms similarly ectopic metaxylem. Conversely, inducing *MIR165A* expression in the stele reduces *PHB* expression and leads to ectopic protoxylem development.[8] Miyashima et al.[9] further identified *miR166A* as a third miRNA expressed in the endodermis in a *SCR*-dependent manner, and that *MIR166A* and *MIR166B* are expressed in the QC in addition to the endodermis. Importantly, the authors also demonstrated that miR165 restricts the *PHB* expression domain and xylem differentiation in a dose-dependent manner. The miRNAs are additionally required for proper ground-tissue and pericycle development. miRNA-dependent restriction of *PHB* expression is required for the proper expression of a C2H2 zinc finger protein *JACKDAW* (*JKD*) in the ground-tissue, which is necessary to restrict SHR movement. In *phb-1d* roots with ectopic *PHB* expression SHR movement is not inhibited by *JKD*, leading to the development of an extra cortex layer. In addition, *PHB* restriction mediated by the miRNAs is necessary for pericycle differentiation, as in both *phb-1d* and *scr-3* the expression of two pericycle identity markers, *AHP6* and *SKOR*, is reduced in the pericycle domain.[9] Thus, stele and the ground-tissue exchange information via mobile SHR and miR165/6 species to coordinate each other's development.

Transport Pathways

Plants have specific mechanisms to exchange information between cells. Long-distance transport occurs via the two conductive tissues, xylem and phloem, whereas for short-range movement of molecules plants use apoplastic/transcellular and symplastic pathways. In apoplastic transport, a molecule is secreted to the extracellular space between cell walls, called the apoplast, where it moves without entering the target cell. In the transcellular transport, molecules moving in the apoplast are transported into the target cells through the plasma membrane by various mechanisms. Polar auxin transport is an example of a trans-cellular transport pathway, where auxin is polarly transported by efflux and influx carriers.[10] The symplastic transport is a unique pathway found only in plants mediated by plasmodesmata (PD), plant-specific structures that enable direct communication between cells (**Fig. 3**). PD are plasma membrane-lined pores that provide cytoplasmic connection by traversing cell walls of adjacent cells. Inside the pore runs a desmotubule which is a tube of tightly packed endoplasmic reticulum (ER). The intervening space between the desmotubule and the plasma membrane, called the cytoplasmic sleeve, contains proteins that

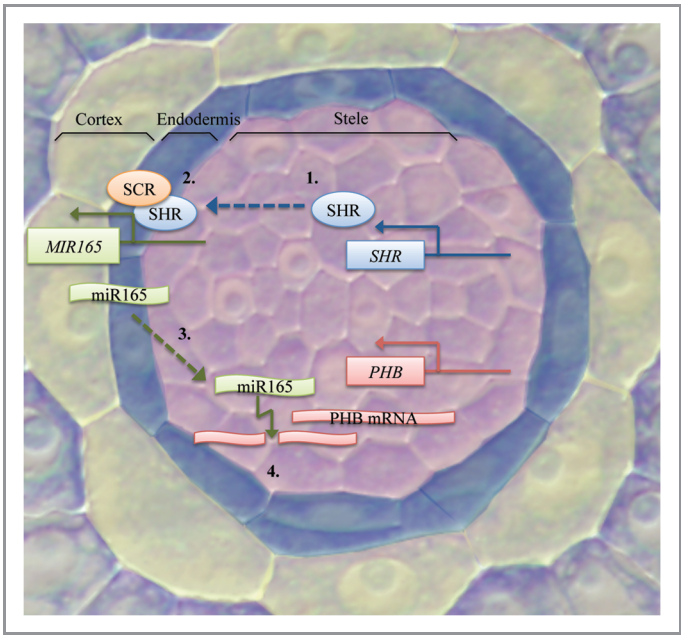

Figure 2. Bidirectional signaling pathway between the stele and the ground-tissue. SHR transcription occurs in the stele (1), after which the protein moves outwards from the stele to the endodermis to activate miRNA transcription together with SCR (2). The miRNAs move to the stele periphery (3) and downregulate *PHB* expression by targeting its mRNA transcripts for cleavage (4). Stele is highlighted in pink, endodermis in blue and cortex in yellow.

are attached to both membranes. These proteins divide the passage into microchannels that are thought to control the movement of molecules.[11-13] PD build a cytoplasmic network throughout the plant body that connects most cell types. PD not only provide a direct route from cell-to-cell, which overcomes the limitations of the rigid cell walls surrounding all plant cells, but also integrate local movement of molecules with long distance transport by functioning in the loading and unloading of phloem. Water and solutes and larger molecules including proteins and RNA move via PD. In addition to endogenous molecules, viruses and some other pathogens have evolved to use PD as their pathway to spread inside plants.[11-13]

The frequency of PD between cells and their permeability is controlled throughout development in a dynamic manner. PD apertures can alternate between closed, open and dilated states. In the closed state, PD are completely sealed from all traffic. In the open state, small molecules less than 1 kDa can diffuse through, whereas in the dilated state, diffusion of larger molecules is possible. The extent of the dilation determines the size exclusion limit (SEL), which is the upper size limit of molecules that can pass through PD. The SEL varies between 30–50 kDa in most growing tissues.[14,15]

Callose Regulates the SEL of PD

PD are structurally complex and their molecular composition as well as mechanisms regulating their permeability are not well known, since their purification is very difficult and mutants with altered PD structure/transport are often lethal.[16,17] However, recent studies have increased this understanding. Regulation of molecular traffic through PD has been associated with modifications in PD structure and callose deposition. Callose is a b-1,3 glucan polymer synthesized from UDP-glucose, which is involved in various biological processes in plants. Callose is synthesized in a number of locations, including cell plates of dividing cells, phloem sieve plates and at PD, and is deposited during normal growth and development, as well as in response to various mechanical and physiological stresses, such as wounding, chemical exposure and pathogen invasion.[18-20] Callose turnover at PD is a key component in controlling PD permeability. Its deposition at PD is thought to physically constrict the aperture, which reduces the SEL, and in some cases blocks it altogether from traffic. The deposition and turnover of callose are very dynamic, and controlled by the joint action of b-1,3-glucanase and b-1,3-glucan synthase, which degrade and synthesize callose, respectively.[21,22]

There are 12 callose synthase (*CALS*) genes encoded in the Arabidopsis genome.[23] *CALS* genes code for large plasma membrane localized proteins, several of which are known to produce callose in specific physiological and developmental processes. The most recent finding is CALS3 (At5g13000) that participates in the biosynthesis of callose in the cell wall surrounding PD. *CALS3* is strongly expressed in the stele and in and around the stem cell niche. *cals3-1d* and *cals3-2d* are allelic gain-of-function mutations in the *CALS3* gene that were identified in a genetic screen for altered vascular patterning. Their phenotype is strikingly similar to that of *phb-7d* and other mutants with defects in the bidirectional signaling pathway mediated by *SHR* and miR165/166. *cals3-d* mutations display ectopic metaxylem formation in the root and are shorter than wild-type roots, similar to *shr* and *phb-7d*. Also, those mutants were found to have impaired phloem unloading as GFP driven by a companion cell specific promoter *SUC2* diffused in more restricted manner compared with wild-type roots. These results suggested that the SEL of PD is altered in these mutants. Furthermore, the *cals3-d* mutations lead to reversible overproduction of callose at PD, which is thought to be caused by altered activity of the CALS3 protein. Root development in *cals3-1d* mutants was partially rescued by driving a β-1,3-glucanase from the *CALS3* promoter.[24]

icals3m: A Tool to Regulate Symplastic Trafficking

Based on the discovery of *cals3-d* mutants, we have designed a tool to study symplastic signaling networks by regulating PD permeability in a spatially and temporally specific manner.[24] *icals3m* is a tissue-specific inducible vector system that

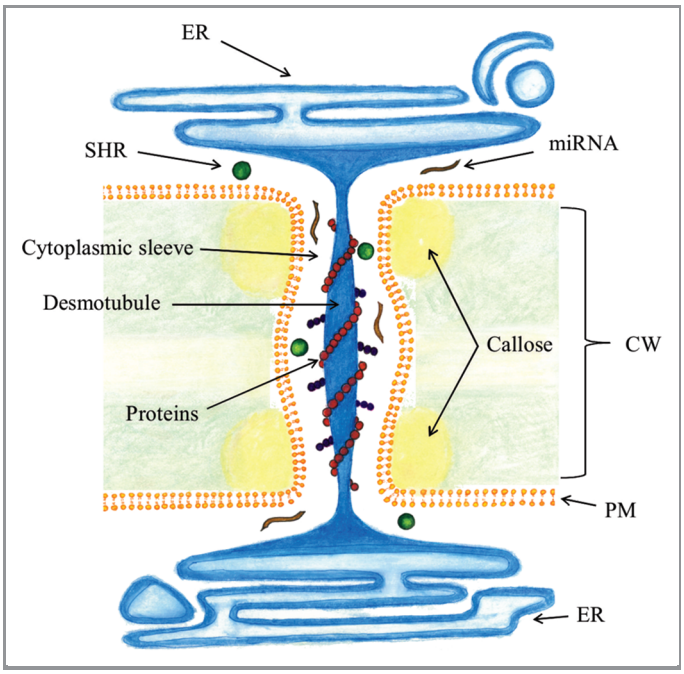

Figure 3. Plasmodesmata. Plasmodesmata connect cytoplasms of adjacent cells by traversing the cell wall. Appressed endoplasmic reticulum, called the desmotubule, runs through the plasma membrane-lined pore. Molecules move via the cytoplasmic sleeve between the desmotubule and plasma membrane. Callose is deposited at the neck region in the cell wall. CW, cell wall; PM, plasma membrane; ER, endoplasmic reticulum.

enables us to overexpress mutated *cals3* (*cals3m*) gene that contains both *cals3-1d* and *cals3-2d* mutations. When the transgene is activated, callose production is enhanced in tissue specific manner, leading to reduced PD apertures. For inducible expression, *icals3m* is controlled by the XVE system activated only with estradiol, enabling the expression of the transgenes to be regulated temporally.[25]

The *icals3m* system when expressed in the vasculature or QC is capable of blocking SHR movement, confirming that the protein moves via PD, and that protein traffic from cell-to-cell can be regulated with *icals3m*.[24] Moreover, microRNA movement was shown to be inhibited by *icals3m*. The mobility of miRNAs was investigated by combining two genetic tools, *icals3m* and *nlsYFP_targetMIR165mu*, a nuclear localized YFP marker targeted by mutated version of miR165 (miR165mu) (**Fig. 4**). First, *UAS::icals3m* construct was introduced into the ground-tissue specific enhancer trap line *(J0571)* established by J. Haseloff (www.plantsci.cam.ac.uk/Haseloff), and previously used by Carlsbecker et al.[8] The *UAS* promoter is activated by GAL4-VP16 protein transcribed in the ground-tissue of this enhancer-trap line. In addition to activating *icals3m*, the line contains GFP marker (**Fig. 4A**). *pSPR1::nlsYFP_targetMIR165mu/UAS::MIR165mu* was then introduced into *J0571; UAS::icals3m* (**Fig. 4B**). *MIR165Amu* was placed under the *UAS* promoter (*UAS::165Amu*) to express it in the ground-tissue, whereas the coding sequence of miR165mu-targeted nuclear-localized YFP was driven by the broadly-expressed *SPIRAL1* (*SPR1*) promoter,[26] (*pSPR1::nlsYFP_165mu_tgt*) to express it throughout the root.

The analysis of miR165mu movement in *J0571; UAS::icals3m/pSPR1::nlsYFP_targetMIR165mu/UAS::MIR165mu* showed that *icals3m* expression in the ground-tissue is able to inhibit the movement of the miRNAs from the ground-tissue to the stele (**Fig. 4C and D**). This was visualized as YFP signal in the stele, which indicated that the miR165mu were not present to cleave their targeted YFP sequences.[24] These results thus demonstrate for the first time that the miRNAs move via PD, and therefore provides new insights into the mechanisms of miRNA movement. This result is in accordance with previous suggestions that small RNAs use PD as their moving route. In addition, the data presented here indicate that the *icals3m* is very effective inhibitor of symplastic trafficking since it is now known the system not only blocks protein movement, but also miRNA movement. This observation provides additional value to the *icals3m* as a tool to study symplastic trafficking and proves its usefulness in visualization assays investigating differential movement of a molecule after PD closure.

The efficiency of the system to block diffusion of small molecules is, however, unclear. Thus, an important future experiment would be to quantify this aspect more precisely. The importance of PD in solute transport is also not well known. Even though water and some solutes can cross the membrane via specific transporter proteins, small molecules such as nutrients and hormones are assumed to move through PD. A recent study showed that symplastic solute transport is more extensive than previously thought.[27] In the study the authors quantified PD flux in the root meristem, and concluded that the flux is 10-fold compared with previous measurements. Presumably with the measured flux rate, symplastic diffusion of sucrose is required for maintaining root growth. These results suggest that symplastic diffusion of solutes is very important, and can be a major route for sucrose transport in the meristem. The authors also assayed solute flux in an Arabidopsis line overexpressing *PDCB1*, a protein that promotes callose deposition at PD, and found out that flux is reduced by approximately one-half. This indicates that enhanced callose deposition at PD also suppresses solute movement. The effect of *icals3m* on solute transport is not known; however, these results raise the possibility that impaired solute diffusion might be occurring.

Toward Revealing Novel Symplastic Networks Using the *icals3m*

Although the data on signaling networks has increased, many questions still remain how developmental processes are regulated non-cell-autonomously. Thus, we envision identifying novel symplastic communication

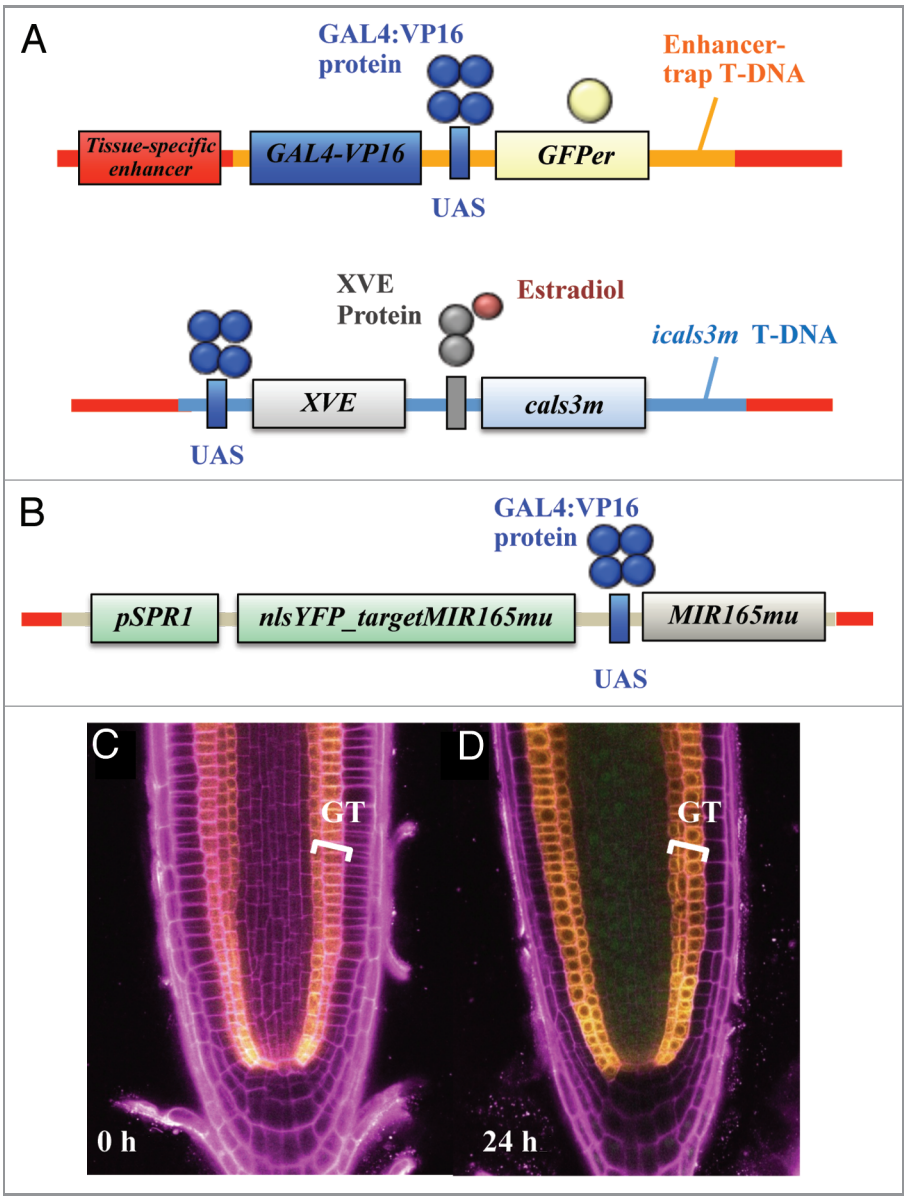

Figure 4. Analysis of miRNA movement with a miRNA sensor system. *J0571; UAS::icals3m* line expressing *cals3m* in an estradiol inducible manner and GFP constitutively in the ground-tissue (**A**) was introduced with a sensor system *pSPR1::nlsYFP_targetMIR165mu/UAS::MIR165mu*. The coding sequence of miR165mu-targeted nlsYFP was driven by the broadly-expressed *SPR1* promoter, whereas the *MIR165mu* gene, which targets the nlsYFP_165mu-tgt, was placed under the *UAS* (**B**). In the resulting line (called *J0571; UAS::icals3m/pSPR1::nlsYFP_targetMIR165mu/UAS::MIR165mu*), YFP signal was not detected under non-induced condition (**C**). In contrast, after 24 h induction period, nlsYFP signal was detected throughout the stele (**D**), indicating that the miRNAs were not present to cleave the nlsYFP sequences and consequently YFP signal was not suppressed. GFP signal was detected in the ground-tissue layer of all of the roots, indicating that miR165mu transcription occurred in both conditions. Yellow, GFP signal; green, YFP signal; magenta, PI signal; GT, ground-tissue.

events that regulate development in Arabidopsis root. As described in the previous section, whether a known mobile molecule moves via the symplastic route can be investigated with *icals3m*. In addition, inhibiting symplastic communication using this system could be used to identify novel regulatory networks relying on symplastic signaling. Cell type- and tissue-specific *icals3m* plants enable us to block symplastic communication from the corresponding spatial domain in an inducible manner. *icals3m* expression in a tissue-specific manner combined with genome-wide expression analyses by microarray or RNA sequencing can reveal regulatory targets of symplastic signals. Especially tissue-enriched genes that are differentially expressed under callose induction are promising candidates involved in the cell specification and differentiation of that tissue. For example, after *icals3m* expression in the ground-tissue, differential expression of stele enriched genes is likely caused by blockage of a regulatory signal originating from the ground-tissue that is unable to transmit into the stele.

After collecting gene expression information following spatially and temporally specific inhibition of symplastic communication, expression profiles of different spatial domains can be compared with the root gene expression map[28] to establish a global symplastic communication map of Arabidopsis root. This map would reveal valuable information on which genes are regulated by symplastic signals and how different tissues of the root, including the stele, ground-tissue and epidermis, regulate gene expression non-cell-autonomously through PD during the development of other root domains. On the other hand, using cell type specific lines within tissues reveals how the various cell types within tissues communicate with each other. Moreover, by looking at the number of affected genes, it is possible to determine which cell types and tissues are major sources and sinks of information signals. In addition, by performing functional analyses of the differentially expressed genes after *icals3m* expression, a deeper understanding of the processes regulated by symplastic signals can be obtained. It is likely that the differentially expressed genes include not only primary targets but also those more downstream. In order to reduce these secondary effects, a time-course analysis to optimize the induction time as short as possible is necessary.

To summarize, plant development is mainly controlled by positional signals, and the symplastic pathway is a major communication route in plants. Callose turnover is a central mechanism to regulate PD traffic, and the spatially and temporally controlled enhancement of callose deposition with *icals3m* is a very effective tool to block symplastic trafficking. At

least protein and smRNA traffic are inhibited, but also small molecule transport may be affected. It is important to quantify exactly the reduction in PD diameter in order to understand if the movement of small molecules is inhibited.

Finally, investigating genome-wide expression changes occurring after manipulating the symplastic connection by using *icals3m* system can be used to find novel symplastic networks that operate between different tissues and cell types of the root.

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