Cell-to-cell communication via plasmodesmata in vascular plants

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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 ical3m, 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 ical3m system for global analyses on symplastic signaling in plants.

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...
endodermis and cortex. The outermost layer is the epidermis with alternating hair cells and non-hair cells.3

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 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 (JDK) 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 JDK, 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 apoplasm, where it moves without entering the target cell. In the transcellular transport, molecules moving in the apoplasm 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...
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.\textsuperscript{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 β-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.\textsuperscript{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.\textsuperscript{21,22}

There are 12 callose synthase (\textit{CALS}) genes encoded in Arabidopsis genome.\textsuperscript{23} \textit{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 \textit{CALS3} (At5g13000) that participates in the biosynthesis of callose in the cell wall surrounding PD. \textit{CALS3} is strongly expressed in the stele and in and around the stem cell niche. \textit{cals3-1d} and \textit{cals3-2d} are allelic gain-of-function mutations in the \textit{CALS3} gene that were identified in a genetic screen for altered vascular patterning. Their phenotype is strikingly similar to that of \textit{phb-7d} and other mutants with defects in the bidirectional signaling pathway mediated by \textit{SHR} and \textit{mir165/166}. \textit{cals3-d} mutations display ectopic metaxylem formation in the root and are shorter than wild-type roots, similar to \textit{sbr} and \textit{phb-7d}. Also, those mutants were found to have impaired phloem unloading as GFP driven by a companion cell specific promoter \textit{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 \textit{cals3-d} mutations lead to reversible overproduction of callose at PD, which is thought to be caused by altered activity of the \textit{CALS3} protein. Root development in \textit{cals3-1d} mutants was partially rescued by driving a β-1,3-glucanase from the \textit{CALS3} promoter.\textsuperscript{24}

\textbf{icals3m: A Tool to Regulate Symplastic Trafficking}

Based on the discovery of \textit{cals3-d} mutants, we have designed a tool to study symplastic signaling networks by regulating PD permeability in a spatially and temporally specific manner.\textsuperscript{24} \textit{icals3m} is a tissue specific inducible vector system that
enables us to overexpress mutated ical3 (icals3m) gene that contains both ical3-1d and ical3-2d 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 icals3 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 SPRI (SPRI) 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 PDCBI, 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.

**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.

**Figure 4.** Cell Adhesion & Migration Volume 7 Issue 1

**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
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 \textit{ical3m}. In addition, inhibiting symplastic communication using \textit{icals3m} plants enable us to block symplastic communication from the corresponding spatial domain in an inducible manner. \textit{ical3m} 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 \textit{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 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 \textit{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 \textit{icals3m} is a very effective tool to block symplastic trafficking. At

\textbf{Figure 4.} Analysis of miRNA movement with a miRNA sensor system. J0571; UAS::icals3m line expressing \textit{icals3m} in an estradiol inducible manner and GFP constitutively in the ground-tissue (A) was introduced with a sensor system \textit{pSPR1::nlsYFP\_targetMIR165mu/UAS::MIR165mu}. The coding sequence of miR165mu-targeted nlsYFP was driven by the broadly-expressed \textit{SPR1} promoter, whereas the \textit{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 indicates GFP signal, green indicates YFP signal and magenta indicates PI signal. GT stands for ground-tissue.
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 ica1-3m system can be used to find novel symplastic networks that operate between different tissues and cell types of the root.

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