Regulation of Vascular Development by CLE Peptide-receptor Systems

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Abstract

Cell division and differentiation of stem cells are controlled by non-cell-autonomous signals in higher organisms. The plant vascular meristem is a stem-cell tissue comprising procambial cells that produce xylem cells on one side and phloem cells on the other side. Recent studies have revealed that TDIF (tracheary element differentiation inhibitory factor)/CLE41/CLE44 peptide signal controls the procambial cell fate in a non-cell-autonomous manner. TDIF produced in and secreted from phloem cells is perceived by TDR/PXY, a leucine-richrepeat receptor kinase located in the plasma membrane of procambial cells. This signal suppresses xylem cell differentiation of procambial cells and promotes their proliferation. In addition to TDIF, some other CLE peptides play roles in vascular development. Here, we summarize recent advances in CLE signaling governing vascular development.

Introduction

Plants have acquired unique morphological features and keen environmental response systems to adapt to their specific ecological niche. The vascular system, the long-distance transport system that links every part of the plant body, has also developed as a component of this adaptive system. In the vascular system, there are two conducting tissues, the phloem and xylem. The vascular meristem, called the procambium/cambium, is located between the phloem and xylem, and gives rise to both phloem and xylem cells in opposite sides (Figure 1).

An important function of the vascular system is the transport of water and nutrients essential for the growth and development of developing tissues and organs. Vessels and tracheids in the xylem transport water and ions absorbed from the soil, whereas sieve tubes in the phloem transport sucrose and amino acids produced in photosynthetic leaves. Vascular tissues also function as pathways for the delivery of signaling molecules, such as cytokinins, synthesized in roots and auxin produced in meristems and young leaves. Thus, the network of the vascular system plays an important role in the integration of plant growth and development at the whole-plant level.

To form the complex tissues of the vascular system, local communication must control the behavior of each cell. Plant hormones are known to act as signaling molecules that mediate in the cell-cell communication pathways. Recently, new players, small secretory peptides have come into the limelight in this regard. CLAVATA3/EMBRYO SURROUNDING REGION-related (CLE) peptides are central players of such cell-cell communication in plants (Diévant and Clark 2004; Matsubayashi and Sakagami 2006; Jun et al. 2008; Fiers et al.


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2007; Fukuda et al. 2007; Butenko et al. 2009). It has now been shown that tracheary element differentiation inhibitory factor (TDIF), a CLE peptide, functions as a signaling molecule mediating vascular cell communication to form well-organized vascular tissues (Ito et al. 2006; Hirakawa et al. 2008). Here, we review recent advances in cell–cell signaling through CLE peptides and their receptors in terms of the regulation of vascular tissue development.

**CLE Peptides and Their Signaling Pathway**

The CLE genes are found extensively in terrestrial plants (Cock and McCormick 2001). In the Arabidopsis thaliana genome, 32 CLE genes have been identified (Jun et al. 2008) and there is (Oryza sativa) genome some 47 genes have been annotated (Kinoshita et al. 2007). CLE family genes encode small proteins of less than 15 kDa with a signal peptide at the N-terminus and a conserved 14-amino-acid domain, called the CLE domain, at or near the C-terminus. Functional CLE peptides of CLV3, CLE2 and TDIF are secreted as peptides composed of 12 or 13 amino acids that contain the last 12 amino acids of the CLE domain; these peptides undergo hydroxylation at two proline residues (Ito et al. 2006; Kondo et al. 2006; Ohyama et al. 2009). Recently Ohyama et al. (2009) identified 12-amino-acid CLE2 and 13-amino-acid CLV3 peptides having three arabinose residues on a hydroxyproline residue as being the most active forms. These studies were based on culture medium derived from submerged whole plants that were overexpressing CLE2 or CLV3 genes. In contrast, functional TDIF is not glycosylated (Ito et al. 2006; Ohyama et al. 2008). Thus, the function of CLE peptides is regulated through various processes involving transcription, translation, processing, hydroxylation, and glycosylation (Figure 2).

**Identification of TDIF and Its Physiological Action**

A Zinnia xylogenic culture is an efficient system for analysis of xylem cell differentiation and has been used to identify various key genes, proteins and signaling molecules involved in xylem differentiation (Fukuda and Komamine 1980; Demura and Fukuda 1994; Yamamoto et al. 2001; Ito and Fukuda 2002; Ohashi-Ito and Fukuda 2003; Motose et al. 2004; Fukuda 2004; Yoshida et al. 2005; Ito et al. 2006). In the culture, isolated Zinnia mesophyll cells transdifferentiate into tracheary elements (TEs), when cultured in medium containing both naphtaleneacetic acid as an auxin and benzyladenine as a cytokinin. Ito et al. (2006) found that TE differentiation was inhibited by extracellular factor(s) in cultured medium of Zinnia

![Figure 1. Radial pattern of the plant vascular system.](image)

(A) A vascular bundle is composed of the phloem (green), the procambium (yellow), and the xylem, which has tracheary elements (blue) and xylem parenchyma cells (red). (Reproduced from Fukuda 2004)

(B) Intervening procambial cells proliferate through periclinal cell divisions to give rise, in opposite directions, to phloem and xylem cells.

![Figure 2. Biosynthetic pathway for a CLE peptide.](image)

The CLE gene encodes a small protein, which contains the N-terminal signal peptide (sp, gray) and the conserved CLE domain at or near the C-terminus (CLE, blue). Translated prepro-CLE protein is modified through processing, hydroxylation, and glycosylation to give rise to a mature CLE peptide.
cells, and they named the unknown factor(s) tracheary element differentiation inhibitory factor (TDIF).

This TDIF was present in a 20% methanol fraction taken from the cultured medium. A bioassay with Zinnia mesophyll cell culture demonstrated that a dodeca-peptide, which is the product of genes from the CLE family, is TDIF. This peptide consists of 12 amino acids, and first and second proline residues are hydroxylated (HEVHyPSPHePHEV). The full-length cDNA corresponding to TDIF from Zinnia cells encodes a protein of 132 amino acids, but only 12 from H120 to N131 match the TDIF sequence. This result indicates that TDIF is produced through the removal of residues M1-A119 at the N-terminus and R132 at the C-terminus. Through homology searches, the TDIF sequence was found to be the same as the Arabidopsis C-terminal 12-amino-acid sequences of CLE41 and CLE44, and highly similar to that of CLE42.

Experiments with synthetic peptides revealed that TDIF functions at as low as 30 pM in the Zinnia xylegenic culture and a dodeca-peptide from CLE42 also functions in suppression of TE differentiation, but the other dodeca-CLE peptides do not (Ito et al. 2006). TDIF peptide was identified in culture medium derived from submerged culture of CLE44-overexpressed transgenic plants, which confirmed that CLE44 and probably CLE41 produce TDIF peptide (Ohyama et al. 2008).

In situ function of TDIF has also been analyzed by the addition of chemically synthesized TDIF to submerged culture of Arabidopsis seedlings (Hirakawa et al. 2008). TDIF caused discontinuity of xylem vessel strands in rosette leaves, although phloem and procambium strands were normal even in vessel strand-discontinuous regions (Figure 3A). This result indicates that TDIF specifically inhibits the differentiation from procambial cells into TEs. This in situ function of TDIF is confirmed by the fact that CLE41- and CLE44-overexpressed transgenic plants showed the same vessel stand-disrupted phenotype as observed with peptide-treated plants.

Surprisingly, TDIF causes an incremental increase in vascular area as well as production of discontinuous vessel strands. This effect is remarkable in hypocotyls. Detailed analysis revealed that this increase in vascular area results from the promotion of procambial cell divisions (Figure 3B). This finding is consistent with the result that TDIF promotes cell proliferation in the Zinnia xylogenic culture (Ito et al. 2006). Therefore, TDIF should have two critical roles: first, to inhibit xylem differentiation, and second to promote procambium proliferation in the vascular system.

Identification of a Receptor for TDIF

As aforementioned, CLV1, which is a receptor for CLV3, belongs to the LRR-RLK family. LRR-RLKs are single-transmembrane protein kinases with extracellular LRR domains. The LRR-RLK family is a major family of plant receptor kinases, and Arabidopsis has more than 200 members and rice has nearly 400 members in their genome (Shiu et al. 2004). Another receptor group, the LRR-RLP, of which CLV2 is a member, lack the kinase domain of the LRR-RLK family. Most LRR-RLKs are categorized into three super-subclades (Cf-9, RPP27, and PSKR) in Arabidopsis, but CLV2 is not classified into any of these subclades (Fritz-Laylin et al. 2005). Some LRR-RLK genes have been implicated in development, disease resistance, and symbiosis, but for most LRR-RLKs, neither their function nor ligand is known (Morillo and Tax 2006). However, public database AtGenExpress and gene profiling of differentiating Zinnia cells (Demura et al. 2002) and of Arabidopsis xylegenic cells (Kubo et al. 2005) indicate that a number of LRR-RLKs are expressed in association with vascular development. This fact suggested that various LRR-RLKs may be involved in cell–cell communication during vascular development (Fukuda et al. 2007).

Hirakawa et al. (2008) selected approximately 60 Arabidopsis genes that appeared to be expressed preferentially in procambium, based on the abovementioned gene expression data. When the T-DNA insertion mutant lines were treated with TDIF, all except one line showed fragmented vein patterns. Three different alleles of this line similarly exhibited TDIF-insensitive phenotypes. The causal gene for these lines encoded an LRR-RLK belonging to the LRR subclass XI, the same subclass as CLV1 (Shiu and Bleecker 2001), and was named putative TDIF RECEPTOR (TDR). This gene was also identified as PHLOEM INTERCALATED WITH XYLEM (PXY) in a forward-genetic analysis (Fisher and Turner 2007).

Further biochemical analysis, using the extracellular domain of TDR expressed in BY-2 cells, and a radiolabelled photoactivatable TDIF derivative, showed the direct and specific binding of TDIF to TDR. tdr mutants exhibited reduced procambial cell proliferation and, sometimes, in the hypocotyl xylem vessels were located adjacent to phloem cells without intervening procambial cells (Figure 3C). This fact can be explained by the loss of sensitivity to endogenous TDIF in procambial cells. Collectively, these experiments demonstrated that TDR is a receptor for TDIF.

The TDIF/TDR system involves a member of the CLE peptide ligand family and its receptor belonging to the subclass XI of the LRR-RLK family. The CLV3/CLV1 system also consists of a CLE peptide and a member of the LRR-RLK family XI. A phylogenetic tree derived from sequences of the extracellular region, to which CLE ligands would bind, displays a clade comprising seven members, TDR/PXY, CLV1, BAM (BARELY ANY MERISTEM)1, BAM2, BAM3, PXL (PXY-like)1, and PXL2 among 26 members of the subclass XI (Figure 4A). On the other hand, CLV3 and TDIF are far among CLE peptides in an unrooted neighbor-joining tree of Arabidopsis dodeca-CLE
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Figure 3. Roles played by tracheary element differentiation inhibitory factor (TDIF) and TDIF RECEPTOR (TDR) in *Arabidopsis* vascular development.

(A) Exogenous application of TDIF results in discontinuity of xylem vessel strands (blue-outline), but not of phloem (yellow) or procambium (red-outline) strands in *Arabidopsis* rosette leaves.

(B) Exogenous application of TDIF promotes procambial cell proliferation in the hypocotyl vascular bundle. Red dots indicate procambial cells.

(C) Three different mutant alleles of *TDR/PXY*, which encodes a single-transmembrane leucine-rich repeat receptor-like kinase (LRR-RLK), show reduced procambial cell proliferation and xylem cells unusually adjacent to phloem cells in vascular bundles. Ph and Xy indicate phloem and xylem cells, respectively (reproduced from Hirakawa et al. 2008).

sequence (Figure 4B). Therefore the seven members may be receptors for most of the CLE peptides in *Arabidopsis*. Indeed, a mature CLE2 peptide with three arabinose residues binds to CLV1 at almost the same efficacy as does the mature CLV3 (Ohyama et al. 2009).

A Phloem-xylem Crosstalk Mediated by the TDIF/TDR Signal

Where are TDIF and TDR produced and where do they function? To answer these questions, the tissue-specific expression patterns of TDIF and TDR genes were analyzed with promoter–GUS reporter gene fusions (*pTDR::GUS*, *pCLE41::GUS*, and *pCLE44::GUS*) (Hirakawa et al. 2008). *pCLE41::GUS* activity was observed along the vascular strands in all organs examined, and restricted to the phloem and the neighboring pericycle cells in the root and hypocotyl. *pCLE44::GUS* expression occurred in not only cells in the phloem, and the adjacent pericycle, but also in endodermal cells. These findings indicated that TDIF is mainly produced in phloem cells from where it is secreted.

Immunohistochemical analysis, using an anti-TDIF antibody, also revealed the localization of mature TDIF peptide in the apoplasm surrounding two phloem precursor cells (Figure 5). In contrast, *pTDR::GUS* was expressed specifically in the procambium in the root and hypocotyl. This result is consistent with procambial cell-specific expression of *TDR* mRNA in xylemogenic cultures (Kubo et al. 2005). Based on these findings, we propose that TDIF functions in a non-cell-autonomous manner (Figure 6A). TDIF is produced in and secreted from phloem cells and perceived by TDR located in the plasma membrane of procambial cells. This signal enhances division of procambial cells and suppresses the differentiation of procambial cells into xylem cells. This means that there must be crosstalk between phloem and xylem formation, mediated by a phloem-derived xylem-differentiation inhibitor, TDIF.

Tracheary element differentiation inhibitory factor might make a gradient from the phloem to the xylem via the procambium (Figure 6B). This gradient might act as positional information for the control of cell division and differentiation within the procambium. That is, procambial cells near the phloem perceive a relatively high concentration of TDIF and are maintained to be undifferentiated and in a proliferative
state. At greater distances from the phloem, the TDIF signal becomes weak, which allows procambial cells to acquire the potential to differentiate into xylem cells. Indeed, exogenously supplied TDIF causes not only procambial cell proliferation but also ectopic xylem cells (Hirakawa et al. 2008). This might result from a rapid decrease in the TDIF gradient resulting from an increase in the number of intervening procambial cells.

Other CLE Peptides that Control Vascular Development

Of the 32 CLE genes that have been identified in Arabidopsis, most genes have yet to be characterized in terms of function. Eighteen CLE genes were overexpressed and their gain-of-function phenotypes were observed (Strabala et al. 2006). The overexpression of 11 genes, including CLV3, showed a growth arrest phenotype at the level of the SAM equivalent to the wus mutant, suggesting similar functions for these CLE peptides. The redundant function of CLE peptides has also been indicated by rescue of the clv3 phenotype by various CLE genes (Hobe et al. 2003; Ni and Clark 2006). In contrast, CLE42- and CLE44-overexpression produced shrub-like dwarf plants that lacked apical dominance, whose phenotype was not observed in the other CLE-overexpressing plants. Therefore, this phenotype might be directly or indirectly related to vascular development. Finally, there may be CLE peptides other than CLE41/CLE44 and CLE42, that are involved in vascular development.

Whitford et al. (2008) reported that overexpression of some CLE genes (CLE6, CLV3 and CLE19), although they could not affect vascular development alone, cooperate with TDIF to enhance procambium proliferation. These CLE genes are known to reduce the size of the SAM and root apical meristem (RAM). In clv1 and clv2 mutants, these CLE peptides do not cause a reduction in meristem size, but they can enhance TDIF-induced procambium proliferation. These results establish that CLE6, CLV3 and CLE19 peptides cooperate with TDIF to enhance procambial cell proliferation through receptors different from CLV1 and CLV2. Therefore, it seems reasonable to suggest that one CLE ligand may bind to a range of receptors. Furthermore, based on results from competition analysis with
Intracellular Signal Transduction from CLE Peptide/Receptor System

Recent studies have revealed that a novel receptor-like kinase, CRN/SOL2, is involved in meristem maintenance, thus indicating that receptor-like kinases other than CLV1 can function in CLE signaling (Miwa et al. 2008; Müller et al. 2008). This extracellular domain-lacking kinase may function with the kinase domain-lacking CLV2 through an interaction via their transmembrane domains (Müller et al. 2008). This finding suggests that the stem cell population in the SAM may be regulated by a complicated signaling perception pathway through the mature CLV3 and CLE2 peptides, a particular LRR-RLK may well recognize some different CLE ligands (Ohyama et al., 2009).

An earlier report indicated that, in Arabidopsis, transcripts for some of the CLE genes (CLE10, CLE26, CLE27) increased during in vitro xylogenesis (Kubo et al. 2005). Brassica napus CLE19 gene is expressed in a few pericycle cells facing the protoxylem poles as well as in organ primordia (Fiers et al. 2004). We have also found that various CLE genes are preferentially expressed in vascular tissues and that some CLE peptides function at different stages of procambium and xylem development (Kondo unpubl., data, 2009). Therefore, CLE peptides other than TDIF may play roles in vascular development. Sophisticated regulation by various CLE ligands and receptors may control the well-organized formation of vascular tissues.
numerous receptors, including CLV1, CLV2 and CRN/SOL2. Also, in vascular development, numerous receptors as well as several CLE peptides may produce a complex cell-cell communication network (Figure 7).

The intracellular signaling pathway downstream of CLE signals is partly resolved in the SAM. Analysis using a transient CLV3 induction system has shown that the CLV3 signal rapidly, but transiently, represses WUS expression, which after a few hours leads to a reduction of endogenous CLV3 expression ( Müller et al. 2006). The signaling pathway between perception of CLV3 and control of WUS expression is still largely unknown (Carles and Fletcher 2003). In contrast, a target of WUS has been reported. WUS directly represses the expression of Type-A ARR (Arabidopsis Response Regulators) genes, which are negative regulators of cytokinin signaling and act as positive regulators of SAM maintenance (Leibfried et al. 2005). Recently, it has also been established that localized perception of cytokinin regulates the expression of WUS (Gordon et al. 2009). Hence, the CLV3 and cytokinin signals cooperate to control SAM maintenance.

In the vascular system, the CLE peptide-enhanced procambial proliferation is positively regulated by auxin, and negatively regulated by NPA (1-naphylphthalamic acid), which is an inhibitor of auxin transport (Whitford et al. 2008). This result suggests that auxin is necessary for enhancement of procambium proliferation by CLE peptides. Cellular proliferation in the procambium/cambium is also controlled by cytokinin and its signaling mediators in Arabidopsis and Poplar (Mähönen et al. 2006; Matsumoto-kitano et al. 2008; Nieminen et al. 2008; Hejátko et al. 2009). These findings suggest that, in general, CLE peptides may regulate the fate of stem cells through crosstalk with signaling pathways mediated by other plant hormones, such as auxin and cytokinin.

Future Perspectives

First and foremost, the relationship between various CLE peptides and their receptors should be clarified biochemically. Another important target for future research is the downstream signaling pathway after the perception of the CLE peptides. This will involve identifying the intracellular phosphor-relay machinery as well as the key transcription factor(s) that function downstream of TDIF/TDR. Here, it is important to reiterate that TDIF/TDR acts at two different events; promotion of procambial cell division and suppression of xylem differentiation. It will be interesting to learn whether a single or two separate downstream signaling pathways are responsible for these two events. Crosstalk between the CLE peptide and plant hormones is also essential. It will be a challenge to identify the molecular mechanism by which these hormonal signals are integrated to control behavior at the single cell level. Regarding production of CLE peptides, it will be crucial to identify the enzymes responsible for cleavage of the peptide precursors and for modification of amino acid residues, such as proline-hydroxylation and glycosylation.

Finally, phloem controls xylem development through TDIF. However, this system is unbalanced. Intercellular signaling derived from the xylem should also control phloem development. Identification of such xylem-derived signals involved in controlling phloem development will further clarify our overall understanding of vascular organization.

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