Short title: Peptide signaling during vascular differentiation

Peptide signaling pathways in vascular differentiation

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One-sentence summary:
CLE peptide and related signaling pathways have been assigned prominent roles in the development of both vascular tissues, xylem and phloem.

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The modern plant vascular system is a central feature of extant angiosperms and consists of xylem, phloem, and the intervening procambium (Lucas et al. 2013). During vegetative growth, the apical meristem serves as a continuous source of procambial initial cells, which produce the primary xylem, the primary phloem, and the procambium. In secondary growth, vascular cambium, which is derived from the procambium, acts as a stem cell reservoir and continues to give rise to secondary xylem and phloem cells. The formation of the vascular tissue is a well-organized plant developmental process, whose central step is the regulation of vascular stem cell fates. It is governed by cell-to-cell communication, and symplastic movement of signaling molecules contributes to vascular development. Various secreted peptides as well as plant hormones play crucial roles in cell-to-cell communication for regulating vascular development (Fukuda and Ohashi-Ito, 2019). Among the peptides, several members of the CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR) (CLE) family act at different points of key processes in vascular development (Fukuda and Ohashi-Ito, 2019; Hazak and Hardtke, 2016). The CLE family genes are conserved among land plants. In the Arabidopsis (Arabidopsis thaliana) genome, there are 32 genes encoding 27 distinct CLE peptides (Yamaguchi et al. 2016). CLE precursor proteins contain an N-terminal signal peptide and at least one conserved C-terminal 12-14 amino-acid CLE domain, from which a mature CLE peptide is produced through proteolytic cleavage. The activity of some processed peptides is increased by modifications such as hydroxylation on proline residues, and in some cases by glycosylation with three arabinose residues (Ito et al. 2006; Ohyama et al. 2009; Matsubayashi, 2011; Shinobara and Matsubayahi, 2013). During vascular development, CLE41/CLE44, CLE45, and CLE9/CLE10 peptides function in distinct processes. In addition, other peptides such as phytosulfokine and EPIDERMAL PATTERNING FACTOR-LIKE (EPFL) peptides are thought to contribute to the regulation of vascular development (Holzwarth et al. 2018; Ikematsu et al. 2017). Crosstalk among peptides and between peptides and plant hormones is an important current topic in vascular development. In this update, we focus on recent advances in our understanding of the regulation of vascular fate with an emphasis on peptide signaling.

The TDIF-TDR/PXY signaling pathway in xylem differentiation

TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF) is a
CLE-family peptide composed of twelve amino acids including two hydroxylated proline residues (Ito et al. 2006). TDIF was first isolated from zinnia (Zinnia elegans) xylogenic culture medium as a factor inhibiting tracheary element differentiation (Ito et al. 2006). The TDIF sequence is conserved in the genome of Euphyllophytes (Hirakawa and Bowman, 2015) and encoded by the CLE41 and CLE44 genes in the Arabidopsis genome (Hirakawa et al. 2008). The activity of TDIF in vascular development is also conserved among extant Euphyllophytes (Hirakawa and Bowman, 2015). TDIF receptor/PHLOEM INTERCALATED WITH XYLEM (TDR/PXY) was demonstrated to be a TDIF receptor (Hirakawa et al. 2008). TDR/PXY belongs to Class XI LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASES (LRR-RLK), which consist of an extracellular LRR domain, a single transmembrane domain for anchorage in the plasma membrane, and a cytoplasmic kinase domain (Fisher and Turner, 2007; Hirakawa et al. 2008). The extracellular domain of TDR forms a twisted right-handed superhelical structure, comprising 22 LRRs and the N-terminus, and specifically recognizes TDIF by its inner concave surface (Morita et al., 2016, Zhang et al., 2016a). The crystal structure of TDR ectodomains contains N-linked glycans (Zhang et al., 2016b). The PXY (TDR) family (PXYf) is comprised of TDR (PXY), PXY-LIKE1 (PXL1), and PXY-LIKE2 (PXL2) (Fig. 1A). PXL1 and PXL2 also participate in vascular development together with TDR, since TDIF binds the ligand binding pocket of both PXL1 and PXL2 (Zhang et al., 2016b), and pxl1 and pxl2 mutations enhance the vascular organization defects in tdr mutants (Etchells et al., 2013; Fisher and Turner, 2007). SOMATIC EMBRYOGENESIS RECEPTOR KINASE (SERK) family proteins (SERK1 and others) act as co-receptors of TDR (Fig. 1A; Zhang et al., 2016b). In the TDIF-TDR-SERK2 complex, TDIF mediates interactions between the LRRs of TDR and SERK2. Similarly, the CLE42 peptide, the closest homolog of TDIF that also displays TDIF activity (Ito et al., 2006), promotes an interaction between PXL2 and SERK2 (Mou et al., 2017). Because SERK2 does not change the basic structure of the TDIF-TDR complex, TDIF functions as a “glue” that joins TDR and a SERK, which may contribute to phosphorylation of a cytoplasmic downstream factor (Zhang et al., 2016b).

In plants, the TDIF-TDR signaling module acts by inhibiting xylem differentiation from procambial cells and promoting procambial cell proliferation, which results in the maintenance of the procambial cell population (Hirakawa et al. 2006).
2008). WOX4, which is a member of the WUSCHEL-RELATED HOMEOBOX (WOX) gene family, is a downstream factor of TDR and promotes procambial cell divisions (Fig. 1A; Ji et al. 2010; Hirakawa et al. 2010; Suer et al. 2011). Moreover, the SK1 [ARABIDOPSIS THALIANA SK1 (ATSK11), ATSK12, and ATSK13] and SK2 [BRASSINOSTEROID-INSENSITIVE2 (BIN2), BIN2-LIKE1 (BIL1), and BIL2] subgroups of GLYCOGEN SYNTHASE KINASE3/SHAGGY-LIKE KINASE proteins (GSK3s) contribute redundantly to suppress xylem differentiation downstream of TDR (Fig. 1A).

**Promotion of procambial/cambial cell proliferation by TDIF-TDR**

TDR is expressed preferentially in procambium and cambium (Hirakawa et al. 2008; Fisher and Turner 2007), while CLE41 and CLE44 are expressed specifically in phloem and more widely in its neighboring cell files, respectively (Fig. 1A; Hirakawa et al. 2008). Defects in TDR or CLE41 cause the depletion of the procambial cells (Fisher and Turner 2007, Hirakawa et al. 2008, 2010, Etchells and Turner 2010). Ectopic expression of CLE41 under different promoters revealed that expression of CLE41 in or around procambial cells is sufficient to drive vascular cell division, but localized expression of CLE41 in the phloem is required for maintaining properly orientated cell divisions (Etchells and Turner 2010). Thus, phloem-synthesized TDIF regulates the orientation of procambial cell divisions in a non-cell-autonomous fashion.

TDIF upregulates WOX4 expression in a TDR-dependent manner (Hirakawa et al. 2010). Genetic analyses showed that WOX4 is required to promote the proliferation of procambial/cambial cells, but not for repressing xylem differentiation in response to the TDIF signal. The WOX14 transcription factor may also function redundantly in Arabidopsis to modulate procambial cell proliferations (Etchells et al., 2013). Moreover, WOX14 may act in xylem fiber differentiation through promotion of gibberellin biosynthesis (Denis et al., 2017), consistent with the requirement of gibberellin for xylem expansion (Ragni et al., 2011) (Fig. 1A). Although WOX4 was shown to interact physically with the HAIRY MERISTEM (HAM) family protein HAM4 (Zhou et al., 2015), it is still unknown how WOX4 regulates gene expression with HAM proteins.

The cambium plays a central role in radial growth of woody plants. In the poplar (*Populus trichocarpa*) genome, orthologs of CLE41, TDR/PXY, and WOX4 are
and \(Pt\)t\(WOX4a\) and \(Pt\)t\(WOX4b\) are specifically expressed in the cambial region during vegetative growth, but not after growth cessation and during dormancy (Kucukoglu et al., 2017). A decrease in \(Pt\)t\(WOX4a/b\) levels achieved by RNAi treatment caused severe reduction of the width of the vascular cambium and greatly diminished secondary growth, although primary growth was not affected.

Because there is no homologous sequence to Arabidopsis \(WOX14\) in poplar or spruce (\(Picea\)) genomes, \(WOX4\) is expected to be a major player of cambial proliferation in woody plants. \(Pt\)PP2::\(Pt\)t\(CLE41\)-\(Pt\)t\(ANT\)::\(Pt\)t\(PXY\) double overexpression lines, in which phloem-specific expression of \(Pt\)t\(CLE41\) and cambium-specific expression of \(PXY\) are induced, exhibited highly organized vascular tissue, comparable to that of wild-type controls (Etchells et al., 2015). Because orthologs of \(CLE41\), \(TDR/PXY\), and \(WOX4\) are widely distributed in angiosperm and gymnosperm tree species (Hirakawa and Bowman, 2015, Kucukoglu et al., 2017), the TDIF-TDR/PXY-WOX4 pathway is an evolutionarily conserved program for the regulation of the vascular cambium activity in wood formation. Recently two groups identified bifacial, strongly proliferating cambial stem cells that feed both xylem and phloem production during secondary growth in Arabidopsis (Shi et al., 2019, Smetana et al. 2019). In fact, in the cambial stem cells, \(TDR/PXY\) and \(WOX4\) genes were actively expressed. Recently, Zhu and others found that \(PtrCLE20\) is expressed in xylem cells and represses cambium activity in poplar (Zhu et al. 2019). The molecular mechanism underlying the xylem-producing CLE peptide-dependent regulation of cambium activity should be analyzed further.

**Interaction of the TDIF-TDR pathway with phytohormones**

The role of auxin in the regulation of vascular cambium activity is well established and the auxin concentration gradient peaks over the cambial region and decreases towards the surrounding secondary tissues in hybrid aspen (\(Populus tremula\) \(L\). \(x\) \(Populus tremuloides\) \(Michx\)) (Tuominen et al., 1997). \(WOX4\) has been shown to be necessary both for the auxin responsiveness of the cambium cells and for the auxin-dependent increase in the cambium cell division activity (Suer et al., 2011). Local auxin signaling in TDR-positive stem cells stimulates cambium activity (Brackmann et al. 2018). An analysis with a highly sensitive auxin response marker revealed a moderate auxin...
response in TDR-positive stem cells and a higher response in xylem and phloem tissues. Whereas the auxin response factors (ARFs) ARF3 and ARF4 act redundantly as general cambium activators, MONOPTEROS (MP)/ARF5 acts as an inactivator specifically in cambium stem cells (Fig. 1A). Indeed, MP induction resulted in both WOX4 repression and the induction of xylem- and phloem-related genes (Brackmann et al. 2018). Recently, Smetana and others proposed that auxin-dependent WOX4 regulation occurs in xylem precursor cells with an auxin maximum, which promotes division of vascular stem cells in a non-cell autonomous manner (Smetana et al., 2019). This idea should be verified from various viewpoints.

Periclinal and radial cell divisions in procambial cells of the root apical meristem (RAM) are also regulated by two bHLH transcription factors, LONESOME HIGHWAY (LHW) and TARGET OF MONOPTEROS 5 (TMO5) (Schlereth et al., 2010). In xylem precursor cells in the RAM, auxin induces TMO5 expression, which allows the formation of heterodimers between LHW and TMO5 (Fig. 1B). The heterodimers directly promote the expression of target genes involved in the final step of cytokinin biosynthesis, LONELY GUY 3 (LOG3) and LOG4 (Ohashi-Ito et al., 2014, De Rybel et al., 2014). Therefore, cytokinin was hypothesized to act as a mobile signal from the xylem to trigger divisions in the neighboring procambium cells. DOF2.1 and its closest homologs, TMO6 and DOF6, may be key transcription factors downstream of cytokinin in terms of procambial cell proliferation (Smet et al., 2019, Miyashita et al., 2019). In xylem precursor cells, however, LHW-TMO5-induced HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6) represses cytokinin signaling to inhibit cell proliferation (Ohashi-Ito et al., 2014, De Rybel et al., 2014).

**Suppression of xylem differentiation from procambial cells**

TDIF signaling releases BIN2 and BIL2, but not BIL1, from TDR, and then activates these GSK3s, which results in the suppression of xylem differentiation (Fig. 1A; Kondo et al., 2014). Conversely, the inhibition of GSK3s with a chemical inhibitor (De Rybel et al., 2009), bikinin, induces xylem differentiation (Kondo et al., 2014, 2015). Bikinin-dependent ectopic xylem differentiation is suppressed in loss-of-function mutants for *BRI1-EMS-SUPPRESSOR 1 (BES1)* (Kondo et al., 2016, Saito et al., 2018), a direct substrate of GSK3s in the brassinosteroid signaling pathway (Yin et al., 2002). By contrast, constitutively active BES1 (*bes1-D*) promotes xylem differentiation.
(Kondo et al., 2014). The loss-of-function mutant of BRASSINAZOLE RESISTANT 1 (BZR1), the closest homolog of BES1, also shows a bes1-like phenotype, but a weaker one (Saito et al., 2018). Thus, BES1 and BZR1, which are suppressed by GSK3s, act redundantly in promoting xylem differentiation (Fig. 1A). Further analysis indicated that the GSK3s-BES1/BZR1 signal not only regulates xylem but also phloem differentiation (Kondo et al., 2016, Saito et al., 2018). This finding is consistent with a previous report showing that both bes1-D and bzr1-D partially rescued the phenotype of the octopus mutant, in which root protophloem differentiation is suppressed (Anne et al., 2015), and with a role of brassinosteroid signaling in phloem sieve element differentiation (Kang et al., 2017). Nevertheless, whether the canonical brassinosteroid pathway effectors also have a role in root protophloem development remains unclear (Kang et al., 2017). Of SK1 and SK2 GSK3s, BIL1 may have a distinct role in vascular development (Fig. 1A). Han and others suggested that BIL1 phosphorylates and activates MP, and the activated MP promotes the expression of two A-type Arabidopsis Response Regulators (ARRs), ARR7 and ARR15, which suppress cambial cell divisions (Han et al., 2018).

The finding that bikinin induces ectopic xylem cell differentiation led to the establishment of a useful tissue culture system for vascular cell differentiation named VISUAL (Vascular cell Induction culture System Using Arabidopsis Leaves). In VISUAL, mesophyll cells in cultured cotyledons or leaf disks differentiate at a high frequency into tracheary elements and sieve elements via the procambial stage in the presence of bikinin (Kondo et al., 2015, 2016). This system enabled the analysis of the roles of various genes in vascular differentiation by using their mutants as starting materials of VISUAL.

Brassinosteroid signaling may interact with the TDIF-TDR-GSK3s-BES1/BZR1 signaling pathway, because GSK3s and BES1/BZR1 are among its key components (Fig. 1A). Indeed, brassinosteroid promotes xylem differentiation (Yamamoto et al., 1997, Caño-Delgado et al., 2004, Kubo et al., 2005), which suggests that brassinosteroid regulates procambial cell fates to counteract TDIF signaling through the reverse regulation of GSK3s. BRASSINOSTEROID INSENSITIVE 1 (BRI1) is the major receptor for brassinosteroid and the BRI1 gene is expressed widely in plants, while its homologs, BRI1-LIKE 1 (BRL1) and 3 (BRL3), are expressed predominantly in vascular tissues. In particular, BRL1 expression is
associated with the procambial cells in inflorescence stems (Caño-Delgado et al., 2004). In procambial cells, therefore, the TDIF and brassinosteroid signals may be balanced via GSK3s to determine procambial cell identity. Because brassinosteroid biosynthesis occurs in procambial cells, which is promoted by auxin and cytokinin (Yamamoto et al., 2001, 2007), brassinosteroid may act as an autocrine factor in xylem differentiation from procambial cells.

**CLE9/CLE10 perception in xylem formation**

*CLE9* and *CLE10*, which encode the same CLE peptide, are preferentially expressed in vascular cells of roots (Fig. 1B; Kondo et al. 2011). *cle9* mutants and *cle9 cle10* double mutants exhibit a phenotype of increased metaxylem cell file numbers, which results from enhanced periclinal cell division of xylem precursor cells in the RAM (Qian et al., 2018). The receptor kinase HAESA-LIKE1 (HSL1) is a CLE9/10 receptor that regulates stomatal lineage cell division, while BARELY ANY MERISTEM (BAM) class receptor kinases are CLE9/10 receptors that regulate periclinal cell division of xylem precursor cells (Fig. 1B; Qian et al., 2018). Both HSL1 and BAM1 bind to CLE9/10, but only HSL1 recruits SERKs as co-receptors in the presence of CLE9/10, suggesting different signaling modes for these receptor systems (Hazak et al., 2017; Qian et al., 2018). Genetic analysis indicated that BAM1, BAM2, and BAM3 are involved in the action of CLE9/10, but BAM1 is most important among them. Their genes are expressed in vascular cells, with BAM1 being expressed in xylem and phloem precursor cells in roots, consistent with the function of CLE9/10 in xylem precursor cells.

Application of CLE9/10 peptide suppressed protoxylem vessel formation in Arabidopsis roots (Kondo et al., 2011). An early event caused by exogenous CLE9/10 peptide was the reduced expression of type-A *ARR* genes such as *ARR5* and *ARR6*, which are negative regulators of cytokinin signaling (Fig. 1B). Consistently, an *arr10* *arr12* double mutant, which is a combination of mutations of B-type ARRs, showed CLE9/10-resistance in terms of protoxylem vessel suppression, and displayed ectopic protoxylem vessel formation (Kondo et al., 2011). Therefore, CLE9/10 signaling may control xylem differentiation through a cytokinin signaling pathway. However, because *cle9 cle10* double mutants did not enhance protoxylem vessel differentiation, other CLE peptide(s) may also be involved redundantly in this process.

**Phytosulfokine peptide perception in procambial cell fate**
Phytosulfokines (PSKs) are sulfated pentapeptides that are perceived by PHYTOSULFOKINE RECEPTOR-1 and -2 (PSKR1/PSKR2) (Matsubayashi et al., 2002). PSK stabilizes the PSKR island domain for recruitment of a SERK (Wang et al., 2015). A recent study revealed a small RECEPTOR-LIKE PROTEIN 44 (RLP44)-mediated interaction between PSKRs and BRI1 that regulates procambial cell fate in roots (Fig. 1B; Holzwart et al. 2018). In the rlp44 loss-of-function mutant, supernumerary metaxylem-like cells are formed frequently outside the primary xylem axis in the position of the procambium. This rlp44 phenotype can be rescued by application of PSK peptide. Moreover, pskr1 pskr2 double mutants displayed an rlp44-like xylem phenotype. Likewise, bri1 mutants show ectopic xylem in the procambial position, while brassinosteroid-deficient mutants do not exhibit an rlp44-like xylem phenotype. Therefore, PSK-PSKR signaling acts to maintain procambial cell identity and RLP44 mediates the interaction between PSKR1 and BRI1. These results may provide a model for the integration of multiple signaling pathways at the plasma membrane by shifting associations of receptors in multiprotein complexes in response to different ligands (Holzwart et al. 2018). Because RLP44 and most PSK genes are expressed preferentially in xylem/phloem and procambium of roots, respectively, this signaling may occur in a non-cell autonomous fashion.

ERECTA and related receptors cooperate with TDR/PXY family receptors

The LRR-RLK encoded by the ERECTA (ER) gene is part of a small gene family together with ER-LIKE1 (ERL1) and ERL2. In er erl1 stems, intervening cambial cells are decreased and phloem cells are frequently located adjacent to xylem cells (Uchida and Tasaka, 2013). This also occurs in tdr (Fisher and Turner, 2007; Hirakawa et al., 2008), suggesting that ER and ERL1, like TDR, act by suppressing xylem differentiation and promoting cambial cell activity. By contrast, er erl1 hypocotyls display a remarkable expansion of the xylem and an increase in xylem fiber cells (Ragni et al., 2011; Ikematsu et al., 2017).

On the one hand, this result supports the idea that ER and ERL1 suppress xylem differentiation but appear to suppress cambial activity. On the other hand, the relative xylem expansion in the Landsberg er (Ler) genotype was found to be due to early cessation of phloem formation and reduced cambial activity (Sankar et al., 2014). The difference between stems and hypocotyls in er erl1 may be explained by the
tissue-specific gene regulation of ERL1 and ERL2: In the er pxy pxl1 pxyl2 genetic background, ERL1 and ERL2 gene expression is reduced greatly in stems, while their expression is strikingly enhanced in hypocotyls (Wang, et al., 2019). These findings clearly indicate a tight interaction between PXY family genes and ER family genes (Fig. 1A). Indeed, the sextuple mutant, er erl1 erl2 pxy pxl1 pxyl2 shows a complete suppression of secondary growth of hypocotyls, supporting the view that both TDR and ER family genes are crucial factors of secondary growth (Wang, et al., 2019).

Both ER and ERL1 are expressed widely in the epidermis, phloem, and xylem (Uchida and Tasaka, 2013), but the vascular defect in er erl1 stems is rescued by phloem-specific activity of ER (Uchida and Tasaka, 2013), consistent with a proposed role of ER in phloem proliferation (Sankar et al., 2014). Therefore, ER ERL1-dependent suppression of xylem precursor cell division should occur in a non-cell autonomous manner (Fig. 1A; Tamashige et al., 2017). ER and ERL1 also function in preventing xylem fiber differentiation, which is mediated by the NAC SECONDARY WALL THICKENING PROMOTING FACTORs (NSTs) in hypocotyls (Ikematsu et al., 2017). Although BREVIPEDICELLUS is required for the hypocotyl to gain competency to respond to gibberellin and trigger fiber differentiation in both the wild-type and er erl1, it is still unknown whether the ER/ERL1 pathway is associated with the gibberellin pathway.

Ligands for ER and EFR1 that function in xylem development have not been identified. However, it is known that EPIDERMAL PATTERNING FACTOR 1 (EPF1), EPF2, and EPF-LIKE9 (EPFL9) peptides function as ligands for ER and ERL1 in stomata development (Han and Torii, 2016). In stem elongation, EPFL4 and EPFL6, which are produced in epidermal cells, act as ligands for phloem-located ER (Abrash et al., 2011; Uchida et al., 2012). In hypocotyls, EPFL4 and EPFL6 are expressed in xylem parenchyma cells and differentiating xylem cells (Wang, et al., 2019). However, there is presently no evidence that EPFL4 and EPFL6 function as ligands for regulating hypocotyl secondary growth. In the Arabidopsis genome, there are 11 EPF/EPFL family members (Hara et al., 2009). It is plausible that some EPF/EPFL family members may function as ligands for ER and ERL1 to suppress xylem development in stems and hypocotyls. TOO MANY MOUTHS (TMM) and SERK family proteins function as co-receptors in ER signaling (Hang and Torii, 2016). Therefore, a specific combination of ER/ERL1, EPF/EPFL peptides, and co-receptors, such as TMM and SERK family
proteins, may function in vascular development in stems and hypocotyls.

Phloem-related CLE peptide signals

An interesting aspect emerging from the analysis of pxy/tdr and related mutants is that they do not lose their capacity to produce proper xylem and phloem tissues, as judged from morphological and molecular-genetic criteria (Fisher and Turner, 2007; Hirakawa et al., 2008). This even holds true for higher order (sextuple) mutants that largely remove redundancy (Wang et al., 2019). Thus, the main role of the PXY/TDR-CLE41/44/TDIF system is likely the organization of vascular patterning, which is, for instance, evident in the regular spacing of phloem strands during hypocotyl expansion (Sankar et al., 2014).

An easier, more accessible system to study a possibly autonomous role of CLE peptide signaling in vascular development is the root tip with its simple diarch vascular organization. Here, two protophloem poles flank a central xylem axis and allow continuous observation of development along a spatio-temporal gradient. Moreover, protophloem expresses key players or markers, such as ALTERED PHLOEM DEVELOPMENT (APL) (Bonke et al., 2003) or COTYLEDON VASCULAR PATTERN 2 (CVP2) (Carland and Nelson, 2004; Rodriguez-Villalon et al., 2015), that are also expressed in secondary phloem development (Lehmann and Hardtke, 2016), indicating that the principal findings in one context likely apply to the other.

Root-active CLE peptides are perceived in the phloem

The root protophloem is also a system where a key question can be answered: Is CLE peptide signaling required to form functional phloem? Early hints that CLE peptides could play a role in root development came from the observation that several CLE genes are expressed in the root (Jun et al., 2010), and that treatments with many chemically synthesized CLE peptides suppress root growth (Fiers et al., 2005; Ito et al., 2006; Kinoshita et al., 2007). Such so-called “root-active” CLE peptides were also employed to identify components that are necessary for CLE peptide perception in the root, and notably identified the receptor-like protein CLV2 and its interacting partner, the pseudokinase SUPPRESSOR OF OVEREXPRESSION OF LLP1-2/CORYNE (SOL2/CRN) (Jeong et al., 1999; Miwa et al., 2008; Muller et al., 2008; Meng and Feldman, 2010).
Both *clv2* and *crn* mutants display substantial resistance to root growth inhibition by a range of root-active CLE peptides, and both the *CLV2* and *CRN* genes are essentially expressed at low levels throughout the root (Hazak et al., 2017). However, recently it was shown that restricted expression of CRN in the developing protophloem is sufficient to fully recover CLE perception in a *crn* null mutant background (Hazak et al., 2017). This matched the observation that root-active CLE peptides *a priori* suppress protophloem sieve element differentiation (Rodriguez-Villalon et al., 2014; Hazak et al., 2017), which is essential for root growth (Furuta et al., 2014; Rodriguez-Villalon et al., 2014). Given that interactions between CLE peptides and their cognate receptors are rather specific (Hohmann et al., 2017), the requirement of the CLV2|CRN heteromer for perception of root-active CLE peptides likely reflects a more general rate-limiting role in receptor complex activity. For example, it has been suggested that CLV2|CRN stabilizes the expression of BAM3, the cognate CLE45 receptor in the root (Kang and Hardtke, 2016; Hazak et al., 2017).

**Suppression of CLE peptide signaling permits root protophloem sieve element differentiation**

CLE45 is one of the few CLE peptides that is expressed in the developing protophloem sieve elements, the others being the related CLE25 and CLE26 peptides (Rodriguez-Villalon et al., 2014; Anne et al., 2018; Ren et al., 2019). Recently, it was suggested that CLE25 is required for protophloem sieve element formation, although a *cle25* mutant did not display a root growth phenotype and only showed a marginal delay in sieve element elongation (Ren et al., 2019). While this might reflect redundancy with other CLE peptides, expression of a supposedly dominant negative mutant version of CLE25 (a G6T exchange in the peptide) abolished protophloem sieve element differentiation as well as one of the formative divisions in the phloem lineage (Ren et al., 2019).

Interestingly, such divisions can also be suppressed by mild auxin antagonist treatment (Rodriguez-Villalon et al., 2014) and appear to be non-essential. However, it often remains unclear which of the two successive periclinal formative divisions in the phloem lineage is affected, and it might very well turn out that one of them is essential for sieve element formation. Nonetheless, the CLE25<sup>G6T</sup> phenotype strongly resembles the so-called Disturbed Protophloem Syndrome observed in backgrounds with elevated
CLE45 signaling (Rodriguez-Villalon et al., 2014; Anne and Hardtke, 2017) and is consistent with simple dominant (rather than dominant negative) action of the CLE25<sup>G6T</sup> version, as observed for other CLE peptides in other contexts (Czyzewicz et al., 2015), including CLE45<sup>G6T</sup> in protophloem formation (Rodriguez-Villalon et al., 2014; Czyzewicz et al., 2015). This interpretation is also consistent with observations in pertinent receptor mutants. For example, neither crn loss-of-function mutants, nor CLE-RESISTANT RECEPTOR KINASE (clerk) loss-of-function mutants, which are both strongly resistant to CLE25, CLE26, and CLE45 application, display any root growth or protophloem sieve element differentiation phenotypes (Hazak et al., 2017; Anne et al., 2018). The same applies to the crn clerk double mutant (Anne et al., 2018).

In summary, current data suggest that CLE peptide signaling is not required per se for sieve element formation in the Arabidopsis root. On the contrary, it seems that suppression of autocrine CLE peptide signaling is a pre-requisite for proper protophloem sieve element differentiation (Breda et al., 2019). This does not, however, preclude a role in other aspects of root development, for example formative divisions or radial organization. For instance, CLE9 and CLE10 redundantly appear to affect formative xylem divisions in the root, through their interaction with the BAM1 and BAM2 receptors (Qian et al., 2018). Such mechanisms may also exist for phloem formation.

**CLE peptide signaling as rapid mediators of stress-induced sink shutdown**

Beyond purely developmental aspects, CLE peptides may also transmit environmental inputs, and these two roles might not be mutually exclusive in the case of individual peptides. Indeed, CLE25 is a prime example in this context. In addition to its expression in the protophloem, CLE25 expression is induced by drought stress and acts as a long-distance signal that is transported through the vasculature to convey this physiological state to the shoot system (Takahashi et al., 2018). Coincidentally, such induction should also suppress protophloem differentiation and thereby root growth (Anne and Hardtke, 2017; Hazak et al., 2017), which could be advantageous in drought conditions. Yet another example is the root-active CLE peptide, CLE14, which is induced upon phosphate starvation and triggers a breakdown of root meristem maintenance and root growth (Gutierrez-Alanis et al., 2017). Since this response requires the CLV2|CRN module, it appears that it is achieved through a shutdown of
protophloem formation (Meng and Feldman, 2010; Gutierrez-Alanis et al., 2017; Hazak et al., 2017).

In summary, beyond any positive developmental roles in phloem development that largely remain to be discovered, root-active CLE peptides might be mainly involved in the sensing of adverse environmental conditions. From the current data, it seems that their upregulation in response to such adverse conditions suppresses protophloem differentiation and thereby halts root growth. From a physiological perspective, such a mechanism would be a very efficient and rapid way to stop growth and eliminate a metabolic sink that finds itself in sub-optimal conditions. Because phloem transport is essentially auto-regulated, this would “automatically” redirect phloem sap to actively growing roots. Thus, CLE peptide regulation may primarily serve to fine-tune and optimize root system exploration of the wildly heterogenous soil matrix, which could represent a substantial adaptive advantage.

Concluding remarks

In summary, peptide signaling pathways have been increasingly implicated in vascular development over recent years (Figure 1 summarizes peptide and hormone signaling pathways discussed in this update). From the current data, it appears clear that various peptides and plant hormones not only function as ligands in vascular cell proliferation, but also in both xylem and phloem cell type fate regulation, and both in a cell-autonomous or non-cell autonomous manner. In addition, the emerging picture is becoming considerably complex since the exact impact of a given signaling pathway seems to depend on the developmental stage or the organ. For example, the TDIF-TDR signaling pathway apparently acts in the cambium and actively proliferating procambium of hypocotyls and stems, but not in the root apical meristem. Moreover, the picture is complicated by the observation that at least in the RAM context, CLE45-BAM3 signaling has to be suppressed to permit phloem differentiation, although CLE peptide signaling might be required to initiate proper phloem formation. From the genetic analyses, it is also apparent that the complex network composed of multi-signaling pathways makes the regulation of procambial/cambial cell identity and proliferation robust. Similar redundancy might exist at the level of differentiation of vascular tissues, and future efforts could focus on uncovering such redundancy through genetic as well as biochemical/cell biological investigations.
FIGURE LEGENDS

Figure 1. Peptide-related signaling pathways that regulate proliferation of procambial/cambial cells and their differentiation into xylem and phloem cells. A: Signaling pathways in hypocotyls and stems. B: Signaling pathways in the root apical meristem. BR: brassinosteroid; CK: cytokinin; AHKs: cytokinin receptors.
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ADVANCES

- Various peptides and plant hormones function as ligands in vascular cell proliferation, as well as in xylem and phloem cell type fate regulation, in both a cell-autonomous or non-cell autonomous manner.
- The role of a given signaling pathway can be context-dependent and depend on the developmental stage or the organ.
- The complex network composed of multiple intersecting signaling pathways renders the regulation of procambial/cambial cell identity and proliferation robust.
- CLE peptide signals might convey environmental inputs as a rapid control of sink organ development.
OUTSTANDING QUESTIONS

• How does crosstalk between different peptide signaling pathways occur mechanistically during vascular development? Do different receptors form a complex on the plasma membrane, and/or are there signaling hubs that connect different signaling pathways?

• Are long-distance peptide signaling pathways controlling vascular development?

• Are the peptide processing and secretion processes regulated as a function of vascular development?

• What are the mechanisms that underlie developmental stage- and organ-specific peptide signaling?

• Where do the processed peptides act at high spatio-temporal resolution?
A  
hypocotyls & stems

B  
root apical meristem
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