A xylem-produced peptide PtrCLE20 inhibits vascular cambium activity in Populus

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Summary

In trees, lateral growth of the stem occurs through cell divisions in the vascular cambium. Vascular cambium activity is regulated by endogenous developmental programmes and environmental cues. However, the underlying mechanisms that regulate cambium activity are largely unknown. Genomic, biochemical and genetic approaches were used here to elucidate the role of PtrCLE20, a CLAVATA3 (CLV3)/embryo surrounding region (ESR)-related peptide gene, in the regulation of lateral growth in Populus. Fifty-two peptides encoded by CLE genes were identified in the genome of Populus trichocarpa. Among them PtrCLE20 transcripts were detected in developing xylem while the PtrCLE20 peptide was mainly localized in vascular cambium cells. PtrCLE20 acted in repressing vascular cambium activity indicated by that upregulation of PtrCLE20 resulted in fewer layers of vascular cambium cells with repressed expression of the genes related to cell dividing activity. PtrCLE20 peptide also showed a repression effect on the root growth of Populus and Arabidopsis, likely through inhibiting meristematic cell dividing activity. Together, the results suggest that PtrCLE20 peptide, produced from developing xylem cells, plays a role in regulating lateral growth by repression of cambium activity in trees.

Introduction

Woody plants feature massive lateral growth dependent on vascular cambium activity, which gives rise to xylem tissue on the inner side (wood) and phloem tissue on the outer side. Xylem tissue, vessel cells transport water from roots to shoots and fibre cells provide mechanical support. While in phloem tissue, sieve elements transport photoassimilates/carbohydrates, metabolites and signalling compounds (hormones and small molecules). Cambium activity is strictly regulated by signal pathways due to developmental programmes (Johnsson and Fischer, 2016; Nieminen et al., 2015) and can also shift in response to environmental cues, such as water availability (Bhalerao and Fischer, 2017).

Intercellular communication and subsequent intracellular signalling have been shown to be required for the regulation of meristem/procambium/cambium activity due to developmental programmes. CLE signalling peptides are known to be involved in the regulation of meristem activity (Jun et al., 2010; Sharma et al., 2003; Whitford et al., 2008). The Arabidopsis thaliana genome contains 32 CLE genes, which encode 27 different CLE peptides with 12 or 13 amino acid residues (Jun et al., 2010; Sharma et al., 2003; Strabala et al., 2006). According to their sequence similarity and functions, CLE peptides are divided into A-type and B-type. CLAVATA3 (CLV3), an A-type CLE, restricts stem cell proliferation in the organizing centre of the shoot apical meristem (SAM) (Schoof et al., 2000; Yadav et al., 2011). The receptor CLV1, which is expressed in the organizing centre and surrounding cells, perceives the CLV3 peptide signal to repress WUS transcription (Brand et al., 2000; DeYoung et al., 2006; Ogawa et al., 2008; Shinozaka and Matsuhashi, 2015).

Meanwhile, genetic evidence showed that a parallel CLV2-CORYNE (CRN) heteromeric complex is also involved in the CLV3 signalling pathway, even though no direct binding between CLV3 and CLV2 is detected (Fiers et al., 2005; Guo et al., 2010; Muller et al., 2008). A similar CLE-LRR-RLK-WUS signalling pathway was also identified in the root apical meristem (RAM) (De Smet et al., 2008; Sarkar et al., 2007). CLE40 is expressed in differentiated columnella cells of the distal meristem and in the stele in the proximal root meristem and perceived by the receptor-like kinase ARABIDOPSIS CRINKLY 4 (ACR4), together with CLV1 (Stahl et al., 2009). CLE40-ACR4 signalling promotes the differentiation of the distal stem cells (Stahl et al., 2009, 2013). Similar to CLV3-CLV1 in the SAM and CLE40-ACR4 in the RAM, CLE peptides are also found to regulate cell proliferation in procambium. On the other hand, CLE41/CLE44/TDF (tracheary element differentiation inhibitory factor) peptides, belonging to B-type, are produced mainly in phloem cells (Etchells and Turner, 2010; Fisher and Turner, 2007; Hirakawa et al., 2008; Ito et al., 2006; Whitford et al., 2008). The leucin-rich repeat receptor-like kinase, TDF/PXY (TDF receptor/phloem intercalated with xylem) is able to recognize CLE41 in regulation of procambial cell proliferation in Arabidopsis (Etchells and Turner, 2010; Fisher and Turner, 2007; Hirakawa et al., 2008; Ito et al., 2006; Whitford et al., 2008).

In trees, vascular tissue is initiated from procambium in SAM which further differentiates into fascicular cambium, and then the fascicular cambium undergoes periclinal division to link together to form a ring of vascular cambium (Little et al., 2002; Mazur et al., 2014; Zhu et al., 2018). The CLE41-PXY signalling pathway is reported to play a role in promotion of cambium proliferation and xylem development in Populus (Etchells et al., 2015a).

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In this study, 52 CLE genes were identified in the genome of *Populus trichocarpa*. Among them, PtrCLE20 is found to be specifically expressed in developing xylem. Evidence from genomic, biochemistry and genetic approaches indicates that PtrCLE20 peptide produced in xylem cells plays a role in regulating vascular cambium activity.

**Results**

**Analysis of CLE genes in *P. trichocarpa***

Using 32 Arabidopsis CLE proteins as queries to search the *P. trichocarpa* genome database through BLAST (Basic Local Alignment Search Tool), 52 genes encoding full-length CLE proteins (referred to as PtrCLE genes) were identified and named following Arabidopsis numbering based on CLE peptide similarity (Table S1). The PtrCLE genes were predicted to encode proteins with sizes ranging from 66 to 162 amino acids (Table S1), including a predicted hydrophobic signal peptide domain at the N-terminal, a highly variable region in the middle and a conserved CLE domain at the C-terminal (Figure S1). Although the sequences of the PtrCLE gene family vary, they contain the conserved signal peptide domain and CLE domain shown by alignment of PtrCLE proteins (Figure S1). The CLE peptides predicted from 52PtrCLE genes can be grouped as A-type (33 peptides) and B-type (6 peptides) in *Populus* (Figure S2). Phylogenetic analysis of CLE peptides of *Populus* and Arabidopsis showed that CLE peptides in A-type were further divided into three subtypes (A-I, A-II and A-III) (Figures S2 and S3).

The expression of 52 PtrCLE genes across multiple tissues in *Populus* was analysed using public available transcriptional data, including secondary xylem, secondary bark, mature leaf and root (Figure S4a) (Xue et al., 2016). B-type PtrCLE41s and PtrCLE44s were highly expressed in secondary phloem, consistent with the results from other studies (Etchells and Turner, 2010). The Type A-III PtrCLE genes were expressed in a variety of tissues but barely in secondary xylem. Interestingly, PtrCLE20, PtrCLE17A/B and PtrCLE13A/B were specifically expressed in secondary xylem. The similar expression pattern was also detected by analysis of the AspWood database (http://aspwood.popgenie.org) (Figure S4b) (Sundell et al., 2017), which prompted us to investigate what functions of these PtrCLE genes perform in xylem tissue and this study mainly focuses on PtrCLE20.

To confirm PtrCLE20 expression pattern, various tissues including shoot tip, young shoot undergoing transition from primary growth to secondary growth, secondary xylem, secondary phloem, leaf and root were collected to measure the PtrCLE20 transcripts using RT-qPCR. PtrCLE20 expression was specifically detected in the samples containing developing secondary xylem tissue but barely in shoot tip, secondary phloem and other tissues without secondary xylem (Figure 1a). To verify the tissue-specific expression pattern of PtrCLE20, a PtrCLE20 promoter of 1.7 kb sequence was cloned and utilized for driving GUS (PtrCLE20pro::GUS) expression in *Populus*. Eighteen independent lines of the transgenic plants were generated for GUS staining analysis. GUS staining was observed in developing xylem but barely in cambium cells (Figure 1b,c). Additionally, in situ hybridization was performed to examine the PtrCLE20 mRNA localization, which showed that PtrCLE20 transcripts were specifically detected in developing xylem (Figure 1d).

**PtrCLE20 peptide was detected in vascular cambium**

To examine the PtrCLE20 peptide localization, specific antibodies against PtrCLE20 peptide were raised as well as antibodies against phloem expressed PtrCLE41 peptide (Figure S5a–d). Both antibodies were able to detect a single band, respectively, in the total proteins isolated from *Populus* young stem without bark (Figure S5e). It was noted that the detected band sizes of both PtrCLE20 (approximately 17 KD) and PtrCLE41B (approximately 23 KD) were larger than the predicted sizes of 8.5 and 14.4 KD, respectively. They were also larger than the recombinant peptides from *E. coli* (approximately 14 and 18 KD, respectively) (Figure S5a–e), suggesting possible occurrence of post-translational modifications of the peptides in *Populus*, like in Arabidopsis and tomato (Matsubayashi, 2014; Ohyama et al., 2009 and Xu et al., 2015). To confirm the authenticity of the detected PtrCLE20, both bands detected from *E. coli* (14 KD) and *Populus* (17 KD) proteins were subject to LC-MS/MS analysis (Figure S5a,e). The PtrCLE20 peptide was identified in both samples (Figure S5f,g), further verifying the antibodies specificity. Using these antibodies, immunolocalization was performed to examine the PtrCLE20 presence in stem vascular tissues. The PtrCLE20 peptide signal was detected in vascular cambium cells and in early developing xylem cells (Figure 1f). In contrast, the PtrCLE41 peptide was localized in vascular cambium and phloem cells (Figure S6b), which is consistent with previous studies (Hirakawa et al., 2008). To further confirm the presence of PtrCLE20 peptide in vascular cambium cells and developing xylem cells, the PtrCLE20 antibodies were applied to purify PtrCLE20 peptides from these *Populus* tissues. LC-MS/MS analysis revealed that the purified peptide has 12 amino acid residues, which is identical to the predicted PtrCLE20 peptide sequence (Figure 1h). Together, these results demonstrated that PtrCLE20 peptide is present in vascular cambium cells.

**PtrCLE20 was involved in secondary growth by regulating vascular cambium activity**

To analyse the function of PtrCLE20 in vascular tissues, 35S::PtrCLE20 overexpression transgenic *Populus* were generated. A total of 36 independent transgenic lines with significantly increasedPtrCLE20 expression were obtained, among which Line 32, Line 51 and Line 45 with different overexpression levels were selected for detailed analysis (Figure 2a–d). Compared to the wild-type plants, the height of 35S::PtrCLE20 plants was reduced 30%–60% due to fewer internodes and shorter internode length (Figure 2a,b,f,g). Meanwhile, 35S::PtrCLE20 plants exhibited

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**Figure 1**  
PtrCLE20 peptide was mainly localized in vascular cambium. (a) Expression levels of PtrCLE20 in diverse tissues of *Populus*. Expression level is shown relative to the expression abundance of *PtrActin1*. Bars represent the means ± SD of three biological replicates and three technical replicates. (b and c) GUS staining in vascular tissue of *PtrCLE20pro::GUS*. Bar, b, 100 μm, c, 20 μm. (d) In situ hybridization of antisense *PtrCLE20* in vascular tissue of wild-type *Populus*. Bar, 50 μm. (e) In situ hybridization of sense *PtrCLE20* in vascular tissue of wild-type *Populus*. Bar, 50 μm. (f) Immunolocalization of *PtrCLE20* peptide in vascular tissue of wild-type *Populus*. Bar, 20 μm. (g) Negative control of immunolocalization. Bar, 20 μm. (h) Identification of *PtrCLE20* peptide in *Populus* vascular cambium and developing xylem. ca, cambium; co, context; pf, phloem fibre; ph, phloem; v, vessel element; xf, xylem fibre; xy, xylem.
reduced stem diameter (Figure 2h). The reduction of plant height and stem diameter was relatively corresponding to the increase of $PtrCLE20$ expression (Figure 2d–h). In addition, smaller and wrinkled leaves were observed in $35S::PtrCLE20$ plants (Figure 2c).

The stem at the 16th internode, where secondary growth was fully developed in wild-type plants, was dissected to investigate how reduction of the stem diameter is caused in the transgenic plants. At this developmental stage, wild-type plants displayed approximate seven layers of dividing cells in the vascular cambium region and approximate 20 layers of differentiated xylem cells, forming a complete ring of secondary vascular tissue (Figure 3a,b,c,j,k). Transgenic Line 51 with a low-level overexpression of $PtrCLE20$ had approximate four layers of dividing cells in cambium region (Figure 3d,j). The radial width of both secondary xylem and secondary phloem tissues was smaller compared to those in the wild type (Figure 3a,d,b,e,k). Meanwhile, the cross-sectional area of vessel elements and fibre cells in secondary xylem were also smaller in $35S::PtrCLE20$ plants (Figure 3l,m).

Figure 2 Morphological phenotypes of overexpression of $PtrCLE20$ in Populus. (a) Whole plants. (b) Stems. (c) Leaf of wild type and three independent lines of $35S::PtrCLE20$. (d) Expression levels of $PtrCLE20$ in wild type and three independent lines of $35S::PtrCLE20$. Expression level is shown relative to the expression abundance of $PtrActin1$. Bars represent the means ± SD of three biological replicates and three technical replicates. (e) Plant height. (f) Number of internodes. (g) Internode length. (h) Stem diameter. Bars in e, f, g and h represent the means ± SD of three biological replicates. Significance testing is conducted using the two samples t-test (*<0.05, **<0.01 and ***<0.001) between wild type and $35S::PtrCLE20$ plants. Bars, a, 10 cm, b, 1 cm, c, 5 cm.

Figure 3 $PtrCLE20$ inhibited vascular cambium activity. Cross sections of the 16th internode in stems of wild type (a, b and c), Line 51 of $35S::PtrCLE20$ (d, e and f) and Line 45 of $35S::PtrCLE20$ (g, h and i). The yellow bars indicate vascular cambium in a, d and g and secondary phloem in b, e and h; the yellow arrows indicate secondary xylem. Ca, cambium, xy, xylem, ph, phloem. Bars: a, d and g, 20 μm, b, e and h, 100 μm, c, f and i, 500 μm. Cell layers of cambium zone (j) and cell layers of xylem in the 16th internode (k). Cross-sectional area of vessels (l) and fibre cells (m). Bars represent the means ± SD of $n = 10$ in j, k and $n = 20$ in m, significance testing is conducted using the two samples t-test (*<0.05, **<0.01 and ***<0.001) between wild type and $35S::PtrCLE20$ plants.
PtrCLE20 peptide represses vascular cambium activity.
secondary vascular tissue at the 16th internode (Figure 3c,f), probably due to defects in cambium dividing activity. This possibility was confirmed by observing vascular cambium development at an earlier stage. Wild type had developed a ring of vascular cambium in the 2nd internode by linking interfascicular and fascicular cambium together in Populus (Figure S7b,d) and showed secondary vascular tissue in the 5th internode (Figure S7f, h). However, Line 51 showed unlinked vascular cambium and isolated fascicular cambium bundles in the 2nd internode (Figure S7a,c). The formation of vascular cambium ring was still uncompleted, and secondary vascular tissues showed delayed development in the 5th internode (Figure S7e,g). The observation indicated that PtrCLE20 overexpression hindered the cambium activity. Furthermore, the degree of the phenotypic changes in the process of secondary vascular tissue development was correlated with the PtrCLE20 expression levels. The transgenic Line 45, which had higher level of PtrCLE20 transcripts, displayed more severe phenotypic changes (Figure 3h,i,j,k). Line 45 showed narrower secondary vascular tissue with smaller vessel elements compared to Line 51 (Figure 3h,i,j,m). These results suggest that PtrCLE20 plays a role in secondary growth, likely through regulation of the cambium activity.

PtrCLE20 peptide acted in regulating cambium activity likely through a peptide signalling pathway

To investigate how PtrCLE20 is associated with meristematic activity in Populus vascular cambium, the cambium cells in Line 32 and Line 51 of 35S::PtrCLE20 and wild-type plants were collected using a laser microdissection system (Figure 4a) (Song et al., 2010). Gene expression in the isolated cells was analysed by qRT-PCR. Compared to wild type, in PtrCLE20 overexpression plants, PtrWOX4, which is a key gene related to cambium activity (Ji et al., 2010; Kucukoglu et al., 2017; Suer et al., 2011), showed a significantly reduced expression (Figure 4b); Expression of PtrRR5 and PtrCycA1, both reflecting cell dividing activity (Leibfried et al., 2005; To et al., 2007), was strongly suppressed, indicating that cell proliferation was restrained by overexpressing PtrCLE20 (Figure 4b) (d’Erfurth et al., 2010; Wang et al., 2004); PtrHB7, an essential gene for xylem differentiation (Zhu et al., 2013), displayed substantially lower expression (Figure 4b); PtrLBD1, a transcription factor involved in secondary vascular tissue development (Yordanov and Busov, 2011; Yordanov et al., 2010), and PtrWND18, a key transcriptional factor gene for secondary cell wall biosynthesis (Zhao et al., 2014), had much lower expression. Furthermore, the PtrCLE20 activities were examined using Populus micro-propagated seedlings. When the seedlings were treated with PtrCLE20 peptide, they developed root in a considerably slower manner and resulted in shorter roots (Figure 5a,b). To examine how PtrCLE20 peptide affects root growth, Arabidopsis seedlings treated with the peptide were employed as a test system (Figure 5c). Similarly, root growth was repressed (Figure 5d). The RAM zone displayed fewer dividing cells (Figure 5e), indicating that the activity of RAM was inhibited.

CLE peptides may be used as signalling ligands to be recognized by specific receptors (Yamaguchi et al., 2016). To test whether PtrCLE20 acts as a receptor ligand in inhibition of the RAM, several Arabidopsis CLE peptide receptor mutants, including clv1, clv2, px, bam1 and bam2, were used to test their response to PtrCLE20 peptides. In the mutant and wild-type seedlings, the length of primary roots was similar without peptide treatment (Figure 6a), consistent with the previous report (Etchells et al., 2015b). After treatment with 0.01 μM PtrCLE20 peptide, clv2 displayed normal primary root length, however, wild type and other mutants (pxy, clv1, bam1 and bam2) displayed dramatically shorter primary roots (Figure 6b). When the peptide concentration was gradually increased from 0.01 to 10.00 μM, the clv2 mutant showed a slight reduction of primary root length, but the other mutants (pxy, clv1, bam1 and bam2) showed a dramatic and similar reduction of primary root growth like the wild type (Figure 6c). These results indicate that clv2 mutant was insensitive to PtrCLE20 peptide, implying that PtrCLE20 peptide could be recognized by a possible receptor that is similar to CLV2. One possible Populus ortholog (Potri.013G087200) of CLV2 was predicted with 90% identity of amino acid sequence. The expression pattern of PtrCLV2 in various Populus tissues has been analysed in Poplar eFP Browser (Sundell et al., 2015), showing that PtrCLV2 expression is more abundant in xylem and root (Figure S8).

Discussion

The data presented here demonstrates that PtrCLE20, a xylem-produced peptide, plays an essential role in regulating vascular cambium activity and may represent a pathway for cell communication in which CLE peptides function to regulate lateral growth in Populus. The CLE gene family has been discovered in green algae to high plants (Han et al., 2016; Liu et al., 2016; Miwa et al., 2009; Oelkers et al., 2008; Strabala et al., 2014; Zhang et al., 2014). In the Arabidopsis genome, 32 CLE genes were identified with different expression specificity in various tissues and classified into two types: A-type and B-type (Jun et al., 2010; Sharma et al., 2003; Whitford et al., 2008). The number of CLE genes has risen

Figure 4 Expression of the genes related to cell proliferation and xylem development in the 35S::PtrCLE20 cambium cells. (a) Cross section of stem for laser microdissection, showing the cambium cells were sampled from the 9th internode of 35S::PtrCLE20 plants. (b) Expression of the genes in the collected cambium cells. Bars represent the means ± SD of two biological replicates and three technical replicates. Significance testing is conducted using the two samples t-test (*<0.05, **<0.01 and ***<0.001) between wild type and 35S::PtrCLE20 plants.

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to 52 in P. trichocarpa, most likely the result of whole-genome duplications, which was supported by presence of more ortholog copies of Arabidopsis and the same or similar CLE motif among the paralogs. For instance, four CLE14 orthologs in A-I type, three CLE13 in A-II type, three CLV3 orthologs in A-III type and four CLE41s and CLE44s, three CLE46s in B-type. Based on CLE domain similarity, PtrCLE peptides were clustered into A-type with three subtypes and B-type. These types were generally preserved when Arabidopsis was included. Gain-of-function phenotypes of many CLE genes in Arabidopsis have shown that similarities among overexpression phenotypes correlated with similarities in their CLE domain and the resulting phenotypes can be categorized into four classes, which correlate with types of CLE peptides in both Arabidopsis and Populus, indicating that CLE peptide in Populus shared similar roles with that in Arabidopsis (Strabala et al., 2006).

CLE peptides are proposed to be able to move through the extracellular apoplast space and be perceived by neighbour cells, which was supported by purification and identification of the first CLE peptides from the growth medium in which plant seedlings or cultured suspension cells had grown (Ito et al., 2006; Kondo et al., 2006). In Arabidopsis, B-type CLE41/CLE44 peptides are secreted from the phloem and distributed through the procambial region, which provides position information for promotion of cambium cell proliferation and division (Etchells and Turner, 2010; Etchells et al., 2015a). Expression of A-type CLE peptides commonly results in growth arrest in the SAM and RAM. For example, loss-of-function mutations in CLV3, an A-type member, caused excess stem cell accumulation in SAM and FM (Clark et al., 1995). In this study, PtrCLE20 peptide, an A-type peptide, was produced in developing xylem which consists of differentiated cells and moved into cambium cells. Overexpression of PtrCLE20 peptide represses vascular cambium activity, which results in decrease of cell division in the vascular cambium zone. Formation of vascular cambium ring is largely dependent on the activity of fascicular cambium (Guo et al., 2009; Little et al., 2002; Mazur et al., 2014; Zhu et al., 2018). Overexpression of PtrCLE20 caused delayed closure of the cambium ring during secondary vascular development. Possibly, this is due to that the

Figure 5  PtrCLE20 peptide inhibits root meristem activity in Populus and Arabidopsis. (a) Root growth of Populus when treated with 0 (left), 0.1 (middle) and 1 μM (right) PtrCLE20 peptide concentrations for 1 week. Bar, 2 cm. (b) Root growth of Populus when treated with 0 (left), 0.1 (middle) and 1 μM (right) PtrCLE20 peptide concentrations for 3 weeks. Bar, 2 cm. (c) Root morphology of Arabidopsis after one-week treatment with 0 (left), 0.1 (middle) and 1 μM (right) PtrCLE20 peptide concentrations. Bar, 60 μm. (d) Meristematic zones (MZ, white line), elongation zones (EZ, blue line) and differentiation zone (DZ, green line) of Arabidopsis root after one-week treatment with 0 (left), 0.1 (middle) and 1 μM (right) PtrCLE20 peptide concentrations. Bar, 60 μm. (e) Cell number in meristematic zone (MZ) and elongation zone (EZ). n = 10. Significance testing is conducted using the two samples t-test (*<0.05, **<0.01 and ***<0.001) between the treatments with 0, 0.1 and 1 μM PtrCLE20 peptide.
revealed that the binding activity between ligands and receptors analysis of the crystal structure of the PXY-CLE44/41 complex

PtrCLE20 times that of trees.

different sources may integrate to coordinate the secondary growth in

PtrCLE41/44 region. The production of led to changes in the orientation of cell division in the cambium

the PtrCLE41/44-PXY signalling pathway in phloem which then

ectopic expression of severe defects in vascular tissue (Figure 3g,h,i). One explanation is

approximately 25 times higher than wild type, Figure 2d) caused more

provides this mobile signalling peptide to repress cambium activity

activity of fascicular cambium was inhibited when PtrCLE20 was upregulated. Overall, the data suggest that developing xylem provides this mobile signalling peptide to repress cambium activity (Figure 7).

Overexpression of PtrCLE20 at higher levels (Line 45, approximately 25 times higher than wild type, Figure 2d) caused more severe defects in vascular tissue (Figure 3g,h,i). One explanation is ectopic expression of PtrCLE20 using the 35S promoter disturbed the PtrCLE41/44-PXY signalling pathway in phloem which then led to changes in the orientation of cell division in the cambium region. The production of PtrCLE41/44 in wild-type plant was 25 times that of PtrCLE20 (Figure S4a) (Xue et al., 2016). In addition, analysis of the crystal structure of the PXY-CLE44/41 complex revealed that the binding activity between ligands and receptors could be partially increased by high concentrations of the ligand

(Morita et al., 2016; Whitford et al., 2008; Zhang et al., 2016). As seen in low overexpression of PtrCLE20 plants (Line 51, Figure 2d), PXY and PXY-LIKE (PXL1 and PXL2) mutants also retained a degree of polarity within the vascular bundle (Fisher and Turner, 2007). It is possible that the PtrCLE20 peptide could bind to the PXY or PXY-LIKE receptors in cambium cells, allowing for the transmission of information necessary for determining the proper cell division plane during vascular development (Etchells and Turner, 2010; Fisher and Turner, 2007; Hirakawa et al., 2008; Ito et al., 2006; Whitford et al., 2008). This suggests additional factors are involved in vascular tissue development mediated by PtrCLE20 peptide. At the same time, primary root growth in pxy mutant seedlings was not sensitive to PtrCLE20 peptide, indicating that the PtrCLE20 signalling pathway may be in parallel with the PXY signalling pathway, with components existing in the PtrCLE20 signalling pathway either having a similar function to or involved in crosstalk with the PXY signalling pathway. PtrCLV2 might be a receptor of PtrCLE20 peptide in Populus as indicated by screening Arabidopsis LRR-RLK mutants and analysing expression patterns of PtrCLV2 in Populus (Figures 6 and S8). 3D structural and interaction models have predicted that the AtCLE20 peptide appears to bind tightly to the CLV2-CRN heteromeric complex (Meng and Feldman, 2010). PtrCLE20 and AtCLE20 peptides are distinguished only by the presence of either a proline or lysine residue, respectively, at position 4 of the peptide, suggesting they may have conformational similarity, thus allowing PtrCLE20 to bind to the CLV2-CRN heteromeric receptor complex. CLV2-CRN heteromeric receptor complexes are present in a ready state in the absence of ligand activation and application of CLE peptide is able to induce additional receptor clustering (Somssich et al., 2015).

In the PtrCLE20 overexpression transgenics, shorter plants were generated mainly due to fewer internodes, which indicates the activity of SAM was inhibited. In addition, both in Populus and Arabidopsis, root length was reduced after PtrCLE20 peptide treatment because of fewer cells in the root dividing zone, suggesting that inhibition on the RAM activity occurred. In the Populus stem, PtrCLE20 peptide is localized in the vascular cambium zone, which contributes on the development of secondary tissues and overexpression of PtrCLE20 led to fewer cambium dividing cells. Likely, the PtrCLE20 inhibition is mediated through a receptor which maybe localized in both lateral

Figure 6 CLV2 was a potential receptor of PtrCLE20 peptide. (a) Roots of wild type (Col and Ler) and mutants pxy, clv1, clv2, bam1 and bam2. (b) Root phenotypes of wild type (Col and Ler) and mutants pxy, clv1, clv2, bam1 and bam2 treated with 0.01 µM PtrCLE20 peptide. (c) Primary root length of wild type (Col and Ler) and mutants pxy, clv1, clv2, bam1 and bam2 treated with PtrCLE20 peptide at gradient concentrations. Bars represent the means ± SD of at least 16 biological replicates. Significance testing is conducted using the two samples t-test (*<0.05, **<0.01 and ***<0.001) between wild type and mutants treated with the same concentrations of PtrCLE20 peptide.

Figure 7 A model of PtrCLE20 function in vascular tissue of Populus. Phloem-derived CLE41 peptide binds to its receptor PXY in cambium cells and up-regulates the expression of WOX4 to promote cambium activity. Xylem-produced CLE20 peptide inhibits cambium activity through the repression of cell proliferation, likely via the CLV2 receptor by down-regulating the expression of WOX4. The two peptide signals from different sources may integrate to coordinate the secondary growth in trees.
obtained CLEs in v3.0 genomes (https://phytozome.jgi.doe.gov/) and sectioned (10 µm thin sections and mounted onto pre-charged slides. A PtrCLE20 specific fragment (221 bp) was amplified using primers (Table S2) and used as a probe for in situ hybridization as described (Gui et al., 2011).

Both PtrCLE20 and PtrCLE41 peptides with 12 amino acids were synthesized and injected into rabbits to raise antibodies and purified antibodies were obtained (Willget Biotech Co., Ltd, Shanghai, China). To examine the specificity of the antibodies, the full-length PtrCLE20 and PtrCLE41B as well as the truncated N-terminal PtrCLE20 and PtrCLE41B without CLE domain were cloned into pET28b vector and expressed in Escherichia coli (BL21). Meanwhile, both the full-length and the truncated genes were recombined with 6-His-tag at the C-terminal to examine expression of proteins. Western blots were performed using total protein extracts of cell lysate induced by IPTG (Figure S5a-d) and total protein extracts from stems without bark of wild-type Populus against PtrCLE20 or PtrCLE41 peptide antibodies (Figure S5e) (diluted for cell lysate: PtrCLE20: 1:1000 and PtrCLE41: blue and fixed with neutral balsam, and then imaged using a light microscope of OLYMPUS BX51. The dividing cell layers in cambium zone and cell layers in xylem tissue of each radial file were counted within cross sections from the 16th internodes (counting from tip). Cross-sectional area of the vessels and fibres was measured within cross sections from the 16th internodes by Image J. For quantitative analysis, 10 radial cell files were counted for cell layers and 10 vessels and 20 fibres were measured for cross-sectional area in each section. The measurements were carried out with at least three biological replicates. Two samples t-test was used to determine statistical significance between wild type and 35S::PtrCLE20 transgenic plants.

RNAs extraction and RT-qPCR

Shoot tips (the 1st and 2nd internode), young shoots undergoing primary growth (the 5th internode), secondary xylem and secondary phloem (the 30th internode), leaf and root tissues were harvested from three independent 3-month-old wild-type Populus (Populus×euramericana cv. ‘Nanlin895’) grown in a phytotron (under a light and dark cycle of 16 h and 8 h, respectively). RNAs were extracted from samples above via the modified CTAB method and subjected to cDNA synthesis using Hieff® First Strand cDNA Synthesis Kit (11120ES72; Yeasen, Shanghai, China). PtrCLE20 and PtrCLE41B transcript levels were determined via RT-qPCR using UNICON™ qPCR SYBR® Green Master Mix (11198ES08; Yeasen, Shanghai, China) and analysed by the 2−ΔΔCT method with the P’rActin1 housekeeping gene. To determine transcript levels of genes involved in PtrCLE20 signalling, vascular cambium cells were harvested from stems (the 9th internode) in wild type and both Line 32 and Line 51 of 35S::PtrCLE20 plants through laser microdissection method. RNA was extracted from the isolated vascular cambium cells and subjected to cDNA synthesis as described in detail in (Song et al., 2010). Three technical repeats were performed for each pair of primers. All primers used for RT-qPCR were listed in Table S2.

GUS staining, in situ hybridization and immunolocalization

Cross sections of stems undergoing secondary growth (the 15th internode) were cut from 18 independent lines of PtrCLE20::GUS plants approximately 3 months old and subjected to staining as described in detail in (Zhu et al., 2013).

The 11th internode from wild-type Populus stems (2 months old) was embedded in paraplast (Sigma-Aldrich) and cut into 10-µm thin sections and mounted onto pre-charged slides. A PtrCLE20 specific fragment (221 bp) was amplified using primers (Table S2) and used as a probe for in situ hybridization as described (Gui et al., 2011).

In summary, PtrCLE20 is produced in developing xylem cells and acts in cambium region to inhibit cambium cell division activity (Figure 7). On the other hand, the phloem-derived CLE41 peptide promotes cambium activity (Etchells and Turner, 2010; Fisher and Turner, 2007; Hirakawa et al., 2008; Ito et al., 2006; Whitford et al., 2008). Possibly, integration of peptide signals from different sources serves as a mechanism to coordinate the secondary growth in trees. Further verification of this possibility would provide more insights into how secondary growth in trees is coordinately controlled through different signals.

Experimental procedures

Gene identification and alignment

To identify CLE genes, BLASTP searches using 32 Arabidopsis CLE protein sequences were conducted in Phytozome against the P. trichocarpa v3.0 genomes (https://phytozome.jgi.doe.gov/pz/portal.html). To ensure identification of all CLE genes, the obtained CLEs in P. trichocarpa were used as queries to search additional genes through BLASTP. CLUSTALX was used to generate multiple sequences alignments. Phylogenetic trees were constructed from multiple sequence alignments using the neighbor-joining method in MEGA 7.

Gene cloning, constructs generation and genetic transformation

In order to generate PtrCLE20pro::GUS, a 1.7 kbs upstream sequence of PtrCLE20 coding sequence was cloned from P. trichocarpa genome using primers listed in Table S2 and then replaced 535 promoter sequence in pCambia1301 vector using HindIII and Ncol enzyme sites. In order to generate the overexpression construct 35S::PtrCLE20, the CDS of PtrCLE20 was cloned from the cDNA of developing xylem of P. trichocarpa using primers listed in Table S2 and inserted into pCambia2300 vector between SacI and PstI enzyme sites. All constructs were transformed into Populus×euramericana cv. ‘Nanlin895’ by Agrobacterium-mediated transformation according to the protocol adopted in our laboratory (Li et al., 2003). For each construct, at least 25 individual transgenic lines were generated. For characterization, the transgenics were clonally propagated for multiple copies which were used for biological replicates.

Plant growth and histological analysis

35S::PtrCLE20 transgenic plants of individual lines were micro-propagated for more than eight copies in order to obtain identically grown plants for multiple biological replicates. Transgenic and wild-type plants were grown in a phytotron with a light and dark cycle of 16 and 8 h at 23 °C for 3 months. Morphological features, including plant height, internode numbers, internode length and stem diameter, were measured from 3-month-old plants with at least three biological replicates. Shoot tips and series of internodes of approximate three-millimetre-length stem were fixed in formaldehyde-acetic acid solution (FAA, formaldehyde: glacial acetic acid: ethanol 1:1:18) for 24 h, then dehydrated in graded ethanol series and embedded into paraffin and sectioned (10 µm in thickness) using a microtome. After deparaffinization, sections were stained with 0.05% toluidine deparaffinization, sections were stained with 0.05% toluidine

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light-dark cycle at 23° C were grown in MS medium plus 0.01, 0.1, and 1 μM of PtrCLE20 peptide in a phytotron with a 16–8 h light-dark cycle at 23° C. The treatment groups were grown in MS medium plus 0.01, 0.1, and 1 μM of PtrCLE20 peptide. Three independent experimental trials were conducted with at least five seedlings for each treatment in each trial.

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Conflict of Interest
The authors declare no conflict of interest.

Author Contributions
Y.Z. and D.S. designed, acquired, analysed, interpreted data and wrote the manuscript. R.Z., L.L., S.C., H.J., I.S. and J.G. designed, acquired and analysed data. L.L. designed, analysed, interpreted data and wrote the manuscript.

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Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Alignment of full-length CLE proteins in Populus trichocarpa.

Figure S2 Two types of CLE peptides from the CLE proteins in Populus trichocarpa.

Figure S3 Phylogenetic analysis of CLE motif in Populus trichocarpa and Arabidopsis.

Figure S4 Expression pattern analysis of PtrCLE genes across multiple tissues.

Figure S5 Specification of PtrCLE20 and PtrCLE41 peptides antibodies.

Figure S6 PtrCLE41 peptide localized in cambium and phloem cells.

Figure S7 Overexpression of PtrCLE20 delayed the formation of vascular cambium at early developmental stages.

Figure S8 Expression pattern analysis of PtrCLV2 in various tissues in Populus.

Table S1 CLE gene family in Populus trichocarpa

Table S2 Primers used in this study