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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. In 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; Shinohara and Matsubayashi, 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 columella 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/TDIF (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, TDR/PXY (TDIF 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).

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 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 predicated 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 52 *PtrCLE* 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 *PtrCLE41*s and *PtrCLE44*s 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).

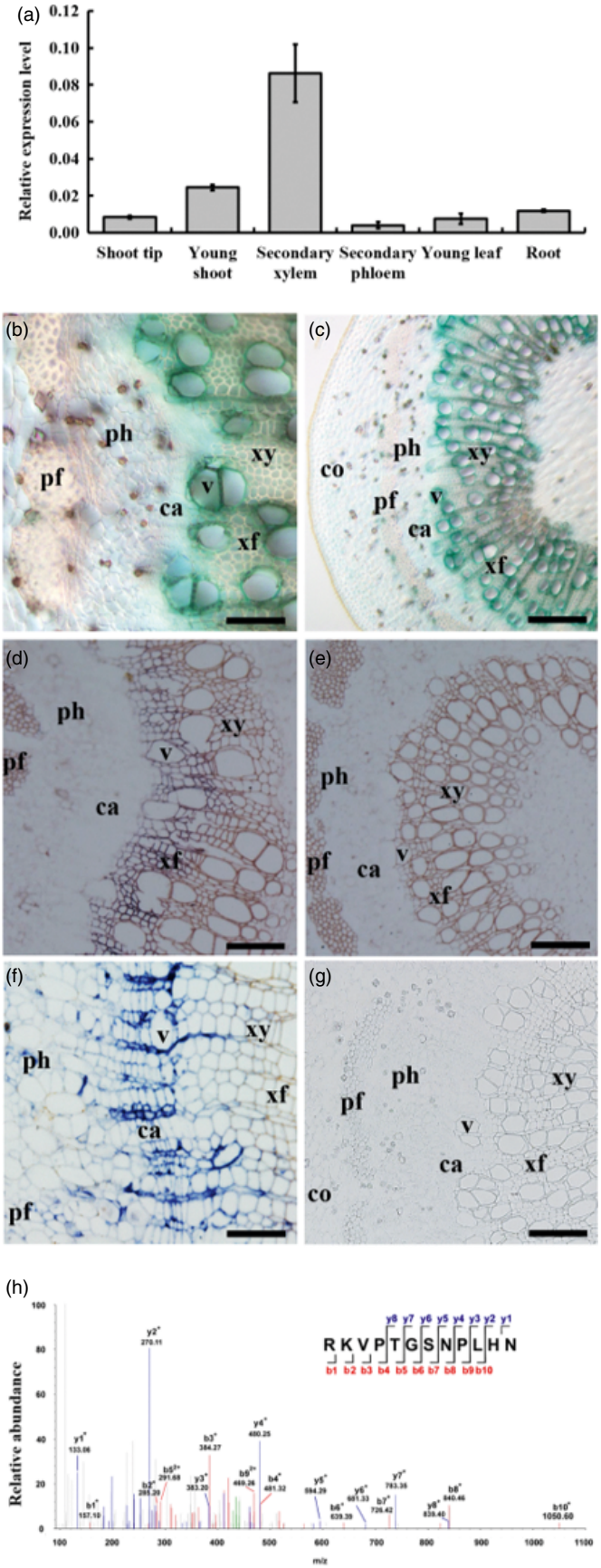
PtrCLE20 peptide was detected in vascular cambium

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 predicated 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 increased *PtrCLE20* 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

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.


(a)
Relative expression level
0.12
0.10
0.08
0.06
0.04
0.02
0.00
Shoot tip
Young shoot
Secondary xylem
Secondary phloem
Young leaf
Root
(b)
ph
xy
pf
v
ca
xf
(c)
co
ph
xy
pf
v
ca
xf
(d)
ph
xy
pf
v
ca
xf
(e)
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v
xf
(f)
v
xy
ph
xf
ca
pf
(g)
ph
xy
pf
v
ca
xf
co
(h)
Relative abundance
m/z
R K V P T G S N P L H N
b1 b2 b3 b4 b5 b6 b7 b8 b9 b10
y8 y7 y6 y5 y4 y3 y2 y1

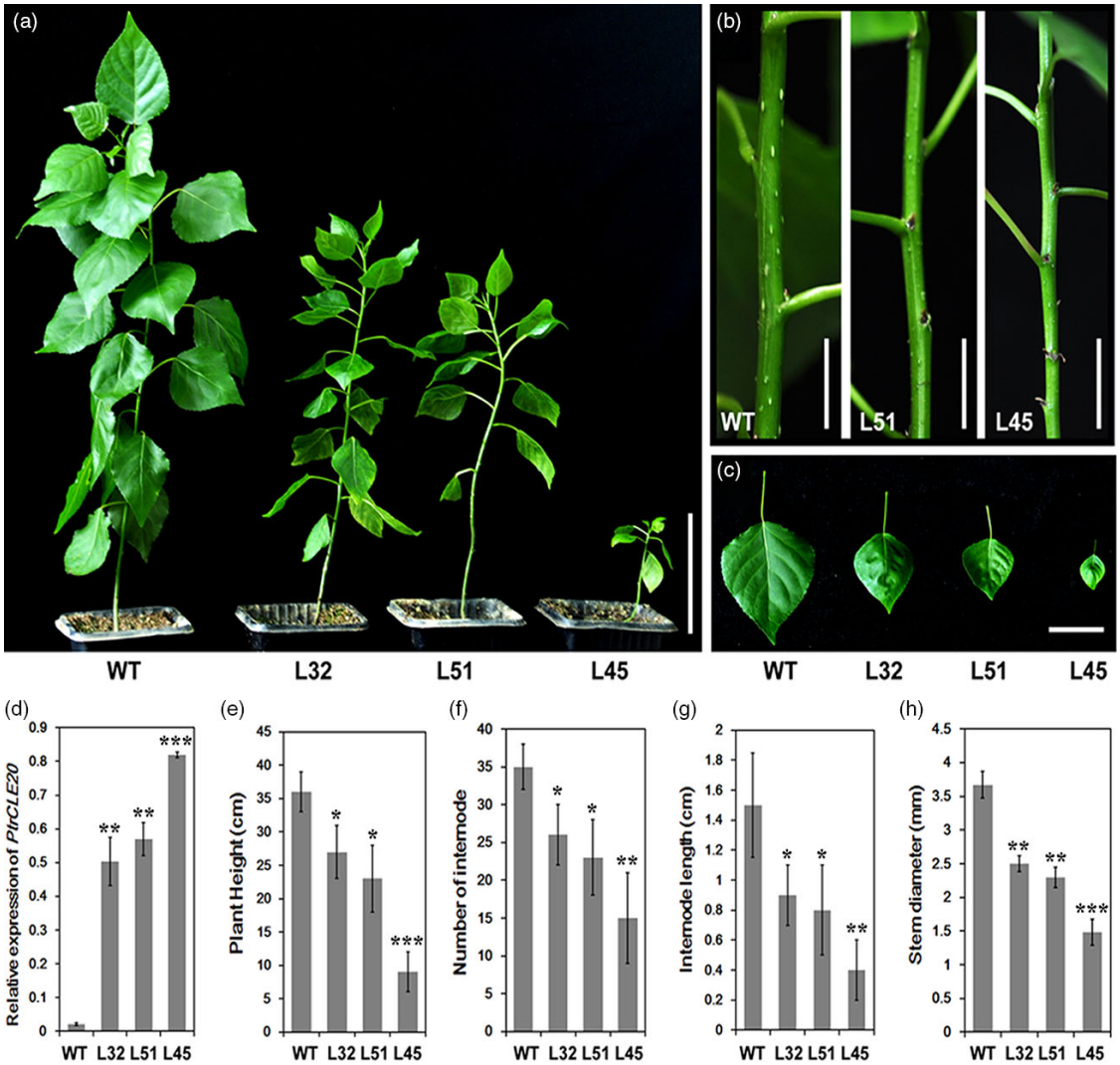
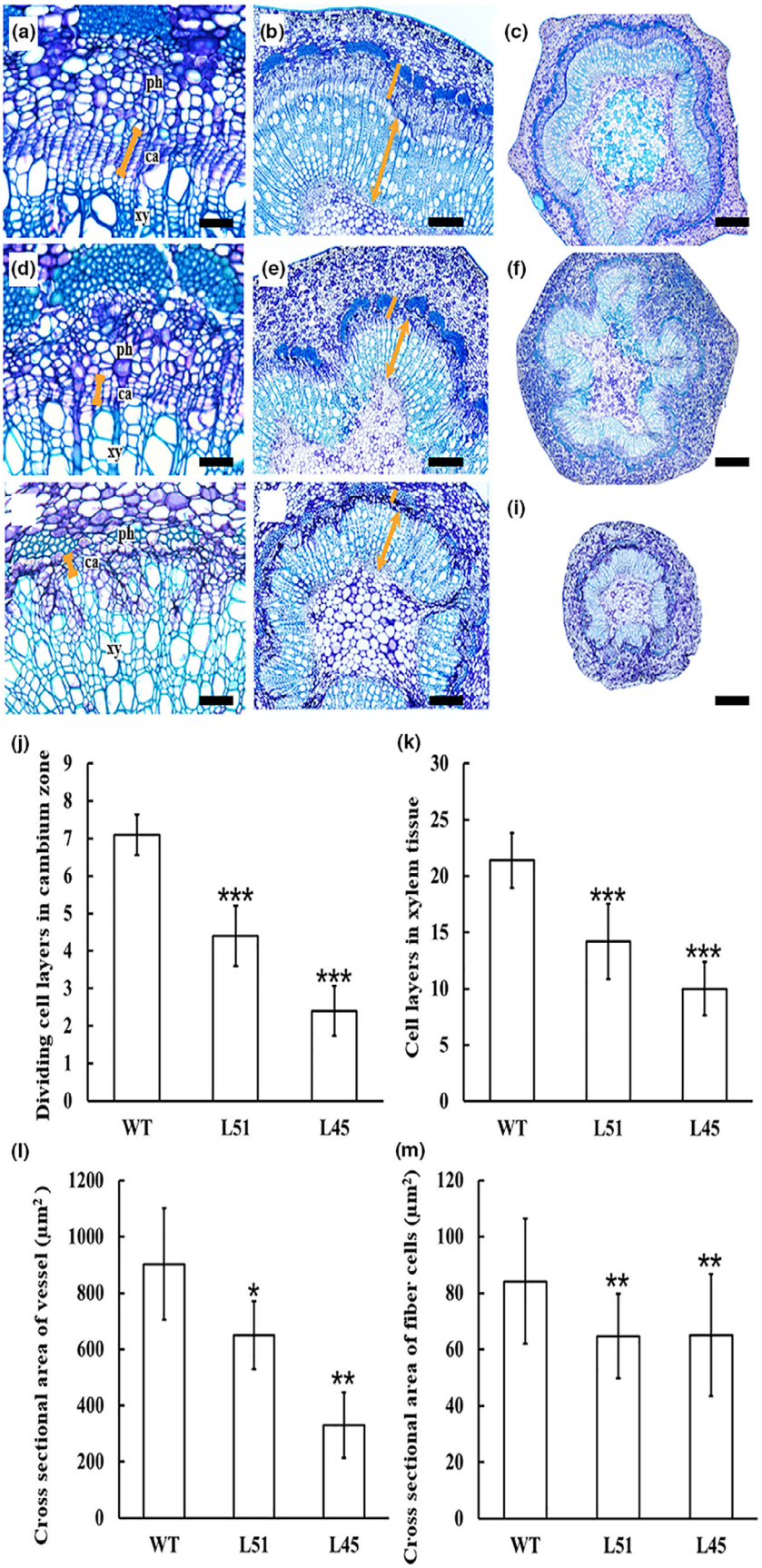


Figure 2 Morphological phenotypes of overexpression of *PtrCLE20* in *Populus*. (a) Whole plants. (b) Stems. (c) Leaf of wild type and three independent lines of *35S::PtrCLE20*. (d) Expression levels of *PtrCLE20* in wild type and three independent lines of *35S::PtrCLE20*. Expression level is shown 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.

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). The cross section of transgenic Line 51 displayed a contorted ring of

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 l 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.


(a)
ph
ca
xy
(b)
(c)
(d)
ph
ca
xy
(e)
(f)
ph
ca
xy
(i)
(j)
Dividing cell layers in cambium zone
***
***
WT
L51
L45
(k)
Cell layers in xylem tissue
***
***
WT
L51
L45
(l)
Cross sectional area of vessel (μm²)
*
**
WT
L51
L45
(m)
Cross sectional area of fiber cells (μm²)
**
**
WT
L51
L45

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,l,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 *PtrCLE2*0 (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 *PtrWND1B*, 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*, *pxy*, *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 0.01μM 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 gradiently 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

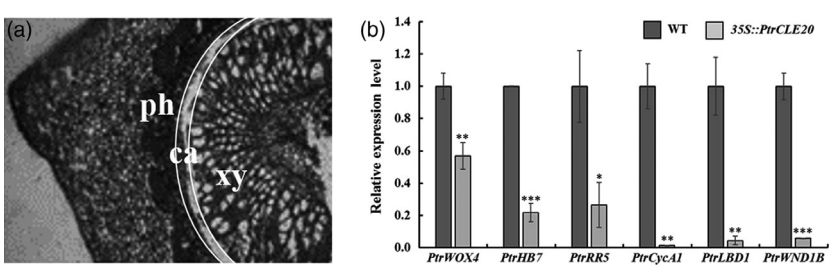


Figure 4 Expression of the genes related to cell proliferation and xylem development in the *35S::PtrCLE20* cambium cells. (a) Cross section of stem for laser microdissection, showing the cambium cells were sampled from the 9th internode of *35S::PtrCLE20* plants. (b) Expression of the genes in the collected 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.

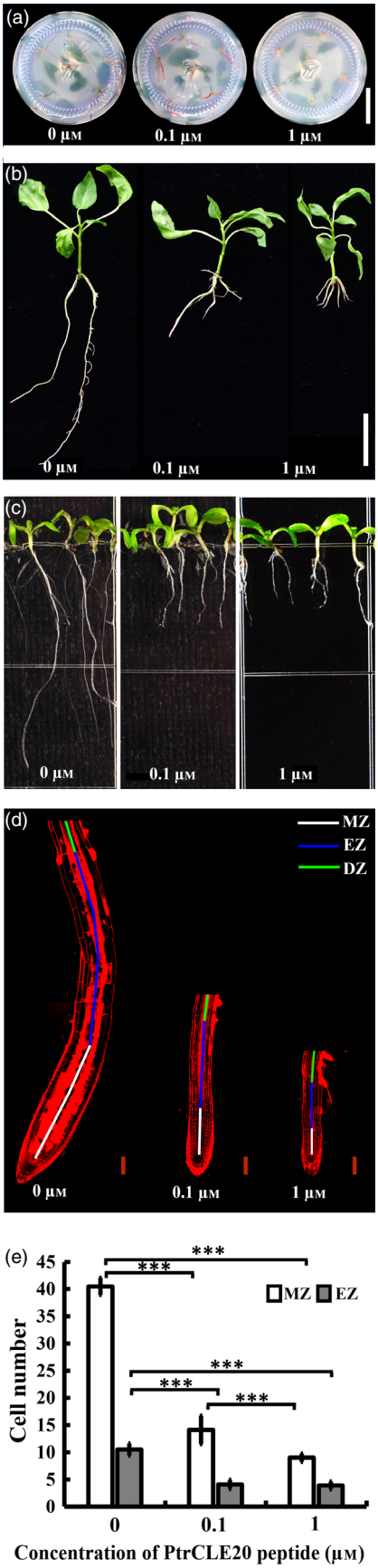


Figure 5 PtrCLE20 peptide inhibits root meristem activity in *Populus* and Arabidopsis. (a) Root growth of *Populus* when treated with 0 (left), 0.1 (middle) and 1 μM (right) PtrCLE20 peptide concentrations for 1 week. Bar, 2 cm. (b) Root growth of *Populus* when treated with 0 (left), 0.1 (middle) and 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. (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.

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* caused fewer layers of cells in cambium zone and in xylem tissue during secondary growth. In agreement with this phenotypic alternations, *PtrWOX4*, which promotes cambium activity (Ji *et al*., 2010; Kucukoglu *et al*., 2017; Suer *et al*., 2011), showed a reduced expression in the cambium region when *PtrCLE20* was overexpressed. This indicates that the PtrCLE20 peptide plays a role in repression of the 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

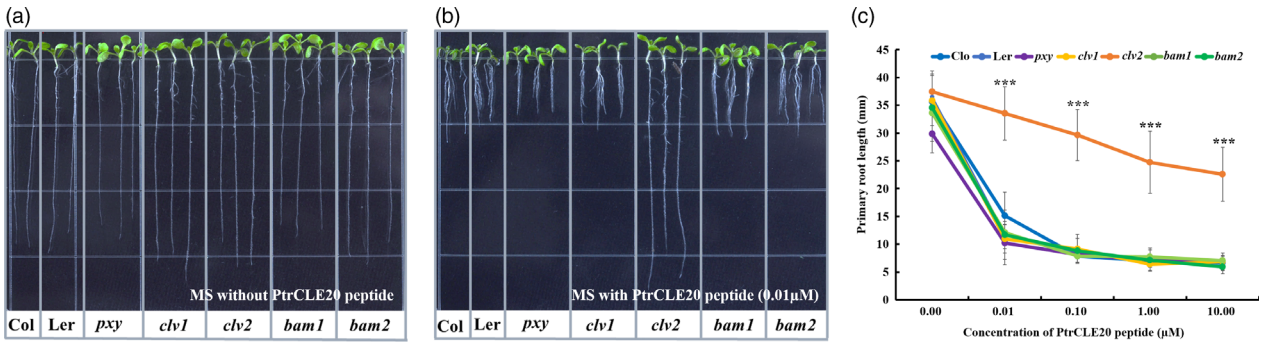


Figure 6 *CLV2* was a potential receptor of PtrCLE20 peptide. (a) Roots of wild type (Col and Ler) and mutants *pxy*, *clv1*, *clv2*, *bam1* and *bam2*. (b) Root phenotypes of wild type (Col and Ler) and mutants *pxy*, *clv1*, *clv2*, *bam1* and *bam2* treated with 0.01 μM PtrCLE20 peptide. (c) Primary 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.

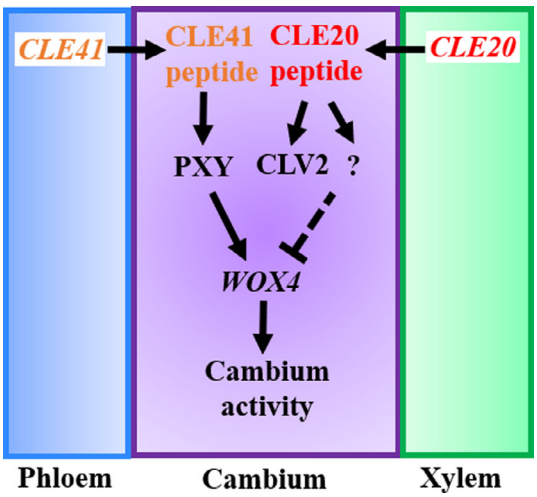


Figure 7 A model of *PtrCLE20* function in vascular tissue of *Populus*. Phloem-derived CLE41 peptide binds to its receptor *PXY* in cambium cells and up-regulates the expression of *WOX4* to promote cambium activity. Xylem-produced CLE20 peptide inhibits cambium activity through the repression of cell 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.

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*s 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

cambium and apical meristems. Further characterization of the receptor and its correct match with PtrCLE20 at specific location would provide a clearer picture regarding how PtrCLE20 acts in different meristems.

In summary, PtrCLE20 is produced in developing xylem cells and acts in cambium region to inhibit cambium cell dividing 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 on the p-distance 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 35S promoter sequence in *pCambia1301* vector using *HindIII* and *NcoI* 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 micropropagated 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 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 $2^{-\triangle Ct}$ method with the *PtrActin1* 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 *PtrCLE20pro::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).

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:

1:1000, for stems: PtrCLE20: 1:1000 and PtrCLE41: 1:1000). The secondary antibodies (linked with alkaline phosphatase; Santa Cruz, CA, USA) were diluted in 1: 5000. The 12th internode of wild-type *Populus* was embedded and sliced into 10-μm thin sections for immunolocalization according to previous protocol (Song *et al*., 2014). The first antibodies were diluted in 1:200 and the secondary antibodies were diluted in 1:1000.

Identification of PtrCLE20 peptide from developing xylem and vascular cambium

The developing xylem and vascular cambium tissues collected from stems of 1-year old trees were ground to fine powder in liquid nitrogen. The powder (100 mg) was homogenized at 4 °C in ice-cold IP buffer (1/5, w/v) containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2% (v/v) Triton X-100, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 1 mM protease inhibitor cocktail (20123ES10, Yeasen). The homogenate was centrifuged at 10 000 g for 10 min at 4 °C. The supernatant (about 1 ml) was combined with the PtrCLE20 peptide antibody (1 μg/μl) with a dilution of 1: 300 and rotated 4 °C overnight. 100 μl of protein A-Sepharose Cl-4B was then added to the mixture, and gently shaken for 2 h at 4 °C with end-over-end rotation. After centrifugation for 30 s at 2000 g, the pellet was washed three times with ice-cold IP buffer and twice with ice-cold washing buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM PMSF, 1 mM protease inhibitor cocktail). The pellet was finally washed with 10 mM NH_4AC (pH 3.0) to release the PtrCLE20 protein, peptides and the antibody. The released protein mixture was neutralized with ammonia and passed through a cut-off ultrafiltration tube (3 KD; Millipore, Tullagreen, Carrigtwohill, Co. Cork, IRL) to remove the antibody and collect the PtrCLE20 peptide. The cut-off solution containing the PtrCLE20 peptide was desalted through lyophilization and the collected peptide was subsequently re-suspended with 5% ACN in 0.1% FA and subjected to LC-MS/MS (Waters UPLC/Thermo Q Exactive). The identification and modification analysis of PtrCLE20 peptide was performed according to our previous procedure (Song *et al*., 2010).

Test of the PtrCLE20 peptide effect using a root system

The following *A. thaliana* genotypes: *clv1* (CS45), *clv2* (CS46), *bam1* (SALK_015302) and *bam2* (CS16306) of Landsberg ecotype and *pxy* of Columbia-0 ecotype (SALK_026128C) were ordered from ABRC stock centre. Homozygotes of mutants above were obtained and determined by PCR genotyping and sequencing. PtrCLE20 peptide with 12 amino acids (RKVPTGSNPLHN) was synthesized with 95% purity and dissolved in H_2O. The seeds were sterilized with 3% sodium hypochlorite for 15 min, rinsed with H_2O for three times and stratified at 4°C for 2 days before the peptide treatment. For the peptide treatment, the seeds of the control group were grown in plates containing MS medium (4.4 g/L MS salts, 10 g/L sucrose, 0.8 g/L agar), while the seeds of the treatment groups were grown in MS medium plus 0.01, 0.1, 1.0 and 10 μM of PtrCLE20 peptide. Three independent experimental trials were conducted with at least 16 seedlings for each treatment in each trial. The plates were incubated vertically in a phytotron with a 16–8 h light-dark cycle at 23 °C for 7 days, then the length of the primary root was measured under regular microscope and confocal microscope. For *Populus* seedlings treatment, young shoots with equal numbers of leaves and internodes of wild type were grown in woody plant medium with 0, 0.1 and 1 μM of PtrCLE20 peptide in a phytotron with a 16–8 h light-dark cycle at 23 °C. 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., C.H., J.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