New Phytologist

Research

Peptide encoding *Populus CLV3/ESR-RELATED 47* (*PttCLE47*) promotes cambial development and secondary xylem formation in hybrid aspen

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Received: *19 September 2019*
Accepted: *8 November 2019*

New Phytologist (2020) **226:** 75–85
doi: 10.1111/nph.16331

Key words: cambium, CLE peptides, *Populus*, *PttCLE47*, secondary growth and development, wood formation.

Summary

- The CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR)-RELATED (CLE) peptide ligands in connection with their receptors are important players in cell-to-cell communications in plants. Here, we investigated the function of the *Populus CLV3/ESR-RELATED 47* (*PttCLE47*) gene during secondary growth and wood formation in hybrid aspen (*Populus tremula* × *tremuloides*) using an RNA interference (RNAi) approach.
- Expression of *PttCLE47* peaks in the vascular cambium. Silencing of the *PttCLE47* gene expression affected lateral expansion of stems and decreased apical height growth and leaf size.
- In particular, *PttCLE47 RNAi* trees exhibited a narrower secondary xylem zone with less xylem cells/cell file. The reduced radial growth phenotype also correlated with a reduced number of cambial cell layers. In agreement with these results, expression of several cambial regulator genes was downregulated in the stems of the transgenic trees in comparison with controls.
- Altogether, these results suggest that the *PttCLE47* gene is a major positive regulator of cambial activity in hybrid aspen, mainly promoting the production of secondary xylem. Furthermore, in contrast to previously characterized *CLE* genes expressed in the wood-forming zone, *PttCLE47* appears to be active at its site of expression.

Introduction

In plants, peptide-receptor signalling modules have important roles in mediating cell-to-cell communications and interactions during growth, development, and responses to environmental stimuli. One of the best-studied gene families encoding such small peptide ligands is the *CLAVATA3* (*CLV3*)/*EMBRYO SURROUNDING REGION* (*ESR*)-*RELATED* (*CLE*) gene family (Clark *et al.*, 1995; Fletcher *et al.*, 1999). *CLE* genes encode small proteins (*c.* 60–120 aa), which carry an N-terminal signal peptide and a C-terminal conserved CLE peptide domain of length 12–13 aa (Oelkers *et al.*, 2008; Ohyama *et al.*, 2008, 2009). The CLE peptide domain is excised from its prepropeptide by serine proteases and carboxypeptidases, and posttranslationally modified (i.e. hydroxylation and arabinosylation) to become biologically active (Fiers *et al.*, 2005, 2006; Ito *et al.*, 2006; Ni & Clark, 2006; Ohyama *et al.*, 2008, 2009; Ni *et al.*, 2011). In addition, the signal peptide is essential for the release of the active CLE peptide into the extracellular space, where it functions (Rojo *et al.*, 2002).

In *Arabidopsis thaliana*, *CLE* genes are expressed with different tissue specificities and mainly interact with the plasma-membrane-associated LEUCINE-RICH REPEAT RECEPTOR-LIKE KINASEs (LRR-RLKs) (Clark *et al.*, 1995; Sharma *et al.*, 2003; Hirakawa *et al.*, 2008; Jun *et al.*, 2010). Prominent examples from this peptide family include CLV3 and CLE40, which are involved in the regulation of stem cell pools in the shoot apical meristem and root apical meristem, respectively, in *Arabidopsis* (Clark *et al.*, 1995; Brand *et al.*, 2000; Schoof *et al.*, 2000; Stahl & Simon, 2009; Stahl *et al.*, 2009). Other than their roles in stem cell homoeostasis in apical meristems, *CLE* genes also regulate a variety of other biological processes, such as expansion of lateral roots in response to nitrogen availability (Araya *et al.*, 2014), embryo and endosperm development (Fiume &

Fletcher, 2012), phloem initiation (Ren *et al.*, 2019) and protophloem development (Rodriguez-Villalon *et al.*, 2014), protoxylem development (Kondo *et al.*, 2011; Qian *et al.*, 2018), stomatal development (Qian *et al.*, 2018) and closure upon dehydration stress (Takahashi *et al.*, 2018; Zhang *et al.*, 2018), pollen–pistil interactions (Endo *et al.*, 2013), and autoregulation of nodulation (Okamoto *et al.*, 2009, 2013; Mortier *et al.*, 2010; Reid *et al.*, 2011).

The secondary xylem (wood) and the secondary phloem of the plants are generated by the proliferative activity of the vascular cambium – a secondary meristematic tissue that contains the vascular stem cells. It has been shown that, similar to the apical meristems, the activity of the vascular stem cells in the procambium/cambium in *Arabidopsis* stems and hypocotyls is also mediated by an interaction between the CLE peptide TRACHEARY ELEMENT DIFFERENTIATION INHIBITORY FACTOR (TDIF)/CLE41/CLE44 and the LRR-RLK PHLOEM INTERCALATED WITH XYLEM (PXY)/TDIF RECEPTOR (TDR) (Ito *et al.*, 2006; Hirakawa *et al.*, 2008; Etchells & Turner, 2010). CLE41 and CLE44 genes are expressed in the phloem tissues and neighbouring cells in the inflorescence stems and hypocotyl (Hirakawa *et al.*, 2008; Etchells & Turner, 2010). The secreted TDIF/CLE41/CLE44 peptide is perceived by TDR/PXY, which shows an expression maximum in procambium and in the xylem side of the cambium (i.e. in the stem cell organizer) (Hirakawa *et al.*, 2008; Etchells & Turner, 2010; Smetana *et al.*, 2019). This interaction promotes the vascular cell divisions, suppresses the xylem cell specification, and controls the patterning of the vascular tissues (Ito *et al.*, 2006; Hirakawa *et al.*, 2008, 2010; Etchells & Turner, 2010; Etchells *et al.*, 2012, 2013).

Reports suggested that the TDIF/CLE41/CLE44-PXY/TDR signalling module is evolutionarily conserved in regulating the secondary growth and wood formation of *Populus* trees (Etchells *et al.*, 2015; Kucukoglu *et al.*, 2017). Aside from TDIF/CLE41/CLE44-like genes, a recent study showed that the xylem-produced peptide *PtrCLE20* negatively regulates cambial activity in *Populus* (Zhu *et al.*, 2019). Poplars are deciduous, hardwood trees and widely cultivated world-wide as bioenergy feed stocks and for production of pulp, paper, packing, and woody material. Accompanied by the available sequenced genome (Tuskan *et al.*, 2006), transformation and *in vitro* propagation techniques (Nilsson *et al.*, 1992), as well as transcriptomic resources (Schrader *et al.*, 2004; Sundell *et al.*, 2017), it is one of the best model plants in understanding the molecular basis of tree growth and development, particularly the formation of wood. Other than the studies already mentioned, functional information regarding the roles of different CLE peptides in this important tree species is scarce. This prompted us to investigate the function of the cambium-expressed *CLE* gene *PttCLE47*in hybrid aspen. Our results demonstrate that *PttCLE47* has a role in controlling stem secondary growth by positively regulating the cell division activity of the vascular cambium in trees at its site of expression. It also affects overall growth and leaf size.

Materials and Methods

Plant material and growth conditions

Transgenic and wild-type (WT) hybrid aspen (*Populus tremula* × *tremuloides* Michx, clone T89) plants were propagated from cuttings in tissue culture for 4 wk and transferred to soil. For phenotypic characterization they were grown under long day conditions (18 h : 6 h, light : dark, 22°C : 18°C) with 60–70% humidity for 3 months. During this period, they were rotated weekly to randomize environmental effects. Plant growth was assessed by measuring plant height, number of internodes, and stem width once a week for 6 wk. Height of the trees was measured from the shoot tips to the bottom of the stems. Stem width was measured and followed in the first visible internodes at the bottom of trees, 20 cm above the soil level. The number of internodes and leaves was counted omitting 3 cm from the shoot tips and a 20 cm long section above the soil (this part of the stem normally grows inside the tissue culture media during clonal propagation of trees, and upon transferring the plants to soil it grows slightly atypical with smaller, nonexpanding leaves independent of genotype; hence, it is omitted). In order to calculate the leaf areas, leaves (numbers 7 and 21, counted 3 cm below the shoot tip) were scanned and leaf area was calculated using ImageJ software (http://imagej.net). All statistical analyses were performed by GraphPad Prism v.8, using recommended tests. Phenotypes were compared using either an unpaired parametric *t*-test (to compare means of two groups at a time; i.e. WT vs RNA interference (RNAi)-*n* in 1 wk) by assuming a Gaussian distribution with Welch's correction (for unequal variance) or a Holm–Sidak multiple comparisons *t*-test (to compare groups of means within each of the rows of data at a time; i.e. WT vs RNAi-*n* over multiple weeks of growth), without assuming consistent standard deviations.

Cloning and transformation of hybrid aspen

The gene accession no. for *PtCLE47* gene is *Potri.017G074600.1* (Han *et al.*, 2016). It was also annotated as *PtrCLE25C* (Zhu *et al.*, 2019). Peptide sequences for PtCLE47 and AtCLE25 (AT3G28455) were obtained from Zhu *et al.* (2019). For *RNAi* plants, a 155 bp long *RNAi* fragment (also targeting the putative predicted active PttCLE47 peptide domain) was amplified from hybrid aspen stem complementary DNA (cDNA) using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The RNAi fragment was cloned via the Gateway Technology (Thermo Fisher Scientific) first into pDONR201 and further transferred into pK7GWIWG2 (I) (Karimi *et al.*, 2002), using the *Escherichia coli* bacterial strain *DH5α*. The binary vector was transformed into *Agrobacterium tumefaciens C58* strain GV3101 pMP90RK, and hybrid aspen trees were transformed as described previously (Nilsson *et al.*, 1992). The primers utilized are listed in Supporting Information Table S1.

Gene expression analysis

For *in silico* gene expression analyses, expression data of *PtCLE47*, *PtCLE24* (*Potri.008G191500.1*) and *PtCLE28* (*Potri.010G039800.1*) were extracted from the ASPWOOD database (http://aspwood.popgenie.org; Sundell *et al.*, 2017). For transcriptional analyses of *PttCLE47* in different tissues of hybrid aspen, a cDNA sample set from 6-month-old trees described previously by Kucukoglu *et al.* (2017) was used. For subsequent gene expression analyses, stem segments from 18-d-old internodes of transgenic *PttCLE47 RNAi* trees and corresponding 18 d-old WT internodes were harvested. As a control, stem segments from 11-d-old internodes of WT trees, which displayed similar thickness to 18 d-old internodes of *PttCLE47 RNAi* trees, were also collected. For expression analysis in stocks segments of grafted plants, internodes 5 cm below the graft junctions were sampled. Total RNA was extracted using the cetyl trimethylammonium bromide method (Chang *et al.*, 1993). Following the extraction, total RNA was treated with DNAse using the Ambion DNA-free™ DNA Removal Kit (Thermo Fisher Scientific) and cDNA was synthesized using the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. Quantitative PCR (qPCR) analysis was performed in 10 µl reactions using the LightCycler® 480 SYBR Green I Master (Roche Life Science) in LightCycler® 480 instrument II (Roche Life Science). Each sample was analysed by three technical replicates. Detected expression levels were normalized against *UBQ* or the geometric mean of expression for four reference genes (*18S*, *UBQ*, *50S*, *TIP41-like*; Livak & Schmittgen, 2001). All statistical analyses were performed using GraphPad Prism v.8 for Mac OS X (GraphPad Inc.). Expression data were compared using an unpaired parametric *t*-test (to compare means of two groups at a time, i.e. WT vs RNAi-*n*) by assuming a Gaussian distribution with Welch's correction (for unequal variance). The primers utilized are listed in Table S1.

Histology

For histological analysis, stem segments from 18-d-old internodes of transgenic *PttCLE47 RNAi* trees and corresponding 18-d-old WT internodes were harvested. Stem segments from 11-d-old internodes of WT trees, which displayed similar thickness to 18-d-old internodes of *PttCLE47 RNAi* trees, were used as a control. These samples were fixed in a 5% formaldehyde, 5% acetic acid, and 50% ethanol mixture, gradually dehydrated, and embedded in Leica HistoResin (Leica Biosystems, Wetzlar, Germany). Thin cross-sections (5 µm) were cut using a Leica RM 2055 rotary microtome (Leica Biosystems) and stained with toluidine blue and ruthenium red. Thick cross-sections (100 µm) were cut using a vibrating-blade microtome (Microm Microtech France, Brignais, France) and stained with safranin and Alcian blue, Bright-field images were taken using a Leica DM2500 optical microscope (Leica Microsystems, Wetzlar, Germany). Secondary xylem and secondary phloem widths were measured using the ImageJ software (http://imagej.net), from the border of the cambium to the outermost cells of the pith and phloem fibres, respectively. Bark size was measured from the border of the cambium to the outermost cells of the cortex. The number of undifferentiated cambial cells and secondary xylem cells was calculated by counting 10 cell files and three cell files, respectively, from three trees per line. All statistical analyses were performed using GraphPad Prism v.8. Tissue thicknesses were compared using a Holm–Sidak multiple comparisons *t*-test (to compare groups of means within each rows of data at a time, i.e. WT-18D vs RNAi-*n*-18D for multiple characteristics, including thickness of secondary xylem, secondary phloem, and bark), without assuming consistent standard deviations. The number of cambial cells was compared using an unpaired parametric *t*-test (to compare means of two groups at a time, i.e. WT vs RNAi-*n*) by assuming a Gaussian distribution with Welch's correction (for unequal variance).

Grafting

Grafting experiments were performed as previously described (Nieminen *et al.*, 2008). Briefly, transgenic and WT hybrid aspens were propagated from cuttings in tissue culture for 4 wk and transferred to soil. After 50 d, 5 cm long scions were excised and trimmed into a triangular shape. Simultaneously, longitudinal splits were made in stock stems, and trimmed scions were inserted. The scion–stock graft junction was secured with parafilm, and grafted plants were covered with plastic bags until after the graft junction to prevent drying. Bags were removed after 2 wk, when grafted scions started producing new leaves. Plant growth was assessed by measuring stem girths 5 cm below the graft junctions for 6 wk. Statistical analysis was performed using GraphPad Prism v.8. Stem widths of stocks were compared using an unpaired parametric *t*-test (to compare means of two groups at a time, i.e. WT/WT vs RNAi/RNAi) by assuming a Gaussian distribution with Welch's correction (for unequal variance).

Results

PttCLE47 is highly expressed in the cambium of hybrid aspen

The *Populus trichocarpa* genome encodes 52 *CLE* genes (Zhu *et al.*, 2019), hereafter referred to as *PtCLE*s (for *P. trichocarpa*) or *PttCLE*s (for *P. tremula* × *tremuloides*, hybrid aspen). Recent studies indicated that 33 *PtCLE*s are expressed, with various levels of specificity, in the *Populus* stem, including secondary phloem, vascular cambium, expanding xylem, and maturing lignified xylem tissues (Han *et al.*, 2016; Zhu *et al.*, 2019). This suggests that members of the *PtCLE* family may potentially regulate a diversity of functions during vascular development in *Populus* trees, such as cambial activity, specification of xylem and phloem cell types, or secondary cell wall formation. Based on these gene expression profiles (Han *et al.*, 2016; Zhu *et al.*, 2019), we decided to focus on the previously uncharacterized *PtCLE47* gene, which shows a strong expression gradient over the vascular cambium (Fig. 1a). We verified the transcript levels of *PttCLE47*

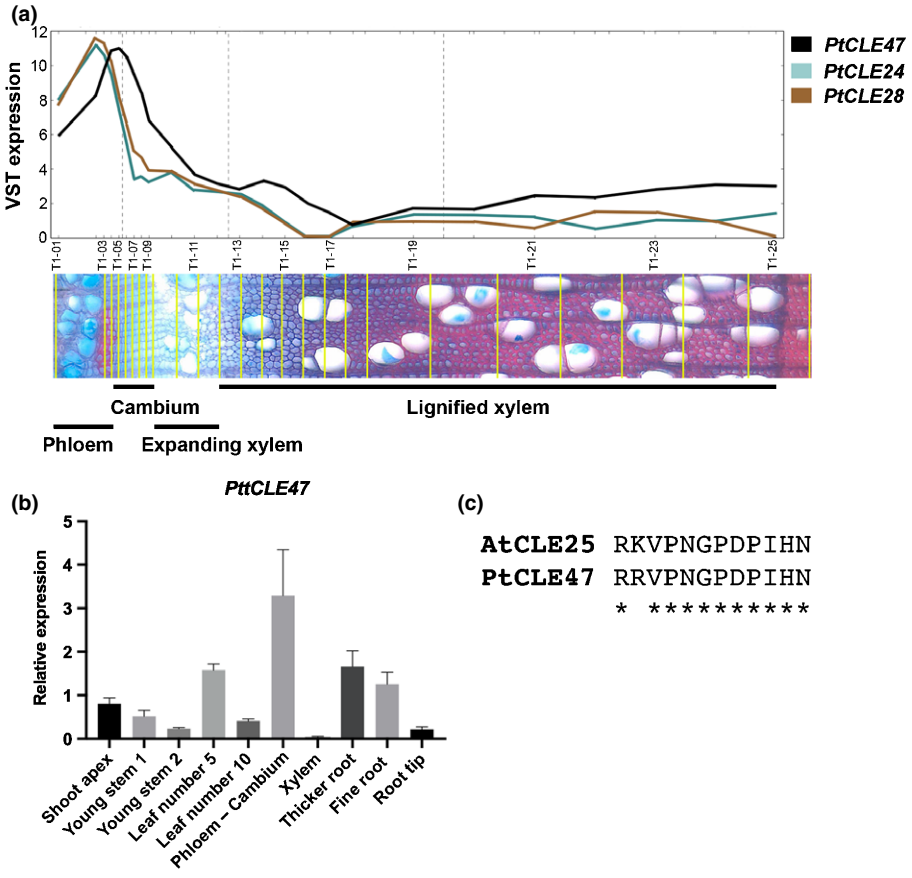

Fig. 1 *PttCLE47* is highly expressed in the cambial zone of *Populus*. (a) Smoothed and micrometre-scaled expression of *PtCLE47* (black), *PtCLE24* (turquoise), and *PtCLE28* (brown) as retrieved from the AspWood RNA-sequencing database (http://aspwood.popgenie.org). The *y*-axis shows average variance-stabilizing transformation (VST) expression, and the *x*-axis shows tangential samples over the wood-forming zone (T1-*nn*). Secondary phloem, cambium, expanding xylem, and lignified xylem are marked. (b) Relative transcript abundance of *PttCLE47*. Expression levels were quantified via quantitative PCR in different tissues of hybrid aspen and normalized against *UBQ* reference gene expression. Average expression of *PttCLE47* in all samples is equalized to one. Values are means ± SE ($n = 3$). (c) Sequence alignment of AtCLE25 and PtCLE47 peptides. Asterisks show conserved amino acid sequences. At, *Arabidopsis thaliana*; Pt, *Populus trichocarpa*.

via qPCR analysis in different tissues of hybrid aspen trees, including shoot apices, leaves, stems, phloem-cambium and xylem tissues, as well as roots (Fig. 1b). *PttCLE47* transcript levels were most abundant in the phloem-cambium samples, which is in agreement with the RNA-sequencing data (Fig. 1a), suggesting that *PttCLE47* may be involved in the regulation of vascular development in hybrid aspen. A lower but detectable *PttCLE47* expression was also observed in shoot apices, young leaves, young stems, and roots (Fig. 1b).

Previous phylogenetic analyses (Han *et al.*, 2016; Zhu *et al.*, 2019) indicated that the *PtCLE47* gene encodes a small A-type peptide (subtype III) similar to *Arabidopsis* CLE25 (Fig. 1c). A-type peptides include CLV3 and similar peptides based on sequence and functional similarity, whereas B-type peptides contain only TDIF/CLE41/CLE44-like peptides (Whitford *et al.*, 2008). *CLE25* is expressed in the vascular tissues of the shoot, root, and young leaf primordia in *Arabidopsis* (Jun *et al.*, 2010; Takahashi *et al.*, 2018), consistent with our findings. Interestingly, though, when investigated in detail, *CLE25* expression was found to follow the development of the phloem cell lineage during vascular development (Ren *et al.*, 2019). Moreover, knockout mutants of *cle25* cause delayed protophloem differentiation in the primary roots of *Arabidopsis*, which suggests that *CLE25* mainly regulates phloem formation. Though the expression of *PtCLE47* peaks over the vascular cambium (Fig. 1a), its closest homologues *PtCLE24* and *PtCLE28* (Han *et al.*, 2016) display slightly shifted expression patterns towards the differentiating phloem in trees (Fig. 1a). Therefore, whereas the *PtCLE24* and *PtCLE28* genes may represent the functional orthologues of the *CLE25* gene in *Populus*, the *PtCLE47* gene may have acquired a different function during *Populus* tree evolution.

Silencing of *PttCLE47* affects apical/lateral growth, and leaf size in hybrid aspen

To investigate the function of *PttCLE47* in trees, we generated transgenic hybrid aspen trees with RNAi-mediated gene-silencing of *PttCLE47* gene expression (Figs 2, S1). A total of 14 transgenic lines were generated, and four lines (lines 1, 4, 8, and 13) with reduced expression of *PttCLE47* compared with WT trees were chosen for further analysis (Fig. 2a). qPCR analysis of *PttCLE47* transcript levels in stems of these four lines displayed that the expression of *PttCLE47* was reduced to 7–17% of the WT levels. In *Populus*, *PtCLE28* and *PtCLE24* genes display high sequence similarity to *PtCLE47* (Han *et al.*, 2016). Expressions of these two genes were affected only minimally in the *PttCLE47 RNAi* plants (Fig. S2a,b), suggesting that the *PttCLE47 RNAi* construct is specific and does not target similar genes.

The *PttCLE47 RNAi* lines and corresponding WT trees were propagated from *in vitro* cuttings and grown on soil under glasshouse conditions. After 10 wk of growth, transgenic trees displayed a small but significant reduction in height growth (Figs 2b,c, S1a,b), and had thinner trunks (Figs 2d, S1c) in

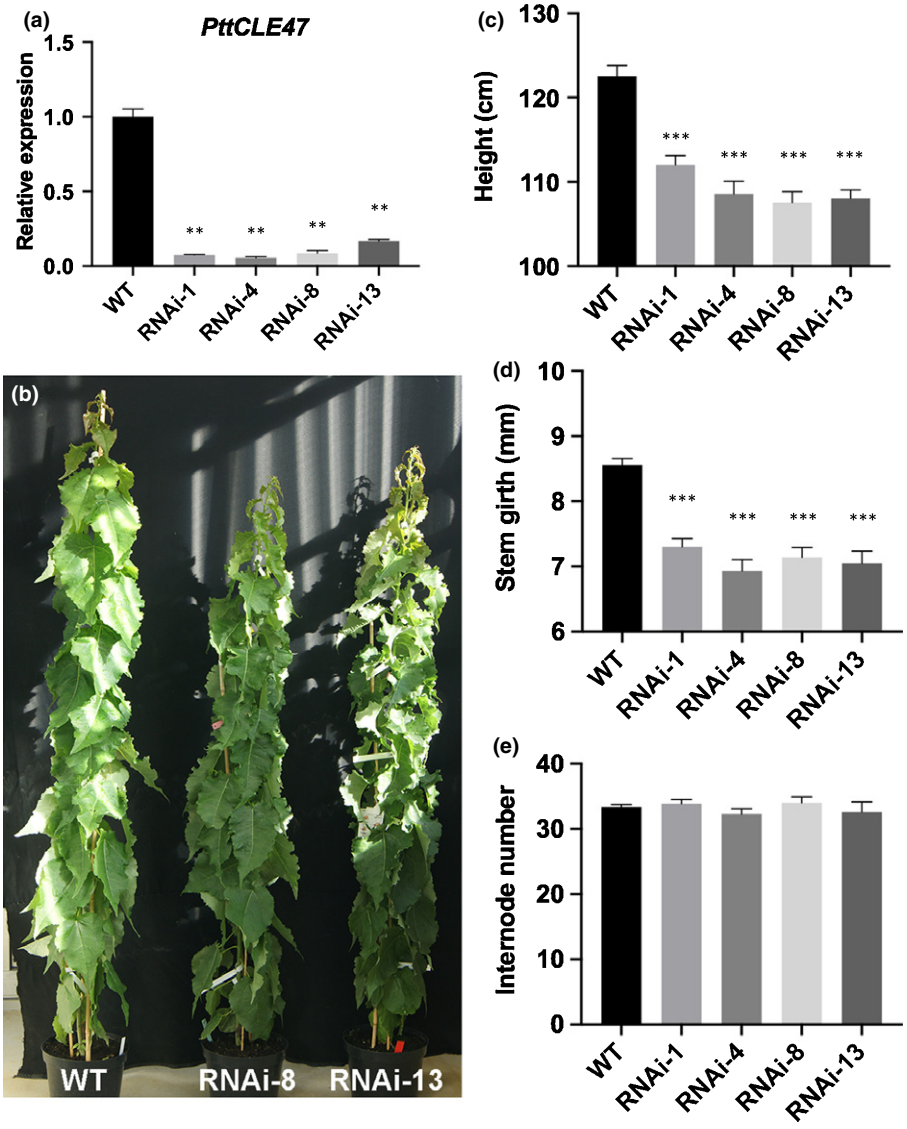

Fig. 2 *PttCLE47 RNA interference* (*RNAi*) transgenic hybrid aspen trees display reduced growth. (a) *RNAi*-mediated downregulation levels of the *PttCLE47* gene in transgenic hybrid aspen plants. Relative transcript abundance of *PttCLE47* was quantified via quantitative PCR and normalized against geometric means of the expression of four reference genes (*18S*, *UBQ*, *50S*, and *Tip41-like*). Values are means ± SE ($n = 3$). (b, c) *PttCLE47 RNAi* trees are shorter in comparison with wild-type (WT) plants: (b) WT (left) and *PttCLE47 RNAi* trees (right); (c) plant height for 10-wk-old plants. (d) *PttCLE47 RNAi* trees are thinner in comparison with WT plants. Stem width for 10-wk-old plants. (e) Internode numbers for 10-wk-old plants. Values are means ± SE ($n = 7$–10 for *PttCLE47 RNAi* plants, $n = 6$ for WT). Statistical significances are determined using an unpaired parametric *t*-test with Welch's correction. *t*-test significance for different means: **, $P < 0.01$; ***, $P < 0.001$.

comparison with WT trees. However, internode numbers of *PttCLE47 RNAi* plants remained unchanged (Figs 2e, S1d), suggesting that the difference in height growth could be related to the differences in internode lengths rather than the initiation rate of leaves at the shoot apex. Taken together, these results suggest that *PttCLE47* promotes both apical and lateral growth in hybrid aspen.

We also observed a difference between the sizes of the *PttCLE47* RNAi and WT leaves (Fig. 3a). The decrease in the leaf size of the *PttCLE47 RNAi* trees was apparent both in young expanding leaves (leaf number 7) and fully grown leaves (leaf number 21), resulting in *c.* 40–50% reductions in final leaf size (Fig. 3b). Although this aspect has not been investigated further, it is worth mentioning that the shape of the *PttCLE47 RNAi* leaves was also slightly different than WT leaves, where *RNAi* leaves displayed more lobes in comparison. In accordance with a role in controlling leaf growth and shape, *PttCLE47* is also expressed in the young expanding leaves (Fig. 1b).

Reduced lateral growth of *PttCLE47 RNAi* trees is due to local effects in the stem

Even though our phenotyping results suggested that *PttCLE47 RNAi* and WT plants display similar shoot apical meristem activities (i.e. initiation rate of leaves at the shoot apex each week were not changed and, as a result, WT and *RNAi* plants display similar numbers of internodes; Figs 2e, S1d), leaves of the transgenic trees were smaller than those of WT trees (Fig. 3). This may have an effect on the total photosynthetic area of *RNAi* trees and, in turn, reduce the amount of carbon available for growth and wood formation. Therefore, to exclude the effects of reduced leaf area on the radial growth of *PttCLE47 RNAi* trees, we performed a reciprocal grafting experiment (Figs 4a, S3a). For this purpose, one transgenic line (line 8) was reciprocally grafted to WT, and lateral thickening of stocks below the graft junctions was monitored. At 8 wk after grafting, as expected, stocks of WT/WT (scion/stock) were still significantly thicker than stocks of *RNAi*/*RNAi* (Fig. 4a). This is consistent with the thin-stemmed

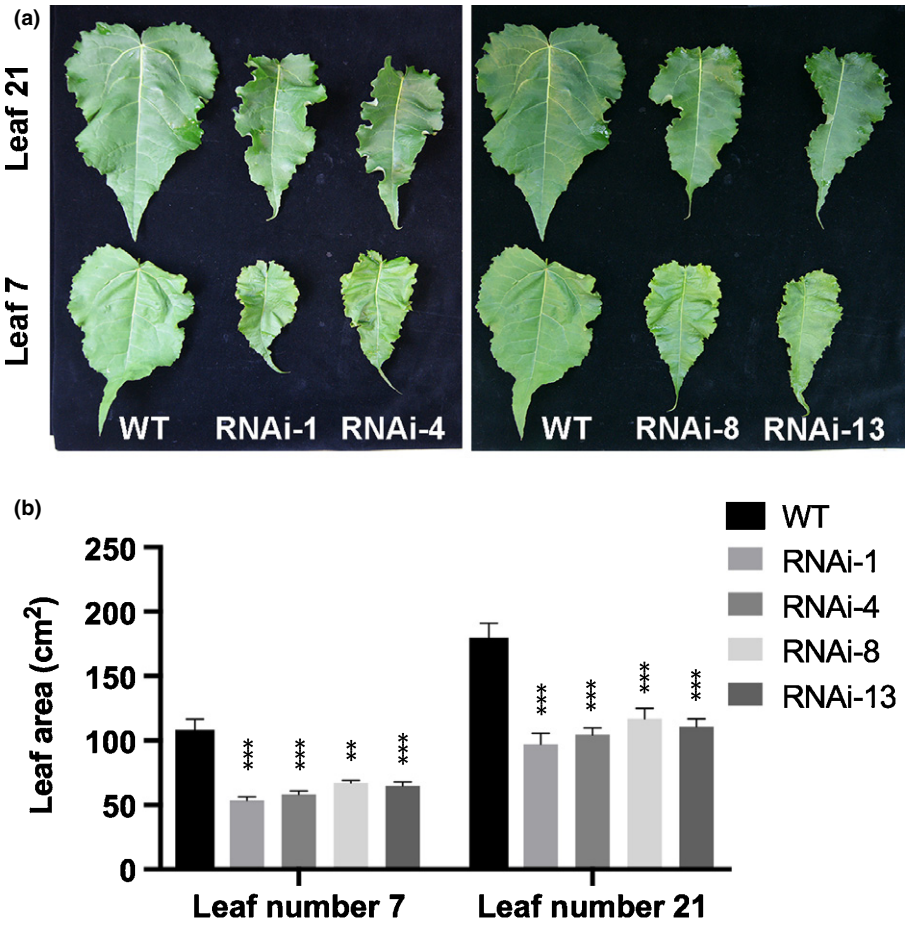

Fig. 3 *PttCLE47 RNA interference* (*RNAi*) transgenic hybrid aspen trees display reduced leaf size. (a) Leaves from wild-type (WT; left) and *PttCLE47 RNAi* trees (right). (b) Leaf areas from WT and transgenic plants. Values are means ± SE ($n = 7$–8 for *PttCLE47 RNAi* plants, $n = 12$ for WT plants). Statistical significances are determined using the Holm–Sidak multiple comparisons *t*-test, without assuming consistent SD. *t*-test significance for different means: **, $P < 0.01$; ***, $P < 0.001$.

phenotype of the ungrafted *PttCLE47 RNAi* plants (Figs 2d, S1c). WT/*RNAi* stocks also displayed compromised stem thickening in comparison with WT/WT stocks, suggesting that WT scions could only partially rescue the thin-stemmed phenotype of *RNAi* stocks. Thus, our data showed that reduced *PttCLE47* activity during secondary growth rather than reduced leaf size is the major cause of the thin-stemmed phenotype of the mutant trees. Interestingly, *RNAi*/WT stocks were also thinner than WT/WT stocks after this growth period, which may be caused by the systemic spread of the *RNAi* signals across the graft junction from transgenic scions to nontransgenic stocks (Palauqui *et al.*, 1997; Voinnet & Baulcombe, 1997; Han & Grierson, 2008). This hypothesis was supported by the fact that expression of *PttCLE47* was downregulated in stocks of *RNAi*/WT plants compared with stocks of WT/WT plants (Fig. S3b).

Downregulation of *PttCLE47* causes reduced wood formation in hybrid aspen

Next, we investigated the effects of *PttCLE47* downregulation on vascular development by histological comparison of cross-sections from stems of *PttCLE47 RNAi* and WT trees (Fig. 4). Our anatomical observations (Fig. 4d) and measurements of the secondary vascular tissues (Fig. 4b) revealed that, in comparison with the WT stems of similar age (18-d-old internodes, denoted as WT-18D), stems of the transgenic trees (18-d-old internodes, denoted as RNAi-18D) exhibited a narrower secondary xylem zone (Fig. 4b,d) with less xylem cells per radial cell file (Fig. 4c). The secondary xylem size of the transgenic trees (18-d-old internodes, denoted as RNAi-18D) was similar to that of WT trees with an earlier developmental stage (11-d-old internodes, denoted as WT-11D; Fig. 4b,d). On the other hand, the size of the secondary phloem in *PttCLE47 RNAi* trees remained unaffected (Fig. 4b,d). Interestingly, the development of the cortex region in the transgenic trees was also impeded, which contributed to the thin-stemmed phenotype (denoted as bark; Fig. 4b). These results suggest that *PttCLE47* acts mainly as a promoter of wood formation in hybrid aspen.

Downregulation of *PttCLE47* causes reduced cambial activity in hybrid aspen

As the number of new xylem and phloem cells is determined by the periclinal divisions of the cambial cells, we next studied the structure of the cambial zone in the stems of *PttCLE47 RNAi* and WT trees (Fig. 5). The cambial cells, localized between the differentiating xylem and phloem cells, can be observed as flat, thin-walled files of cells. Our data demonstrated that, in the *PttCLE47 RNAi* trees, the vascular cambium consists of fewer cell layers than in WT trees of similar age (Fig. 5a,b). Altogether,

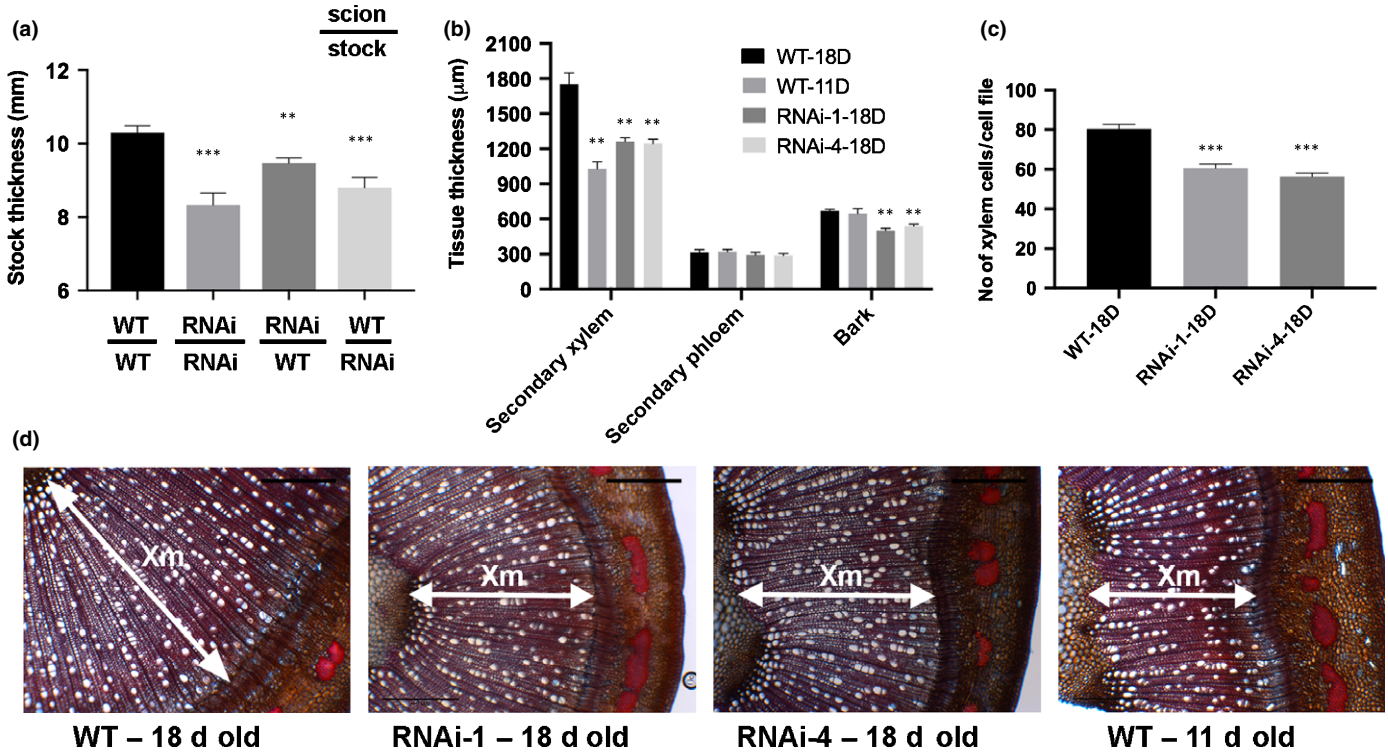

Fig. 4 *PttCLE47 RNA interference* (*RNAi*) transgenic hybrid aspen trees display reduced secondary xylem development. (a) Reciprocal grafts between wild-type (WT) and *PttCLE47 RNAi* trees (line 8). Graph shows the stock width 8 wk after the graft junction was formed. The WT scion only partially complements the *RNAi* stocks. Values are means ± SE ($n = 7$–11). (b) Total widths of the secondary xylem, secondary phloem, and bark. Measurements were taken from the 18-d-old or 11-d-old internodes (from the shoot tips) Values are means ± SE ($n = 3$). (c) Number of xylem cells/cell file in 18-d-old internodes from WT and *PttCLE47 RNAi* plants. Values are means ± SE ($n = 9$, three biological replicates/line). (d) Anatomical comparison between WT and *PttCLE47 RNAi* plants. Cross-sections were taken from the 18-d-old internodes of the *PttCLE47 RNAi* plants and corresponding WT plants (18-d-old stem segments displaying similar developmental age, 11-d-old stem segments displaying similar developmental stage/size). Double-sided arrows (white) indicate secondary xylem regions. Bars, 500 µm. Statistical significances are determined using an unpaired parametric t-test with Welch's correction for (a, c) and the Holm–Sidak multiple comparisons *t*-test, without assuming consistent SD, for (b). *t*-test significance for different means: **, $P < 0.01$; ***, $P < 0.001$. 18D, 18 d old; 11D, 11 d old; Xm, secondary xylem.

these results indicate that *PttCLE47* is a positive regulator of cambial activity in hybrid aspen, and mainly promotes the secondary xylem production.

To further characterize the molecular function of *PttCLE47*, we studied the expression of important cambial regulator genes in the stems of *PttCLE47 RNAi* and WT trees (Fig. 5c). The expression levels of the *class III homeodomain-leucine zipper* (*HD-Zip III*) transcription factor genes *PttHB4* (Zhu *et al.*, 2018) and *PttHB7* (Zhu *et al.*, 2013) was dramatically reduced in the stems of *RNAi* lines, both with respect to similar age and developmental stage of WT stems (Fig. 5c). Additionally, expression of *Populus WUSCHEL-RELATED HOMEOBOX4*-like genes (*PttWOX4a/b*; Kucukoglu *et al.*, 2017), which are positive regulators of cambial activity and identity in *Populus*, and their upstream regulator *PttPXYa* (Etchells *et al.*, 2015; Kucukoglu *et al.*, 2017), were also reduced in the stems of transgenic plants (Fig. 5c). Thus, these results suggest that *PttCLE47* could act upstream of these *HD-Zip IIIs* and the *PXY-WOX4* module in hybrid aspen.

Discussion

Previous work on the roles of CLE peptides has been mainly limited to *Arabidopsis*, and only little is known about their functions in different plant species, including trees. This motivated us to investigate the role of *PttCLE47*, a putative peptide-encoding gene, in hybrid aspen. We showed that *PttCLE47* is predominantly expressed in the vascular tissues, particularly in the vascular cambium of trees. To analyse the function of the *PttCLE47* gene, we generated knockdown trees with decreased expression of the *PttCLE47* gene. Repression of this gene led to many defects in the mutant plants, including reduced apical growth and leaf size and impaired radial growth. Through grafting, we showed that reduction in the leaf size of the transgenic plants cannot explain the decrease in the lateral growth. A detailed anatomical examination revealed that *RNAi* plants had reduced secondary xylem formation because the vascular cambium of these plants contains fewer cell layers than that of WT. Altogether, our results demonstrated that *PttCLE47* gene is a major positive regulator of cambial activity and secondary xylem formation in hybrid aspen. Notably, the action of *PttCLE47*, likely through the active PttCLE47 peptide, seems to be cell autonomous for the vascular cambium, functioning locally at the site of expression. Although most CLE peptides act noncell autonomously – that is, CLV3 (Clark *et al.*, 1995; Brand *et al.*, 2000; Schoof *et al.*, 2000), CLE40 (Stahl & Simon, 2009; Stahl *et al.*, 2009), and TDIF/CLE41/CLE44 (Ito *et al.*, 2006; Hirakawa *et al.*, 2008; Ohyama

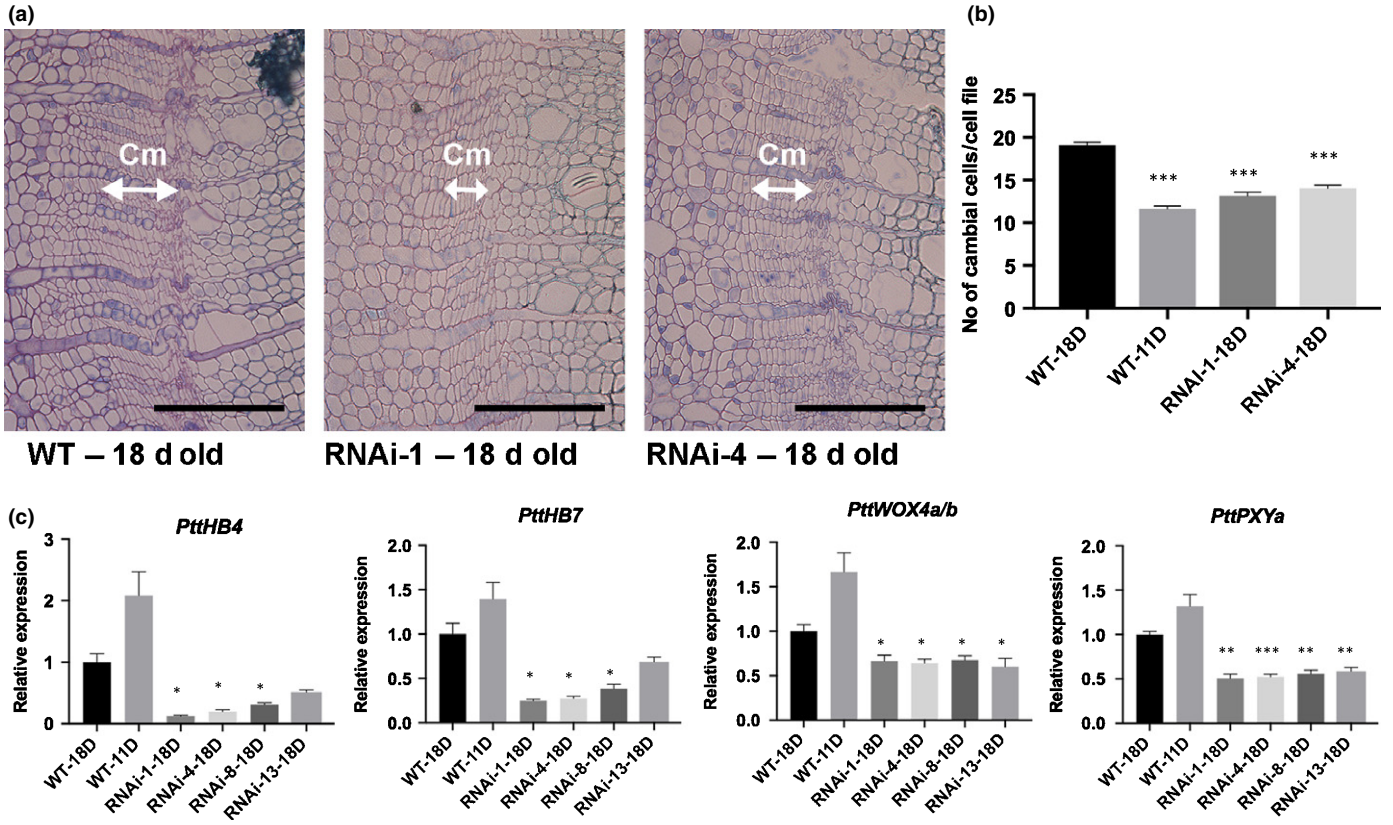

Fig. 5 Reduced secondary xylem size results from the reduced cambial activity in *PttCLE47 RNA interference* (*RNAi*) transgenic hybrid aspen trees. (a) Anatomical comparison between cambiums of wild-type (WT) and *PttCLE47 RNAi* plants. Cross-sections were taken from 18-d-old transgenic stems and corresponding WT plants (18-d-old stem segments displaying similar developmental age). Bars, 100 μm. Double-sided arrows (white) indicate cambial region. (b) Number of cambium cells/cell file in 18-d-old internodes from WT and *PttCLE47 RNAi* plants. Values are means ± SE ($n = 30$, three biological replicates/line). (c) Cambial regulator genes are downregulated in *PttCLE47 RNAi* lines. Relative transcript abundance of *PttHB4*, *PttHB7*, *PttWOX4a/b*, and *PttPXYa* were quantified via quantitative PCR and normalized against geometric means of the expression of four reference genes (*18S*, *UBQ*, *50S*, and *Tip41-like*). Values are means ± SE ($n = 3$). Statistical significances are determined using an unpaired parametric *t*-test with Welch's correction against WT-18D. *t*-test significance for different means: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Cm, vascular cambium; 18D, 18 d old; 11D, 11 d old.

et al., 2008; Etchells & Turner, 2010) – it has also previously been observed that both the ligand CLE45 and the receptor BARELY ANY MERISTEM 3 are active in the same tissue in the primary roots to control protophloem development (Rodriguez-Villalon *et al.*, 2014). Therefore, a similar mechanism may be active here as well. Notably, in the *PttCLE47 RNAi* trees, size of the cortex region was also slightly reduced compared with that of WT trees (Fig. 4b), which was partly responsible for the thin-stemmed phenotype. Therefore, it will be interesting in the future to study the expression of *PttCLE47* in the cortex to

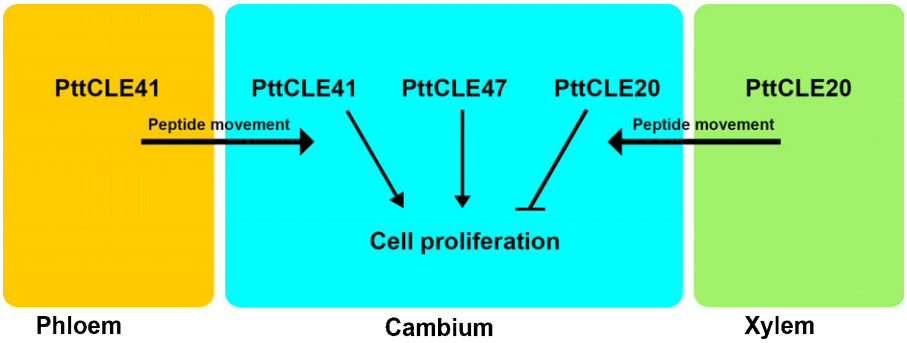

Fig. 6 Model for regulation of cambial activity through CLAVATA3 (CLV3)/EMBRYO SURROUNDING REGION (ESR)-RELATED (CLE) peptides in *Populus*. Phloem produced PttCLE41, and cambial-expressed PttCLE47 regulates cambial activity (cambial cell proliferation) positively in *Populus*. Xylem-derived PttCLE20 inhibits cambial activity negatively in *Populus*. Secondary phloem is indicated in orange, vascular cambium is indicated in blue, and secondary xylem is indicated in green. Arrows show peptide movement and positive or negative function in regulating cambial cell proliferation.

determine if PttCLE47 peptide functions locally in this region as it does in the vascular cambium, or if it works noncell autonomously from the vascular cambium to affect the cortex development.

Previous phylogenetic analysis revealed that *PtCLE47* together with its homologous genes *PtCLE24* and *PtCLE28* groups with *Arabidopsis CLE25* gene (Han *et al.*, 2016). *CLE25* mainly regulates phloem formation in *Arabidopsis* and is expressed in the phloem cell lineage of stems and roots (Ren *et al.*, 2019). Interestingly, our results showed that, in comparison with developing phloem-expressed *PtCLE24* and *PtCLE28*, expression of *PtCLE47* is diverged and highest in the vascular cambium of trees. This points to the fact that true orthologues of *CLE25* in poplar are *PtCLE24* and *PtCLE28* genes. Analysis of the *Populus* genome assembly provided evidence for two whole genome duplication events: one as a result of the salicoid event roughly 65 Ma, and an older eurosid duplication event, which coincided with the divergence of *Populus* and *Arabidopsis* lineages (Tuskan *et al.*, 2006). These duplication events may have contributed to the expansion of the *CLE25* gene clade in poplar, subsequent changes in gene expression patterns between *PtCLE47* vs *PtCLE24* and *PtCLE28*, and rewiring of the gene regulatory networks downstream of these genes. As a result, this may have caused the functional differentiation of *PtCLE47* during evolution.

Previous studies using *Populus* as a model system for wood formation identified some important regulators of cambial development, including *PtrHB4* (Zhu *et al.*, 2018), *PtrHB7* (Zhu *et al.*, 2013), *PttWOX4a/b* (Kucukoglu *et al.*, 2017), and *PttPXYa* (Etchells *et al.*, 2015). Hence, we investigated the expressions of these key players in our mutant trees. *PtrHB4* encodes an *HD-Zip III* transcription factor similar to *PHABULOSA* and *PHAVOLUTA* in *Arabidopsis* (Zhu *et al.*, 2018). In Arabidopsis, *HD-Zip III*s have recently been shown to position and maintain cambial stem cells (Smetana *et al.*, 2019). In poplar, *HD-Zip III* transcription factors are required for cambium development (Robischon *et al.*, 2011; Zhu *et al.*, 2013, 2018). In particular, *PtrHB4* is critical for the formation of a closed vascular cambium ring from fascicular and interfascicular cambium (Zhu *et al.*, 2018). Additionally, *PtrHB7*, a homologue of *AtHB8*, controls a balanced differentiation between secondary xylem and secondary phloem tissues during lateral growth in *Populus*, such that *PtrHB7*-silenced plants display a reduction in xylem but increase in phloem size (Zhu *et al.*, 2013). Consistent with our mutant phenotypes, the expressions of both *PttHB7* and *PttHB4* are downregulated in the stems of *RNAi* trees, both in comparison with WT stems at the same developmental stage or at the same age. *PttWOX4a/b* and *PttPXYa* genes, which are both key regulators of cambial activity and identity in *Populus* (Etchells *et al.*, 2015; Kucukoglu *et al.*, 2017), also showed a modest but significant downregulation in the stems of mutant trees. Taken together, these results indicate that *PttCLE47* might be part of a complex network of regulators of cambium activity and secondary growth.

Finally, it appears that there are many CLE peptides that affect cambial activity from different sides of the cambium, including *PttCLE41* on the phloem side (Etchells *et al.*, 2015; Kucukoglu *et al.*, 2017), *PttCLE20* on the xylem side (Zhu *et al.*, 2019), and *PttCLE47* in the cambial zone itself (Fig. 6). Previously, it was shown that, by overexpressing *PttCLE41* and its receptor *PttPXY* in their original expression domains, one can significantly improve woody biomass production. Hence, our findings on the proliferative role of another CLE peptide in regulating cambial development and radial stem growth may contribute to the development of more efficient plant biomass production systems if/when used together with their signalling receptors. However, to achieve this, a more detailed characterization is required to elucidate the exact mechanism of how *PttCLE47* controls cambial development in *Populus*.

Acknowledgements

We thank Katja Kainulainen, Marie Nygren, Marta Ibañez Lazaro, and Veronica Bourquin for excellent technical assistance. This research was supported by grants from the Swedish Research Council Formas, the Swedish Foundation for Strategic Research (SSF), and the Swedish Governmental Agency for Innovation Systems (Vinnova) to ON, and from the Academy of Finland to YH, APM and MK (postdoctoral researcher, no. 326036).

Author contributions

ON and MK designed the research. MK and SC performed the experiments, collected and analysed the data, and together with ON interpreted the results. MK, SC, BZ, APM, YH and ON co-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.

Fig. S1 *PttCLE47 RNAi* transgenic hybrid aspen trees display reduced growth (Extended graphs).

Fig. S2 Relative expression of *PttCLE24* and *PttCLE28* in *PttCLE47 RNAi* transgenic hybrid aspen lines.

Fig. S3 Reciprocal grafts between WT and *PttCLE47 RNAi* transgenic hybrid aspen trees.

Table S1 Primers used in this study.

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