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Thermospermine levels are controlled by an auxin-dependent feedback loop mechanism in *Populus* xylem

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SUMMARY

Polyamines are small polycationic amines that are widespread in living organisms. Thermospermine, synthesized by thermospermine synthase ACAULIS5 (ACL5), was recently shown to be an endogenous plant polyamine. Thermospermine is critical for proper vascular development and xylem cell specification, but it is not known how thermospermine homeostasis is controlled in the xylem. We present data in the *Populus* model system supporting the existence of a negative feedback control of thermospermine levels in stem xylem tissues, the main site of thermospermine biosynthesis. While over-expression of the *ACL5* homologue in *Populus*, *POPACAULIS5*, resulted in strong up-regulation of *ACL5* expression and thermospermine accumulation in leaves, the corresponding levels in the secondary xylem tissues of the stem were similar or lower than those in the wild-type. *POPACAULIS5* over-expression had a negative effect on accumulation of indole-3-acetic acid, while exogenous auxin had a positive effect on *POPACAULIS5* expression, thus promoting thermospermine accumulation. Further, over-expression of *POPACAULIS5* negatively affected expression of the class III homeodomain leucine zipper (HD-Zip III) transcription factor gene *PttHB8*, a homologue of *AtHB8*, while up-regulation of *PttHB8* positively affected *POPACAULIS5* expression. These results indicate that excessive accumulation of thermospermine is prevented by a negative feedback control of *POPACAULIS5* transcript levels through suppression of indole-3-acetic acid levels, and that PttHB8 is involved in the control of *POPACAULIS5* expression. We propose that this negative feedback loop functions to maintain steady-state levels of thermospermine, which is required for proper xylem development, and that it is dependent on the presence of high concentrations of endogenous indole-3-acetic acid, such as those present in the secondary xylem tissues.

Keywords: *POPACAULIS5*, *ACAULIS5* (*ACL5*), class III homeodomain leucine zipper transcription factors (HD-Zip III), wood development, polyamine, *Populus tremula* × *Populus tremuloides*, *Populus trichocarpa*.

INTRODUCTION

Polyamines are essential organic polycationic amines that have been implicated in several processes in plants, such as biotic and abiotic stress responses (Alcázar *et al.*, 2006; Yamaguchi *et al.*, 2006; Kusano *et al.*, 2007; Naka *et al.*, 2010; Gonzalez *et al.*, 2011; Wang *et al.*, 2011; Sagor *et al.*, 2012), wound responses (Perez-Amador *et al.*, 2002), nitric oxide signaling (Flores *et al.*, 2008), fruit development (Nambeesan *et al.*, 2010; Trénor *et al.*, 2010) and stem growth and elongation (Hanzawa *et al.*, 2000; Alcázar *et al.*, 2005). The most common polyamines are the diamine putrescine, the triamine spermidine and the tetramines spermine and thermospermine. Putrescine is produced from ornithine by ornithine decarboxylase or from arginine by arginine decarboxylase. Spermidine and spermine

production is catalysed by aminopropyltransferases, which transfer an aminopropyl residue from the decarboxylated *S*-adenosylmethionine to an amine acceptor on putrescine or spermidine to produce triamines and tetraamines, respectively. Thermospermine is a structural isomer of spermine that was only recently identified in plants (Knott *et al.*, 2007; Naka *et al.*, 2010; Rambla *et al.*, 2010). It is synthesized by the thermospermine synthase ACAULIS5 (ACL5) (Knott *et al.*, 2007) that is expressed specifically in early developing xylem vessel elements (Muñiz *et al.*, 2008). Disruption of the function of ACL5 in Arabidopsis leads to plants with impaired stem elongation and thinner veins in leaves, as well as lack of secondary growth (Hanzawa *et al.*, 1997, 2000; Clay and Nelson, 2005; Kakehi *et al.*, 2008; Muñiz *et al.*, 2008).

The events downstream of *ACL5* expression have been subject of intensive study in recent years. At least two extragenic suppressors of the *acl5* mutation have been described. One of them disrupts an upstream open reading frame and enhances translation of a basic helix-loop-helix (bHLH) transcription factor encoded by *SUPPRESSOR OF ACAULIS51* (*SAC51*), and the second (*sac52-1d*) affects RPL10a, an important component of the large ribosomal subunit (Imai *et al.*, 2006, 2008). However, the upstream events that regulate *ACL5* expression are largely unknown. Class III homeodomain-leucine zipper transcription factors (HD-Zip III transcription factors) have been hypothesized to play a role in transcriptional control of *ACL5*, as both HD-Zip III transcription factors and *ACL5* have been implicated in control of metaxylem development (Muñiz *et al.*, 2008; Carlsbecker *et al.*, 2010). *AtHB8* is a particularly good candidate for control of *ACL5* as it is the HD-Zip III family member that shows highest transcriptional alterations in the *acl5* mutant background (Imai *et al.*, 2006). Another important factor is auxin, which is well known for its role in xylem development (Uggla *et al.*, 1996; Tuominen *et al.*, 1997; Nilsson *et al.*, 2008) as well as in transcriptional activation of both *ACL5* (Hanzawa *et al.*, 2000; Imai *et al.*, 2006; Rambla *et al.*, 2010) and *AtHB8* (Baima *et al.*, 1995). A model for thermospermine regulation of xylem differentiation involving auxin has been proposed, in which it was suggested that HD-Zip III transcription factors mark the procambial cells that are destined for xylem specification in an auxin-dependent manner, leading to up-regulation of *ACL5* and concomitant differentiation of xylem vessel elements (Vera-Sirera *et al.*, 2010; Takano *et al.*, 2012). However, the role of the HD-Zip III transcription factors in control of *ACL5* expression has not been studied in detail.

In the present study, we wished to elucidate the relationship between *ACL5*, auxin and HD-Zip III transcription factors. *Populus* trees were selected as the model system due to extensive development of xylem, which is the site of thermospermine production as well as auxin transport in plants. Tree stems therefore allow isolation of large amounts of tissues that are enriched in xylem elements transporting auxin and expressing *ACL5*. The *ACL5* orthologue, *POPACAULIS5*, was cloned from hybrid aspen (*Populus tremula* × *Populus tremuloides*) and black cottonwood (*Populus trichocarpa*), and thermospermine levels were altered in trees by manipulating the expression levels of *POPACAULIS5*. In addition, *PttHB8*, a hybrid aspen homologue of *AtHB8*, was over-expressed in hybrid aspen to investigate the effect on *POPACAULIS5* transcript levels. The results on expression levels of *POPACAULIS5* and *PttHB8*, thermospermine accumulation and indole-3-acetic acid (IAA) measurements in the transgenic trees support a novel regulatory mechanism, mediated through auxin and *PttHB8*, for maintenance of thermospermine homeostasis in secondary xylem tissues of the stem.

RESULTS

POPACAULIS5 is the *Populus ACAULIS5* orthologue

In Arabidopsis, *ACL5* encodes thermospermine synthase (Knott *et al.*, 2007). Our search in the *Populus* genome retrieved a putative *ACL5* sequence from *P. trichocarpa*, POPTR_0006s23880 (Phytozome *Populus* version 2; Goodstein *et al.*, 2012) with 86.1% identity to *ACL5*. Two other sequences were found in the *P. trichocarpa* genome, POPTR_0008s15120 and POPTR_0010s09940, which show higher similarity amongst themselves than to *ACL5* or to POPTR_0006s23880 (Figure 1a). BLAST searches of POPTR_0006s23880 against the *Populus* DB (http://www.populus.db.umu.se/; Sterky *et al.*, 2004) retrieved a 496 bp EST (GI:3853671, GenBank EST database) from *P. tremula* × *P. tremuloides*, with 95% identity over 99% of the sequence (Figure 1b). Alignment of ACL5 amino acid sequences from Arabidopsis, *Populus* and other land plants showed that POPTR_0006s23880, hereafter called *POPACAULIS5*, is the sequence most similar to *ACL5*.

The relationship of POPACAULIS5 with other known and predicted ACL5 sequences was inferred from a phylogenetic analysis (Figure 1a), in accordance with previously reported relationships amongst plant aminopropyltransferases (Minguet *et al.*, 2008; Rodriguez-Kessler *et al.*, 2010). The tree shows two major clusters, one identified as predicted thermospermine synthase proteins and the other as thermospermine synthase-like proteins (Figures 1a and S1). The POPACAULIS5 putative sequence from *P. trichocarpa* and *P. tremula* × *P. tremuloides* PttACL5 group within the former cluster, in which the Arabidopsis ACL5 sequence is also included, suggesting that this is the most likely candidate to be thermospermine synthase. We confirmed that POPACAULIS5 is a true orthologue of ACL5 by demonstrating its thermospermine synthase activity in yeast cells (Figure S2).

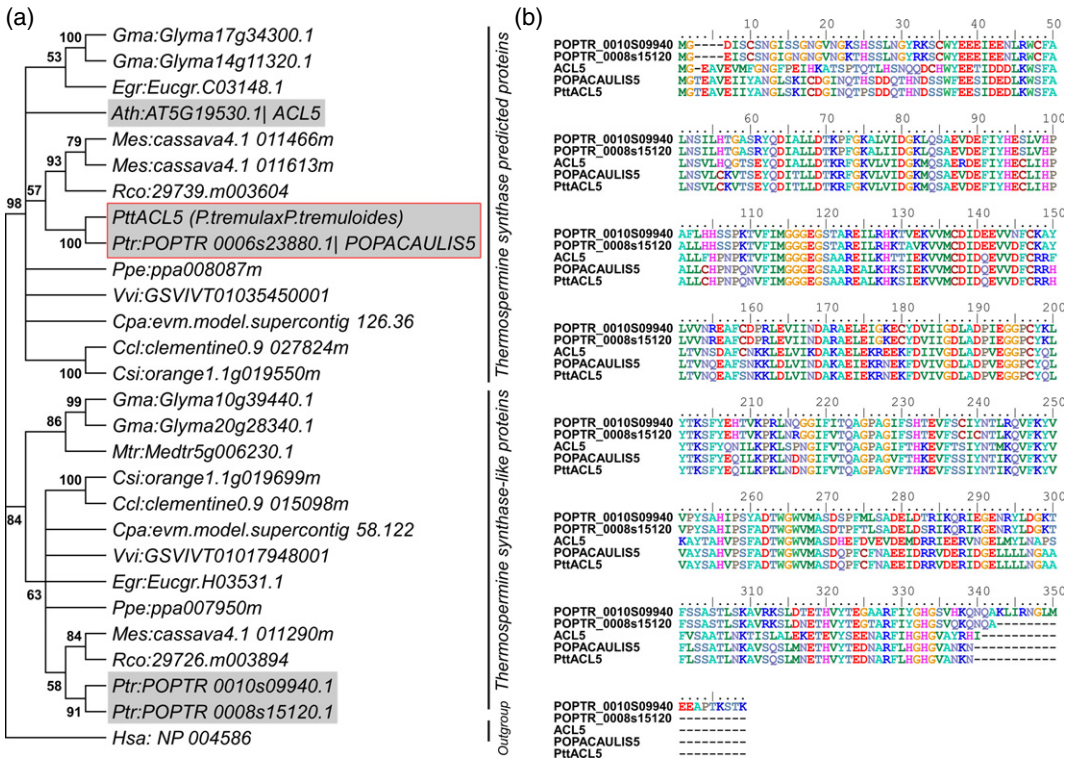

Figure 1. Phylogenetic and sequence analysis of ACAULIS5 from *Populus*, Arabidopsis and other taxa.
(a) Phylogenetic tree showing relationships among ACL5-predicted or ACL5-like amino acid sequences. ACAULIS5 from *Arabidopsis thaliana* (Ath; ACL5), *Populus trichocarpa* (Ptr; POPACAULIS5) and *P. tremula* × *P. tremuloides* (Ptt; PttACL5) were used together with putative thermospermine synthase proteins from other land flowering plants, including Mes (*Manihot esculenta*), Rco (*Ricinus communis*), Vvi (*Vitis vinifera*), Csi (*Citrus sinensis*), Ccl (*Citrus clementina*), Ppe (*Prunus persica*), Cpa (*Carica papaya*), Egr (*Eucalyptus grandis*), Mtr (*Medicago truncatula*) and Gma (*Glycine max*). The spermine synthase sequence from Hsa (*Homo sapiens*) was used as an out-group. The sequences and corresponding ID are shown in Figure S1. The numbers at the branches represent bootstrap values (Felsenstein, 1985).
(b) Alignment of the amino acid sequences of Arabidopsis ACL5, *Populus trichocarpa* POPACAULIS5 (POPTR_0006s23880) and ACL5-like proteins POPTR_0008s15120 and POPTR_0010s09940, and *P. tremula* × *P. tremuloides* PttACL5 (GenBank accession number JX444689).

35S::*POPACAULIS5* trees show reduced overall growth and slight defects in xylem development

We isolated and cloned *POPACAULIS5* cDNA from *P. trichocarpa* and *P. tremula* × *P. tremuloides* (*PttACL5*) for over-expression under the control of the 35S CaMV constitutive promoter. Transformation of hybrid aspen allowed recovery of 90 kanamycin-resistant transgenic lines for 35S::*POPACAULIS5*, and 39 kanamycin-resistant transgenic lines for 35S::*PttACL5*. Ectopic expression of *POPACAULIS5* from both constructs resulted in the same dramatic changes in shoot and root development, and the transgenic lines obtained were grouped according to the severity of observed phenotypes (Figures 2a and S3a–f). Dwarf transgenic plants from group B (Figure 2a) were able to develop a rooting system and showed partial restoration of the wild-type phenotype once transferred to a plant growth regulator-free medium (Figure 2b). For this reason, we selected the transgenic lines 35S::*POPACAULIS5*-B2, B4, B13, B14 and B15 from group B for further analysis of the effects of *POPACAULIS5* over-expression in trees (Figure 2c).

Tree height was significantly reduced in 35S::*POPACAULIS5* trees grown in the greenhouse for a period of 2 months (Figure 2c,d). In addition, the diameter and internode length of the mature stem as well as the leaf size were smaller in the transgenic plants than in the wild-type (Figure 2e–g). Strong phenotypic variation was observed between independent transgenic lines (Figures 2c and S4a) and even in individual trees from the same transgenic line. Maturation of secondary xylem was screened along the stem by searching for the youngest internode with fully lignified xylem fibres, detected by the appearance of highly autofluorescing tissues in the secondary xylem. In wild-type, fully lignified secondary xylem fibres were found in the 25th internode. Xylem maturation was delayed in the transgenic lines as fully lignified secondary xylem fibres were only observed in the 32nd internode in B2 and the 39th internode in B14, and were absent from the stem in lines B4 and B13. We performed further analyses at internode 45 from the top (the so-called reference internode; see Experimental procedures). Xylem anatomy and cell morphology were

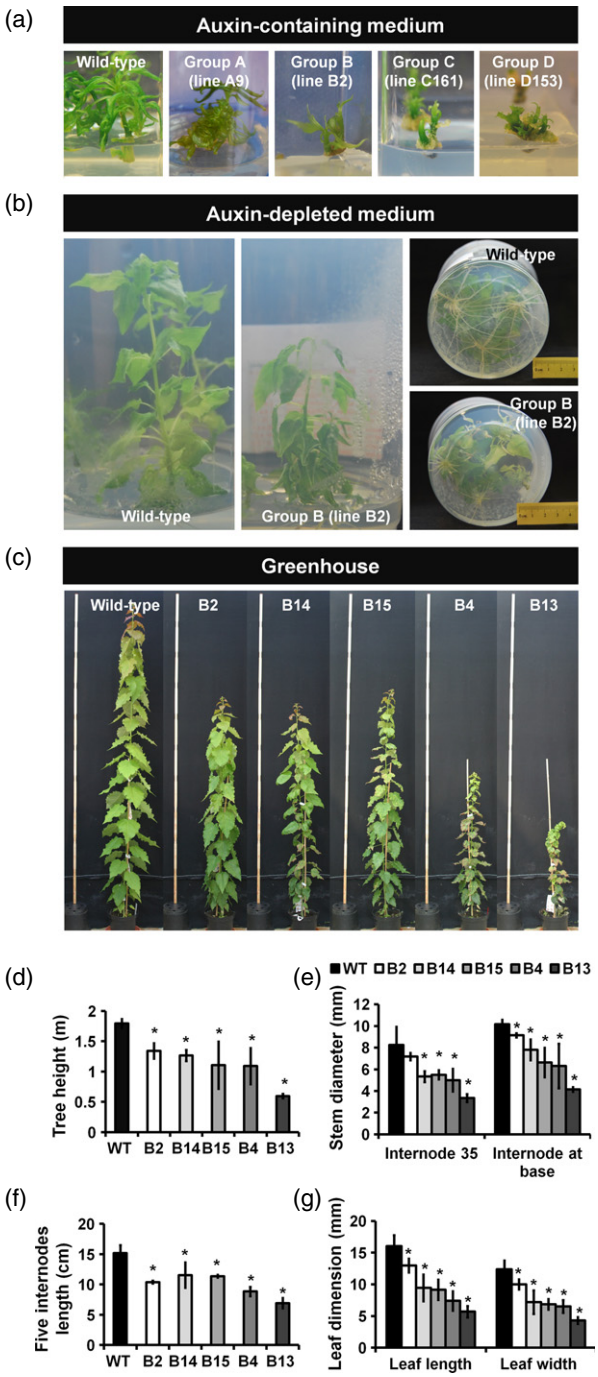

Figure 2. Phenotypic characterization of transgenic lines expressing 35S::*POPACAULIS5* in *Populus*.
(a) Effect of *POPACAULIS5* over-expression on hybrid aspen grown *in vitro* on auxin-containing medium. A9 is representative of transgenic lines with a mild phenotype closer to the wild-type (group A). B2 represents transgenic lines with a dwarf phenotype that have elongation and rooting defects when grown on auxin-containing medium, but that partially recover once transferred to auxin-free medium (group B). C161 and D153 represent transgenic lines with a dwarf phenotype that totally lack roots; these plants did not survive when transferred to auxin-free medium (groups C and D). The increasing severity of abnormal phenotypes found in C161 and D153 correlated well with the increased *POPACAULIS5* transcript level (Figure 4c). Dwarf plants were observed for transgenic lines recovered from six independent transformation assays using *ACL5* homologue cDNA from both *P. trichocarpa* (35S::*POPACAULIS5*) and *P. tremula* × *P. tremuloides* (35S::*PttACL5*).
(b) Five-week-old *in vitro* grown 35S::*POPACAULIS5* line B2 showing the partially restored wild-type phenotype.
(c) Two-month old transgenic trees (from 35S::*POPACAULIS5* lines B2, B14, B15, B4 and B13).
(d–g) Comparison of height (d), stem diameter at internode 35 and at the stem base (e), the mean length of five internodes (f) and the mean dimensions of five fully expanded leaves around internode 35 (two above and three below) (g). Values are means ± SD of at least three biological replicates (each replicate sampled from one individual tree). Asterisks indicate significant differences compared with the wild-type ($P < 0.05$, Mann–Whitney *U* test). The greenhouse experiment was performed twice.

analysed at the reference internode from transgenic lines B2 and B14, which showed a intermediate phenotype in xylem maturation as well as the various growth parameters (Figures 3a and S4a,b). The absence of ACL5 function in the *acl5* mutants of Arabidopsis has a profound effect on xylem maturation, resulting in premature cell death of xylem elements and reduction in the complexity of secondary cell-wall patterning (Muñiz *et al.*, 2008). By nitroblue tetrazolium staining of stem transverse sections, we observed that the width of the living xylem zone in the reference internode of the transgenic lines was similar to that of the wild-type (Figure 3b). The shorter distance from pith to cambium together with the equal width of the xylem living zone observed in the transgenic lines indicate that cell death in xylem fibres may be slightly delayed in the transgenic lines compared to the wild-type (Figure 3b). No major alterations were observed in the size (Figure S5a–f) or cell-wall patterning of xylem elements in the transgenic tree stem samples collected from the reference internode (Figures S6a and S7). However, a slight increase in the proportion of the primary xylem vessel types was observed (Figure S6b,c). Altogether, the lack of major defects in secondary xylem development as a consequence of manipulating *POPACAULIS5* raised the question as to what the level of *POPACAULIS5* over-expression was, and whether thermospermine was over-produced in the secondary xylem tissues of the 35S::*POPACAULIS5* woody stems.

Thermospermine accumulation is suppressed in the 35S::*POPACAULIS5 Populus* woody stem, but not in leaves

The contents of the main polyamines putrescine, spermidine, spermine and thermospermine were measured in samples collected from leaves and scrapings of the living zone of the secondary xylem in the stem. Spermidine and spermine were highly abundant in all plant tissues, while putrescine and thermospermine were less abundant but still easily detectable (Figure 4a,b). As expected, the thermospermine content was higher in leaves of the transgenic lines compared to the wild-type. However, similar or even lower levels of thermospermine were observed in the

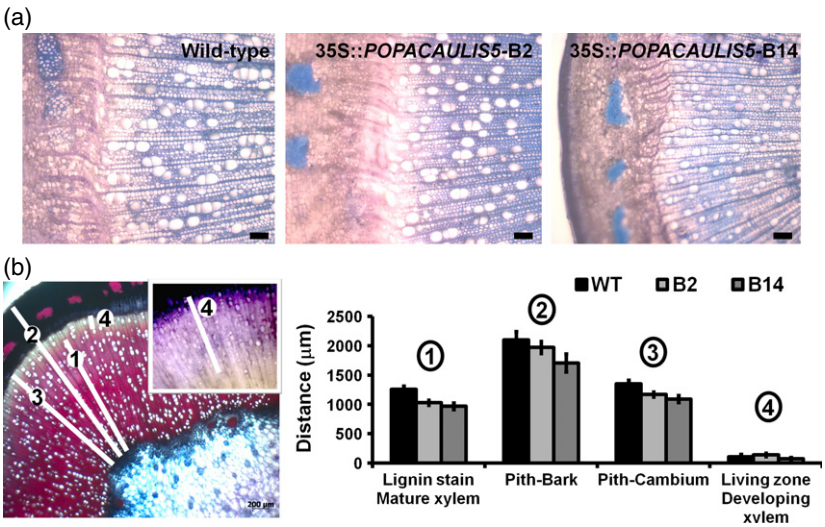

Figure 3. Anatomy of stem tissues and xylem development in transgenic *Populus* trees.
(a) Transverse sections taken from the reference internode of stems of wild-type and transgenic 35S::*POPACAULIS5* B2 and B14 trees and stained with toluidine blue O.
(b) Schematic representation of measurements taken from stem transverse sections. 1, width of the mature xylem (as observed by phloroglucinol staining); 2, distance from the outer bark to the pith; 3, distance from the vascular cambium to the pith; 4, width of living zone of the xylem on the basis of the nitroblue tetrazolium viability stain (inset). Scale bars = 100 μm. Values are means ± SD from six (wild-type) and four (B2 and B14) biological replicates. For each tree cross-section, the measurements were taken at four approximately equidistant positions around the circumference of the stem.

secondary xylem tissues collected from the same tree stems (Figure 4a). Similar results with higher levels of thermospermine in the leaves but not in the stem were obtained in plants grown *in vitro* on auxin-free medium (Figure 4b). It was also interesting to note that the levels of the other polyamines were not increased in the stem or xylem samples of the transgenic lines (Figure 4a,b), making it unlikely that the lack of thermospermine over-accumulation in these samples is due to back-conversion of thermospermine to spermidine or putrescine. A decrease in spermidine and spermine levels was observed in several samples (Figure 4b), which may be related to increased overall polyamine catabolism, as recently proposed in Arabidopsis 35S::*ACL5* plants (Marina *et al.*, 2013).

Expression of *POPACAULIS5* paralleled the changes in the thermospermine levels of the transgenic trees. Quantitative RT-PCR analysis of the same samples analysed for polyamine content showed approximately 40-fold and higher increases in *POPACAULIS5* expression in leaves of the transgenic trees, but the expression in xylem tissues was unaltered in lines B2, B13 and B4 and even reduced in lines B14 and B15, compared to the wild-type (Figure 4a). To exclude the possibility that this result was due to inactivity of the 35S promoter in the secondary xylem, we analysed transgenic trees carrying a 35S::*GUS*:*GFP* construct, and demonstrated by histochemical GUS assay that the 35S promoter is active in the secondary xylem tissues (Figure S8). The lack of *POPACAULIS5* over-expression in the secondary xylem explains the lack of major phenotypic changes in the xylem tissues of the transgenic trees, but also raises a question on what is the mechanism leading to suppression of *POPACAULIS5* transgene expression in xylem tissues but not in leaves. Interestingly, increased expression of *POPACAULIS5* was observed in the stem of plants grown *in vitro* on auxin-containing medium (Figures 2a and 4c), which led us to hypothesize that auxin levels somehow control accumulation of *POPACAULIS5* transcripts and question whether the transgenic 35S::*POPACAULIS5* trees had lower levels of auxin.

POPACAULIS5 over-expression suppresses endogenous auxin levels in the secondary xylem

IAA levels were measured in leaves and stems of *in vitro* grown plants and in leaves and secondary xylem tissues from greenhouse-grown trees. Plants grown *in vitro* showed similar IAA levels in leaves of wild-type and transgenic lines, but a decrease was found in the young stems compared to the wild-type (Figure 5a). In greenhouse-grown trees, IAA levels were strongly reduced in the leaves and in the secondary xylem tissues of the transgenic trees (Figure 5b). This was even more pronounced in lines with more severe reduction in growth. The low levels of auxin and the lack of *POPACAULIS5* over-expression in xylem tissues of the transgenic 35S::*POPACAULIS5* plants is suggestive of a negative feedback mechanism whereby increased *POPACAULIS5* expression functions to reduce IAA levels, which in turn prevents further expression of *POPACAULIS5*. The reduction in the IAA levels in the 35S::*POPACAULIS5* leaves (Figure 5b) most probably reflects functioning of the feedback mechanism in the leaf vasculature, which is the main site of auxin accumulation in this organ (Ljung *et al.*, 2001; Teichmann *et al.*, 2008; Petrásek and Friml, 2009).

We tested our hypothesis by studying whether exogenous auxin affects expression of *POPACAULIS5*. Auxin levels were modulated in young stem tissues of wild-type and two transgenic lines, B2 and B15, by exogenous application of indolebutyric acid (IBA) (Figure 6a–c). Stem pieces were first depleted of auxin for 16 h. As expected,

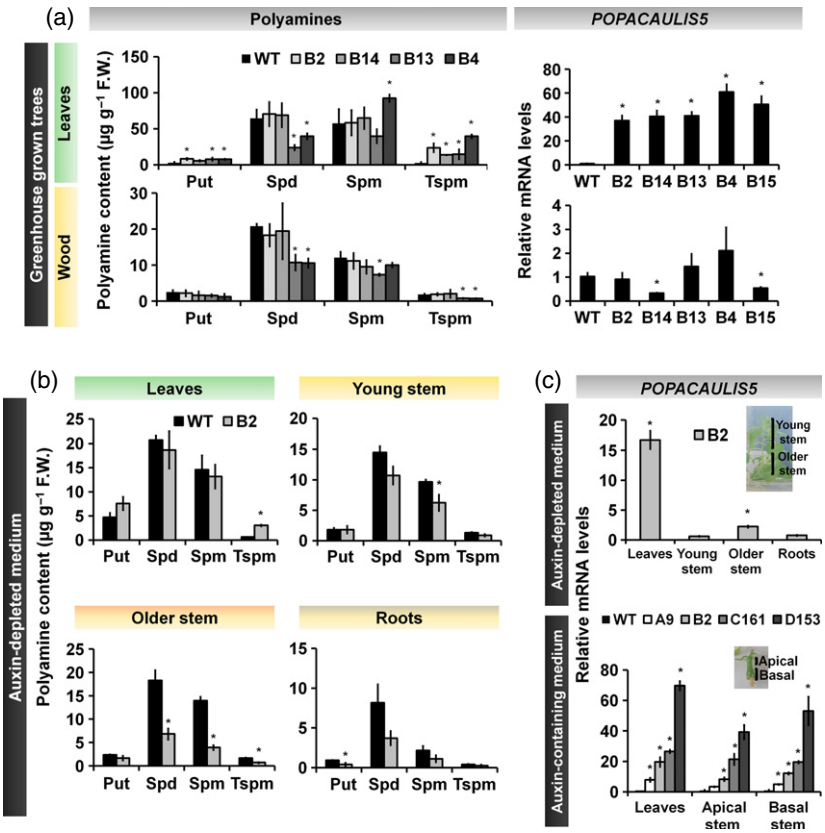

Figure 4. Thermospermine content and *POPACAULIS5* transcript levels of *in vitro*- and greenhouse-grown transgenic *Populus* trees.
(a) Polyamine levels (left panel) and *POPACAULIS5* transcript levels analysed by quantitative RT-PCR (right panel) in leaves and secondary xylem.
(b) Polyamine levels in organs of 5-week-old B2 transgenic plants grown *in vitro* in auxin-depleted medium.
(c) Relative *POPACAULIS5* transcript levels in organs of B2 transgenic plants grown *in vitro* on auxin-depleted medium (upper panel) and in transgenic lines grown on auxin-containing medium (lower panel). Values are means ± SD of three biological replicates (sampled as three pools of 6–10 individual plants for *in vitro* grown material, and as replicates sampled from one individual tree for greenhouse-grown trees) and three technical replicates. Transcript levels are given relatively to the wild-type level in each tissue. Asterisks indicate statistically significant differences compared with the wild-type ($P < 0.05$, Mann–Whitney *U* test). Put, putrescine; Spd, spermidine; Spm, spermine; Tspm, thermospermine. The experiments for gene expression analysis were performed twice.

exogenous supply of auxin resulted in a strong increase in *POPACAULIS5* transcript levels after 4 h in both transgenic lines but not in the wild-type. Twenty-four hours after auxin treatment, the transcript levels decreased in the transgenic stems (40 h point; Figure 6b,c). To exclude the possibility that the auxin-induced increase in *POPACAULIS5* expression was due to induction of the 35S promoter itself, transgenic *Populus* trees carrying a 35S::*GUS*:*GFP* construct were analysed and shown to be non-responsive to exogenous IBA on the basis of expression analyses of the GUS and GFP genes by quantitative PCR (Figure S9). Together, these findings suggest that auxin stimulates *POPACAULIS5* expression at the post-transcriptional level.

Class III HD-Zip *PttHB8* over-expression stimulates *POPACAULIS5* expression

HD-Zip III family member AtHB8 is a good candidate for control of *ACL5* expression in Arabidopsis (Baima *et al.*, 2001; Imai *et al.*, 2006; Carlsbecker *et al.*, 2010). We therefore tested whether the autoregulatory feedback mechanism of *ACL5* expression proceeds through AtHB8. In *Populus*, the closest homologue to *AtHB8* is *PttHB8* (Ko *et al.*, 2006a). First, an *in silico* analysis was performed using the PlantPAN database (Chang *et al.*, 2008) to analyse the target promoter region of *POPACAULIS5*, which resulted in identification of several regulatory homeodomain *cis*-elements (Figure S10a). The search predicted that the transcription factor ATHB9/PHV, which has high affinity *in vitro* for the pseudo-palindromic sequence GTAAT(G/C)ATTAC (Sessa *et al.*, 1998), targets *POPACAULIS5*. Next, the FootprintDB database (Contreras-Moreira, 2010) was interrogated to identify HD-Zip III AtHB8 and PttHB8 DNA-binding signatures. We found that PttHB8 had a similar DNA-binding signature to the one identified for AtHB9/PHV, which suggests that AtHB8/PttHB8 binds to the *POPACAULIS5* promoter (Figure S10b,c). In addition, we found other putative regulatory elements, such as an auxin response factor *cis*-element, which is known to be enriched in the 5′ flanking region of genes up-regulated by IAA (Ulmasov *et al.*, 1999; Goda *et al.*, 2004).

In the *in vitro* transgenic 35S::*POPACAULIS5* lines grown on auxin-containing medium, *PttHB8* expression was significantly suppressed compared to the wild-type (Figure 7a). In the greenhouse-grown trees, *PttHB8* expression was suppressed in the leaves from most of the lines but was unaltered or slightly up-regulated in xylem tissue in comparison to the wild-type (Figure 7b). These results demonstrate that the *POPACAULIS5* over-expression observed in the plants grown *in vitro* and in the leaves of greenhouse-grown trees (Figure 4a,c) suppresses *PttHB8* expression (Figure 7a,b). We also showed that expression of *PttHB8* was rapidly induced by exogenous auxin

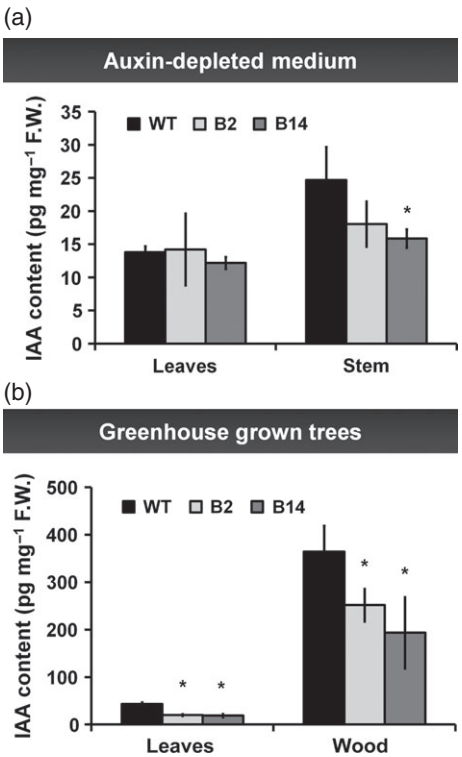

Figure 5. IAA endogenous levels analysis in (a) leaves and stem tissues of *in vitro*-grown plants and (b) leaves and scraped living xylem tissues from greenhouse-grown trees. Values are means ± SD of three biological replicates, each representing three technical replicates. Asterisks indicate statistically significant differences from the wild-type ($P < 0.05$, Mann–Whitney *U* test). The experiment was performed twice.

(Figure 6d–f), and it is therefore possible that the suppression of *PttHB8* expression by *POPACAULIS5* over-expression is mediated through IAA.

To further understand *PttHB8* involvement in *POPACAULIS5* regulation, we expressed a miRNA165/166 mis-regulated form of *PttHB8* under the control of the 35S promoter in hybrid aspen. Three 35S::*PttHB8*-miRNAd transgenic lines, L175, L176 and L179, were obtained. As previously observed for 35S::*POPACAULIS5* trees, up-regulation of *PttHB8* occurred only in leaves but not in the stem (Figure 8). This suggests that *POPACAULIS5* and *PttHB8* expression levels are controlled by the same mechanism. Most importantly, over-expression of *PttHB8* in the leaves resulted in increased levels of *POPACAULIS5*, suggesting that PttHB8 activates expression of *POPACAULIS5* either directly or indirectly.

DISCUSSION

This work focused on POPACAULIS5, which was shown to be the true orthologue of Arabidopsis thermospermine synthase ACL5. Our results provide evidence that thermospermine levels are strictly controlled by a negative feedback mechanism involving *POPACAULIS5*, auxin and the HD-Zip III transcription factor PttHB8. The evidence for the existence of the feedback mechanism is based on the surprising inability to increase the levels of *POPACAULIS5* transcript or thermospermine in xylem tissues by ectopic expression of *POPACAULIS5* under the control of the CaMV 35S promoter in transgenic *Populus* trees (Figure 4a). This inability correlated with reduced accumulation of auxin (Figure 5b), suggesting that over-production of thermospermine suppresses biosynthesis of auxin. This is in accordance with the opposite situation observed in the Arabidopsis *acl5* mutant, in which increased auxin levels were present in young seedlings (Vera-Sirera *et al.*, 2010). However, auxin is known to induce expression of *ACL5* (Hanzawa *et al.*, 2000; Rambla *et al.*, 2010), and this was also shown for *POPACAULIS5* in the *Populus* stem (Figures 4c and 6b,c). Therefore, thermospermine and auxin are part of a feedback loop that involves a negative effect of thermospermine on auxin and a positive effect of auxin on thermospermine. The presence of a negative feedback loop was first noticed when it was observed that *acl5* mutants had increased expression levels of *ACL5* (Hanzawa *et al.*, 2000; Imai *et al.*, 2006; Muñiz *et al.*, 2008). Here, we have identified auxin as a mediator of this feedback control.

The negative feedback loop functions to suppress thermospermine levels specifically in secondary xylem tissues, as high expression levels of *POPACAULIS5* from the CaMV 35S promoter were observed in the leaves of the transgenic *Populus* trees (Figure 4a,b). It is probable that the feedback mechanism operates specifically in the xylem vessel elements, due to the fact that both *ACL5* and *HB8* are specifically expressed in such elements (Baima *et al.*, 2001; Muñiz *et al.*, 2008; Zhang *et al.*, 2011). The absence of the feedback loop in other cell types leads to high levels of thermospermine (Figure 4) that appear to be detrimental to overall growth of the plants (Figures 2 and S3). The decrease in the height of the 35S::*POPACAULIS5* trees may be a direct effect on apical growth but also a secondary effect of the smaller leaf size, impaired photosynthetic capacity or impaired water transport capacity of the transgenic trees, for example. This decrease is somewhat surprising considering that suppression of *ACL5* expression in Arabidopsis causes dwarfism of the inflorescence stem (Hanzawa *et al.*, 2000; Clay and Nelson, 2005; Muñiz *et al.*, 2008). Hence, one would expect an increase rather than decrease in height of the stem in an *ACL5* over-expressor. We can only speculate about the reasons for this, but it is a general phenomenon that plant hormones function in a dose-dependent manner until a threshold level, after which increases in hormonal concentrations become inhibitory (Srivastava, 2002). The thermospermine levels resulting from 35S::*POPACAULIS5* expression appear to exceed the threshold level for thermospermine action in control of height growth. It is quite probable that

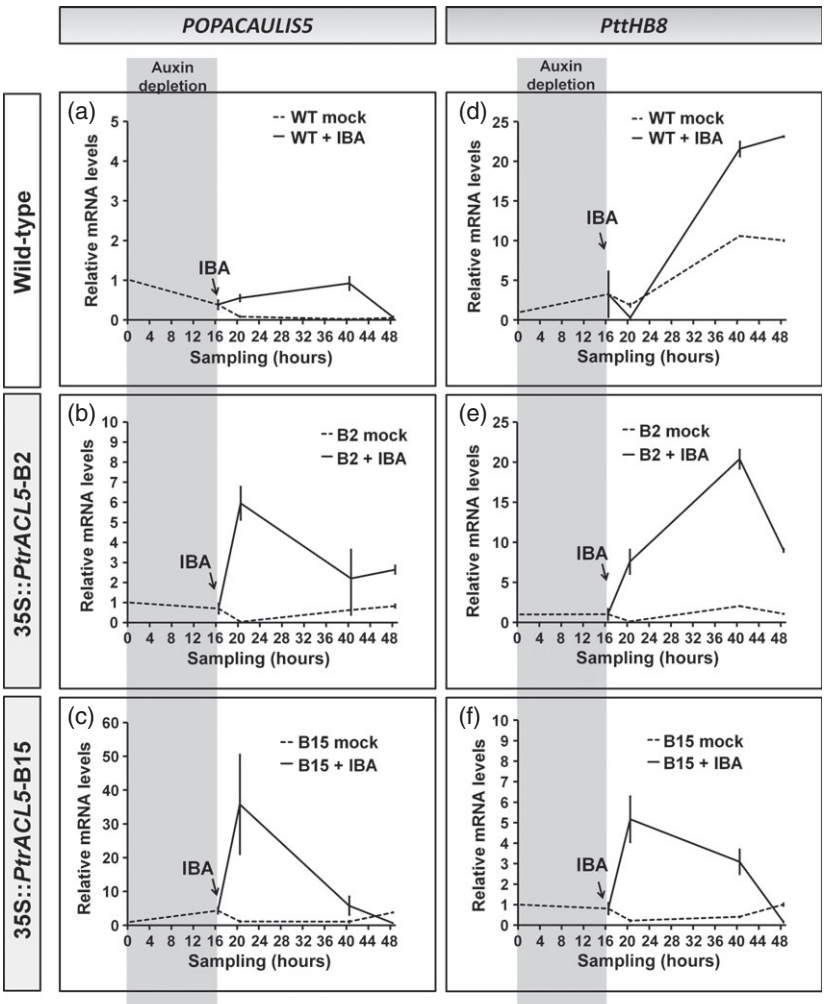

Figure 6. Time-course analysis of *POPACAULIS5* and *PttHB8* expression in response to exogenous auxin.

Five-week-old *in vitro* grown stem segments of wild-type, 35S::*POPACAULIS5* B2 and B15 transgenic plants were depleted of their auxin levels by decapitation and incubation on half-strength MS medium without auxin for 16 h, after which stems were transferred to IBA-containing medium or kept in auxin-free medium (mock). Samples were taken at 0 h (point 0), after 16 h depletion (point 16), and 4 h (point 20), 24 h (point 40) and 32 h (point 48) after transfer to IBA or mock medium. Expression of *POPACAULIS5* (a–c) and *PttHB8* (d–f) was assayed by quantitative RT-PCR. Values represent relative transcript levels normalized to expression values at the start of the experiment (point 0). Values are means ± SD of three replicate samples, consisting of six pooled stems from six individual plants at each time point. The experiment was performed twice.

the threshold for optimal thermospermine levels is very low or maybe even very close to zero in cells other than xylem vessel elements as they normally do not synthesize any thermospermine. In conclusion, we propose that any increase in thermospermine levels of non-vessel cells is detrimental for growth, while a decrease in the thermospermine concentration in the xylem vessel elements, such as in the *acl5* mutant, also leads to reduced growth of the inflorescence stem due to problems in xylem specification.

Several previous reports have demonstrated the importance of polyamines for cambial development and xylem differentiation (Vera-Sirera *et al.*, 2010; Tisi *et al.*, 2011; Waduwara-Jayabahu *et al.*, 2012). Disruption of *ACL5* in Arabidopsis results in over-proliferation of xylem vessel elements with spiral or reticulate secondary wall thickenings, a smaller size of the vessel elements and lack of xylem fibres (Muñiz *et al.*, 2008). However, only modest defects in xylem development were observed in the transgenic 35S::*POPACAULIS5 Populus* trees. Expansion of the secondary xylem was reduced in the 35S::*POPACAULIS5* trees, but this was not surprising considering the severe reduction in the overall height growth of these trees. Despite this, the morphology of xylem elements was scarcely altered, as the size and abundance of the various xylem cell types as well as the type of secondary cell-wall thickenings of vessel elements were similar in wild-type and transgenic trees. The unaltered xylem morphology correlates well with the fact that thermospermine levels were not increased in the secondary xylem tissues of the transgenic trees. It is also possible that the slight defects observed in xylem development are due to increased thermospermine levels in the early stages of plant growth in the auxin-rich environment *in vitro* (Figure S3). Alternatively, thermospermine levels may fluctuate slightly during growth of the trees, and measurement of the thermospermine levels in the pool of xylem tissues perhaps did not reveal these fluctuations. Fluctuations are expected to occur as a result of the presumable strong over-expression of *POPACAULIS5* from the 35S promoter, which must be counteracted by the negative feedback loop. We observed high variation in growth within transgenic lines that may reflect these fluctuations. Similar variation was previously

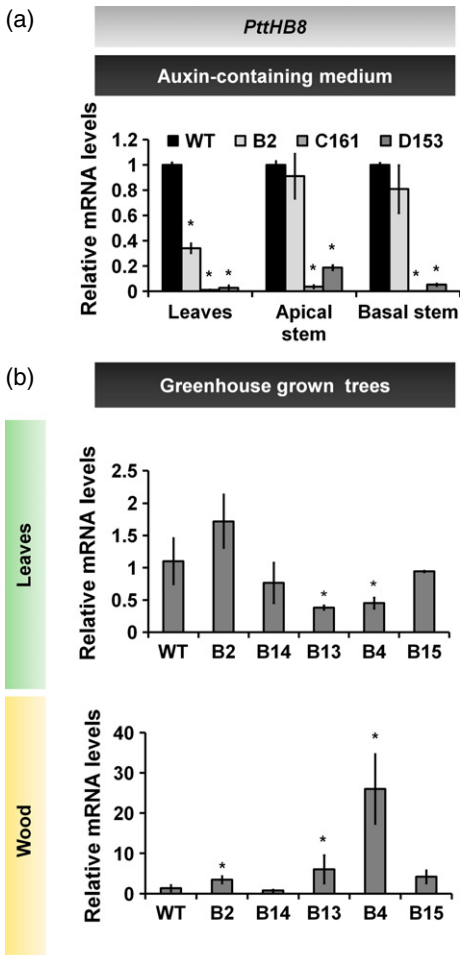

Figure 7. *PttHB8* expression in transgenic 35S::*POPACAULIS5* trees.
Relative expression of *PttHB8* is shown for dwarf transgenic plants from lines B2, C161 and D153 grown *in vitro* on auxin-containing medium (a) and in leaves and woody tissues of greenhouse-grown trees (b). Values are means ± SD of three replicate samples (from a pool of ten) for *in vitro*-grown plants and of three biological replicates each consisting of three technical replicates for the greenhouse-grown trees. The asterisks indicate statistically significant differences compared with the wild-type ($P < 0.05$, Mann–Whitney *U* test). The experiment was performed twice.

observed within transgenic lines in *Populus* trees, in which expression levels of the HD-Zip III transcription factor family member *POPREVOLUTA* were increased (Robischon *et al.*, 2011).

An interesting question is whether the decrease in secondary growth of the stem of the transgenic lines is due to the low IAA levels found there. IAA is known to be a central regulator of cambial growth and xylem specification (Ohashi-Ito and Fukuda, 2010; Ursache *et al.*, 2013), and alterations in levels of auxin have long been known to have severe effects on xylem development (Gälweiler *et al.*, 1998; Hardtke and Berleth, 1998). It was also recently reported that 2,4-dichlorophenoxy acid and other auxin synthetic analogues induce ectopic xylem vessel differentiation in *acl5* but not in wild-type Arabidopsis (Yoshimoto *et al.*, 2012a,b). The authors suggested that xylem differentiation is controlled by auxin, and that thermospermine acts to suppress IAA synthesis and/or sensitivity. Our data provide the evidence for suppression of IAA levels by thermospermine. However, we also showed that inclusion of auxin in the growth medium *in vitro* did not alleviate growth defects but instead further reduced xylem differentiation in the transgenic 35S::*POPACAULIS5* trees (Figure S3c,e), supporting the function of thermospermine rather than auxin as the central regulator of xylem differentiation during secondary growth of the stem.

On the basis of our data, we propose operation of a mechanism in secondary xylem tissues to maintain thermospermine at safe levels in order to facilitate its fundamental role in xylem differentiation. In this proposed mechanism, IAA mediates *POPACAULIS5* expression through *PttHB8*, and thermospermine levels feedback control *PttHB8* and consequently *POPACAULIS5* transcript levels through repression of IAA in a loop (Figure 9). How POPACAULIS5 or thermospermine functions to suppress IAA biosynthesis is not clear currently. Another open question is how the interaction between PttHB8 and *POPACAULIS5* takes place. Our results on up-regulation of *POPACAULIS5* expression as a result of over-expression of

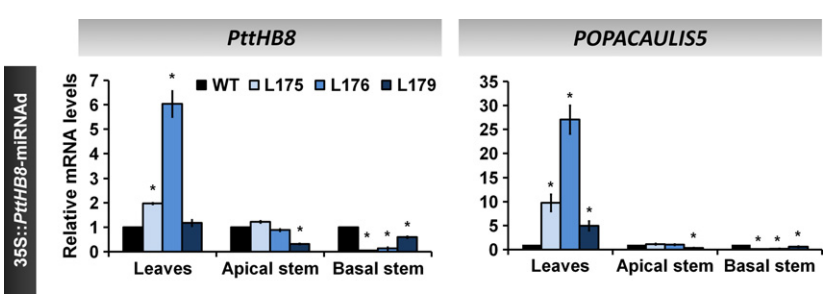

Figure 8. *PttHB8* and *POPACAULIS5* relative transcript levels in transgenic *Populus* plants expressing dominant, gain-of-function *PttHB8*.
Relative expression of *PttHB8* is shown in leaf, apical and basal stem tissues of the transgenic 35S::*PttHB8*-miRNAd lines L175, L176 and L179. Values are means ± SD of three replicate samples (from pools of tissues from six *in vitro*-grown plants for each genotype). Asterisks indicate statistically significant differences compared with the wild-type ($P < 0.05$, Mann–Whitney *U* test). The experiment was performed twice.

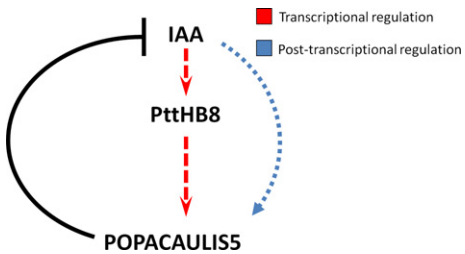

Figure 9. Proposed model for the feedback control mechanism of thermospermine homeostasis in *Populus* secondary xylem tissues.
POPACAULIS5 expression is induced by auxin through PttHB8. POPACAULIS5 suppresses biosynthesis of IAA, which in turn results in reduced activation of *PttHB8* transcription and therefore *POPACAULIS5* expression. Auxin is also proposed to mediate post-transcriptional regulation of the stability of *POPACAULIS5* mRNA. The feedback loop mechanism operates specifically in the xylem as a safeguard mechanism against damaging effects of increased thermospermine levels. The black line indicates the negative effect of *POPACAULIS5* on IAA. The red dotted lines indicate transcriptional regulation. The blue dotted line indicates post-transcriptional regulation.

PttHB8, as well as identification of several regulatory homeodomain *cis*-elements in the promoter of *POPACAULIS5* suggests that *POPACAULIS5* expression may be under direct transcriptional regulation by PttHB8. Transcriptional control of *POPACAULIS5* levels by auxin and PttHB8 does not explain the lower levels of *POPACAULIS5* transcript and thermospermine in the secondary xylem tissues of the 35S::*POPACAULIS5* trees. We propose therefore that *POPACAULIS5* is regulated by auxin in the transgenic 35S::*POPACAULIS5* trees through post-transcriptional control of mRNA stability. Hence, *POPACAULIS5* over-expression in the xylem elements of the 35S::*POPACAULIS5* trees reduces auxin levels, which in turn results in destabilization of the *POPACAULIS5* transcripts. The feedback mechanism cannot cope with excessive amounts of auxin, as inclusion of auxin in the *in vitro* medium resulted in high levels of *POPACAULIS5* expression and damaging levels of thermospermine in the 35S::*POPACAULIS5* stems. Whether post-transcriptional regulation of *POPACAULIS5* also occurs for the endogenous transcript in the wild-type plants is not known, but is feasible as it would allow rapid alterations in thermospermine homeostasis. It is therefore possible that auxin mediates transcriptional activation of *ACL5* through HB8, and that the post-transcriptional control allows rapid alterations in thermospermine signalling, especially in situations where IAA concentrations are excessive. Similar kind of mechanisms involving both transcriptional and post-transcriptional control of gene expression by IAA have been shown in the context of other genes as well, such as the *AUX/IAA* genes (Benjamins and Scheres, 2008). In any case, it is clear from our data that thermospermine levels must be tightly controlled in the secondary xylem tissues of the stem to ensure proper xylem differentiation, and that auxin is a central component in this control.

EXPERIMENTAL PROCEDURES

Plant material, growth conditions and sampling

Hybrid aspen (*Populus tremula* L × *P. tremuloides* Michx.; clone T89) was sub-cultured on MS basal salt medium at half-strength (Murashige and Skoog, 1962) (termed auxin-depleted medium). The *Populus trichocarpa* Nisqually-1 clone was maintained in the greenhouse. Plants were grown in growth chambers at 21°C under a 16 h light/8 h dark photoperiod. Transgenic and wild-type plants were transferred to soil, and trees were grown for 2 months in the greenhouse at 21°C under a 18 h light/6 h dark photoperiod. The greenhouse growth experiment was performed twice.

Sampled tissues were directly frozen in liquid nitrogen when collected and stored at −80°C. Leaves, the first internode (apical stem) and the internode closest to the base (basal stem) of plants grown on auxin-containing medium were collected and pooled in groups of ten from each line for gene expression analysis. Leaves, the stem between the third and the seventh internode from the top (young stem), the stem between the seventh and the basal internode (older stem), and root apices from plants grown *in vitro* on auxin-depleted medium were ground to powder and portioned for gene expression, polyamine and IAA quantification analyses. For greenhouse-grown trees, the five youngest fully expanded leaves were collected. Secondary xylem tissues were obtained between stem internodes 40 and 45 (from the top) by peeling off the bark and scraping the surface of the frozen woody core until emergence of fully mature wood. As no fully mature wood was present in lines B4 and B13, the whole xylem part of the stem was used. The tissues were ground to powder and portioned for gene expression, polyamine and IAA quantification analyses.

Sequence analysis

To identify the *P. trichocarpa* and *P. tremula* × *P. tremuloides* putative ACL5 coding regions, *POPACAULIS5* and *PttACL5*, respectively, we performed BLAST/browse searches in various databases [JGI *Populus trichocarpa* version 1.1 (http://genome.jgi-psf.org/Poptr1_1/Poptr1_1.home.html; Tuskan *et al.*, 2006)], [Phytozome *Populus* version 2 (http://www.phytozome.net/poplar.php; Goodstein *et al.*, 2012), and Populus DB (http://www.populus.db.umu.se/; Sterky *et al.*, 2004)] using the Arabidopsis *ACL5* sequence (GI:145358223; AT5G19530) as the query. Predicted amino acid sequence alignments were performed using ClustalX (http://www.clustal.org/clustal2/) or MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/). For the phylogenetic analysis, putative POPACAULIS5 (POPTR_0006s23880, Phytozome *Populus* version 2; Goodstein *et al.*, 2012; GI:224088768, Genbank), ACL5-like (POPTR_0008s15120, GI:224102051; POPTR_0010s09940, GI:224108055) and *P. tremula* × *P. tremuloides* (PttACL5; GenBank accession number JX444689) sequences were used together with predicted sequences from genomes within the rosid clade, comprising representative orders of angiosperms: evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). Phylogenetic analyses were performed using MUSCLE and MEGA4 (Edgar, 2004; Tamura *et al.*, 2007). The alignment is provided in Figure S1.

Identification of transcription factor-binding sites in the *POPACAULIS5* gene promoter

The PlantPAN database (http://plantpan.mbc.nctu.edu.tw/; Chang *et al.*, 2008) was used to identify *cis*-elements related to HD-Zip III transcription factors in the *POPACAULIS5* putative promoter region, ranging from −1 to −3487 bp upstream of the translation

starting site. The FootprintDB (http://floresta.eead.csic.es/; Contreras-Moreira, 2010) was scanned using the PtrHB8 protein sequence (POPTR_0006s25390) to identify DNA-binding proteins that bind to similar DNA motifs and to identify the amino acids residues that interact with DNA.

Isolation of *POPACAULIS5* and *PttHB8* coding regions

Aliquots (1 μg) of total RNA, extracted using an RNeasy plant mini kit (Qiagen, http://www.qiagen.com/) from shoot apices of *P. tremula* × *P. tremuloides* and *P. trichocarpa*, were used for cDNA synthesis using a 1st Strand cDNA synthesis kit for RT-PCR (Roche, http://www.roche-applied-science.com/) and oligo(dT), according to the manufacturer's instructions. A 1028 bp sequence downstream of the start codon of *PttACL5* and *POPACAULIS5* was amplified from the cDNA of *P. tremula* × *P. tremuloides* and *P. trichocarpa*, respectively, and cloned into the pCR2.1 vector (Invitrogen, http://www.invitrogen.com/). The primers used were *POPACAULIS5* forward, 5′-ATGGGTACTGAGGCAGTTGAG-3′ and reverse 5′-CCCACCAGCAAAGGTATGAG-3′. Similarly, a 2817 bp sequence downstream of the start codon of *PttHB8* was isolated from hybrid aspen using the primers *PttHB8* forward, 5′-ATCTCTAATCCGATCTACGCCAGG-3′ and reverse 5′-GCTCCCAAAGGTTTTTAGGC-3′, by amplification from cDNA and cloned into pCR2.1 vector. Sequence identity was confirmed by sequencing.

Site-directed mutagenesis of the *PttHB8* miRNA 165/166 binding site

A site-directed mutagenesis approach was used to prevent miRNA165/166 from cleaving the *PttHB8* transcript (Emery *et al.*, 2003; Zhong and Ye, 2004; Kim *et al.*, 2005). In the miRNA binding site of the isolated *PttHB8* cDNA sequence, two nucleotides (T and G) were replaced by A nucleotides by PCR amplification. The complete pCR2.1 target plasmid bearing the HD-Zip III isolated sequence was amplified using Phusion Hot-Start DNA polymerase (Finnzymes, http://www.thermoscientificbio.com/finnzymes/). The mutations were introduced using the mutated forward primer 5′-CTGGGATGAAGCCTGGACCAGATTCCATTGG-3′ (point mutations are underlined) and the reverse primer 5′-GCATTTGGACCCACTCCACAGCAGTTCCAGT-3′. The mutated PCR product (named *PttHB8*-miRNAd) was then re-circularized by ligation using T4 Quick DNA ligase (New England Biolabs, https://www.neb.com/). The synonymous point mutations were confirmed by sequencing, and the *PttHB8*-miRNAd cDNA sequence was used to construct the vector for over-expression under the control of the constitutive CaMV 35S promoter as described below.

Hybrid aspen transformation

Cloned sequences were sub-cloned into pDONOR221, recombined with the Gateway vector pK7GW2.0 (Invitrogen, http://www.invitrogen.com) for *POPACAULIS5*, *PttACL5* and *PttHB8* over-expression (Karimi *et al.*, 2002), and introduced into *Agrobacterium tumefaciens* strain GV3101 pMP90 (Koncz and Schell, 1986). Hybrid aspen was transformed as previously described (Nilsson *et al.*, 1992). Transformant selection and shoot elongation were performed using MS medium, with 20 g l^{-1} sucrose, 0.1 μg ml^{-1} IBA, 0.2 μg ml^{-1} 6-benzylaminopurine, 500 μg ml^{-1} cefotaxime and 80 μg ml^{-1} kanamycin monosulfate (auxin-containing medium). To confirm insertions, PCR was performed using the primers *POPACAULIS5* forward 5′-ATGGGTACTGAGGCAGTTGAG-3′ and reverse 5′-TCAATTTTTGTTAGCCACCCCATG-3′, *PttHB8* forward 5′-ATCTCTAATCCGATCTACGCCAGG-3′ and reverse 5′-GAAAGACAGTGTAAGGAG-3′, 35S forward 5′-CTCATCAAGACGATCTACCCGAG-3′ and reverse, 5′-TGGGCAATGGAATCCGAGGAGGT-3′, *NPTII* forward 5′-GAATCGGGAGCGGCGATACCGTAAA-3′ and reverse 5′-CAAGATGGATTACACGCAGGTTCTC-3′, and for false-positive screening the *virBG* forward 5′-GCGGTGAGACAATAGGCG-3′ and reverse 5′-GAACTGCTTGCTGTCGGC-3′ primers. After shoot elongation, plants were transferred to half-strength MS medium for rooting.

Anatomical and ultrastructural analysis

Tree height, internode length, leaf dimensions and stem diameter at internode 35 and stem base (15 cm above soil) were measured. The maturation internode was determined as the youngest internode where the xylem showed signs of complete maturation based on the presence of fully lignified, highly autofluorescing xylem fibres as described by Bollhöner *et al.* (2012). The 45th internode (from the apex) found below the maturation internode in wild-type, B2 and B14 lines was selected as the reference internode. Viability of the xylary cells was determined by staining 0.5–1 mm stem sections with 10 mg ml^{-1} nitroblue tetrazolium in buffered succinate (Berlyn and Miksche, 1976; Gahan, 1984). Lignin staining was performed using a saturated solution of phloroglucinol (Sigma, http://www.sigmaaldrich.com/) in 20% HCl (Jensen, 1962). Sections were observed using a Zeiss Axioplan light microscope, and images were captured using a Axioplan digital camera and Axiovison 4.8 software (Zeiss, http://www.zeiss.com/). Measurements were taken at four positions around the circumference of the stem using Axiovision 4.8 software. Stem segments were fixed in FAA [50% (v/v) ethanol, 5% (v/v) acetic acid, 5% (v/v) formaldehyde] overnight, dehydrated in an ethanol series and gradually infiltrated into LR White (TAAB, http://www.taab.co.uk/). A Leica RM2155 microtome (Leica Microsystems, http://www.leica-microsystems.com) was used for sectioning. Sections were heat-fixed to slides, stained with toluidine blue O (Sigma-Aldrich, http://www.sigmaaldrich.com/), and mounted in mounting medium for observations. Tree growth, growth parameter and microscopy analyses were performed twice.

Electron microscopy

Electron microscopy images of fibre and vessel elements were taken from stem segments from the reference internode, fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, embedded in Spurr resin (Sigma) according to Rensing (2002), and examined using a Hitachi H-7000 transmission electron microscope (Hitachi, http://www.hitachi.com/).

Fibre and vessel element measurements

Stem segments 1 cm long were collected below the reference internode. Pieces of wood were cut to exclude inner pith, outer bark and vascular cambium. The wood samples were immersed in a maceration alkaline solution as described by Berlyn and Miksche (1976). The wooden blocks were mechanically disaggregated. Xylem cell suspensions were observed with a light microscope as described above. Measurements of length and width of at least 200 fibre and 50 vessel elements were performed manually for at least four individual trees, and classified according to the secondary wall thickening patterns (Esau, 1977).

Histochemical GUS staining

Hand sections of stem segments were placed in 90% acetone for 30 min at −20°C, washed twice in distilled water, and incubated in X-Gluc staining solution (1 mM X-Gluc, 1% Triton X-100, 10 mM EDTA, in phosphate buffer) at 37°C in darkness until staining was visible. Stem segments were washed with distilled water, dehydrated in an ethanol series to 50%, fixed for 10 min in 5%

formaldehyde/5% acetic acid/50% ethanol, washed with 50% ethanol for 2 min, cleared in 100% ethanol, incubated overnight in 70% ethanol at 4°C, mounted in 50% glycerol, and observed with a Zeiss Axioplan microscope.

Quantitative reverse transcriptase RT-PCR

Total RNA was extracted from 100 mg of frozen powdered tissues from *in vitro* grown plants using an RNeasy plant mini kit (Qiagen) as described above, and extracted from the tree tissues as described by Chang *et al.* (1993). cDNA synthesis was performed on 1 μg DNase-treated total RNA using a Transcriptor HF cDNA synthesis kit (Roche) with oligo(dT) primers. Quantitative PCR was performed using a LightCycler 480 PCR system with LightCycler480 SYBR Green I Master Mix (Roche) to monitor double-stranded DNA products. Specific primer pairs were designed to generate amplicons of *POPACAULIS5* and *PttHB8* (POPTR_0006s25390). *Pt1* (POPTR_0002s12910) or *CYP2* (POPTR_0009s13270) were used as reference genes (Czechowski *et al.*, 2005; Gutierrez *et al.*, 2008). The primers for the *NAC* gene family member *PNAC058* (POPTR_0013s11740) were as described by Hu *et al.* (2010). The amount of target transcripts was normalized using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). For all experiments, the mean of triplicate quantitative PCR reactions was determined, and at least three biological replicates or pooled biological samples were used. The experiments were performed at least twice. The primers used were: *POPACAULIS5* forward 5′-AAGATGCAGAGTGCCGAAGT-3′ and reverse 5′- GACTTGTGCTTGAGGGCTTC-3′, *PttHB8* forward 5′-ATCTCTAATCCGATCTACGCCAGG-3′ and reverse 5′-CGCATAGAGCTTGGCTTAGG-3′, *Pt1* forward 5′-GCGGAAAGAAAAACTGCAAG-3′ and reverse 5′-TGACAGCACAGCCCAATAAG-3′, and *CYP2* forward 5′- TAAGACCGAATGGCTTGACG-3′ and reverse 5′- AGAACGCACCCCAAAACTACTA-3′.

Quantification of polyamines

Polyamines were extracted from approximately 100 mg of frozen tissues collected as described above, purified as described previously (Rambla *et al.*, 2010), derivatized as described previously (Fernandes and Ferreira, 2000), and identified and quantified as described by Rambla *et al.* (2010). Representative mass spectra for the heptafluorobutyric derivatives of thermospermine, spermine, spermidine and putrescine are shown in Figure S11.

POPACAULIS5 thermospermine activity assays in yeast

The *POPACAULIS* coding sequence was extracted from the pCR2.1-*POPACAULIS5* plasmid as an *Eag*I fragment, and cloned into yeast expression vector pCM190 (Gari *et al.*, 1997). The pCM190-*POPACAULIS5* vector and control empty vector were introduced into yeast using the Yeastmaker yeast transformation system 2 (Clontech, http://www.clontech.com/). After lysis by intense vortexing with 100 μl of 0.5 mm diameter glass beads, polyamine levels in yeast extracts were determined by gas chromatography-mass spectrometry as described above.

Quantification of IAA

Tissues from trees and from *in vitro* grown plants were collected as described above, and 10–20 mg were used for quantification of free IAA content. Sample extraction and purification was performed as described by Andersen *et al.* (2008), with 500 pg $^{13}C_6$-IAA internal standard being added to each sample before extraction. After derivatization, the samples were analysed by gas chromatography-selected reaction monitoring-mass spectrometry as described previously (Edlund *et al.*, 1995).

Auxin treatments for expression analysis

Stem segments 3 cm long from 5-week-old *in vitro* grown wild-type and 35S::*POPACAULIS5* transgenic lines were cut between internodes. Six segments from six individual plants were immediately frozen after cutting, representing the pooled control (0 h). All remaining stem segments were placed on auxin-free half-strength MS medium to deplete them from auxin for 16 h, after which pools of six segments derived from six individual plants were grouped and sampled (16 h). Half of the remaining segments were then placed in fresh half-strength MS medium (mock) and the other half were placed in the same medium containing 20 μM IBA, from which further sets of six stem segments, derived initially from six individual plants, were pooled after mock/IBA treatment for 4 h (20 h point), 24 h (40 h point) and 32 h (48 h point). As negative controls for the auxin treatment experiments, stems from two 35S::*GUS*:*GFP* *Populus* lines were used to monitor the response to 2 and 20 μM IBA. Total RNA, cDNA synthesis and quantitative RT-PCR were performed as described above. The experiment was performed twice.

Statistical analysis

The non-parametric Mann–Whitney *U* test was used to assess significant differences in gene expression, polyamine and IAA contents and tree growth parameters. An α value of 0.05 was considered significant. Statistical analyses were performed using Statistica software (Statsoft Inc., http://www.statsoft.com/) as described by Zar (1998).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Amino acid sequence alignment from taxa used in the phylogenetic analysis.

Figure S2. Thermospermine synthase activity validation assays in yeast.

Figure S3. Phenotypic characterization of 35S::*POPACAULIS5* transgenic lines grown on auxin-containing medium.

Figure S4. Light microscopy and xylem anatomical analysis of stem sections from the transgenic trees.

Figure S5. Length and width of fibres and secondary xylem vessels, and frequency distribution.

Figure S6. Xylem vessel type distribution in the woody stem.

Figure S7. Secondary wall deposition observed by transmission electron microscopy.

Figure S8. 35S promoter activity in xylem tissues observed by histochemical GUS analysis.

Figure S9. Time-course analysis of the response to auxin in 35S::*GUS*:*GFP* plants.

Figure S10. *In silico* analysis of putative *cis*-elements present in the *POPACAULIS5* gene promoter.

Figure S11. Extracted ion chromatogram of ions *m*/*z* 226 and *m*/*z* 254 in an extract of wild-type *Populus* young stem.

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