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Scots pine aminopropyltransferases shed new light on evolution of the polyamine biosynthesis pathway in seed plants

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• **Background and Aims** Polyamines are small metabolites present in all living cells and play fundamental roles in numerous physiological events in plants. The aminopropyltransferases (APTs), spermidine synthase (SPDS), spermine synthase (SPMS) and thermospermine synthase (ACL5), are essential enzymes in the polyamine biosynthesis pathway. In angiosperms, SPMS has evolved from SPDS via gene duplication, whereas in gymnosperms APTs are mostly unexplored and no *SPMS* gene has been reported. The present study aimed to investigate the functional properties of the SPDS and ACL5 proteins of Scots pine (*Pinus sylvestris* L.) in order to elucidate the role and evolution of APTs in higher plants.
• **Methods** Germinating Scots pine seeds and seedlings were analysed for polyamines by high-performance liquid chromatography (HPLC) and the expression of *PsSPDS* and *PsACL5* genes by *in situ* hybridization. Recombinant proteins of PsSPDS and PsACL5 were produced and investigated for functional properties. Also gene structures, promoter regions and phylogenetic relationships of *PsSPDS* and *PsACL5* genes were analysed.
• **Key Results** Scots pine tissues were found to contain spermidine, spermine and thermospermine. PsSPDS enzyme catalysed synthesis of both spermidine and spermine. PsACL5 was found to produce thermospermine, and *PsACL5* gene expression was localized in the developing procambium in embryos and tracheary elements in seedlings.
• **Conclusions** Contrary to previous views, our results demonstrate that SPMS activity is not a novel feature developed solely in the angiosperm lineage of seed plants but also exists as a secondary property in the Scots pine SPDS enzyme. The discovery of bifunctional SPDS from an evolutionarily old conifer reveals the missing link in the evolution of the polyamine biosynthesis pathway. The finding emphasizes the importance of pre-existing secondary functions in the evolution of new enzyme activities via gene duplication. Our results also associate PsACL5 with the development of vascular structures in Scots pine.

Key words: Aminopropyltransferase, gene duplication, gymnosperm, pathway evolution, *Pinus sylvestris*, polyamine biosynthesis, Scots pine, spermidine synthase, spermine synthase, thermospermine synthase.

INTRODUCTION

Polyamines (PAs) are ancient low molecular weight polycations which are found in all living organisms. In addition to the universal occurrence, the fundamental role of PAs in cell metabolism is emphasized by the conservation of PAs across evolution as well as by the complexity of compensatory mechanisms that are invoked to maintain PA homeostasis in cells (Wallace *et al.*, 2003). The cellular functions of PAs may result from their unique chemical characteristics, from the structural changes of RNA when PA–RNA complexes are formed or from the PA-dependent alterations in the structure and function of DNA and further in gene expression (Childs *et al.*, 2003; Igarashi and Kashiwagi, 2010). In plants, PAs have been considered as growth regulators or as secondary messengers (Kakkar and Sawhney, 2002) and they are involved in several physiological events, such as organogenesis, embryogenesis, floral initiation and development, leaf senescence, fruit development and ripening, as well as in abiotic and biotic stress responses (Galston and Sawhney, 1990; Kumar *et al.*, 1997; Malmberg *et al.*, 1998; Bouchereau *et al.*, 1999; Bagni and Tassoni, 2001; Alcázar *et al.*, 2006; Kusano *et al.*, 2008).

The major naturally occurring PAs are the diamine putrescine (Put), the triamine spermidine (Spd) and the tetra-amine spermine (Spm) (Tiburcio *et al.*, 1997). In addition, thermospermine (tSpm), a less abundant structural isomer of Spm, is widely detected in the plant kingdom (Takano *et al.*, 2012). In mammalian cells, Put is synthesized from ornithine by ornithine decarboxylase (ODC; EC 4.1.1.17), and in many fungi ODC is the

sole pathway for Put production (Coleman *et al.*, 2004). Plants are also able to produce Put from arginine by arginine decarboxylase (ADC; EC 4.1.1.19) through two intermediate stages (Tiburcio *et al.*, 1997), and there is also evidence of ADC activity in certain fungal species (Khan and Minocha, 1989; Biondi *et al.*, 1993; Sannazzaro *et al.*, 2004). Put can be metabolized further to the higher PAs by the aminopropyltransferases (APTs), which transfer the aminopropyl moiety (derived from decarboxylated *S*-adenosylmethionine) to an amine acceptor on another PA (Shao *et al.*, 2012) (Supplementary Data Fig. S1). Spd is formed from Put by spermidine synthase (SPDS; EC 2.5.1.16). It has been suggested that the extant core of the eukaryotic PA biosynthetic pathway in the last eukaryotic common ancestor consisted of the ODC and SPDS enzymes, because Put and Spd are the only PAs produced in all PA-synthesizing eukaryotes (Michael, 2016). Spd is an asymmetrical molecule, which can be further aminopropylated at either end, forming Spm or tSpm. Spermine synthase (SPMS; EC 2.5.1.22) transfers an aminopropyl group to the N^8-(aminobutyl) end of Spd to make a symmetrical Spm molecule, whereas thermospermine synthase (ACL5; EC 2.5.1.B4) transfers an aminopropyl group to the N^1-(aminopropyl) end of Spd which results in an asymmetrical tSpm molecule (Pegg and Michael, 2010) (Supplementary Data Fig. S1). To distinguish Spm and tSpm, benzoylation of the PAs is needed followed by separation by high-performance liquid chromatography (HPLC) (Naka *et al.*, 2010).

Several reports have indicated the necessity of Spd for the viability of eukaryotes, whereas eukaryotes deficient in SPMS and thus lacking Spm are mostly viable, even if they may suffer different degrees of dysfunction (Pegg and Michael, 2010). One of the essential functions of Spd is its role as a substrate for hypusine modification of the eukaryotic translation initiation factor eIF5A (Chattopadhyay *et al.*, 2003, 2008; reviewed by Wolff and Park, 2015). In arabidopsis (*Arabidopsis thaliana* L.), the *spds1-1 spds2-1* double mutant had abnormally shrunken seeds, and embryos that were arrested morphologically at the heart–torpedo transition stage (Imai *et al.*, 2004*b*). In contrast, Spm was not essential for survival of arabidopsis under normal growth conditions (Imai *et al.*, 2004*a*), but Spm-deficient mutants were hypersensitive to drought and salt stress (Yamaguchi *et al.*, 2006, 2007). Loss-of-function mutations of the *AtACL5* gene resulted in a severely dwarfed phenotype in arabidopsis due to a specific defect in the growth of stem internodes (Hanzawa *et al.*, 2000). These observations suggest that Spd may act in the basic metabolism of eukaryotic cells and is essential for cell viability, whereas tSpm may play a role in the development of the structures of multicellular eukaryotes and Spm may improve the tolerance to different kinds of stresses. Thus, the slight structural difference between tSpm and Spm seems to be important for their function.

APTs share a high degree of sequence similarity at the putative active sites even if they display different substrate specificities (Rodríguez-Kessler *et al.*, 2010). In plants, SPMS seems to have evolved via gene duplication and change of the function of an ancestral SPDS in angiosperms, whereas the *ACL5* gene may have been acquired by an algal ancestor of plants through a horizontal gene transfer from bacteria or archaea (Minguet *et al.*, 2008). All plant species, including gymnosperms and *Physcomitrella patens* (Hedw.) Bruch & Schimp, have been found to possess sequences identifiable as *SPDS* or *ACL5*, but no *SPMS* genes have been reported from gymnosperms so far (Minguet *et al.*, 2008).

The APTs in angiosperms are well known (reviewed by Shao *et al.*, 2012), whereas APTs in gymnosperms have mostly been an unexplored area. *Pinus* species of the gymnosperms form an evolutionarily old group of vascular plants that shared a common ancestor with angiosperms about 285 million years ago (Bowe *et al.*, 2000). In the present study, we hypothesized that at some point of seed plant evolution both SPDS and SPMS activities might have coexisted in a single protein, and therefore our focus was specifically on the *SPDS* and *ACL5* genes of Scots pine (*Pinus sylvestris* L.). We produced recombinant proteins of PsSPDS and PsACL5 to investigate their functional properties. We also studied the expression of *PsSPDS* and *PsACL5* genes in Scots pine seeds and seedlings by mRNA *in situ* hybridization. Furthermore, gene structures, promoter regions and phylogenetic relationships of *PsSPDS* and *PsACL5* genes were analysed in order to elucidate the evolution of APTs in higher plants.

MATERIALS AND METHODS

Plant material

Mature Scots pine seeds were sterilized overnight in 3 % Plant Preservative Mixture™ (Plant Cell Technology, Washington, DC, USA). Seeds were germinated on moist filter papers in Petri dishes in a growth chamber at +20 ºC, in 100 % moisture and under continuous light. For the PA analyses, the embryos and megagametophytes of the seeds were excised after 2 d imbibition, and the cotyledons, hypocotyls and roots of the seedlings were excised after 16 d of germination. The embryos for RNA extraction were frozen in liquid nitrogen and stored at –80 ºC until use. For the *in situ* hybridization assays of *PsSPDS* and *PsACL5* transcripts, seeds (without seed coat) and hypocotyls were fixed immediately after sampling as described previously in Vuosku *et al.* (2015).

Three populations from different latitudes were used to sequence the *PsSPDS* and *PsACL5* genes: Kolari (northern Finland, latitude 67°11’N, 24°03’E), Punkaharju (southern Finland, latitude 61°48’N, 29°19’E) and Radom (Poland, latitude 50°41’N, 20°05’E). Seeds collected from eight open-pollinated (mostly half-sibs) families of each population were used to sequence the genes as well as their promoter regions.

PA analyses in pine tissues with the benzoylation method

The presence of PAs (Put, Spd, Spm and tSpm) in Scots pine tissues was discovered by using the benzoylation method described by Naka *et al.* (2010). The cotyledon, hypocotyl, root, embryo and megagametophyte samples which were pooled from many seedlings or seeds were extracted in 5 % (w/v) $HClO_4$ and the benzoylated PAs in the tissue samples and in the reaction mixture were separated by HPLC and detected at 254 nm according to Naka *et al.* (2010). The standard for tSpm was kindly provided by Professor Masaru Niitsu.

Production and functional studies of PsSPDS and PsACL5 recombinant proteins

Total RNA was extracted from embryos using the automatic magnetic-based KingFisher™ mL method (Thermo Labsystems, Helsinki, Finland) with the MagExtractor® total RNA purification kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The RNA samples were treated with RNase-free DNase (Invitrogen, Carlsbad, CA, USA) and purified with the NucleoSpin® RNA Clean-Up kit (Macherey-Nagel, Düren, Germany). RNA yields were measured by a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). cDNA was reverse-transcribed from 1 µg of total RNA by using the SuperScript® VILO™ cDNA Synthesis kit (Invitrogen) with the SuperScript® III Reverse Transcriptase in a reaction volume of 20 µL.

The coding region of *PsACL5* was amplified by PCR, using the upstream primer 5′-TATGCC**ATG**GGGGAAGTAGCAGC-3′ (the translation start codon is in bold and the *Nco*I site is underlined) and downstream primer 5′-TATGCGGCCGC**TTA**TGATTTATGGCAC-3′ (the translation stop codon is in bold and the *Not*I site is underlined). The amplified PCR product was restricted with *Nco*I and *Not*I and ligated into the *Nco*I/*Not*I site of the expression vector pET-32a (Novagen, Madison, WI, USA). The coding region of *PsSPDS* was amplified by using the upstream primer 5′-TAGATATCGGATCC**ATG**GCCGAGAAC-3′ (the translation start codon is in bold and the *Bam*HI site is underlined) and downstream primer 5′-TATGCGGCCGC**TTA**AGATAATGGTGG-3′ (the translation stop codon is in bold and the *Not*I site is underlined). The amplified PCR product was restricted with *Bam*HI and *Not*I and ligated into the *Bam*HI/*Not*I site of the expression vector pET-32a. The resulting recombinant plasmids were confirmed by sequencing using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The *PsSPDS* and *PsACL5* sequences have been deposited in GenBank under accession numbers KX761190 and HM236828, respectively.

The recombinant plasmids were transferred into *Escherichia coli* BL21 (DE3) cells (Novagen). The cells were grown in Luria–Bertani liquid medium in the presence of ampicillin (100 µg mL^{-1}) at 30 °C until the D_{600} of the culture reached 0.6. Protein expression was induced by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and the cultures were further incubated at 16 °C for 18 h. The *E.coli* cells were harvested by centrifugation (4000 *g* for 20 min) and resuspended in buffer (50 mM sodium phosphate buffer, pH 7.0, containing 600 mM NaCl, 15 mM β-mercaptoethanol, 10 % glycerol, 1 % Tween-20 and 20 mM imidazole). The cells were disrupted using lysozyme (1 mg mL^{-1}) and sonication (Type UP50H; Dr. Hielscher GmbH, Teltow, Germany). The lysate was centrifuged at 17 000 *g* for 30 min and the supernatant was collected for purification of recombinant protein using Ni-NTA agarose (Qiagen, Hilden, Germany). Unbound proteins were washed away with the buffer described above, after which the recombinant proteins were eluted with an elution buffer (50 mM sodium phosphate buffer, pH 7.0, containing 600 mM NaCl, 15 mM β-mercaptoethanol, 10 % glycerol and 250 mM imidazole). The purified enzyme solution was buffer-exchanged to assay buffer (0.1 M Tris–HCl, pH 7.5, containing 15 % glycerol) using a PD-10 column (GE Healthcare Bio-Sciences, Little Chalfont, UK). The purity of the recombinant proteins was verified by SDS–PAGE using a Mini-Protean II electrophoresis system (Bio-Rad, Hercules, CA, USA). Proteins were visualized with Coomassie Brilliant Blue R-250 (Merck, Darmstadt, Germany).

Analyses of PAs were performed to confirm the SPDS/SPMS activity of the purified PsSPDS protein. The assay mixture (200 µL) contained 0.1 M Tris–HCl (pH 7.5), 5 mM decarboxylated *S*-adenosyl-L-methionine (dcSAM, kindly provided by Professor Keijiro Samejima), substrate (5 mM Put or Spd) and 34 µg of purified PsSPDS protein. The reaction mixtures were incubated for 2 h at 30, 40 or 50 °C. After the 2 h incubation, 16 µL of 60 % (w/v) $HClO_4$ was added into 200 µL of the reaction mixture and analysed immediately for PAs or stored at –80 °C until analysed. For PA analyses, the samples were dansylated (Flores and Galston, 1982), stored at –20 °C and analysed within 1 week by HPLC (Merck Hitachi, Darmstadt, Germany) and fluorescence spectrophotometry (Merck Hitachi F-1050) at excitation and emission wavelengths of 365 and 510 nm, respectively. The PAs were separated by using a LiChrospher 100 RP-18 5 µm column and methanol–water gradient as described by Sarjala and Kaunisto (1993). Putrescine dihydrochloride (Sigma-Aldrich, St. Louis, MO, USA), spermidine trihydrochloride (Sigma-Aldrich) and spermine tetrahydrochloride (Sigma-Aldrich) were used as standards.

The assay of the tSpm activity of the purified PsACL5 protein was performed in the reaction mixture (200 µL) containing 0.1 M Tris–HCl (pH 7.5), 100 nM Spd, 100 nM dcSAM (kindly provided by Dr Yoshihiko Ikeguchi) and 14 µg of purified enzyme. The reaction mixture was incubated for 1 h at 30, 40 and 50 °C. The presence of PAs in the reaction mixture was revealed by using the benzoylation method as described by Naka *et al.* (2010).

mRNA in situ *hybridization assay of* PsSPDS *and* PsACL5 *transcripts*

The localization of *PsSPDS* and *PsACL5* transcripts in Scots pine seeds and hypocotyls was performed as described in Vuosku *et al.* (2015). The primers used for the synthesis of a 275 bp probe for *PsSPDS* transcripts were 5′-CACATGCCCATCATTGAAGA-3′ (upstream) and 5′-CCTATCGCCCTTCTAGCAAA-3′ (downstream). The 332 bp long *ACL5* probe was amplified with the primers 5′-GCCGAGCTCGAGAGTAGAGA-3′ (upstream) and 5′-TCGATTTCTTCGGCGTCTAT-3′ (downstream). In seed sections, the digoxigenin (DIG)-labelled probes were detected by Anti-DIG-AP Fab fragments and NBT/BCIP substrate (Dig Nucleic acid detection Kit, Roche Molecular Biochemicals, Mannheim, Germany) as previously described in Vuosku *et al.* (2015). In hypocotyl sections, the probes were visualized by incubating the slides for 30 min at 37 ºC with 1:200 anti-DIG–rhodamine Fab fragments (Roche Molecular Biochemicals) in the dark. Thereafter, the slides were washed four times in Tris/NaCl buffer for 10 min, dehydrated in a graded series of ethanol, air-dried and mounted with coverslips using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). The hypocotyl sections were examined in a confocal laser scanning microscope (LSM 5 Pascal, Carl Zeiss) with an HBO 103 mercury lamp. Adobe Photoshop CS was used to adjust contrast, brightness and colour uniformly for entire images.

Analysis of PsSPDS *and* PsACL5 *gene structures and copy numbers*

Primers (Supplementary Data Table S1) were designed to sequence full *PsSPDS* and *PsACL5* genes and their promoters. Total genomic DNA was extracted from the haploid megagametophyte tissue of the seeds (see 'Plant material') with Nucleospin Plant II (Macherey-Nagel) with buffer PL1. DNA quality and quantity were checked with agarose gel electrophoresis and a NanoDrop ND1000 spectrophotometer. All of the fragments belonging to the same gene were amplified from a single megagametophyte. The MinElute PCR purification kit (Qiagen, Chatsworth, CA, USA) or the FastAP Thermosensitive Alkaline Phosphatase and Exonuclease I (Fermentas, Vilnius, Lithuania) were used to purify the PCR products. The Genome Walker Universal kit (Clontech, Mountain View, CA, USA) was used according to the manufacturer's instructions to increase the length of the promoter regions. Sequencing reactions were carried out with an ABI Prism 3730 DNA Analyzer (Applied Biosystems) with a Big Dye Terminator kit v3.1 (Applied Biosystems). Following sequencing, manual checking and editing were done in Sequencher 4.7 (Gene Codes Corporation). Sequences were aligned using MUSCLE (Edgar, 2004) in Geneious version 6.1 (created by Biomatters, available from http://www.geneious.com). The genomic sequences of *PsSPDS* and *PsACL5* have been deposited in GenBank under accession numbers KX788070 and KX788071, respectively.

No fully sequenced genome of Scots pine is available to date. Thus, in order to predict the copy number of the *SPDS* and *ACL5* genes in the Scots pine genome, the current assembly (version 2.01 in March 2017) of the loblolly pine (*Pinus taeda* L.) genome was downloaded from the UC Davis Dendrome website (http://dendrome.ucdavis.edu/ftp/Genome_Data/genome/pinerefseq/Pita/) and used for full BLAST search on a local server. The results of the BLAST search were analysed for the occurrences of complete gene sequences of *SPDS* and *ACL5* using the Geneious software. To confirm that the best-matching sequences were putative SPDS and ACL5 proteins, BLAST searches were performed against the NCBI nr or UniProtKB/Swiss-Prot databases. In addition, the deduced PsSPDS and PsACL5 proteins were used for BLAST search against loblolly pine transcripts and protein sequences.

Functional analysis of SPDS *and* SPMS *promoter regions*

The genomic sequences of 3 kb upstream of the *SPDS* and *SPMS* genes in arabidopsis (*Arabidopsis thaliana* L.) and black cottonwood (*Populus trichocarpa*) were extracted from The Arabidopsis Information Resource (TAIR; http://www.arabidopsis. org/) and The Populus Genome Integrative Explorer (PopGenIE; http://popgenie.org/), respectively. The promoter sequence of *PsSPDS* (KX788070, 1201 bp) was extended to 3 kb with the 1810 bp promoter sequence of loblolly pine from conifer Genome Integrative Explorer (ConGenIE; http://congenie.org/). Transcription factor-binding sites (TFBSs) and the corresponding transcription factors (TFs) within the promoter regions of *SPMS* and *SPDS* genes as well as their functions were searched using the PlantPAN2.0 database (http://plantpan2.itps.ncku.edu.tw) (Chang *et al.*, 2008; Chow *et al.*, 2016). The comparison of the presence and absence of the identified *cis*-regulatory elements in the promoter regions of the *SPDS* and *SPMS* genes was carried out manually by generating presence–absence data (Supplementary Data Table S2). The functions of TFBSs/TFs were determined in the cases where a TFBS was present in *PsSPDS* but was absent in at least one of the arabidopsis or black cottonwood *SPDS*/*SPMS* genes, as well as in the cases where a TFBS was absent in *PsSPDS* (Supplementary Data Table S3). The similarity of the TFBS content among the promoter regions of the *SPDS* and *SPMS* genes in Scots pine, arabidobsis and black cottonwood was investigated with global non-metric multidimensional scaling (GNMDS) in the library vegan (Oksanen *et al.*, 2016) of the statistical program R (R Core Team, 2015) using presence–absence data. The analysis was performed with 50 separate random starts with function metaMDS using the dissimilarity index Raup–Crick.

Phylogenetic analysis of plant SPDS, SPMS *and* ACL5 *genes*

For the phylogenetic analysis of plant APT genes, BLAST searches against NCBI databases (http://www.ncbi.nlm.nih.gov) were carried out against the coding sequences of the *PsSPDS* and *PsACL5* genes. In the case of other conifers, expressed sequence tag (EST) information was used to reconstruct a contig containing the complete coding sequence, when no unigene sequences were available. In addition, sequences were chosen on the basis of previous literature on phylogenetic analysis. The nucleotide sequence alignments of APT genes were performed with ClustalX (Thompson *et al.*, 1997) (Supplementary Data Fig. S2). The phylogenetic trees were inferred using the maximum likelihood (ML) method based on the Tamura–Nei model (Tamura and Nei, 1993). Initial trees for the heuristic search were obtained by applying the Neighbor–Joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. All codon positions were included. The positions containing gaps and missing data were eliminated from the data set. The bootstrap method (Felsenstein, 1985) with 1000 replicates was used to evaluate the confidence of the reconstructed trees. Bootstrap values between 70 and 100 % have been suggested to indicate significant support for a branch (Soltis and Soltis, 2003). Phylogenetic analyses were conducted in MEGA 5.05 (Tamura *et al.*, 2011).

RESULTS

Scots pine tissues contain both Spm and tSpm

The PA content was measured in several tissues of Scots pine seedlings and seeds. In particular, we were interested in the co-presence of Spm and tSpm. Both Spm and tSpm were detected in the cotyledons, hypocotyls and roots of seedlings, as well as in the embryos and megagametophytes of mature seeds. The tSpm concentration of the tissues varied from 0.3 to 7.7 nmol g^{-1} (Table 1). The content of tSpm was 2–31 % of the content of Spm. In embryos and megagametophytes, the proportion of tSpm was higher than in other Scots pine tissues.

Identified Scots pine SPDS and ACL5 proteins

The identified *P. sylvestris SPDS* gene (*PsSPDS*) had an open reading frame (ORF) of 1038 bp encoding a protein of 345 amino acids with a calculated molecular weight of 37.90 kDa and a theoretical p*I* of 4.94. The predicted amino acid sequence of *PsSPDS* showed high similarity to the SPDS proteins of angiosperms. PsSPDS shared 72 % identity with SPDSs identified from rice (*Oryza sativa* L.) and clementine (*Citrus clementina* Hort. ex Tan.), 71 % with SPDS of black cottonwood (*P. trichocarpa*), 70 % with SPDS1 of apple (*Malus* × *domestica*) and 67 % with arabidopsis SPDS1 (Supplementary Data Fig. S3). The PsSPDS sequence contained conserved motifs shown to be binding sites for SAM and dcSAM in all members of the APT family.

The *P. sylvestris ACL5* gene (*PsACL5*) had an ORF of 1125 bp that is predicted to encode a protein of 374 amino acids with a calculated molecular weight of 41.78 kDa and a p*I* of 5.98. The predicted amino acid sequences of PsACL5 showed 66 % identity with ACL5 identified earlier in black cottonwood, 61 % with ACL5 of clementine, 60 % with ACL5 proteins of rice and apple (*M. sylvestris*) and 59 % with the arabidopsis ACL5 protein (Supplementary Data Fig. S4). Moreover, the PsACL5 sequence contained the conserved motifs of the APT family.

Bifunctional PsSPDS enzyme produces both Spd and Spm

To study the enzymatic functions of the proteins encoded by *PsSPDS* and *PsACL5*, the coding regions of the genes were expressed in *E. coli* strain BL21 with the pET-32a expression vector. The SDS–PAGE analysis of the purified recombinant proteins resulted in bands with a molecular mass of approx. 55 kDa for the protein encoded by *PsSPDS* and approx. 60 kDa for the protein encoded by *PsACL5* (Fig. 1A) which coincides with the calculated molecular masses of recombinants with an N-terminal His-tag (SPDS protein, 55.5 kDa; ACL5 protein, 58.9 kDa).

We found that purified PsSPDS catalyses the synthesis of both Spd and Spm, while the enzyme encoded by *PsACL5* produced tSpm but not its isomer Spm (Fig. 1B). The PsSPDS enzyme reaction mixture containing Put as a substrate produced Spd, whereas the PsSPDS enzyme reaction mixture containing Spd as a substrate produced Spm (Fig. 1C). Raising the incubation temperature from 30 to 50 °C significantly increased Spm formation (*P*-value 0,00015). Spd or Spm were not produced in the control reactions lacking PsSPDS enzyme (Fig.1B). Incubation of the reaction mixture with the PsACL5 protein confirmed that tSpm was produced at all incubation temperatures, 30, 40 and 50 °C (Supplementary Data Fig. S5).

Table 1. *Polyamine content (nmol g f. wt^{-1}) in Scots pine seedlings and seeds*

Scots pine tissue	Put	Spd	Spm*	tSpm*	tSpm/Spm (%)
Roots	22.7	11.8	6.0	0.4	6.7
Hypocotyls	31.4	21.5	8.3	0.3	3.5
Cotyledons	125.3	68.3	22.5	0.5	2.0
Embryos	47.3	134.4	24.4	7.7	31.5
Megagametophytes	23.3	53.7	10.9	2.7	24.7

Put, putrescine; Spd, spermidine; Spm, spermine; tSpm, thermospermine.

*Spm and tSPM concentrations in different Scots pine tissues were measured as benzoylated polyamines according to Naka *et al.* (2010).

PsSPDS *and* PsACL5 *expression in developing vascular structures*

In the Scots pine seed, the embryo lies within the corrosion cavity of the megagametophyte, which houses the majority of the storage reserves of the seed. In the germinating embryo, intense *PsSPDS* expression was localized in the procambial cells continuing from cotyledons to the hypocotyl, but slight *PsSPDS* expression was found in all cells of the embryo (Fig. 2A). Procambium consists of a few layers of tightly packed cells, where *PsSPDS* is also expressed during cell division (Fig. 2B–E). Unlike the *PsSPDS* gene, *PsACL5* was found to be expressed specifically only in procambial cells in the embryo (Fig. 2F, G). The specificity of the antisense *PsSPDS* and *PsACL5* probes was confirmed by the absence of signals in the sections hybridized with the sense *PsSPDS* and *PsACL5* probes, respectively (Fig. 2H, I). Non-specific hybridization signal generated by fragmented nucleic acids was observed in the embryo surrounding region (ESR) of the megagametophyte, as previously described in Vuosku *et al.* (2010).

In the hypocotyls of Scots pine seedlings, both *PsSPDS* and *PsACL5* were expressed specifically in developing tracheary elements (Fig. 3A, B, D, E). No signals were detected in the hypocotyl sections hybridized with the sense *PsSPDS* (Fig. 3C) and *PsACL5* (Fig. 3F) probes.

Conserved exon–intron structure of PsSPDS *and* PsACL5 *genes*

The full genomic sequence of *PsSPDS* contained nine exons (Fig. 4A) vs. ten for *PsACL5* (Fig. 4B). *PsSPDS* harboured overall larger introns leading to a gene size of between 5995 and 6135 bp for *PsSPDS* vs. between 2641 and 2653 bp for *PsACL5* from the start codon to the stop codon in the 24 samples we sequenced. The intron insertion phases were also very different between the two genes.

The comparison of the exon–intron structure between *PsSPDS* and the *SPDS* genes of angiosperms revealed that the gene structure has been conserved during evolution in seed plants, suggesting an orthologous origin for the genes. The orthologues accumulate much fewer structural differences, unlike the paralogues (Xu *et al.*, 2012). All *SPDS* orthologues harboured nine exons of the same size except for exons I and IX, but the size of the large introns was highly variable (Fig. 4A). For the *ACL5* orthologues, with the exception of *AtACL5*, the situation was similar, with ten exons of identical sizes except for exons I and X (Fig. 4B). Considering the very long divergence time between gymnosperms and angiosperms, the conserved gene structure among seed plants seems remarkable, suggesting the important role of these APTs.

BLAST search against the assembly version v2.01 of the loblolly pine (*Pinus taeda* L.) genome resulted in one full-length copy of *ACL5* (in scaffold 80063) and one full-length copy of *SPDS* (in scaffold 114828). The full-length *PtACL5* showed 95 % sequence identity with the full-length *PsACL5*, while CDS (coding DNA sequence) regions showed

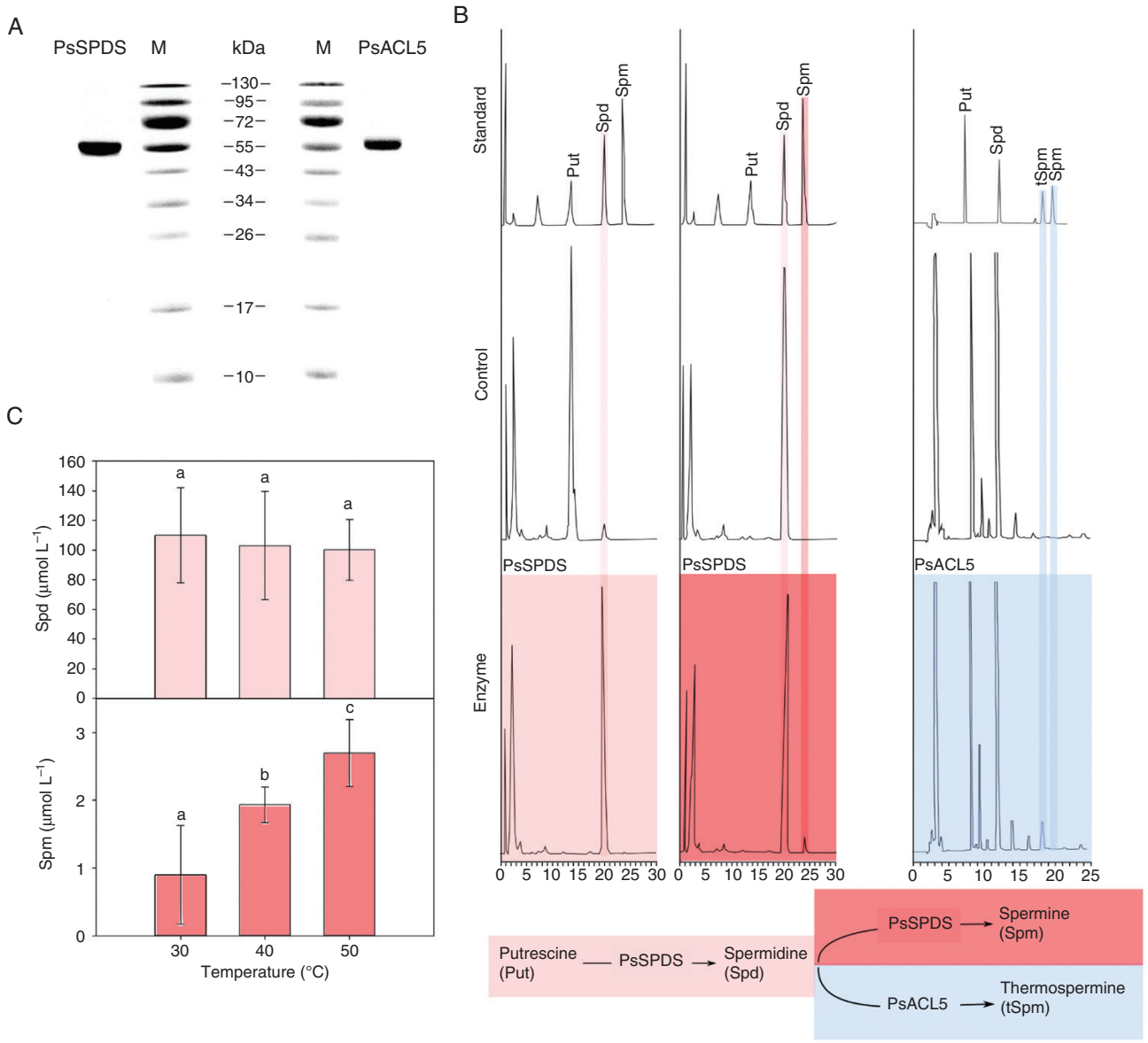

FIG. 1. Size and enzymatic functions of Scots pine spermidine synthase (PsSPDS) and thermospermine synthase (PsACL5) proteins. (A) SDS–PAGE analysis of recombinant PsSPDS and PsACL5 proteins expressed in *E. coli*; purified PsSPDS and PsACL5 proteins with a protein molecular mass marker. (B) Identification of the reaction products after conversion of putrescine (Put) and spermidine (Spd) by PsSPDS and Spd by PsACL5. The HPLC chromatograms show the dansylated polyamine standard of Put, Spd and spermine (Spm) (upper chart); reactions without enzyme as a control (middle chart); reactions with PsSPDS enzyme after 1 h incubation under 50 °C with Put and Spd as substrate, respectively, and a reaction with the PsACL5 enzyme after 1 h incubation under 50 °C with Spd (lower chart). (C) Spd and Spm formation by PsSPDS at 30, 40 and 50 °C incubation temperatures with Put and Spd as a substrate, respectively. Columns (means ± SD) labelled with different letters indicate statistically significant differences ($n = 3$, $P < 0.05$, pairwise t-test, R Software Package 3.3.2).

98.9 % sequence identity. Likewise, there were 97 and 98.9 % sequence identities between the full-length genes and the CDSs of loblolly pine and Scots pine *SPDS*, respectively. BLAST search against the loblolly pine transcript and protein sequences also resulted in one sequence for *PsSPDS* (PITA_000022967-RA). No result was obtained for *PsACL5*; however, the transcript and protein sequences were available only for the genome assembly version v1.01, and this situation may evolve with subsequent updates. Together, these results suggest that there are only single copies of the *PsSPDS* and *PsACL5* genes.

Transcription factor-binding site (TFBS) composition of the PsSPDS *promoter region*

In order to obtain an overview of the roles of bifunctional *PsSPDS* in Scots pine and the separate *SPDS* and *SPMS* genes in angiosperms, putative TFBSs were searched from their promoter regions. The function of TFBSs, when possible, was found out by literature search. Promoter regions of the Scots pine *PsSPDS* gene and the *SPDS* and *SPMS* genes of black cottonwood (*PtSPDS* and *PtSPMS*) and arabidopsis (*AtSPDS* and *AtSPMS*) were used for the analyses. In the presence–absence

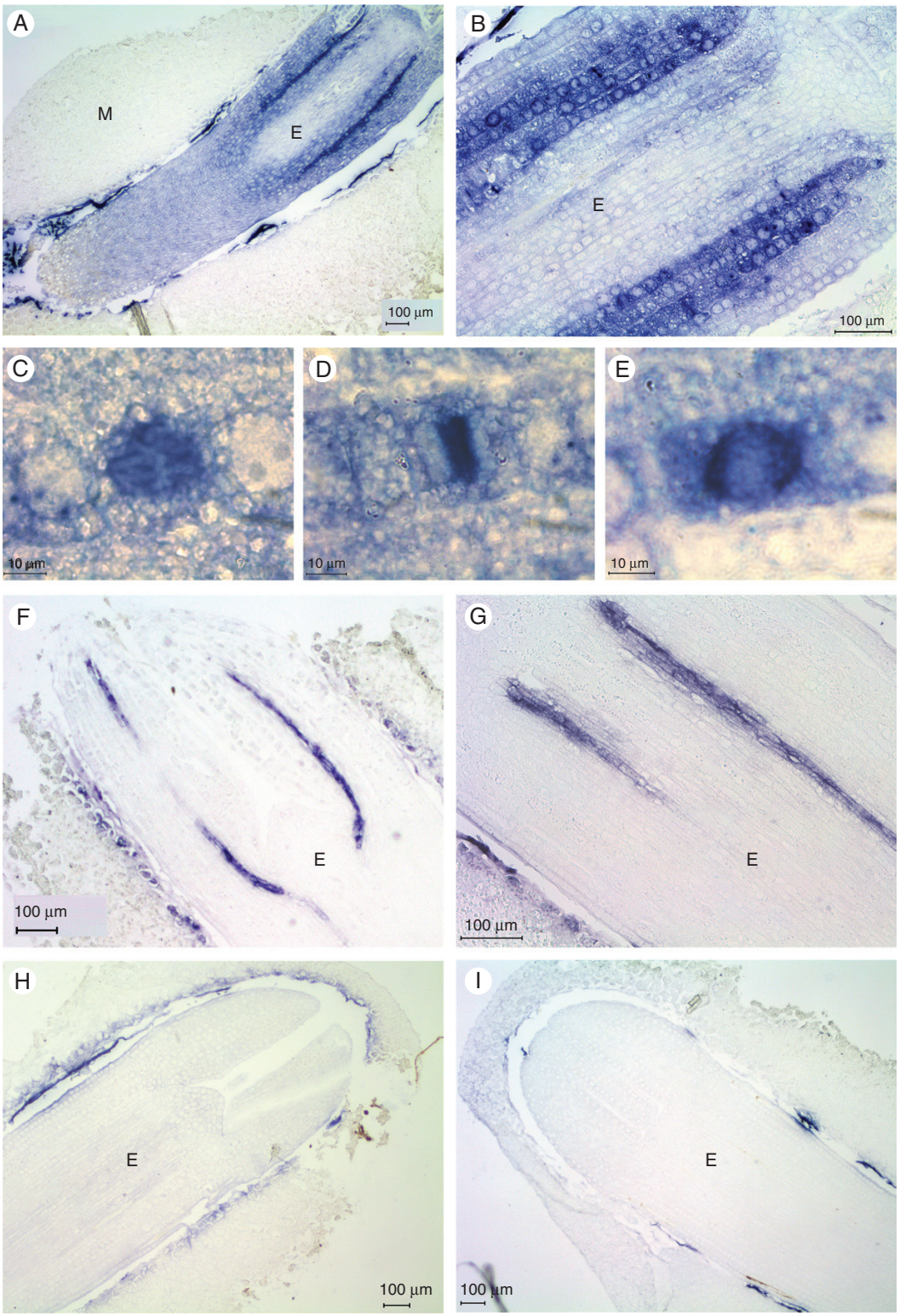

FIG. 2. Localization of spermidine synthase (*PsSPDS*) and thermospermine synthase (*PsACL5*) expression in germinating Scots pine seeds. (A) The localization of *PsSPDS* transcripts in the embryo by mRNA *in situ* hybridization assay using DIG-labelled RNA probes with NBT/BCIP detection (blue signal). (B) Intense *PsSPDS* expression in the procambial cells of the embryo. (C–E) *PsSPDS* expression in the dividing procambial cells. (F and G) *PsACL5* expression in the procambial cells of the embryo. (H) A seed section hybridized with the sense *PsSPDS* probe as a negative control. (I) A section hybridized with the sense *PsACL5* probe as a negative control. E, embryo; M, megagametophyte.

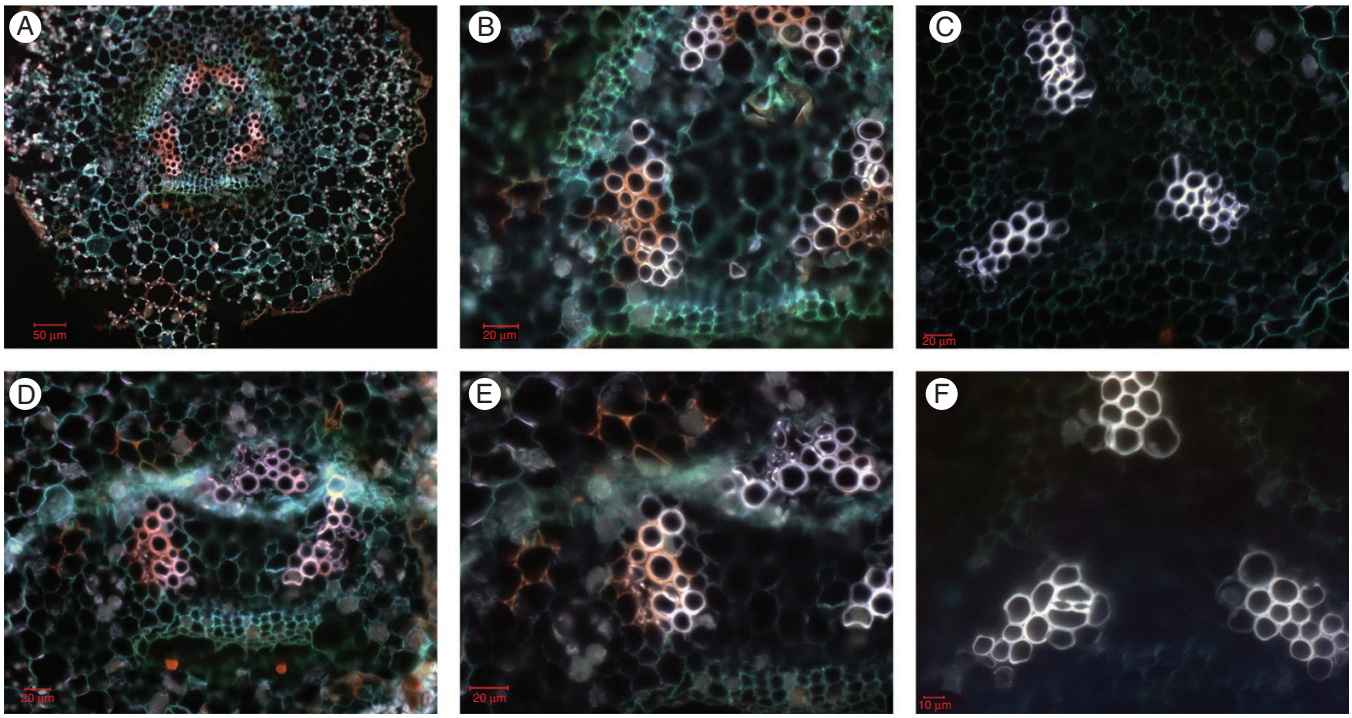

FIG. 3. Localization of spermidine synthase (*PsSPDS*) and thermospermine synthase (*PsACL5*) expression in hypocotyls of Scots pine seedlings. (A and B) The localization of *PsSPDS* transcripts in developing tracheary elements by mRNA *in situ* hybridization assay using DIG-labelled RNA probes with rhodamine detection (red signal). (C) A hypocotyl section hybridized with the sense *PsSPDS* probe as a negative control. (D and E) *PsACL5* expression in developing tracheary elements. (F) A hypocotyl section hybridized with the sense *PsACL5* probe as a negative control.

data of the total of 81 TFBSs detected in the promoter regions of the *SPDS* and *SPMS* genes, 16 TFBSs were not present in the *PsSPDS* promoter (Supplementary Data Table S2). Instead, all TFBSs found from the *PsSPDS* promoter also exist in at least one gene in angiosperms. None of the detected TFBSs was present only in the SPMS promoters. The functions of the angiosperm-specific TFBSs were mostly related to embryogenesis, development and abiotic stress responses (Supplementary Data Table S3). The GNMDS analysis revealed that the TFBS composition of *PsSPDS* resembles more the TFBS composition of the *SPMS* genes of black cottonwood and arabidopsis than the *SPDS* genes of those species (Fig. 4C). The results suggest that PsSPDS carries out many functions, which have been delegated to SPMS after the evolution of separate *SPDS* and *SPMS* genes in angiosperms.

Phylogenetic relationship of plant SPDS, SPMS *and* ACL5 *genes*

The phylogenetic analysis of plant *SPDS*, *SPMS* and *ACL5* sequences confirmed the close relationship between the *SPDS* and *SPMS* sequences, which is in accordance with the view that in angiosperms *SPMS* genes have evolved from *SPDS* genes via gene duplication, whereas the *ACL5* gene seems to have a different evolutionary origin. *SPDS* and *SPMS* sequences divided into separated phylogenetic groups with high bootstrap support (100 %). The *PsSPDS* sequence formed the SPDS group together with the *SPDS* sequences of other conifers, angiosperms and the moss *Physcomitrella*. Furthermore, the phylogenetic analysis confirmed that *PsACL5* belonged to the same main branch as the *ACL5* sequences from other seed plants and *Physcomitrella* (Fig. 5).

DISCUSSION

In the present study, we revealed that Scots pine possesses a bifunctional SPDS enzyme, which is able to produce both Spd and Spm, and that Scots pine tissues contain Spd, Spm and tSpm. To our knowledge, Spm and tSpm have been previously detected in gymnosperms only in ginkgo (*Ginkgo biloba*) nuts (Takano *et al.* 2012) and, so far, the *SPMS* gene has not been found in gymnosperms (Minguet *et al.*, 2008). Thus, differing from the current view, our results demonstrate that SPMS activity is not a novel characteristic which evolved only in the angiosperm line of the seed plants but also exists in the evolutionarily old gymnosperms. The Scots pine *PsSPDS* gene and the *SPDS* genes of the angiosperm species showed a highly similar gene structure, high sequence similarity at the amino acid level as well as a close phylogenetic relationship, providing evidence for a common evolutionary history of the genes.

Only one *SPDS* gene was found in the loblolly pine genome, whereas angiosperm plant species tend to have several copies of *SPDS* genes. The duplication of *SPDS* genes has been suggested to be the origin of a variety of new activities such as SPMS. During evolution, *SPMS* genes have arisen from *SPDS* independently at least three times, in animals, fungi and angiosperm plants (Minguet *et al.*, 2008). Currently, the existence of a separate SPMS gene that may also contribute to Spm synthesis in gymnosperms in addition to the bifunctional SPDS revealed in the present study cannot be definitely excluded

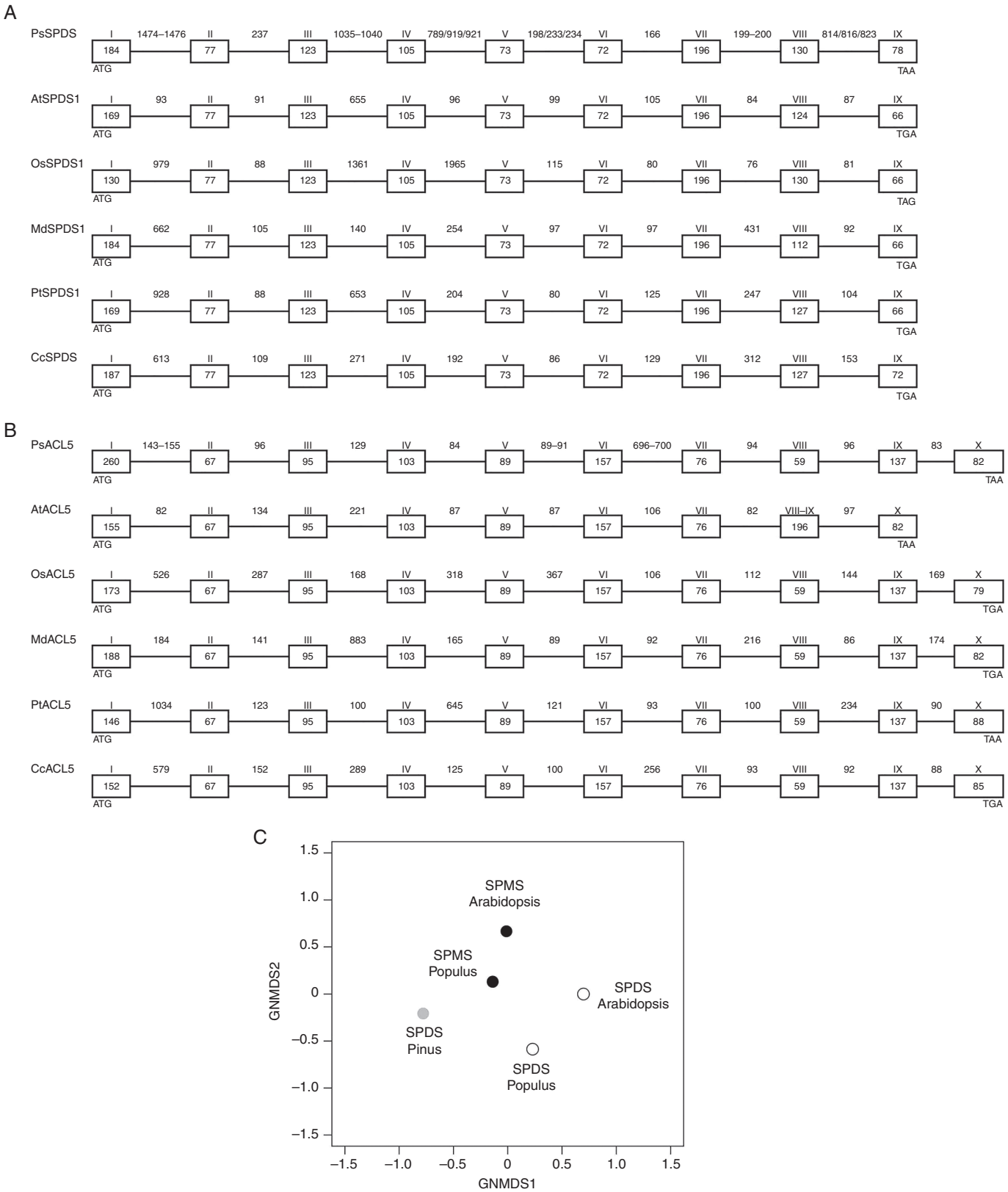

FIG. 4. Structure of Scots pine spermidine synthase (*PsSPDS*) and thermospermine synthase (*PsACL5*) genes and functional analysis of the *PsSPDS* promoter region. (A and B) The exon–intron structures of *PsSPDS* and *PsACL5* were compared with previously characterized plant homologues. Introns are shown as solid lines, while boxes representing exons are numbered from I to X. (C) Global non-metric multidimensional scaling (GNMDS) analysis of the *SPDS* and spermine synthase (*SPMS*) promoter regions. The GNMDS is based on the presence–absence data of the putative transcription factor-binding sites (TFBSs) in the promoter regions of the *SPDS* and *SPMS* genes of *Pinus sylvestris*, *Arabidopsis thaliana* and *Populus trichocarpa*.

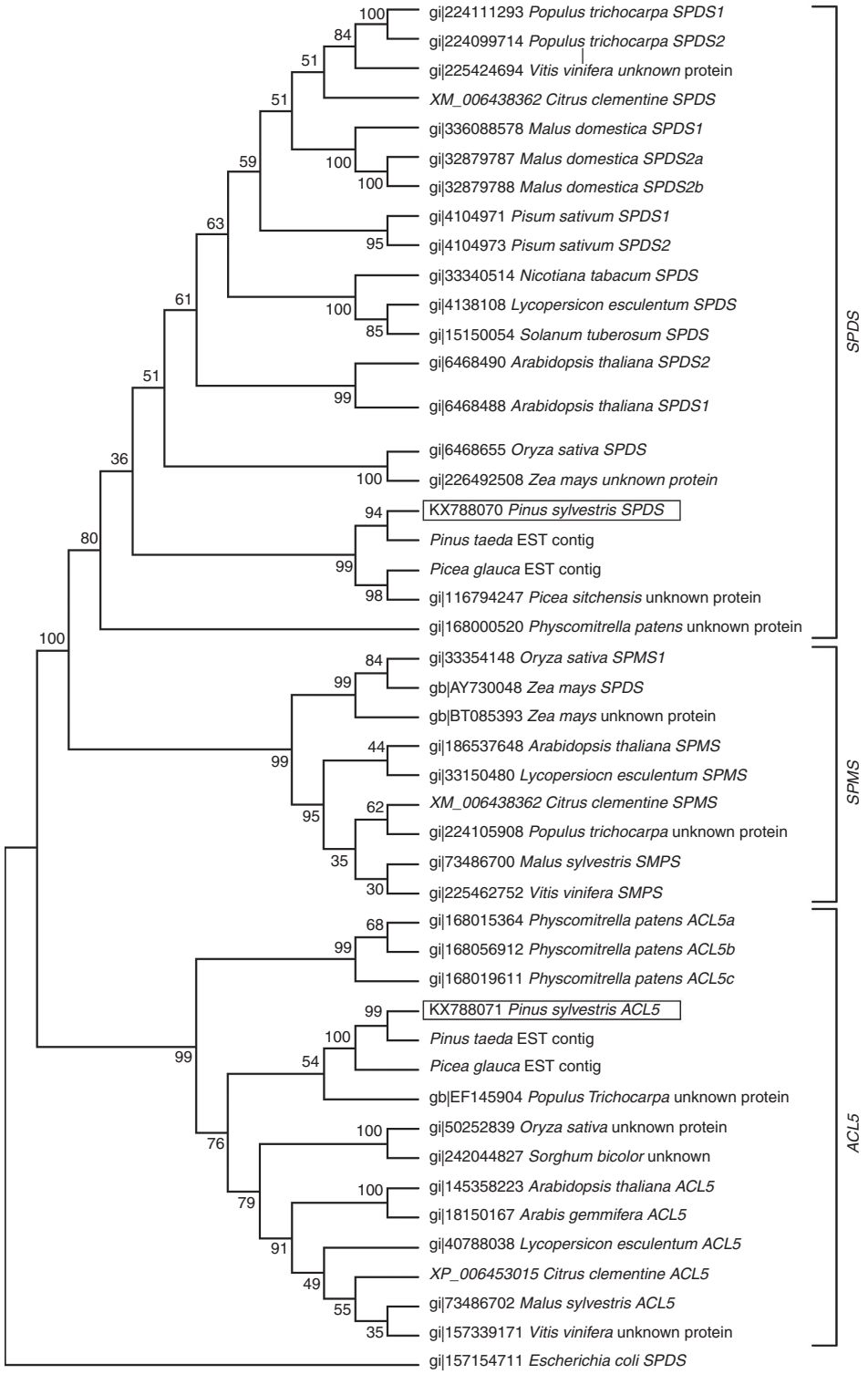

FIG. 5. Phylogenetic analysis of spermidine synthase (*SPDS*), spermine synthase (*SPMS*) and thermospermine synthase (*ACL5*) genes in plants.

due to the imperfect annotation of conifer genomes. Generally, gene duplication has been considered as a major source of new genes and functional innovations, which can originate in different ways, including mutations that directly impart new functions (neofunctionalization), sub-division of ancestral functions (sub-functionalization) and selection for changes in gene dosage (Ohno,1970; reviewed, for example, by Conant and Wolfe, 2008; Hahn, 2009). However, several studies have suggested that most new genes have no novel functions (Prince and Pickett, 2002) and, actually, the predominant fate of most duplicates is loss (Li, 1983; Maere *et al.*, 2005; Hanada *et al.*, 2008).

The mechanisms such as sub-functionalization that do not require the evolution of new functions may play an important role in the initial retention of duplicate genes (Panchy *et al.*, 2016). The patchwork model of enzyme pathway evolution suggests that ancestral enzymes were unspecific and therefore capable of catalysing chemically similar reactions (Fani and Fondi, 2009). After duplication, two genes may be preserved if both members of the pair experience degenerative mutations that result in complementary loss of sub-functions and thus in the portioning of the functions of an ancestral gene among daughter copies (Force *et al.*, 1999; Lynch and Force, 2000). Against this background, it seems likely that the broad substrate specificity of the ancient *SPDS* genes may have favoured the repeated evolution of *SPMS* genes from *SPDS* genes via gene duplications detected in several eukaryotic taxonomic groups by Minguet *et al.* (2008).

There is only little experimental evidence for the various theoretical models of evolution after gene duplication mostly due to incomplete information on functional properties of the progenitor gene, because these ancient genes and the proteins they encode usually no longer exist. Therefore, the bifunctional *PsSPDS* gene found in the present study from an evolutionarily old gymnosperm species together with the separate *SPDS* and *SPMS* genes in angiosperms provide a valuable example of the preservation and evolution of duplicated genes. Our findings suggest that SPMS activity already existed as a secondary property in the *SPDS* progenitor gene. After the *SPDS* duplication in the angiosperm lineage, the functions of the progenitor gene became divided to the daughter copies and SPMS activity co-opted a primary role. In the gymnosperm lineage, the *SPDS* gene remained bifunctional during evolution (Fig. 6).

Sub-functionalization may have occurred simply as a result of accumulation of neutral degenerative mutations that have removed either SPDS or SPMS activity from each gene copy after the duplication of the bifunctional progenitor gene in angiosperms. However, both SPDS and SPMS activities may not have been able to be optimized simultaneously in the bifunctional enzyme due to the different roles of Spd and Spm in plant cells. Thus, separate SPDS and SPMS enzymes might have been a solution to the conflict and provided a clear adaptive advantage. In eukaryotic organisms, hypusine synthesis defines an absolute requirement for Spd (Chattopadhyay *et al.*, 2003, 2008; reviewed by Wolff and Park, 2015). The arabidopsis *spds1-1 spds2-1* double mutant lacking Spd shows an embryo lethal phenotype (Imai *et al.*, 2004*b*). Our findings in the present study supported the essential role of Spd in basic cell functions, since *PsSPDS* expression was detected in all cells of the Scots pine embryo. Moreover, *PsSPDS* expression was localized in dividing cells, where *ADC*, the enzyme catalysing the preceding step in the PA biosynthesis pathway, has also been shown to be expressed (Vuosku *et al.*, 2006). Unlike Spd, Spm is not essential for life but has generally been considered as a stress-protective molecule in plants (Yamaguchi *et al.*, 2007; Do *et al.*, 2013). Arabidopsis plants with blocked activity of *spm* mutants and without Spm show a similar phenotype to the wild type under optimal growth conditions, but the mutants are very sensitive to stresses (Imai *et al.*, 2004*a*). In pine tissues, Spm also accumulates under abiotic stresses (Islam *et al.*, 2003; Muilu-Mäkelä *et al.*, 2015). Taken together, our results propose that the evolution of separate *SPDS* and *SPMS* genes in angiosperms has provided an adaptive advantage not only by making the Spm production more efficient but also by making the regulation of the SPDS and SPMS activities more flexible, whereas the loss of SPMS activity after gene duplication would have led to a fitness cost. The TFBS profile of the *PsSPDS* promoter resembles more the promoter TFBS profiles in *SPMS* genes than *SPDS* genes in angiosperms. The result suggests that bifunctional PsSPDS due to the production of Spm as a secondary function also possesses a wide variety of other functions, which have been acquired by *SPMS* genes in angiosperms. Furthermore, the presence of separate *SPDS* and *SPMS* genes may have released the regulation of the *SPDS* genes to evolve more freely compared with bifunctional *PsSPDS*.

Both PsSPDS and PsACL5 also retained catalytic activity at temperatures which are remarkably high for plant enzymes. Furthermore, PsSPDS seemed to have increasing affinity for Spd as a substrate at high temperatures. Raising the incubation temperature for PsSPDS from 30 to 50 °C tripled Spm formation, whereas there was no significant change in the amount of Spd produced. The result suggests the importance of Spd and especially Spm production at elevated temperatures and under heat stress in Scots pine, which is in agreement with previous reports on angiosperm species (Kasukabe *et al.*, 2006; Cheng *et al.*, 2009; Sagor *et al.*, 2013; Sang *et al.*, 2017). During the seasonal fluctuation of free PAs in Scots pine needles, low Spm/tSpm concentrations coincided with low temperatures in winter (Sarjala and Savonen, 1994; Sarjala and Kaunisto, 1996), which also supports the connection between environmental temperatures and Spm/tSpm production.

The *ACL5* in eukaryotes seems to be an ancient plant-specific gene, which has been proposed to originate from a horizontal gene transfer from Archaea or Bacteria to plants (Minguet *et al.*, 2008). In arabidopsis, tSpm has been identified as a novel plant growth regulator that represses xylem differentiation and promotes stem elongation by preventing premature death of developing xylem vessel elements (Kakehi *et al.*, 2008; Muniz *et al.*, 2008; Vera-Sirera *et al.*, 2010). Our findings in the present study enhance the role of ACL5 and tSpm in development of vascular structures in conifers. *PsACL5* and also *SPDS* transcripts were localized in the procambial cells in Scots pine germinating embryos and, moreover, in the developing tracheary elements of hypocotyls in young Scots pine seedlings. All vascular tissues are derived from undifferentiated, meristematic cells, and the body plan for the vasculature in the adult plant is already established in the embryo (Ye, 2002). Xylem is a specialized vascular tissue that serves as a conduit of water and nutrients, and provides mechanical strength for upright growth (Růžička *et al.*, 2015). The important part of mature xylem is composed of tracheary elements, which fall into two broad categories: tracheids, typically found in lycophytes, ferns and gymnosperms; and vessel elements, which reach their peak of diversity in the angiosperms (Wilson, 2013). Altogether, the role of ACL5 in the development of tracheary elements in angiosperm and gymnosperm species, but also the presence of three *ACL5* copies in the non-vascular moss *Physcomitrella* (Rodríguez-Kessler *et al.*, 2010), suggest that ACL5 and tSpm perform multiple tasks in the development of land plants.

In conclusion, our results revealed that an evolutionarily old gymnosperm, Scots pine, has a bifunctional SPDS able to produce both Spd and Spm, while angiosperms seem to depend on

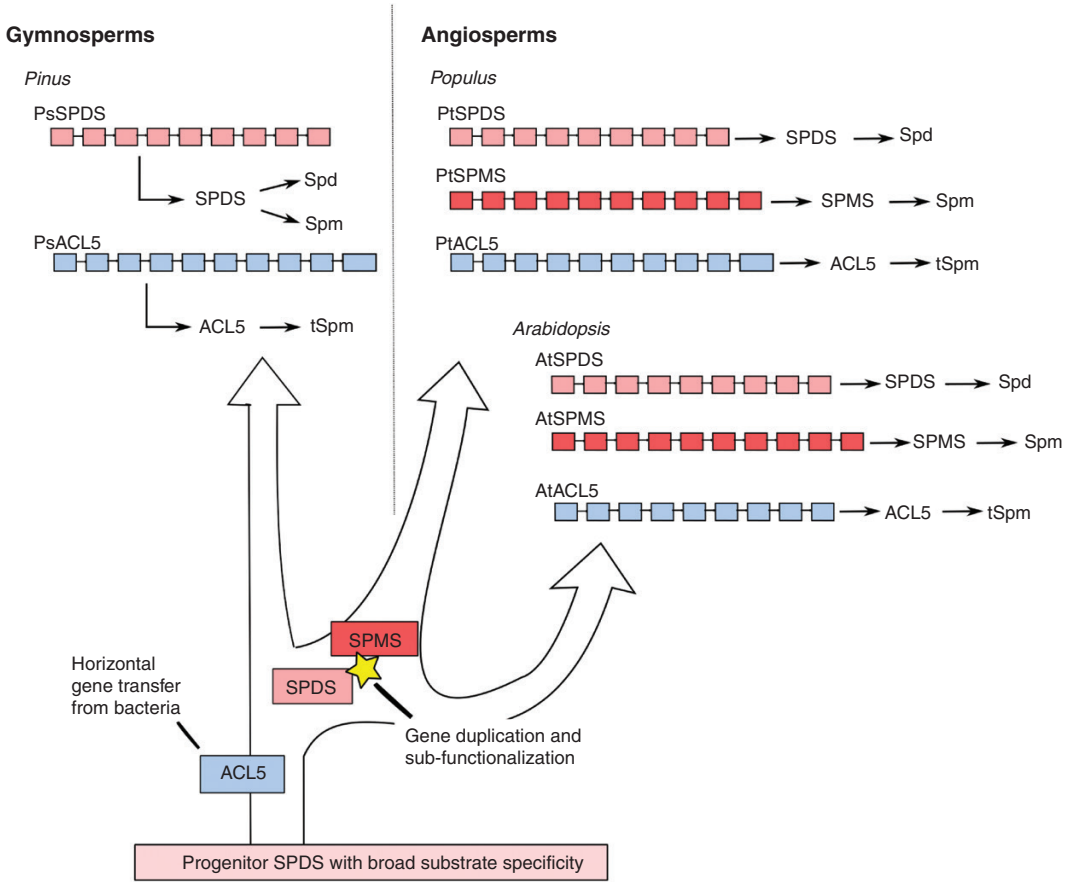

FIG. 6. Proposed model for evolution of aminopropyltransferases in seed plants. In ancient seed plants, the spermidine synthase (SPDS) enzyme possessed a broad substrate specificity and showed spermine synthase (SPMS) activity as a secondary property. After the duplication of the *SPDS* gene in the angiosperm lineage, the functions of the progenitor enzyme were divided to the daughter copies, and SPMS activity co-opted a primary role in one copy, whereas the bifunctional form was preserved in the gymnosperm lineage. The thermospermine synthase (*ACL5*) gene seems to have a different evolutionary origin and may have been acquired by plants via horizontal gene transfer.

separate enzymes in Spd and Spm biosynthesis. The *PsACL5* gene was found to encode a protein with ACL5 activity catalysing the production of tSpm, and the expression of *PsACL5* was associated with the development of vascular structures during early development of Scots pine. Altogether, the bifunctional SPDS and vascular-associated ACL5 enzymes of Scots pine shed new light on the evolution and function of APTs in seed plants.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: polyamine biosynthesis pathway in plants. Figure S2: the alignment of the *SPDS*, *SPMS* and *ACL5* sequences for phylogenetic analysis. Figure S3: comparison of the predicted amino acid sequence of the *P. sylvestris* PsSPDS protein with previously characterized homologues from angiosperm. Figure S4: comparison of the predicted amino acid sequence of the *P. sylvestris* PsACL5 protein with previously characterized homologues from angiosperm. Figure S5: area of the tSpm HPLC peaks in 100 nM reaction mixture with PsACL5 enzyme and Spd as a substrate after 1 h incubation under three different temperatures (30, 40 and 50 °C). Table S1: primers for PCR and sequencing of the *PsSPDS* and *PsACL5* genes from Scots pine. Table S2: presence–absence data generated from the transcription factor-binding sites (TFBSs) found in the 3 kb promoter regions of *SPDS* and *SPMS* genes of *Populus trichocarpa* (*PtSPDS* and *PtSPMS*), *Arabidopsis thaliana* (*AtSPDS* and *AtSPMS*) and *Pinus sylvestris* (*PsSPDS*) using the PlantPAN 2.0. database. Table S3: transcription factor-binding sites (TFBSs) and their functions found in the 3 kb promoter regions of *SPDS* and *SPMS* genes of *Populus trichocarpa* (*PtSPDS* and *PtSPMS*), *Arabidopsis thaliana* (*AtSPDS* and *AtSPMS*) and *Pinus sylvestris* (*PsSPDS*) using the PlantPAN 2.0. database.

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