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Original article

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

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Running title: Evolution and function of Scots pine aminopropyltransferases

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ABSTRACT

- **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 analyzed for polyamines by 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 analyzed.
- **Key Results** Scots pine tissues were found to contain spermidine, spermine and thermospermine. PsSPDS enzyme catalyzed 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 view, our results demonstrate that SPMS activity is not a novel feature developed solely in the angiosperm lineage of seed plants but also exist as a

secondary property in the Scots pine SPDS enzyme. The discovery of bifunctional SPDS from an evolutionary 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 diamine putrescine (Put), triamine spermidine (Spd) and tetra-amine spermine (Spm) (Tiburcio *et al*., 1997). In addition, thermospermine (tSpm), a less abundant structural isomer of Spm, is widely detected in plant kingdom (Takano *et al*., 2012). In mammalian cells, Put is synthesized from ornithine by ornithine decarboxylase (ODC; EC 4.1.1.17) and also in many fungi ODC is the sole pathway for Put production (Coleman *et al*., 2004). Plants are able to produce Put also 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 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 unsymmetrical 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 unsymmetrical tSpm molecule (Pegg and Michael, 2010) (Supplementary Data Fig. S1). To distinguish Spm and tSpm, benzylation of the PAs is needed followed by separation with HPLC (Naka *et al*., 2010).

Several reports have indicated the necessity of Spd for the viability in eukaryotes, whereas eukaryotes deficient in SPMS and thus lacking Spm are mostly viable, even if they may suffer different degrees of dysfunctions (Pegg and Michael, 2010). One of the essential functions of Spd is its role as a substrate for the 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*., 2004b). In contrast, Spm was not essential for survival of Arabidopsis under normal growth conditions (Imai *et al*., 2004a), but Spm-deficient mutants were hypersensitive to drought and salt stress (Yamaguchi *et al*., 2006, 2007). The 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 coexist 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 analyzed 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 TM (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 days imbibition, and the cotyledons, hypocotyls and roots of the seedlings were excised after 16 days 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 benzylation method

The presence of PAs (Put, Spd, Spm and tSpm) in Scots pine tissues was discovered by using benzylation 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 concentration of the benzylated PAs in the tissue samples and in the reaction mixture was separated by HPLC and detected at 254 nm according to Naka *et al*. (2010). The standard for tSpm was kindly provided by Prof. 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 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 upstream primer 5'-TATGCC**ATGG**GGGAAGTAGCAGC-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 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* sequence has 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) at 30 °C until the D_{600} of the culture reached 0.6. Protein expression was induced by 0.5 mM IPTG and the cultures were further incubated at 16 °C for 18 hours. 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).

PA analyses were performed for confirming 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 Prof. Keijiro Samenima), substrate (5 mM Put or Spd) and 34 µg purified PsSDPD protein. The reaction mixtures were incubated for two hours at 30 °C, 40 °C, or 50 °C. After two hours 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 one week with HPLC (Merck Hitachi, Darmstadt, Germany) and fluorescence spectrophotometry (Merck Hitachi F-1050) at excitation and emission wavelengths of 365 nm and 510 nm, respectively. The PAs were separated by using 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 Prof. Yoshihiko Ikeguchi) and 14 µg purified enzyme. The reaction mixture was incubated for one hour at 30 °C, 40 °C, and 50 °C. The presence of PAs in the reaction mixture was discovered by using benzylation 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 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 color uniformly to 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 the 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 Big Dye Terminator kit v3.1 (Applied Biosystems). Following sequencing, manual checking and edition 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 UCDavis Dendrome website (http://dendrome.ucdavis.edu/ftp/Genome_Data/genome/pinerefseq/Pita/) and used for full BLAST search on local server. The results of the BLAST search were analyzed 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 (TFBS) and the corresponding transcription factors (TF) within the promoter regions of *SPMS* and *SPDS* genes as well as their functions were searched using 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 TFBS/TF 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 a statistical program R (R Core Team, 2015) using presence-absence data. The analysis was performed with 50 separate random starts with function metaMDS using 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, 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 to the 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 dataset. 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. Especially, we were interested in the copresence 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. 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 coding 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 with 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 (*Populus trichocarpa*), 70 % with SPDS1 of apple (*Malus x domestica*), and 67 % identity 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 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 % identity 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 *Escherichia coli* strain BL21 with pET-32a expression vector. The SDS-PAGE analysis of the purified recombinant proteins resulted in bands with a molecular mass of ~55 kDa for protein encoded by *PsSPDS* and ~60 kDa for protein encoded by *PsACL5* (Fig. 1A) which coincides with the calculated molecular masses of recombinants with N-terminal His-tag (SPDS protein, 55.5 kDa; ACL5 protein, 58.9 kDa).

We found that purified PsSPDS catalyzes 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 °C 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 reaction mixture with the PsACL5 protein confirmed that tSpm was produced in all incubation temperatures at 30 °C, 40 °C and 50 °C (Supplementary Data Fig. S5).

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* also expressed during cell division (Fig. 2B-E). Unlike the *PsSPDS* gene, *PsACL5* was found to express 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 Ps*ACL5* 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) versus ten for *PsACL5* (Fig 4B). *PsSPDS* harbored overall larger introns leading to gene size of between 5995 bp and 6135 bp for *PsSPDS* versus between 2641 bp and 2653 bp for *PsACL5* from start codon to 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 the evolution in seed plants, suggesting an orthologous origin for the genes. The orthologs accumulate much fewer structural differences unlike the paralogs (Xu *et al.,* 2012). All *SPDS* orthologs harbored nine exons with same sizes except for exons I and IX, but the size of the large introns was highly variable (Fig. 4A). For the *ACL5* orthologs, 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 regions showed 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 composition of PsSPDS promoter region

In order to get an overview about the roles of bifunctional *PsSPDS* in Scots pine and the separate *SPDS* and *SPMS* genes in angiosperms, putative transcription factor binding sites (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 data of the total 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 exist also at least in one gene in angiosperms. None of the detected TFBSs were 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 global non-metric multidimensional scaling (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 on 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 with 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 evolved only in the angiosperm line of the seed plants but SPMS activity also exists in the evolutionary old gymnosperms. The Scots pine *PsSPDS* gene and the *SPDS* genes of the angiosperm species showed highly similar gene structure, high sequence similarity at amino acid level as well as close phylogenetic relationship providing evidence for a common evolutionary history of the genes.

Only one *SPDS* gene was found from 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 also may contribute to Spm synthesis in gymnosperms in addition to the bifunctional SPDS revealed in the present study cannot be definitely excluded 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), subdivision of ancestral functions (subfunctionalization) and selection for changes in gene dosage (Ohno,1970; reviewed e.g. 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 subfunctionalization 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 to catalyze 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 subfunctions 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 favored 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 evolutionary 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 gymnosperm lineage, the SPDS gene remained bifunctional during evolution (Fig. 6).

Subfunctionalization 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 and 2008; reviewed by Wolff and Park, 2015). Arabidopsis *spds1-1*

spds2-1 double-mutant lacking Spd shows embryo lethal phenotype (Imai *et al*., 2004b). Our findings in the present study supported the essential role 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 also *ADC*, the enzyme catalyzing the preceding step in the PA biosynthesis pathway, has 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 *spms* and without Spm show similar phenotype as the wild type under optimal growth conditions, but the mutants are very sensitive to stresses (Imai *et al*., 2004a). 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 by making not only the Spm production more efficient but also the regulation of the SPDS and SPMS activities more flexible, whereas the loss of SPMS activity after gene duplication would have led to fitness cost. The TFBS profile of the *PsSPDS* promoter resembles more the promoter TFBS profiles in *SPMS* genes than *SPDS* genes in angiosperm. 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 to bifunctional *PsSPDS*.

Both PsSPDS and PsACL5 retained catalytic activity also at temperatures, which are remarkable 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 °C to 50 °C tripled Spm formation, whereas there was no significant change in the amount of

produced Spd. The result suggest the importance of Spd and especially Spm production in elevated temperatures and heat stress in Scots pine, which is in agreement with the 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 support 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 TEs in angiosperm and gymnosperm species, but also the presence of three *ACL5* copies in non-vascular moss *Physcomitrella* (Rodríguez-

Kessler *et al*., 2010), suggest that ACL5 and further tSpm perform multiple tasks in the development of land plants.

In conclusion, our results revealed that an evolutionary old gymnosperm, Scots pine, has a bifunctional SPDS able to produce both spd and spm, when angiosperms seem to lean on separate enzymes in spd and spm biosynthesis. The *PsACL5* gene was found to code a protein with ACL5 activity catalyzing 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 aminopropyltransferases in seed plants.

SUPPLEMENTARY DATA

Supplementary data are available in online and consist of the following. Fig. S1: Polyamine biosynthesis pathway in plants. Fig S2: The alignment of the *SPDS*, *SPMS* and *ACL5* sequences for phylogenetic analysis. Fig. S3: Comparison of the predicted amino acid sequence of the *P. sylvestris* PsSPDS protein with previously characterized homologues from angiosperm. Fig. S4: Comparison of the predicted amino acid sequence of the *P. sylvestris* PsACL5 protein with previously characterized homologues from angiosperm. Fig. S5: Area of the tSpm HPLC peaks in 100 nM reaction mixture with PsACL5 enzyme and Spd as a substrate after 1h 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 3kb promoter regions of *SPDS* and *SPMS* genes of *Populus trichocarpa* (*PtSPDS* and *PtSPMS*), *Arabidopsis thaliana* (*AtSPDS* and *AtSPMS*) and *Pinus sylvestris* (*PsSPDS*) using PlantPAN 2.0. database. Table S3: Transcription factor binding sites (TFBSs) and their functions found in the 3kb promoter regions

of *SPDS* and *SPMS* genes of *Populus trichocarpa* (*PtSPDS* and *PtSPMS*), *Arabidopsis thaliana* (*AtSPDS* and *AtSPMS*) and *Pinus sylvestris* (*PsSPDS*) using PlantPAN 2.0. database.

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FIGURE LEGENDS

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 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 dansylated polyamine standard of Put, Spd and spermine (Spm) (upper chart); reactions without enzyme as a control (middle chart); reactions with PsSPDS enzyme after 1h incubation under 50°C degrees with Put and Spd as a substrate, respectively, and a reaction with PsACL5 enzyme after 1h incubation under 50°C degrees with Spd (lower chart). (C) Spd and Spm formation by PsSPDS at 30°C, 40°C and 50°C incubation temperatures with Put and Spd as a substrate, respectively. Columns labeled with different letters indicate statistically significant differences (n=3, $p<0.05$, pairwise t-test, R Software Package 3.3.2).

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, D and E) *PsSPDS* expression in the dividing procambial cells. (F and G) Ps*ACL5* 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 thermoseprmine 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.

FIG 4. Structure of Scots pine spermidine synthase (*PsSPDS*) and thermospermine synthase (*PsACL5*) genes and functional analysis of *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.

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 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.

TABLE 1. *Polyamine content (nmol g FW^{-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 benzylated polyamines according to Naka *et al*. (2010).

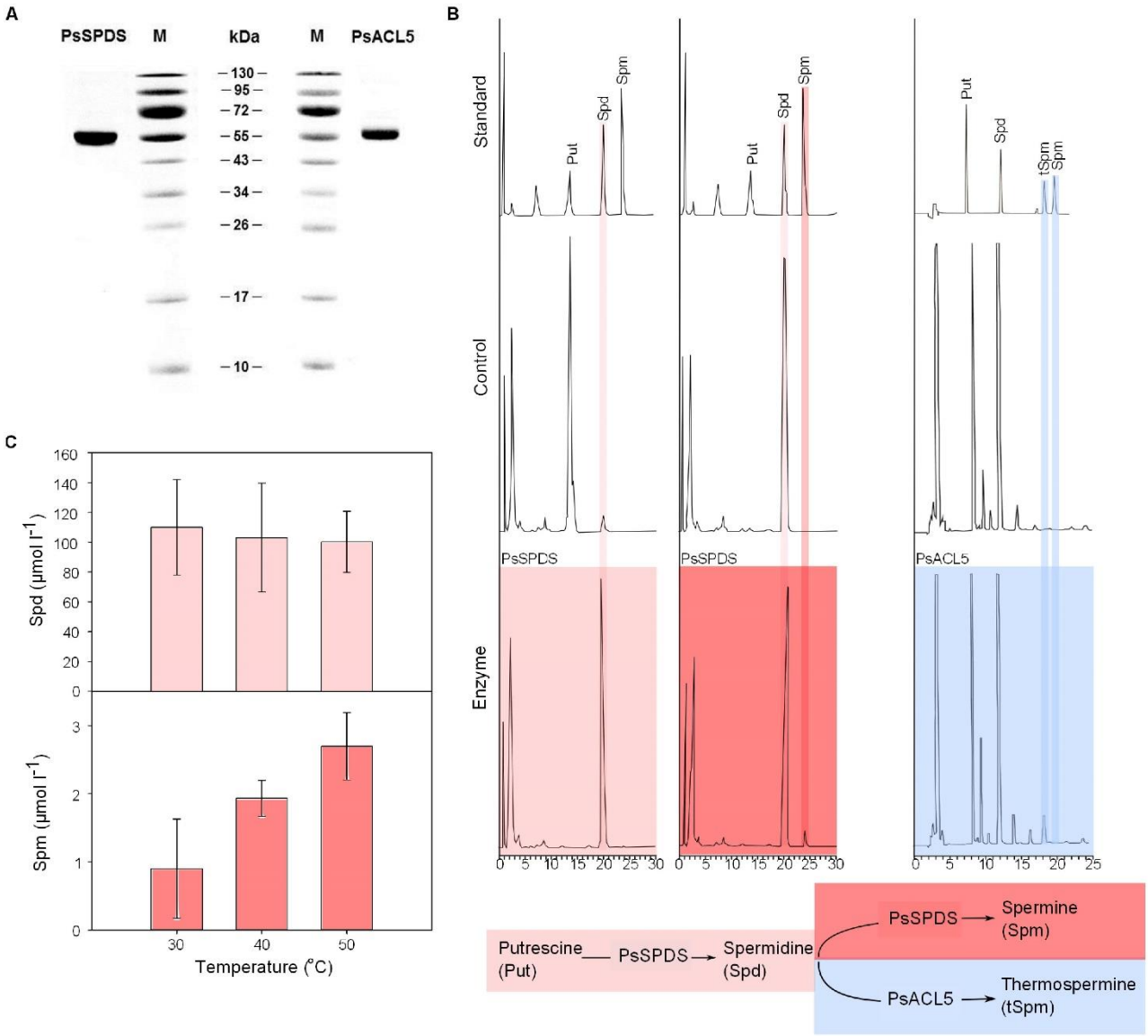


FIG 1. Size and enzymatic functions of Scots pine spermidine synthase (PsSPDS) and thermospermine synthase (PsACL5) proteins. A, SDS-PAGE analysis of recombinant PsSPDS and PsACL5 proteins expressed in *E. coli*; purified PsSPDS and PsACL5 proteins with protein molecular mass marker. B, Identification of the reaction products after conversion of putrescine (Put) and spermidine (Spd) by PsSPDS and Spd by PsACL5. The HPLC chromatograms show dansylated polyamine standard of Put, Spd and spermine (Spm) (upper chart); reactions without enzyme as a control (middle chart); reactions with PsSPDS enzyme after 1h incubation under 50°C degrees with Put and Spd as a substrate, respectively, and a reaction with PsACL5 enzyme after 1h incubation under 50°C degrees with Spd (lower chart). C, Spd and Spm formation by PsSPDS at 30°C, 40°C and 50°C incubation temperatures with Put and Spd as a substrate, respectively.

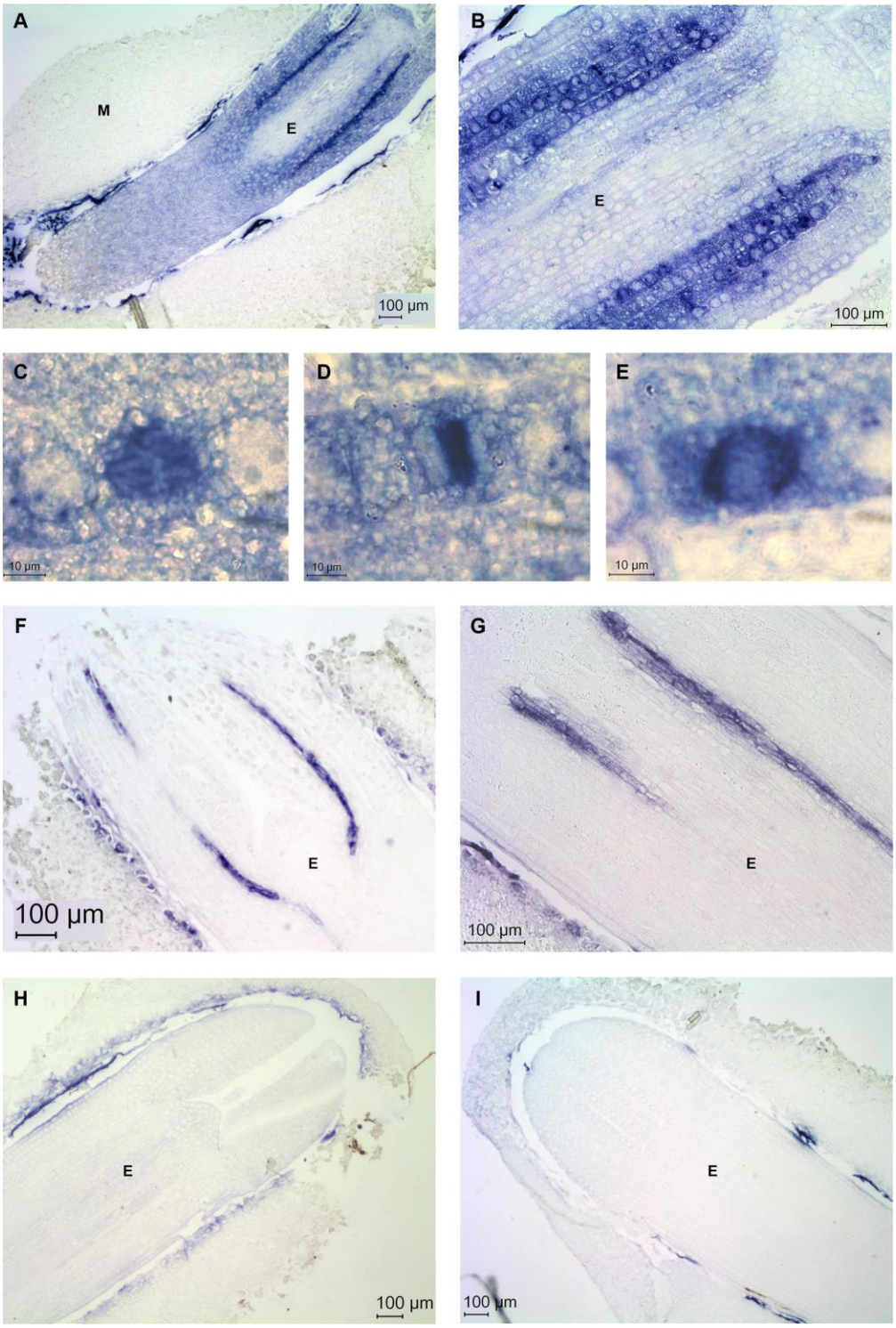


FIG 2. Localization of spermidine synthase (*PsSPDS*) and thermospermine synthase (*PsACL5*) expression in germinating Scots pine seeds. A, The localization of *PsSPDS* transcripts in the embryo by mRNA *in situ* hybridization assay using DIG-labelled RNA-probes with NBT/BCIP detection (blue signal). B, Intense *PsSPDS* expression in the procambial cells of the embryo. C, D, E, *PsSPDS* expression in the dividing procambial cells. F, G, Ps*ACL5* 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.

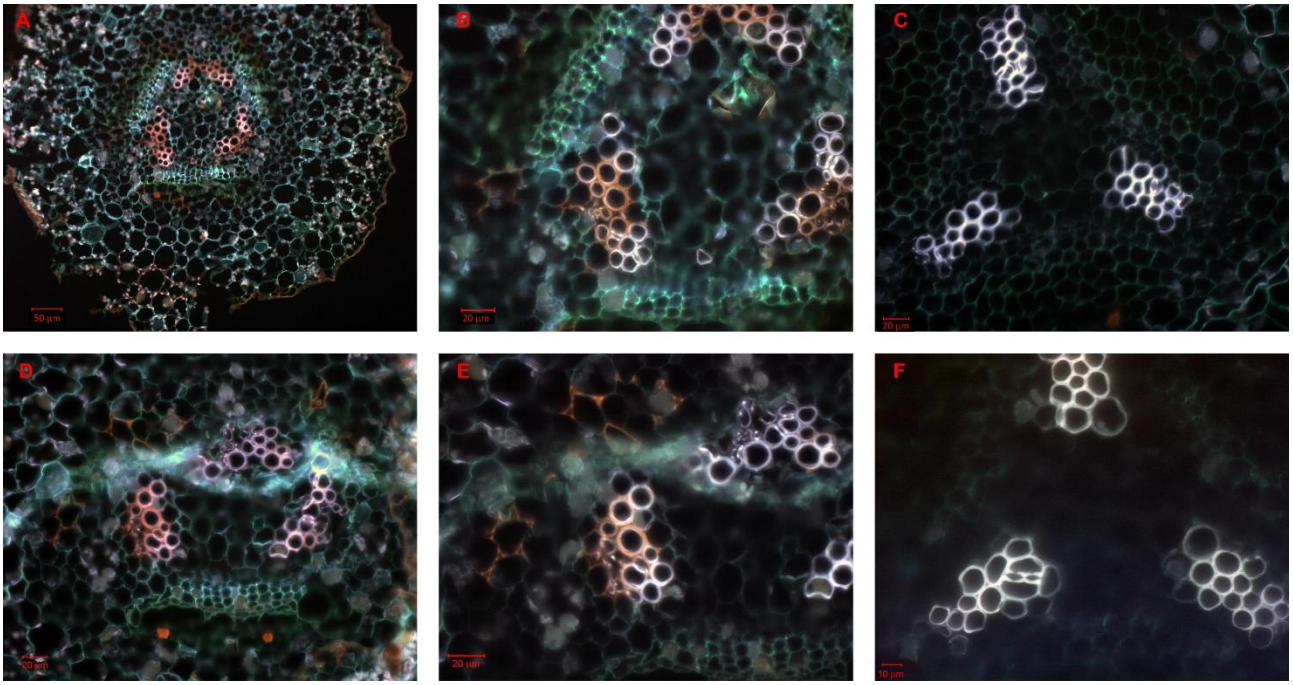


FIG 3. Localization of spermidine synthase (*PsSPDS*) and thermoseprmine synthase (*PsACL5*) expression in hypocotyls of Scots pine seedlings. A, B, The localization of *PsSPDS* transcripts in developing tracheary elements by mRNA *in situ* hybridization assay using DIG-labelled RNA-probes with rhodamine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

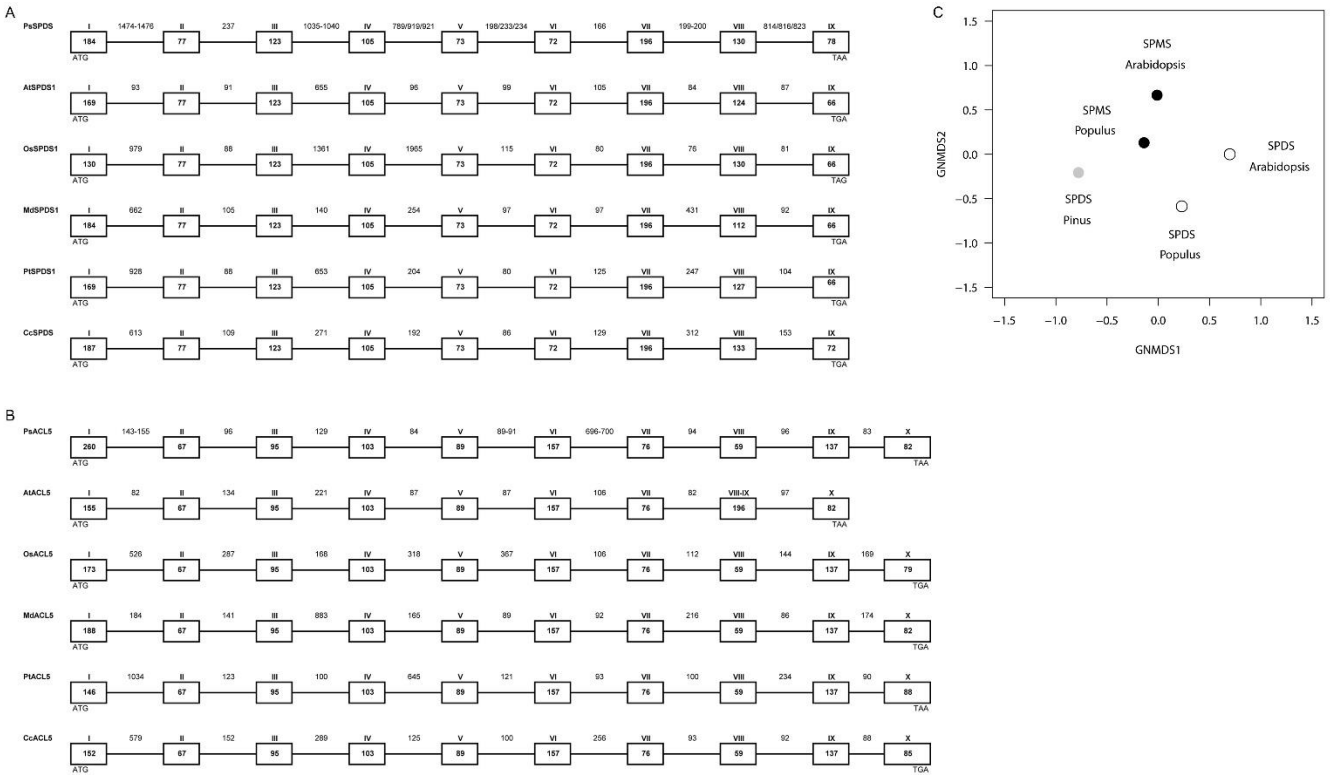


FIG 4. Genomic structure of Scots pine spermidine synthase (*PsSPDS*) and thermospermine synthase (*PsACL5*) genes and functional analysis of *PsSPDS* promoter region. A,B, The exon-intron structures of *PsSPDS* and *PsACL5* were compared with previously characterized plant homologues. Introns are shown 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*.

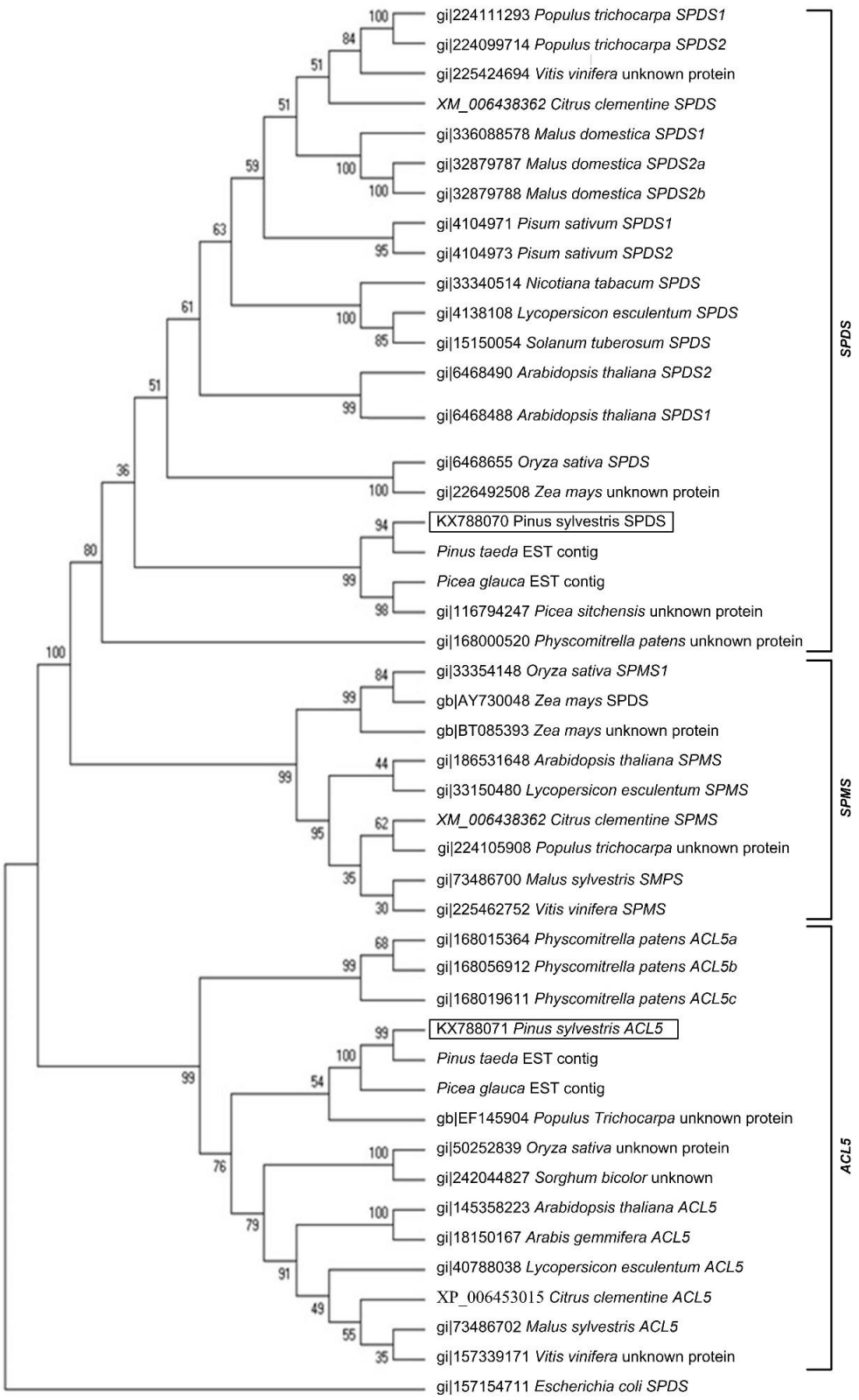


FIG 5. Phylogenetic analysis of spermidine synthase (*SPDS*), spermine synthase (*SPMS*) and thermospermine synthase (*ACL5*) genes in plants.

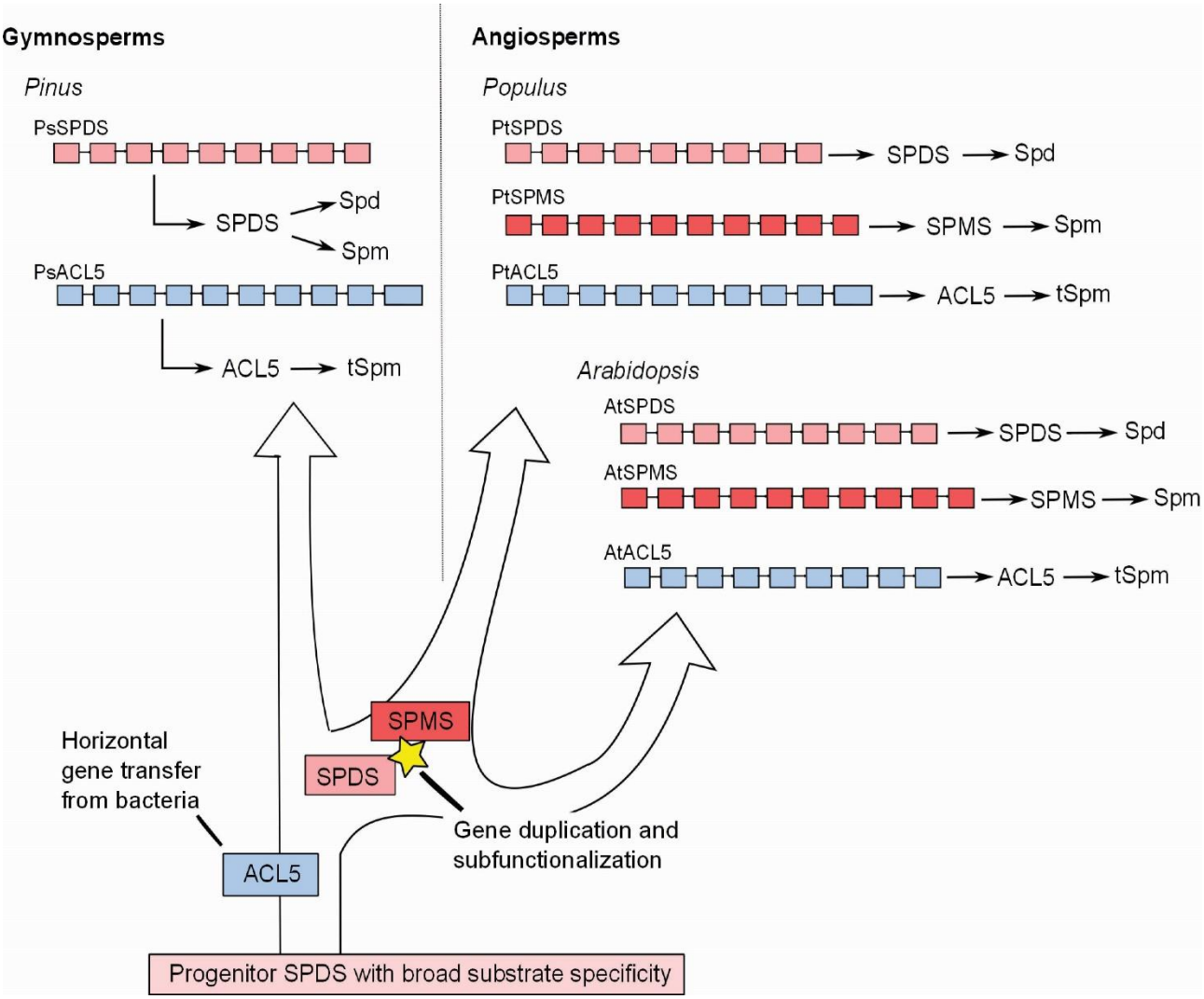
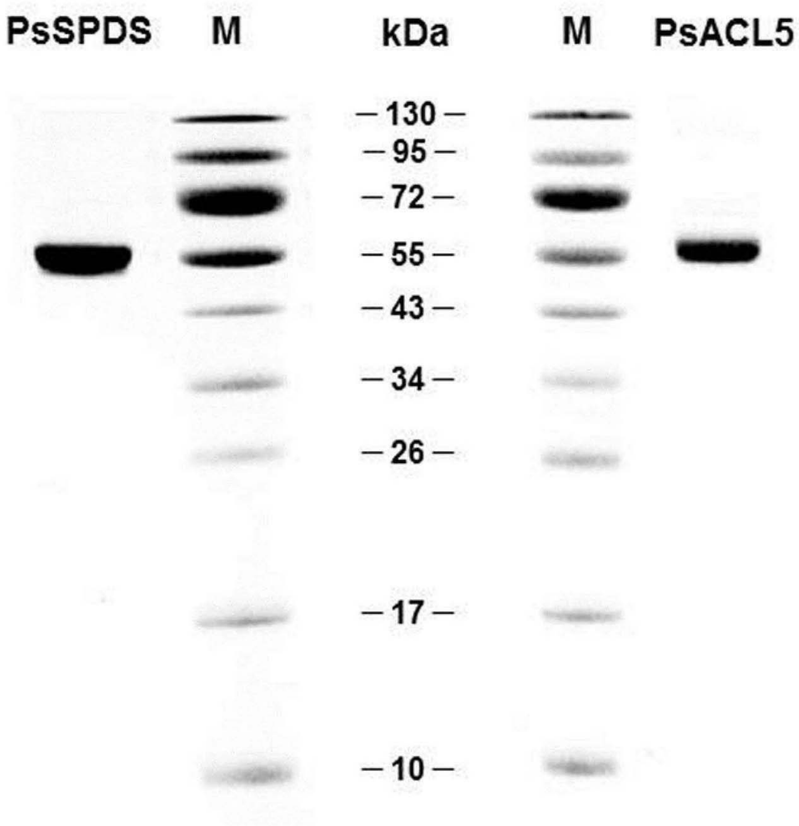
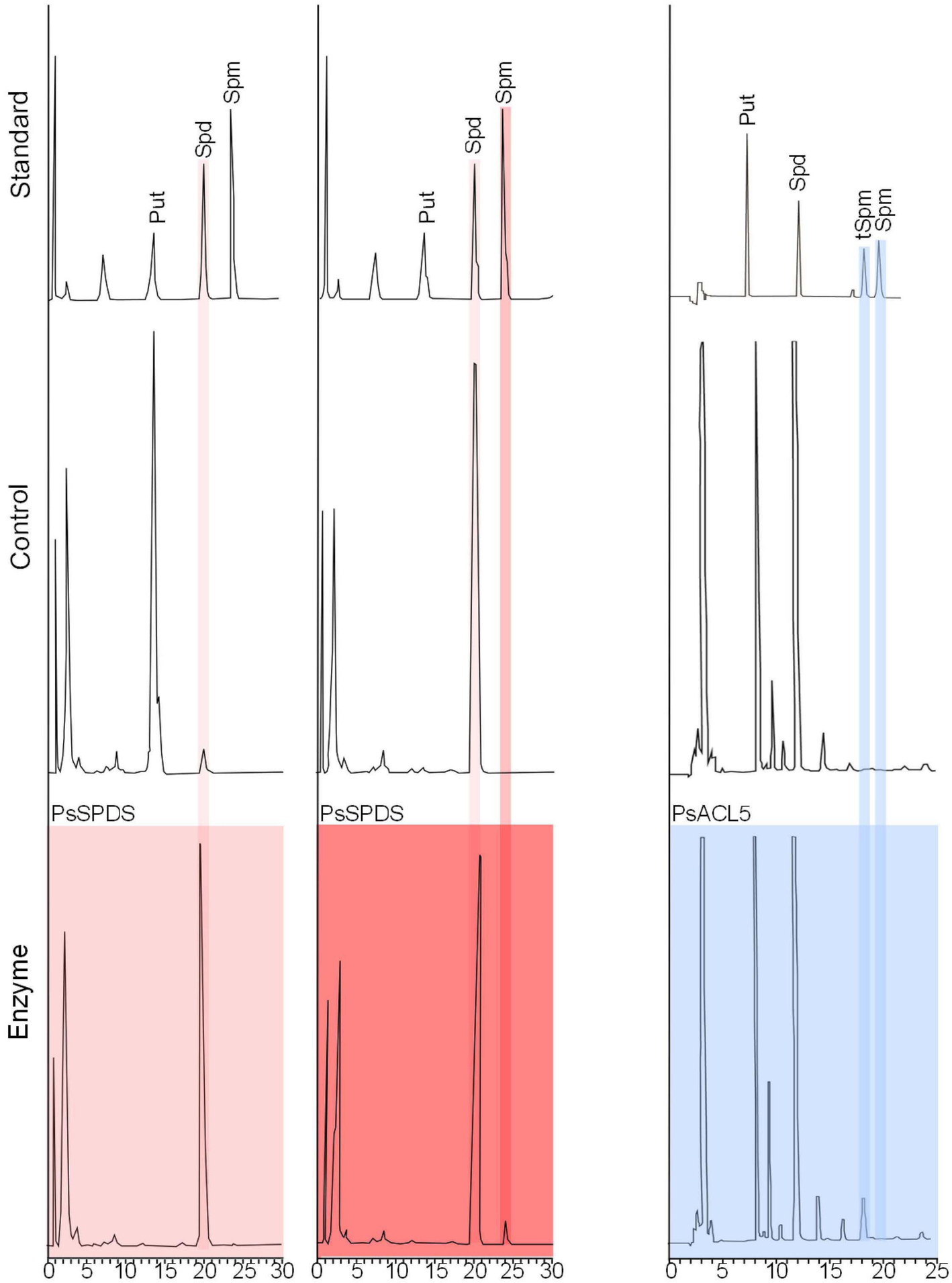


FIG 6. Proposed model for evolution of aminopropyltransferases in seed plants. In ancient seed plants, the spermidine synthase (SPDS) enzyme possessed a broad substrate specificity and showed spermine synthase (SPMS) activity as a secondary property. After the duplication of the *SPDS* gene in the angiosperm 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 gymnosperm lineage. The thermospermine synthse (*ACL5*) gene seems to have a different evolutionary origin and may have been acquired by plants via horizontal gene transfer.

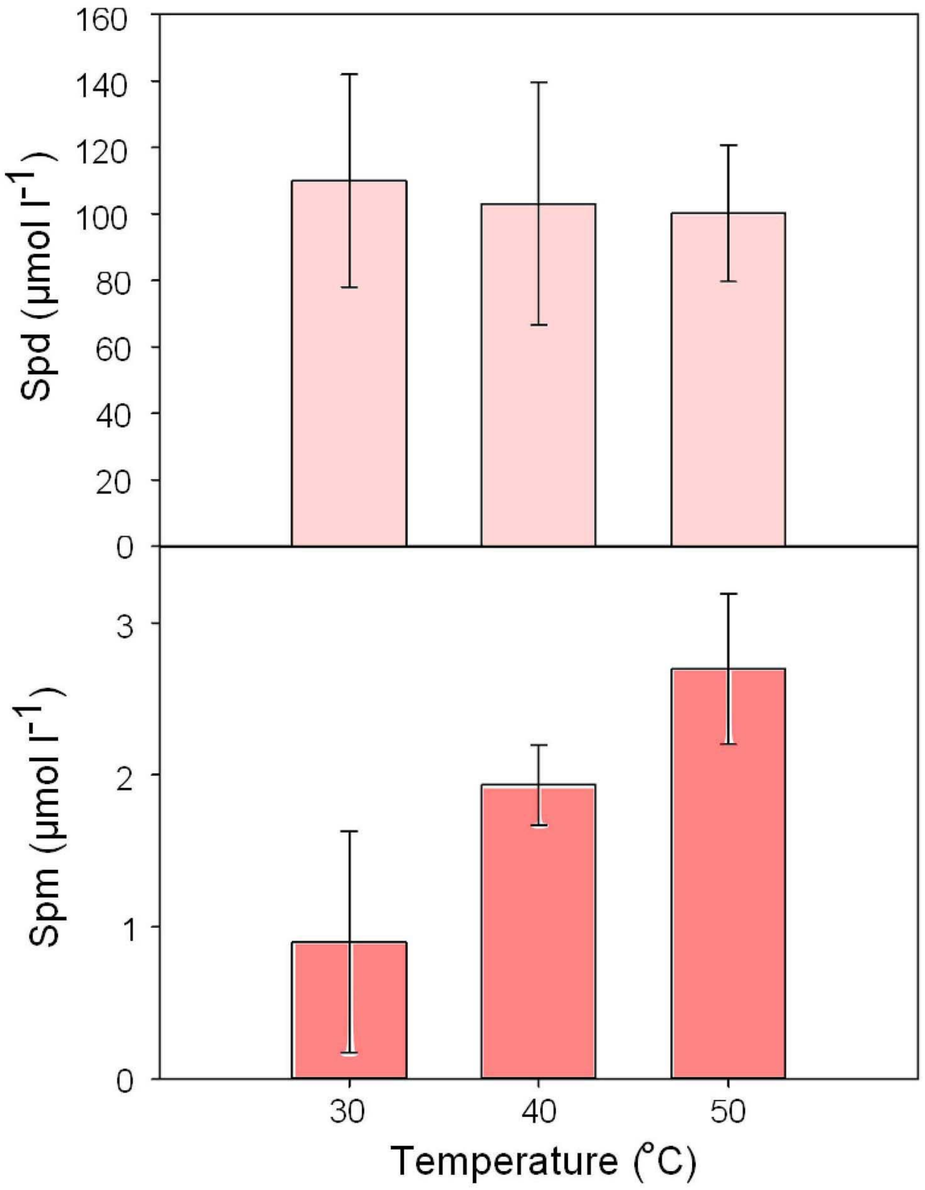
A

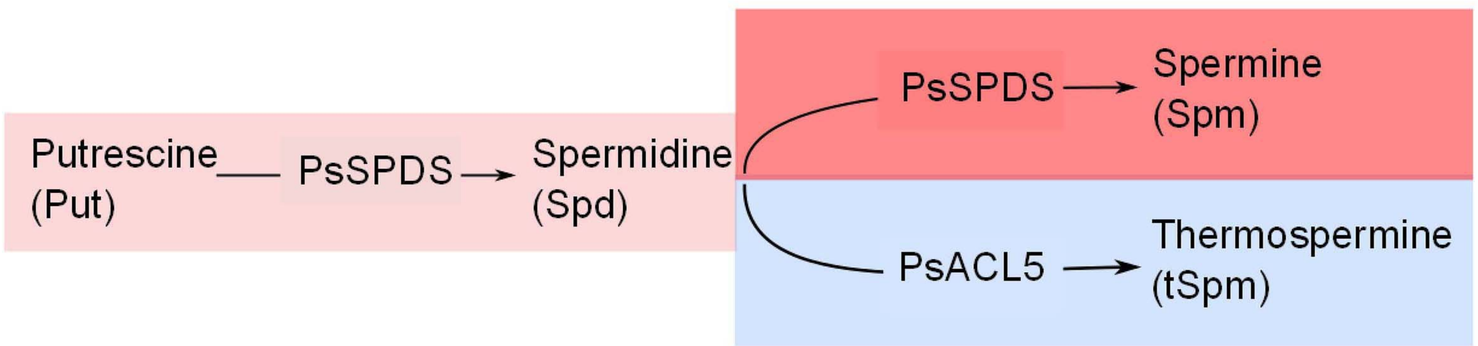

PsSPDS
M
kDa
M
PsACL5
130
95
72
55
43
34
26
17
10

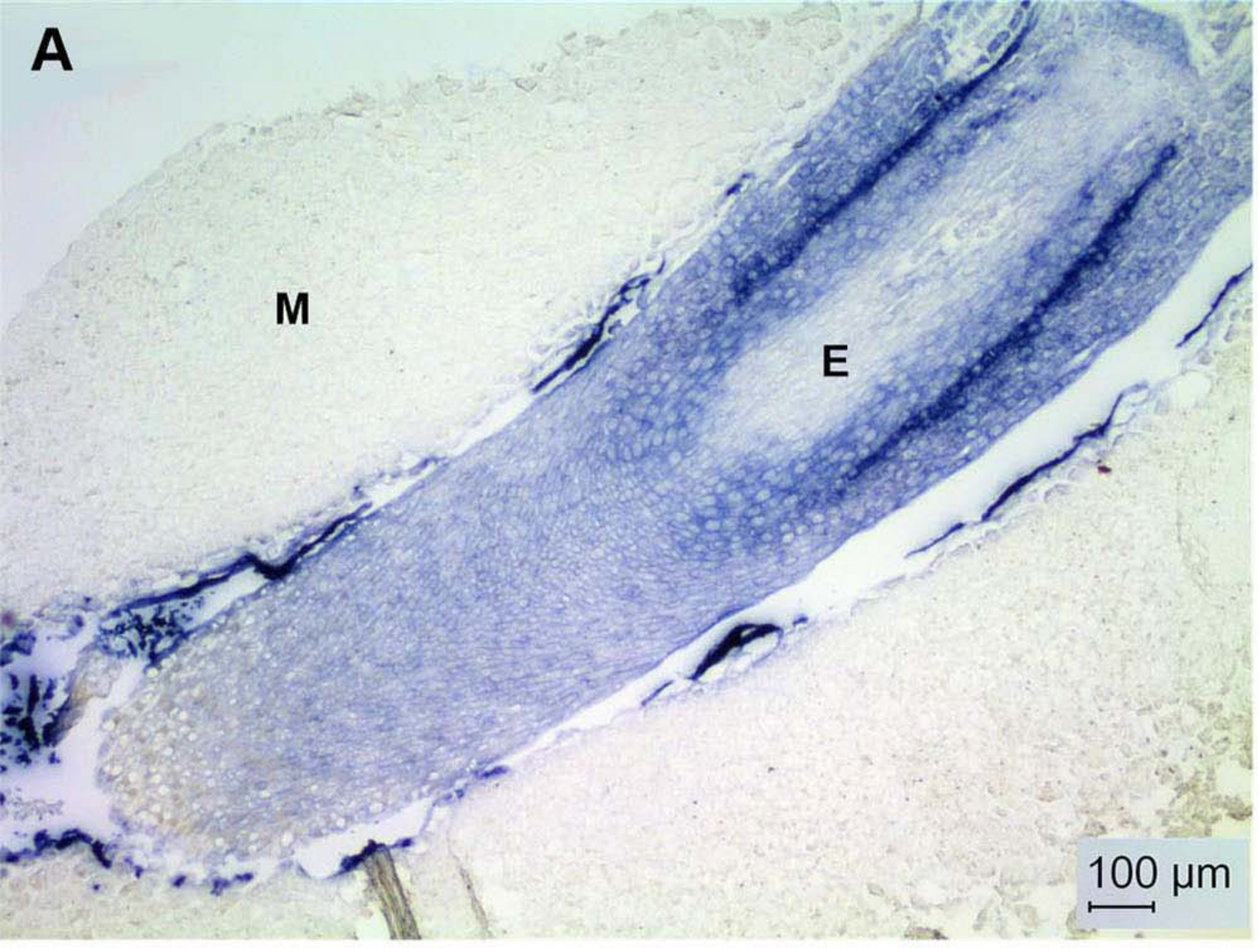
B

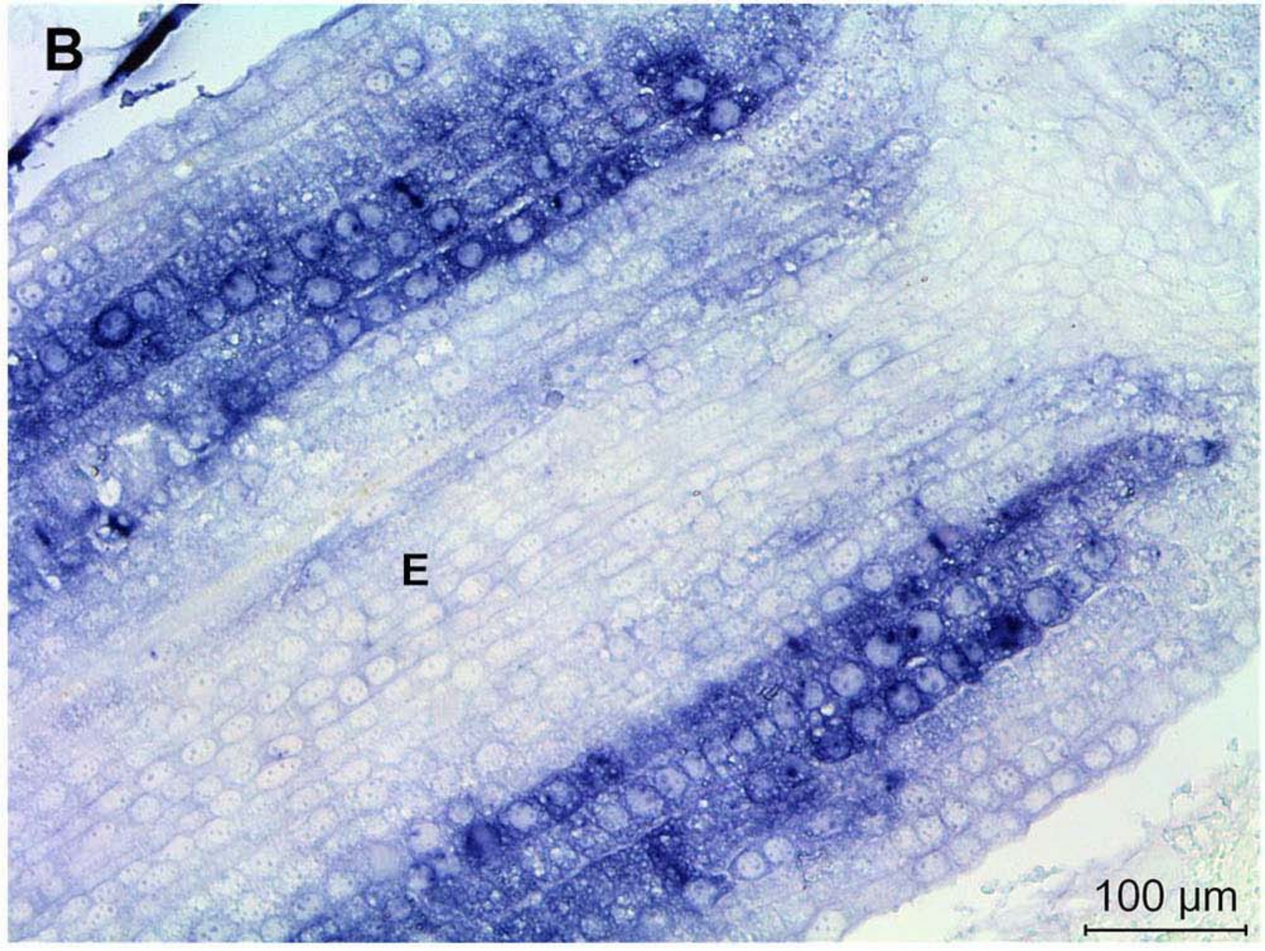

Standard
Control
Put
Spd
Spm
tSpm
PsSPDS
PsSPDS
PsACL5
Enzyme
0 5 10 15 20 25 30
0 5 10 15 20 25 30
0 5 10 15 20 25

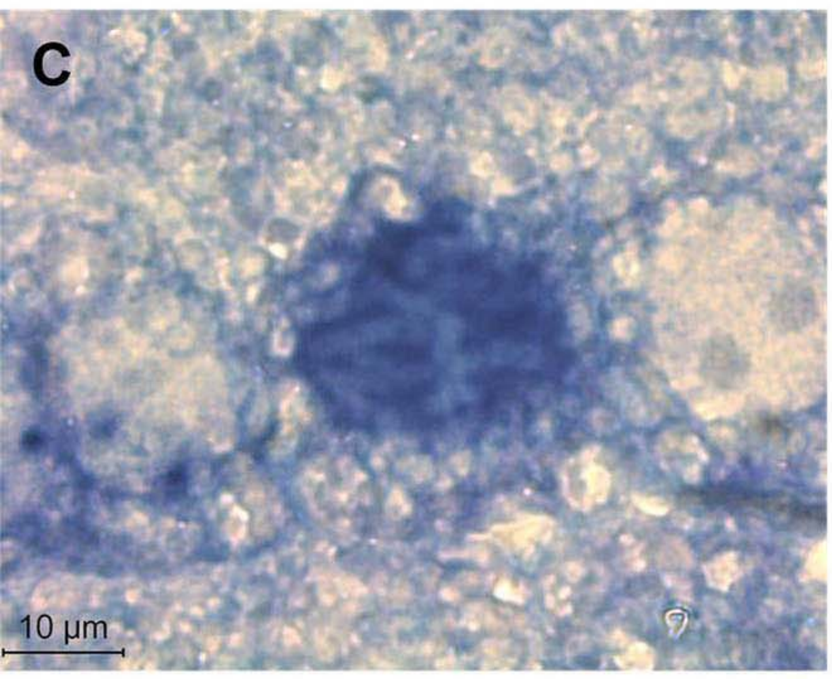
C

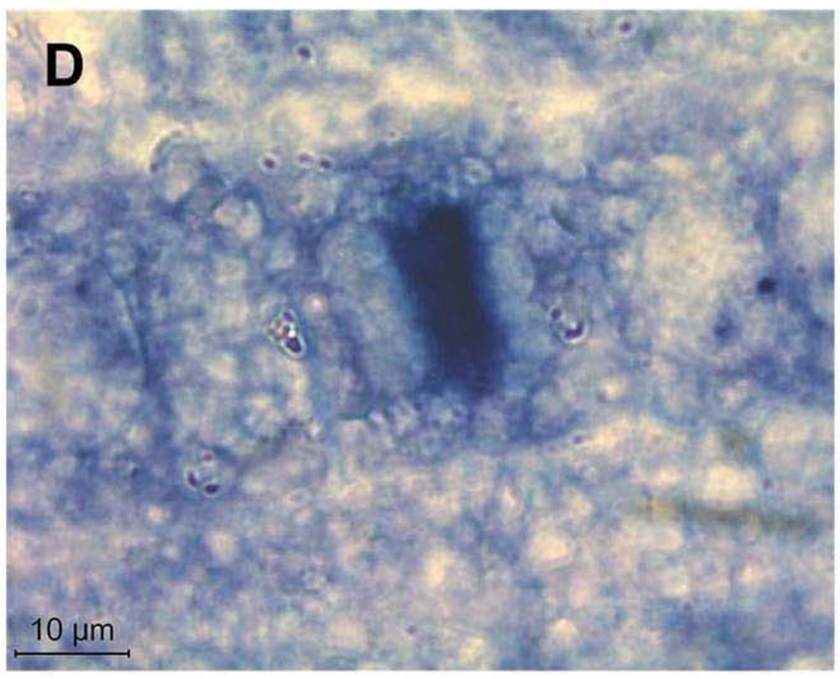

Spd (µmol l-1)
160
140
120
100
80
60
40
20
0
Spm (µmol l-1)
3
2
1
0
30
40
50
Temperature (°C)


Putrescine (Put)
PsSPDS
Spermidine (Spd)
PsSPDS
Spermine (Spm)
PsACL5
Thermospermine (tSpm)


A
M
E
100 µm


B
E
100 µm


C
10 µm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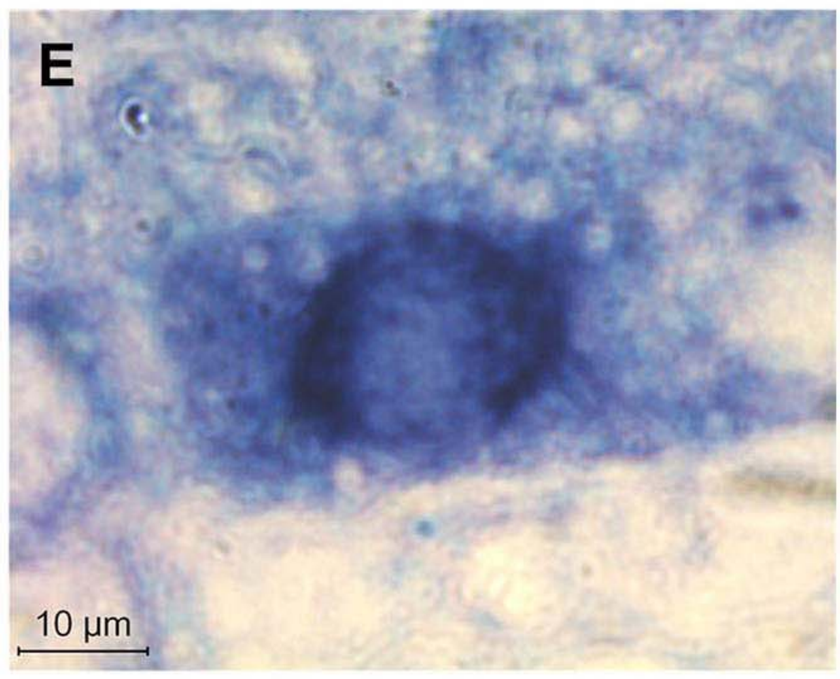
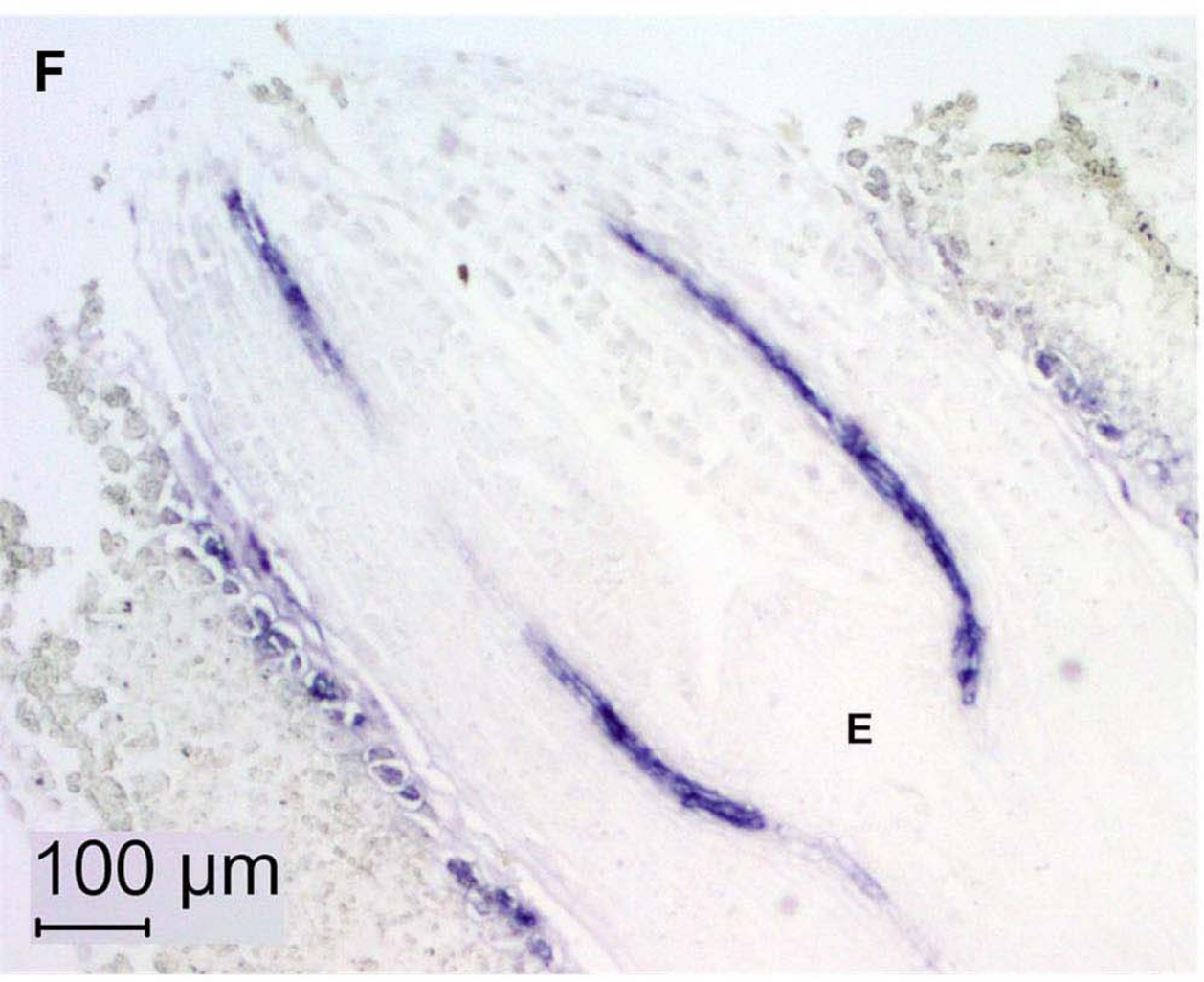
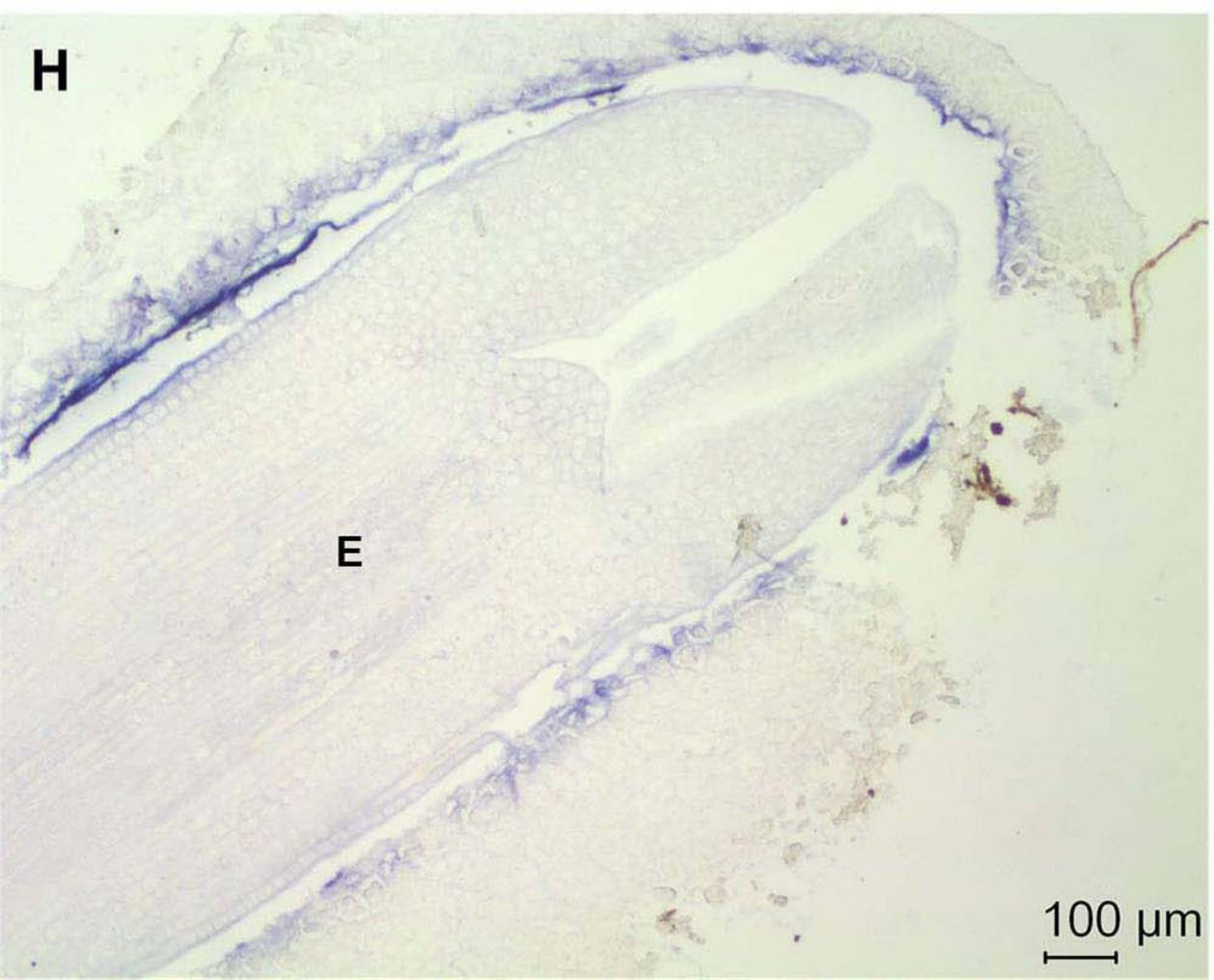
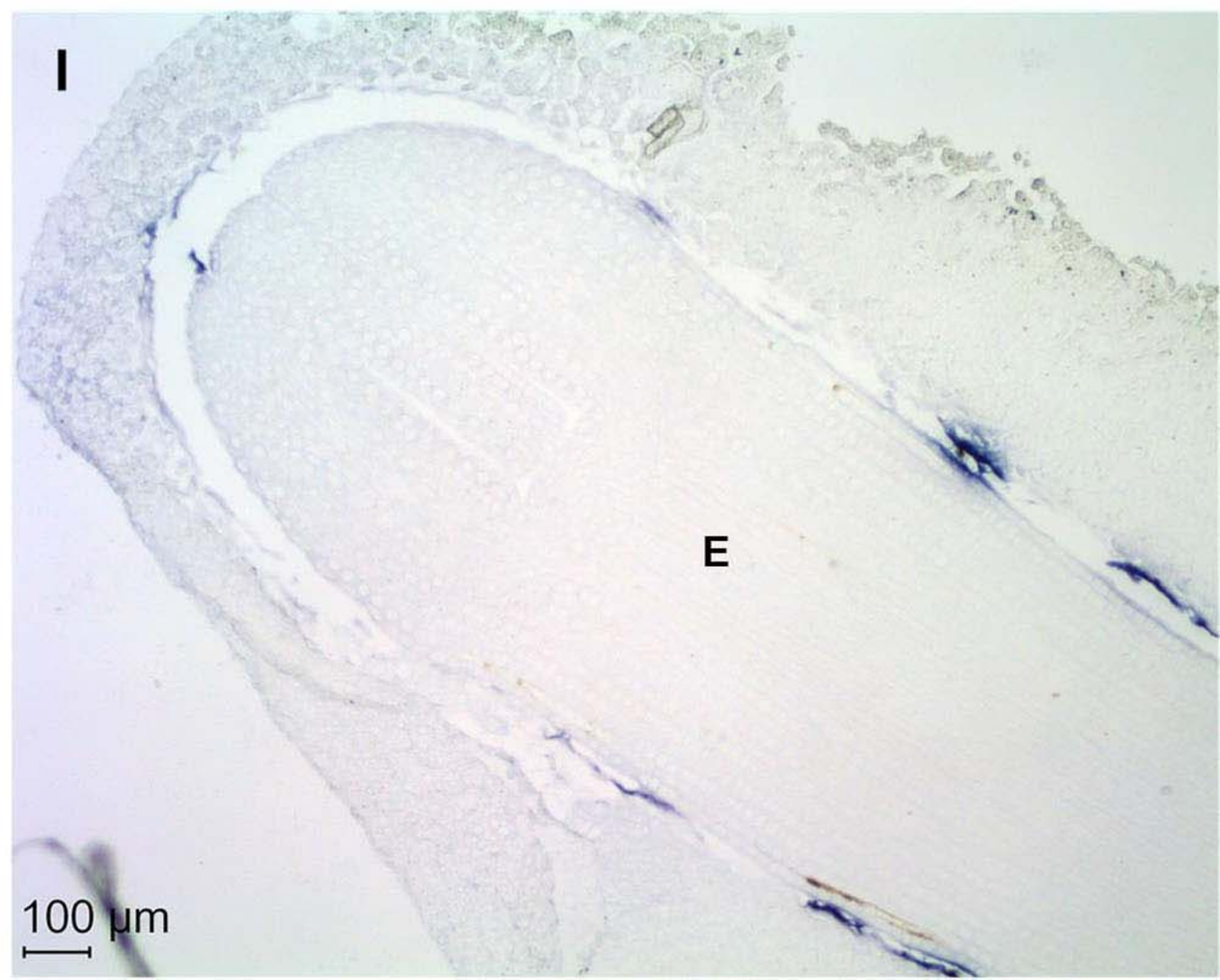
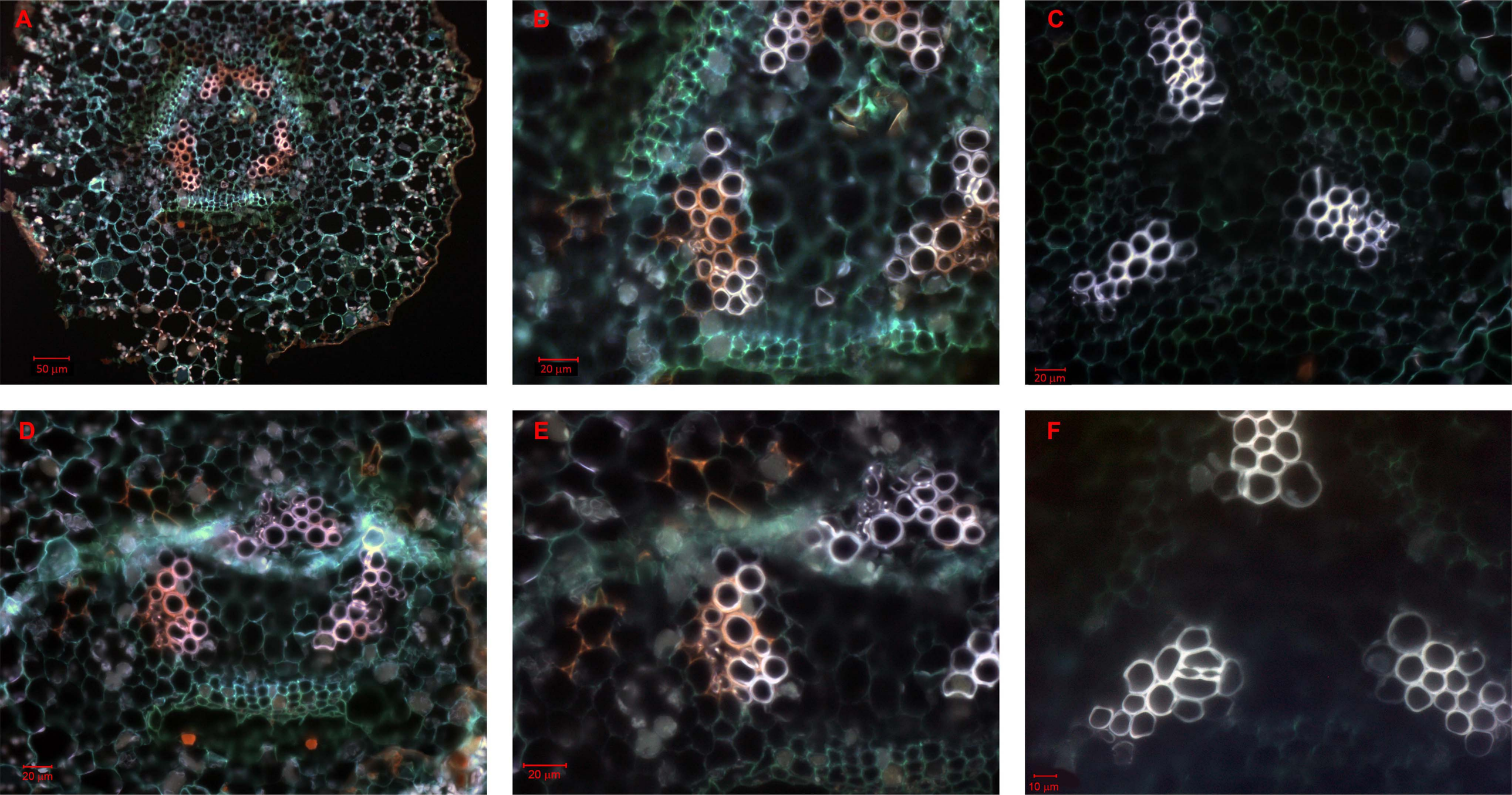

E
10 µm

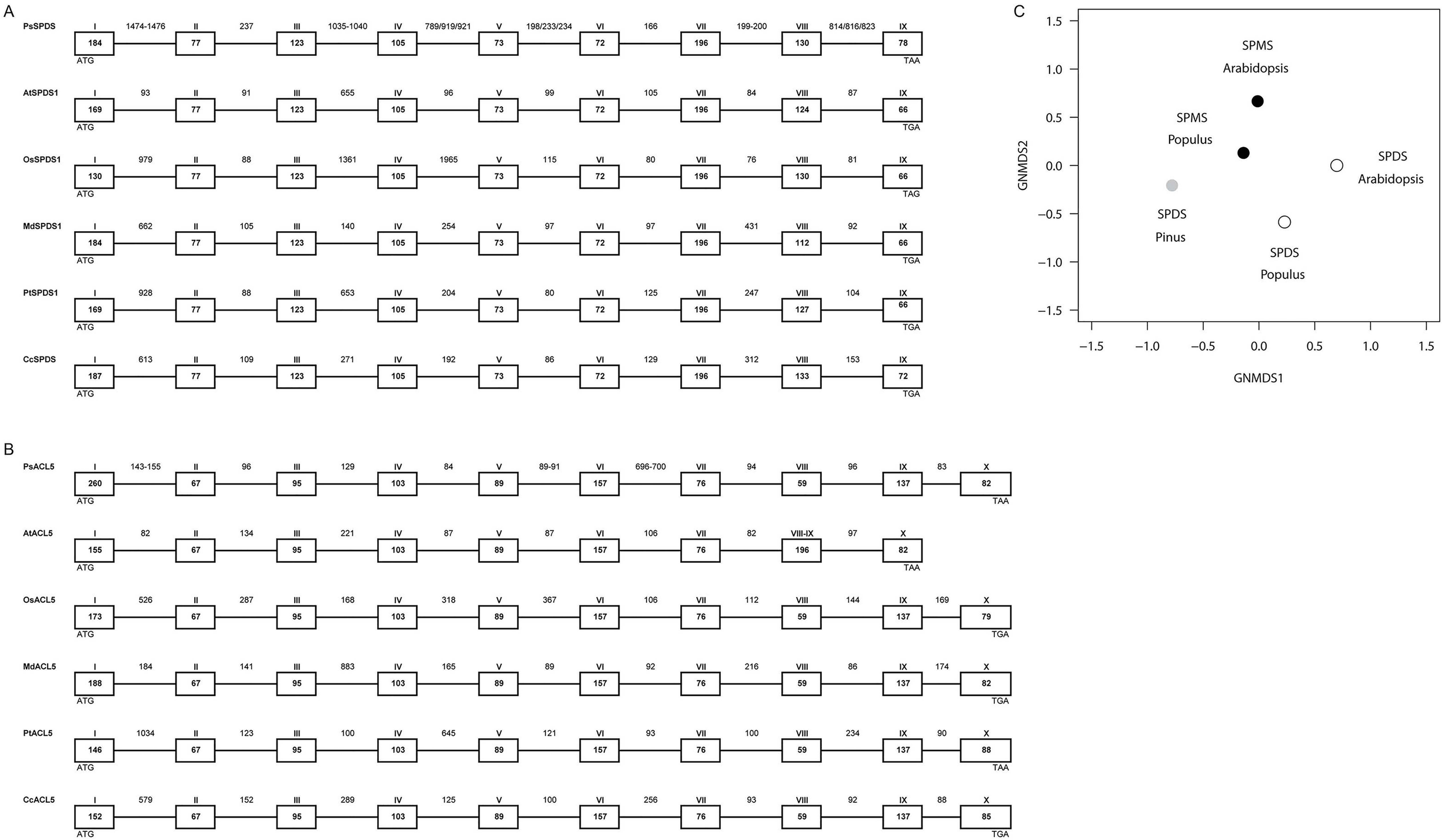

F
E
100 µm

G
E
100 µm


H
E
100 µm


I
E
100 µm


A
50 µm
B
20 µm
C
20 µm
D
20 µm
E
20 µm
F
10 µm


A
PsSPDS
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1474-1476
II 77
237
III 123
1035-1040
IV 105
789/919/921
V 73
198/233/234
VI 72
166
VII 196
199-200
VIII 130
814/816/823
IX 78 TAA
AtSPDS1
I 169 ATG
93
II 77
91
III 123
655
IV 105
96
V 73
99
VI 72
105
VII 196
84
VIII 124
87
IX 66 TGA
OsSPDS1
I 130 ATG
979
II 77
88
III 123
1361
IV 105
1965
V 73
115
VI 72
80
VII 196
76
VIII 130
81
IX 66 TAG
MdSPDS1
I 184 ATG
662
II 77
105
III 123
140
IV 105
254
V 73
97
VI 72
97
VII 196
431
VIII 112
92
IX 66 TGA
PtSPDS1
I 169 ATG
928
II 77
88
III 123
653
IV 105
204
V 73
80
VI 72
125
VII 196
247
VIII 127
104
IX 66 TGA
CcSPDS
I 187 ATG
613
II 77
109
III 123
271
IV 105
192
V 73
86
VI 72
129
VII 196
312
VIII 133
153
IX 72 TGA
B
PsACL5
I 260 ATG
143-155
II 67
96
III 95
129
IV 103
84
V 89
89-91
VI 157
696-700
VII 76
94
VIII 59
96
IX 137
83
X 82 TAA
AtACL5
I 155 ATG
82
II 67
134
III 95
221
IV 103
87
V 89
87
VI 157
106
VII 76
82
VIII-IX 196
97
X 82 TAA
OsACL5
I 173 ATG
526
II 67
287
III 95
168
IV 103
318
V 89
367
VI 157
106
VII 76
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VIII 59
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IX 137
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X 79 TGA
MdACL5
I 188 ATG
184
II 67
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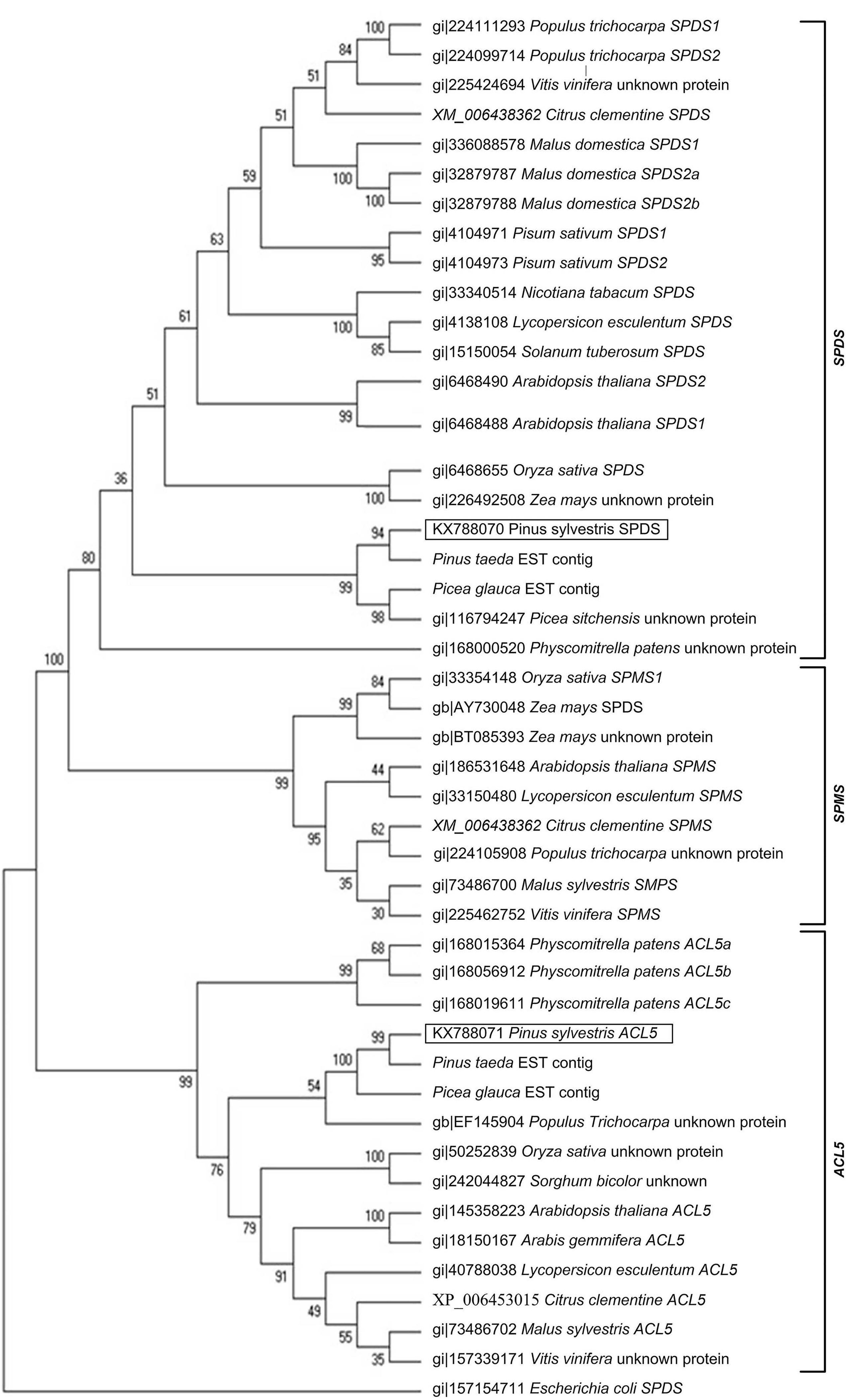
III 95
883
IV 103
165
V 89
89
VI 157
92
VII 76
216
VIII 59
86
IX 137
174
X 82 TGA
PtACL5
I 146 ATG
1034
II 67
123
III 95
100
IV 103
645
V 89
121
VI 157
93
VII 76
100
VIII 59
234
IX 137
90
X 88 TAA
CcACL5
I 152 ATG
579
II 67
152
III 95
289
IV 103
125
V 89
100
VI 157
256
VII 76
93
VIII 59
92
IX 137
88
X 85 TGA
C
GNMDS2
GNMDS1
1.5
1.0
0.5
0.0
−0.5
−1.0
−1.5
SPMS
Arabidopsis
SPMS
Populus
SPDS
Arabidopsis
SPDS
Pinus
SPDS
Populus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gi|224099714 Populus trichocarpa SPDS2
gi|225424694 Vitis vinifera unknown protein
XM_006438362 Citrus clementine SPDS
gi|336088578 Malus domestica SPDS1
gi|32879787 Malus domestica SPDS2a
gi|32879788 Malus domestica SPDS2b
gi|4104971 Pisum sativum SPDS1
gi|4104973 Pisum sativum SPDS2
gi|33340514 Nicotiana tabacum SPDS
gi|4138108 Lycopersicon esculentum SPDS
gi|15150054 Solanum tuberosum SPDS
gi|6468490 Arabidopsis thaliana SPDS2
gi|6468488 Arabidopsis thaliana SPDS1
gi|6468655 Oryza sativa SPDS
gi|226492508 Zea mays unknown protein
KX788070 Pinus sylvestris SPDS
Pinus taeda EST contig
Picea glauca EST contig
gi|116794247 Picea sitchensis unknown protein
gi|168000520 Physcomitrella patens unknown protein
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gb|BT085393 Zea mays unknown protein
gi|186531648 Arabidopsis thaliana SPMS
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XM_006438362 Citrus clementine SPMS
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gi|225462752 Vitis vinifera SPMS
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gi|168056912 Physcomitrella patens ACL5b
gi|168019611 Physcomitrella patens ACL5c
KX788071 Pinus sylvestris ACL5
Pinus taeda EST contig
Picea glauca EST contig
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gi|50252839 Oryza sativa unknown protein
gi|242044827 Sorghum bicolor unknown
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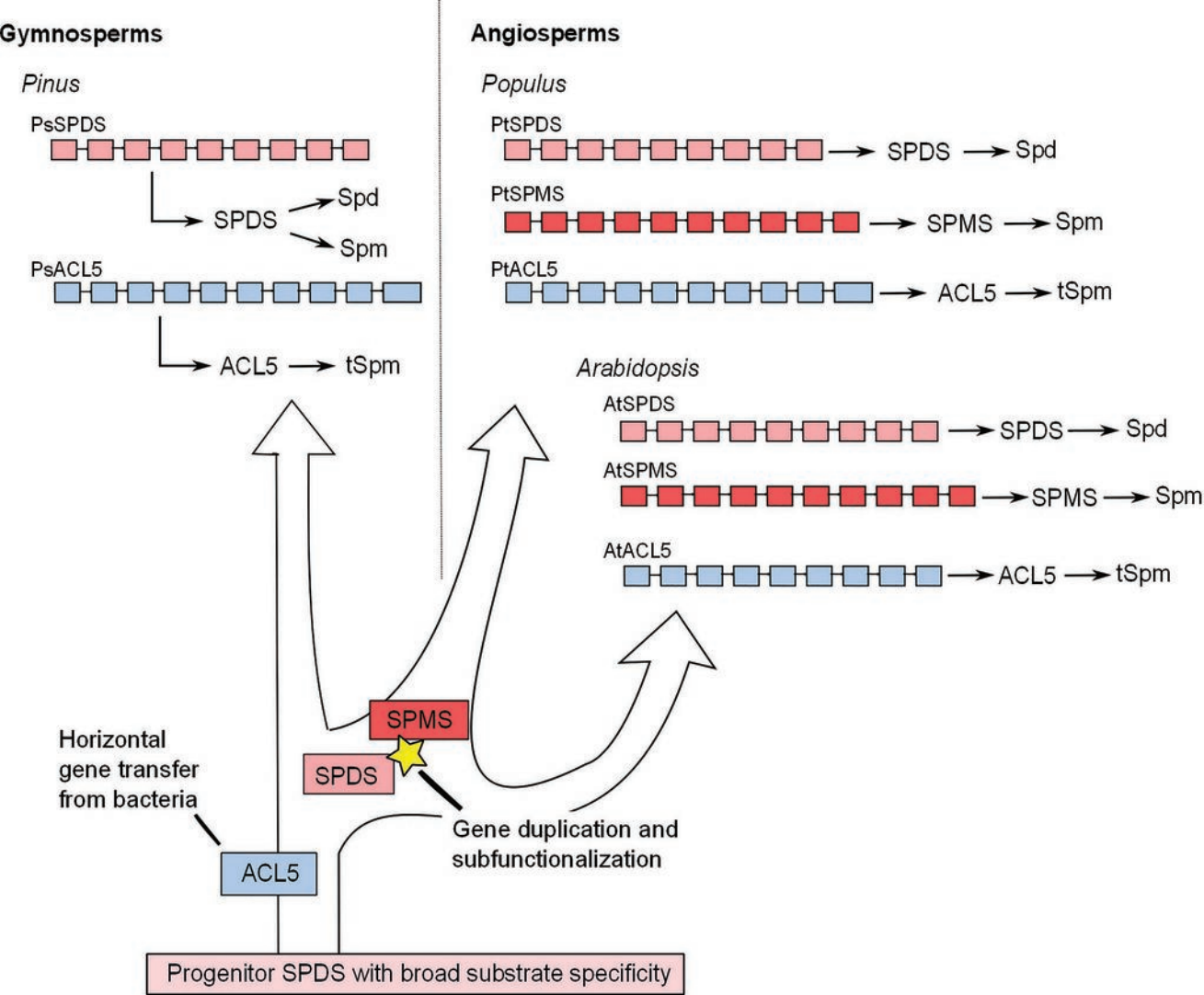
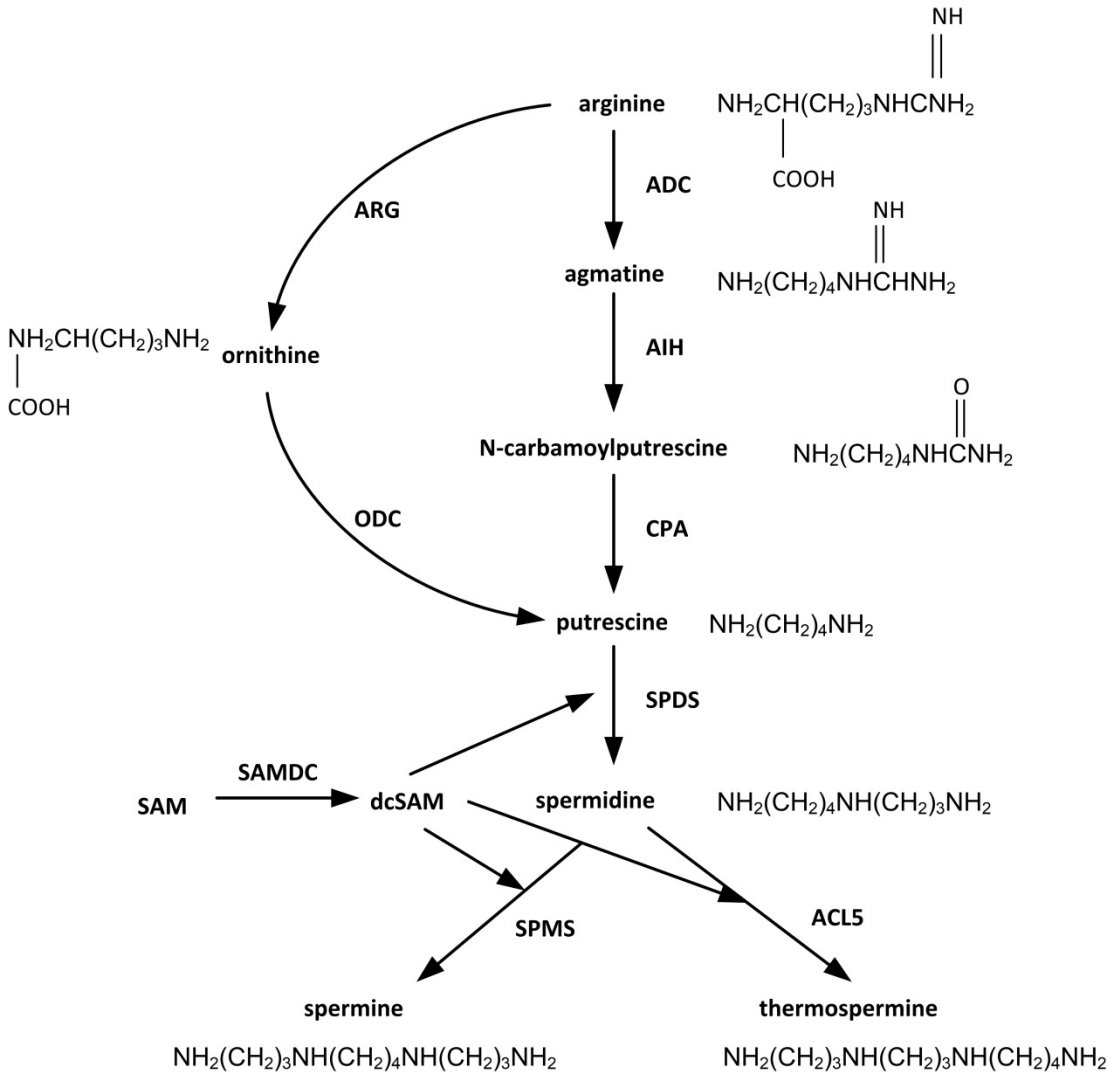
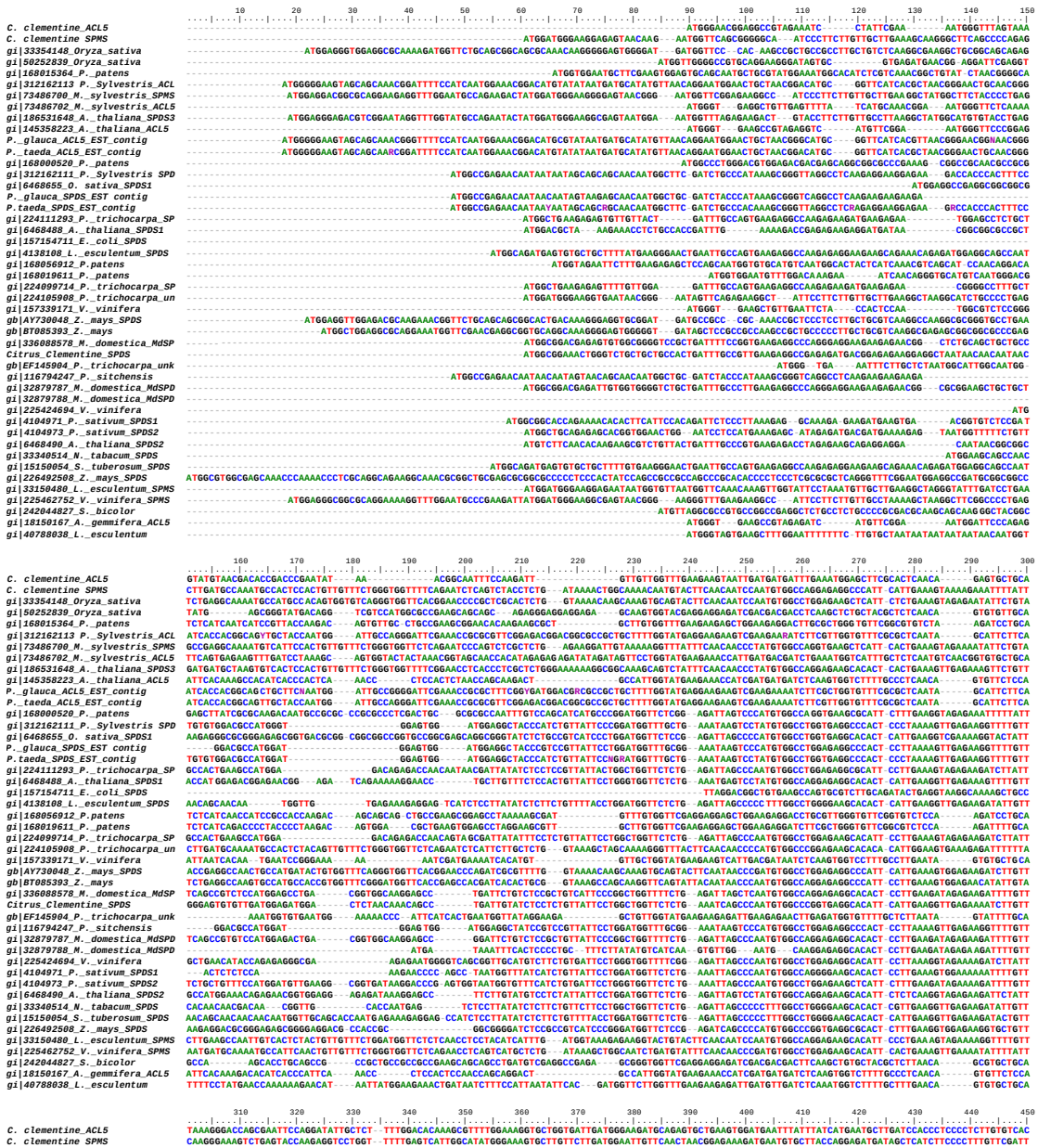
SPDS
SPMS
ACL5

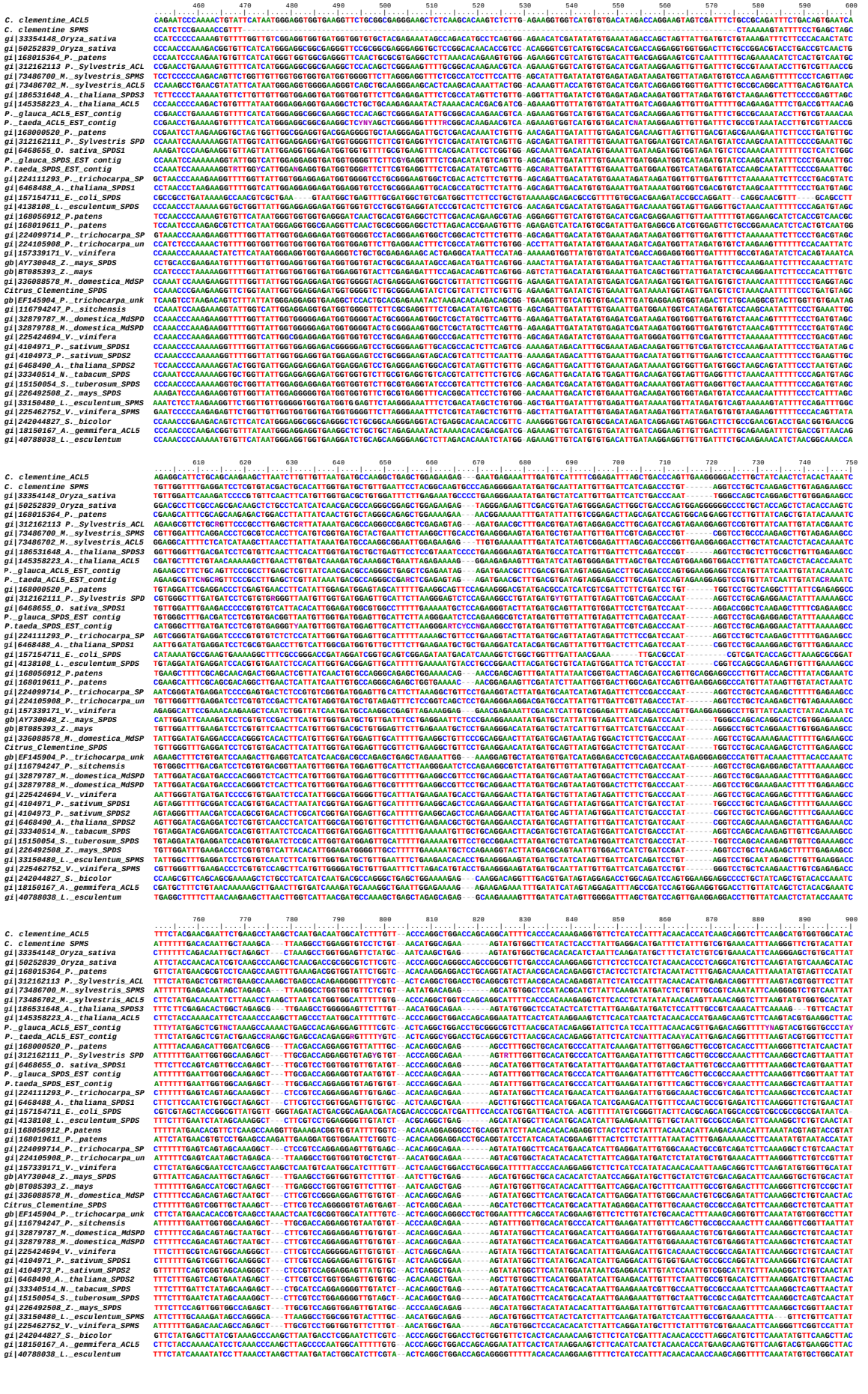

Gymnosperms
Angiosperms
Pinus
Populus
PsSPDS
SPDS
Spd
Spm
PsACL5
ACL5
tSpm
PtSPDS
SPDS
Spd
PtSPMS
SPMS
Spm
PtACL5
ACL5
tSpm
Arabidopsis
AtSPDS
SPDS
Spd
AtSPMS
SPMS
Spm
AtACL5
ACL5
tSpm
SPMS
SPDS
Horizontal
gene transfer
from bacteria
Gene duplication and
subfunctionalization
ACL5
Progenitor SPDS with broad substrate specificity



Supplementary Figure S1. Polyamine biosynthesis pathway in plants. The enzymes in the pathways are arginase (ARG), ornithine decarboxylase (ODC), arginine decarboxylase (ADC), agmatine imidohydrolase (AIH), N-carbamoylputrescine amidohydrolase (CPA), spermidine synthase (SPDS), spermine synthase (SPMS), thermospermine synthase (ACL5) and S-adenosylmethionine decarboxylase (SAMDC).

Supplementary Figure S2. The alignment of *SPDS*, *SPMS* and *ACL5* sequences for phylogenetic analysis.




C. clementine_ACL5
C. clementine_SPMS
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gi|50252839_Oryza_sativa
gi|168015364_P._patens
gi|312162113_P._Sylvestris_ACL
gi|73486700_M._sylvestris_SPMS
gi|73486702_M._sylvestris_ACL5
gi|186531648_A._thaliana_SPDS3
gi|145358223_A._thaliana_ACL5
P._glauca_ACL5_EST_contig
P._taeda_ACL5_EST_contig
gi|168000520_P._patens
gi|312162111_P._Sylvestris_SPD
gi|6468655_O._sativa_SPDS1
P._glauca_SPDS_EST_contig
P.taeda_SPDS_EST_contig
gi|224111293_P._trichocarpa_SP
gi|6468488_A._thaliana_SPDS1
gi|157154711_E._coli_SPDS
gi|4138108_L._esculentum_SPDS
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Citrus_Clementine_SPDS
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gi|6468490_A._thaliana_SPDS2
gi|33340514_N._tabacum_SPDS
gi|15150054_S._tuberosum_SPDS
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gi|225462752_V._vinifera_SPMS
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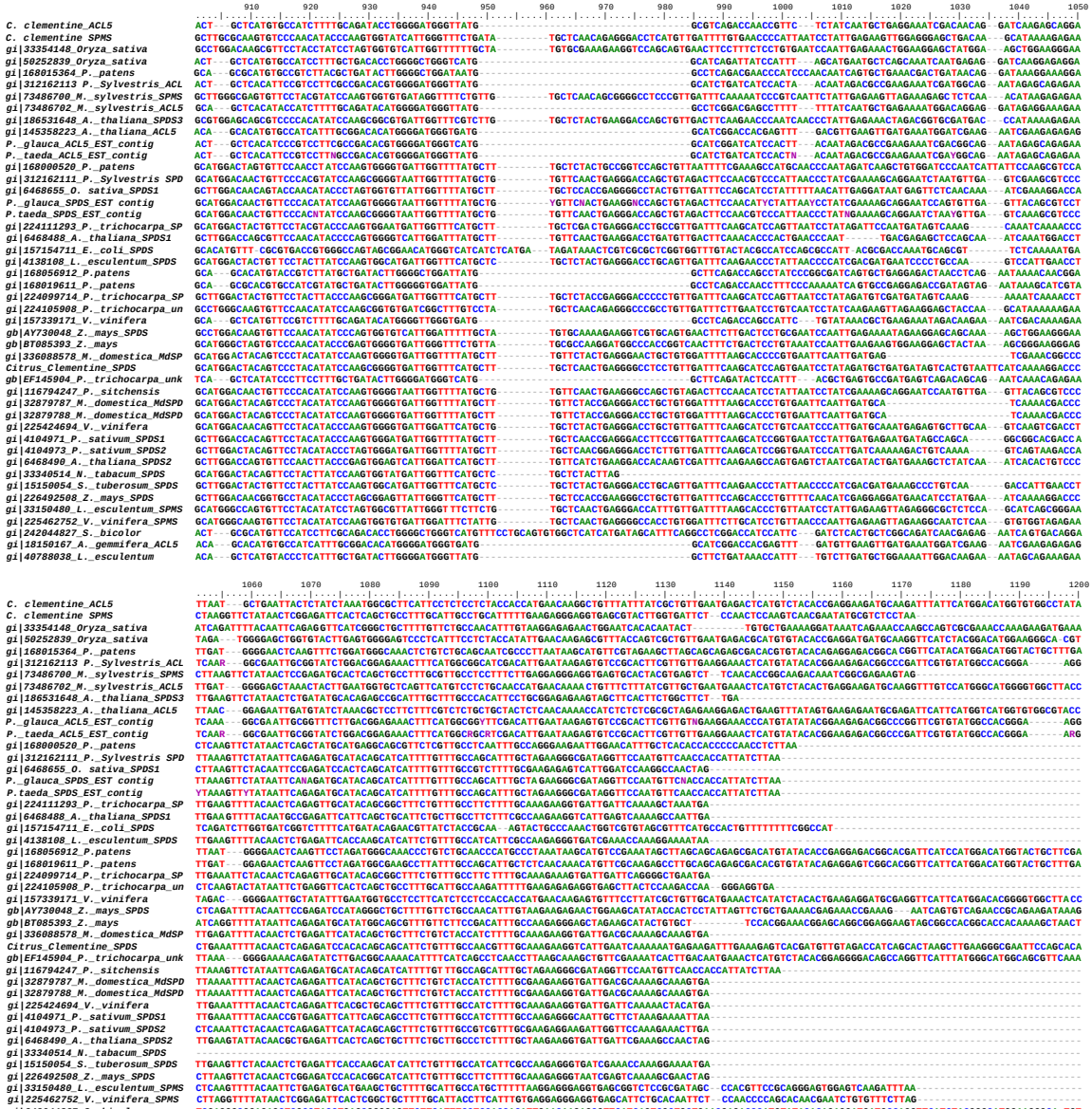
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C. clementine SPMS
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P._glauca_ACL5_EST_contig
P._taeda_ACL5_EST_contig
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P.taeda_SPDS_EST_contig
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gi|157339171_V._vinifera
gb|AY730048_Z._mays_SPDS
gb|BT085393_Z._mays
gi|336088578_M._domestica_MdSP
Citrus_Clementine_SPDS
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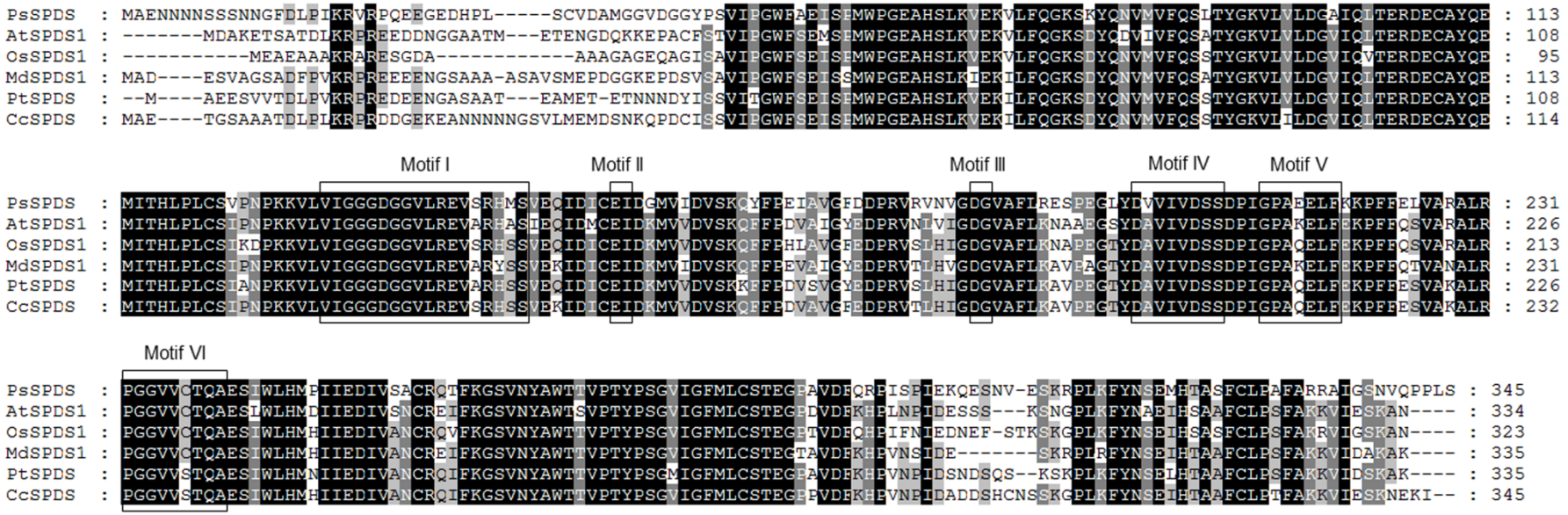
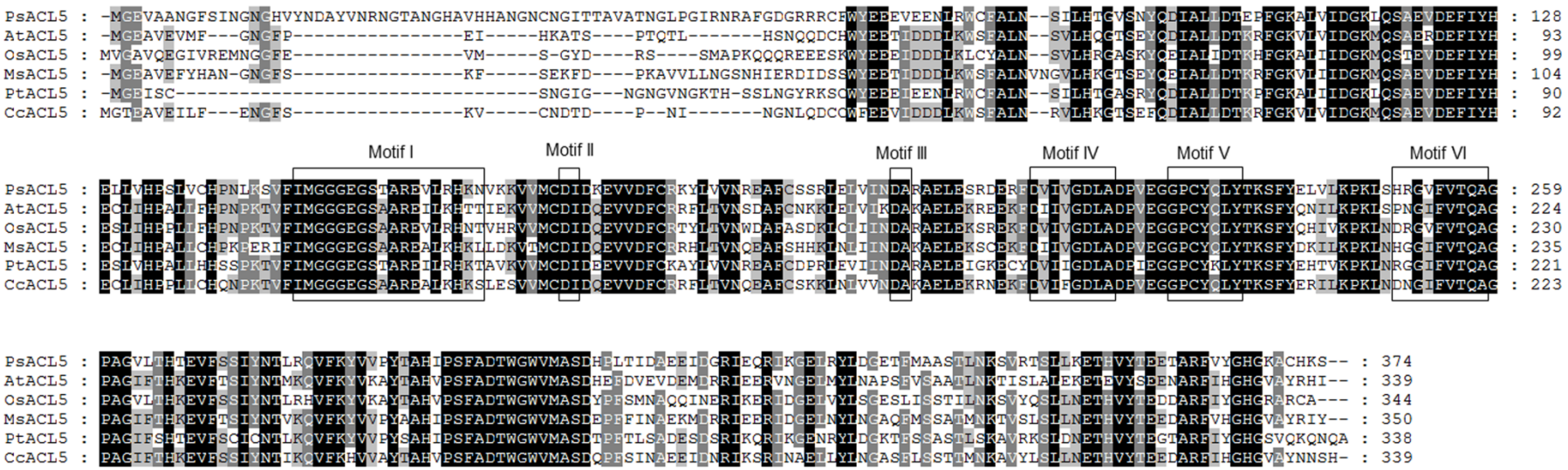
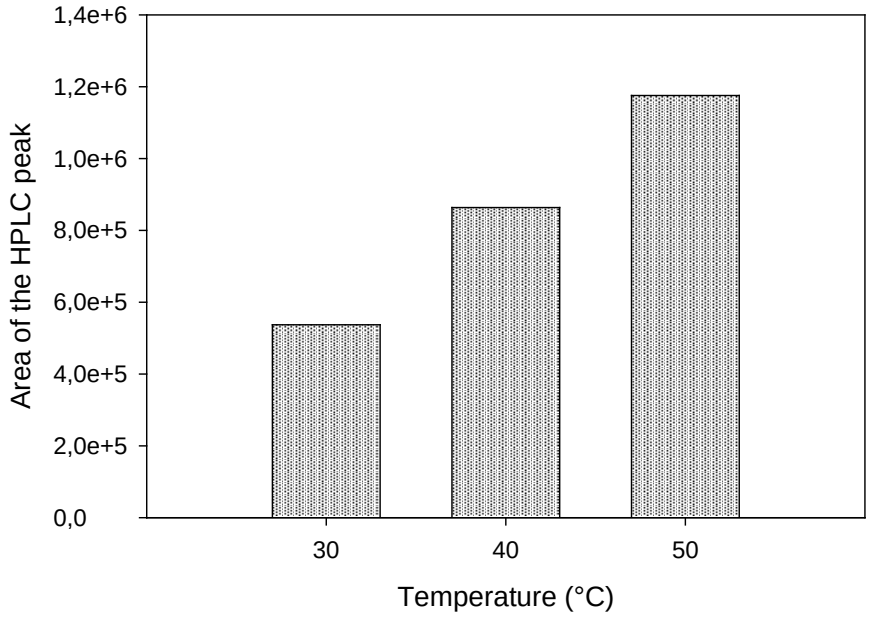


Supplementary Figure S3. Comparison of the predicted amino acid sequence of the *P. sylvestris* PsSPDS protein with previously characterized homologues from angiosperms. The proposed binding sites for SAM and dcSAM (motifs I-VI) are showed in the alignment. The GenBank accession numbers are as follows: *A. thaliana* AtSPDS1 (CAB61614), *O. sativa* OsSPDS1 (CAB61629), *M. domestica* MdSPDS1 (BAC20170), *P. trichocarpa* PtSPDS (XP_002315806) and *C. clementina* CcSPDS (XP_006483825). The *P. sylvestris* PsSPDS sequence has been deposited in GenBank under accession number KX761190.



Supplementary Figure S4. Comparison of the predicted amino acid sequence of the *P. sylvestris* PsACL5 protein with previously characterized homologues from angiosperms. The proposed binding sites for SAM and dcSAM (motifs I-VI) are showed in the alignment. The GenBank accession numbers are as follows: *A. thaliana* AtACL5 (NP_568376), *O. sativa* OsACL5 (NP_001046395), *M. sylvestris* MsACL5 (BAE19759), *P. trichocarpa* PtACL5 (ABK94019) and *C. clementina* CcACL5 (XP_006453015). The *P. sylvestris* PsACL5 sequence has been deposited in GenBank under accession number HM236828.



Supplementary Figure S5. Area of the tSpm HPLC peaks in 100 nM reaction mixture with PsACL5 enzyme and Spd as a substrate after 1h incubation under three different temperatures (30, 40 and 50 °C).

Supplementary Table S1. Primers for PCR and sequencing of the *PsSPDS* and *PsACL5* genes from Scots pine.

Forward primer	Primer sequence 5'-3'	Reverse primer	Primer sequence 5'-3'	Type of primer[a]
SPDS_F2	GTGTACCCAGGCAGAAAGTATTTG	SPDS Right	CTTCAAACTTAAGATAATGGTGGTGG	PCR & seq (F2-Right)
SPDS_F5	CTGTGTCTATCATTGGTTTAATCACC	SPDS_R6	GCTCCACTTAATAGTGCAGTTTCTC	seq (F2-Right)
SPDS_7F	TTCGGAGATAGAGTGGAATAAGTTG	SPDS_7R	AGAGCAAAGTGGAAGATGTGTTATC	PCR & seq (7F-7R)
SPDS_8F	ACTTATGGAAAGGTTCTGGTGCT	SPDS_8R	ACATTAACCCTCACACGAGGA	PCR & seq (8F-8R)
SPDS_9F	ACATATGTCAGTTGAGCAGATTGAT	SPDS_9R	GATACGTGGGAACAGTTGTCCAT	PCR & seq (9F-9R)
SPDS_F10	ATAGAGTGGATAGCTTGCTCCC	SPDS_R10	AGAGCTATAGTTGGTAAGGCGGT	seq (7F-7R)
SPDS_F11	TCAGGTGTGCTTCCATCTCCAGC	SPDS_R11	GGAGTTCAACCATGACAGCACAACA	PCR & seq (F11-R11)
SPDS_F12	GCTAACTGATTGGGGCATGAGGGT			seq (9F-9R)
SPDS_F13	CCCTGTGGATTGGTTAAACCT	SPDS_R13	AGAGTCTTTAGGTGGTTTGTTCAG	seq (8F-8R)
SPDS_F12b	TGGGGCATGAGGGTAACTAACTG			seq (9F-9R)
		SPDS_R13b	TGCAGTGAGACTTCCAAATTCCTC	seq (8F-8R)
SPDS_F14	ACAATTTCGGAGGAATGCTGGGGTC	SPDS_R14	ACCATCCAGCACCAGAACCTTTCC	PCR & seq (F14-R14)
SPDS-Prom_F1	GATCAAACCTCACGGGGGGTGGTTG	SPDS-Prom_R1	CACGGTGATACGGACAGTTTTTGGC	PCR & seq (SPDS-Prom_F1-R1)
SPDS-Prom_F2	GGTGTGCGTAGTGATGGAATCCCAC	SPDS-Prom_R2	CTCCTTCCTCTTGAGGCCTAACCCG	PCR & seq (SPDS-Prom_F2-R2)
ACL5_5F	AGCATCCATAGCTTTTGCCTACT	ACL5_5R	CCAGCAGTTCGTGATAGATAAACTC	PCR & seq (5F-5R)
ACL5_6F	TACCAGGATATAGCACTACTTGATACA	ACL5_6R	TCACACATGACCACTTTCTTGAC	PCR & seq (6F-6R)
ACL5_7F	CTCCACAGCTCGGGAAGTTTT	ACL5_7R	GGCTCAGCTTTGGCTTCAGTA	PCR & seq (7F-7R)
ACL5_8F	CTTTGACGTGATAGTAGGAGACCTT	ACL5_8R	CCTTTGATTCTCTGCTCTATTCTGC	PCR & seq (8F-8R)
ACL5_9F	CTTATACTGCTCACATTCCGTCCT	ACL5_9R	GAGGCCTGTGTACACTGTTTATTTT	PCR & seq (9F-9R)
ACL5_F10	CAGTTCCCGCCTTGAGCTCGTTA	ACL5_R10	AGAGATCAACCGCTCCTCTATCTTGA	PCR & seq (F10-R10)
		ACL5_R11	AAACTTCCCGAGCTGTGGAGCCT	seq (6F-6R)
ACL5_F12	GCATCCATCGCTCGTCTGTCACC	ACL5_R12	GTCTAGTAGCAACCTAGGAAGCATCA	PCR & seq (F12-R12)
ACL5-Prom_F1	TGGGAGGAAAAAAATGAAGACTCGGC	ACL5-Prom_R1	TGCCGTGGTGATCCCGTTGC	PCR & seq (ACL5-Prom_F1-R1)
ACL5-Prom_seqF	TCCACCATCTCGCAGATGGGAATG	ACL5-Prom_seqR	TGTCATCATCCATCCGCCAATGG	seq (ACL5-Prom_F1-R1)

[a]PCR indicates the forward and the reverse primers used to obtain the PCR product, and seq indicates the primer used to sequence the obtained PCR product.

Supplementary Table S2. Presence-absence data generated from the transcription factor binding sites (TFBS) found in the 3kb promoter regions of *SPDS* and *SPMS* genes of *Populus trichocarpa* (*PtSPDS* and *PtSPMS*), *Arabidopsis thaliana* (*AtSPDS* and *AtSPMS*) and *Pinus sylvestris* (*PsSPDS*) using PlantPAN 2.0 database.

TFBS	***PtSPMS***	***PtSPDS***	***AtSPMS***	***AtSPDS***	***PsSPDS***
Alpha-amylase	1	1	1	1	1
AP2	0	1	1	1	1
AP2;B3	1	0	1	1	0
AP2;B3;RAV	1	1	1	1	1
AP2;ERF	1	1	1	1	1
AP2;RAV	0	0	1	1	0
AP2;RAV;B3	1	1	1	1	1
AT-Hook	1	1	1	1	1
Aux/IAA	1	0	1	0	1
B3	1	1	1	1	1
B3;ARF;	1	1	1	0	1
BES1	0	1	0	1	1
Bet_v_1	0	1	0	1	1
bHLH	1	1	1	1	1
bHLH;bZIP	1	1	0	1	1
bZIP	1	1	1	1	1
bZIP;B3	0	1	1	1	1
bZIP;Homeodomain;HD-ZIP	1	1	1	1	1
C2H2	1	1	1	1	1
CG-1;CAMTA	1	1	1	1	0
CSD	1	1	1	1	1
Cupin_1	0	1	0	0	1
Dehydrin	1	1	1	1	1
Dof	1	1	1	1	1
E2F	0	0	1	1	0
E2F/DP	0	1	0	1	0
EIN3	1	1	1	1	1
EIN3;EIL	0	1	1	1	0
ERF	0	1	0	0	1
FAR1	0	0	1	1	1
GATA	1	1	1	1	1
GATA;tify	1	1	1	1	1
GATA;Dof	0	0	1	0	1
GRAS	0	1	0	1	0
HB-PHD	1	1	1	1	1
HD-ZIP	0	1	1	1	1
Homeodomain	1	1	1	1	1
Homeodomain;bZIP;HD-ZIP	1	1	1	1	1
Homeodomain;bZIP;HD-ZIP;WOX	1	1	1	1	1

Homeodomain;HB-PHD	0	1	1	1	1
Homeodomain;HD-ZIP	1	1	1	1	1
Homeodomain;HD-ZIP;bZIP	1	1	1	1	1
Homeodomain;TALE	1	1	1	1	1
Homeodomain;WOX	1	0	1	1	0
Homeodomain;ZF-HD	1	1	1	1	1
HSF	1	1	1	1	0
LEA type 1	0	0	1	1	1
LEA_5	1	0	1	1	0
LIM	0	1	0	0	0
LOB;LBD	0	0	0	0	1
MADF	1	1	1	1	1
MADF;TRIHELIX	1	1	1	0	1
MADS box	1	1	1	1	1
MADS box;MIKC	1	1	1	1	1
MADS box;MIKC;M-type	0	1	0	1	0
MYB	1	1	1	1	1
Myb/SANT	1	1	1	1	1
NAC;NAM	1	1	1	1	1
NAC	0	0	1	0	0
NF-YB;NF-YA;NF-YC	1	1	1	1	1
PsaH	0	1	1	0	1
Ribosomal protein L21P	1	1	1	1	1
SBP	1	1	1	1	1
Sox	0	1	1	1	0
Sox;YABBY	1	1	1	1	1
SRS	1	1	1	1	1
Storekeeper	0	1	1	1	0
TBP	1	1	1	1	1
TCP	1	1	1	1	1
TCR	1	0	1	0	1
TCR;CPP	1	1	1	1	1
Trihelix	1	1	1	1	1
trp	1	1	1	1	1
Tryp_alpha_amyl	1	1	1	1	1
WOX	1	1	1	1	1
VOZ	0	1	1	1	0
WRC;GRF	0	0	0	0	1
WRKY	1	1	1	1	1
YABBY	1	0	1	1	1
ZF-HD	1	1	1	1	1
ZF-HD	1	1	1	1	1

Supplementary Table S3. Functions of transcription factor binding sites (TFBS) found in the 3kb promoter regions of *SPDS* and *SPMS* genes of *Populus trichocarpa* (*PtSPDS* and *PtSPMS*), *Arabidopsis thaliana* (*AtSPDS* and *AtSPMS*) and *Pinus sylvestris* (*PsSPDS*) using PlantPAN 2.0 database.

Gategories of TFBS by function	TFBS ID	TFBS	PtSPMS	PtSPDS	AtSPMS	AtSPDS	PsSPDS	Function of TFBS/TF	Corresponding TF ID (gene ID)	References
Cellular fuctions	TF_motif_seq_0131	AP2					**x**	TF is required for the development of female gametophyte. It is also involved in organs initiation and development, including floral organs.	AT4G37750	Elliot *et al*., 1996; Klucher *et al*., 1999; Mathelier *et al*., 2014
	TFmatrixID_0164, TFmatrixID_0558, TFmatrixID_0507, TFmatrixID_0505, TFmatrixID_0497, TFmatrixID_0170, TFmatrixID_0166	bHLH;bZIP		**x**		**x**	**x**	TF regulates negatively chlorophyll biosynthesis and seed germination in the darkness and modulates responses to abscisic acid (ABA) and Gibberellic acid (GA).	AT2G20180	Mathelier *et al*., 2014; Weirauch *et al*., 2014
								TF is required for the dehiscence of fruit when it matures.	AT5G67110	
								TF is acting positively in the phytochrome signaling pathway.	AT1G09530	
								TF promotes the shade-avoidance response and promotes ethylene activity in the dark. It might also be involved in controlling the circadian and photomorphogenic processes.	AT3G59060	
								Transcription factor that plays a role in floral organogenesis.	AT4G36930	
								TF is required during the fertilization of ovules by pollen.	AT4G00050	
	TFmatrixID_0195	bZIP;B3		**x**	**x**	**x**	**x**	TF is involved in floral development.	AT1G68640	Mathelier *et al*., 2014; Weirauch *et al*., 2014
	TFmatrixID_0299	Homeodomain; WOX	**x**		**x**	**x**		TF has a possible function in developmental processes	AT1G20700	Weirauch *et al*., 2014
	TFmatrixID_0305, TFmatrixID_0306, TF_motif_seq_0320	MADF;Trihelix	**x**	**x**	**x**		**x**	TF may act as a molecular switch in response to light signals	AT1G13450, AT3G25990	Villain *et al*., 1996; Weirauch *et al*., 2014
	TFmatrixID_0499, TFmatrixID_0503	MADS-box;MIKC; M-type		**x**		**x**		Most TFs participate in regulation of flowering.	e.g. AT4G22950, AT1G26310	Mathelier *et al*., 2014; Matys *et al*., 2006
	TF_motif_seq_0442	NAC				**x**		TF is a central longevity regulator. Overexpression enhances tolerance to various abiotic stresses.	AT2G43000	Wu *et al*., 2014
Cellular fuctions	TF_motif_seq_0151	VOZ		**x**	**x**	**x**		TFs might be involved in the development of phloem and pollen tissues. TF can also act positively in the phytochrome B signaling pathway.	AT2G42400	Mitsuda *et al*., 2004

	TFmatrixID_0633	YABBY	**x**		**x**	**x**	**x**	TF is involved in the abaxial cell fate determination during embryogenesis and organogenesis, also required during flower formation and development.	AT2G45190	Franco-Zorrilla *et al*., 2014
Stress responses	TFmatrixID_0623, TFmatrixID_0625, TFmatrixID_0626	AP2		**x**		**x**	**x**	TF may be involved in the regulation of gene expression abiotic and biotic stresses.	AT2G28550, AT5G60120, AT5G60120	Franco-Zorrilla *et al*., 2014; Mathelier *et al*., 2014
	TFmatrixID_0063, TFmatrixID_0057	AP2;RAV			**x**	**x**		TFBS is a common sequence (CCAAT box) and it participates in increasing the heat shock promoter activity. For example, corresponding TFs have roles in embryogenesis, embryo maturation and/or in the blue light and ABA signaling pathways.	e.g. AT5G47670, AT1G21970	Rieping & Schöff, 1992; Haralampidis *et al*., 2002; Wenkel *et al*., 2006
	TF_motif_seq_0338	Bet_v_1		**x**		**x**	**x**	TFBS is required for elicitor responsiveness. It is found in the promoters of parsley (*Petroselinum crispum*) pathogenesis-related *PR1*-genes.	U48862, U48863	Rushton *et al*., 1996
	TF_motif_seq_0356	ERF		**x**			**x**	AtERF proteins are stress signal-response factors that affect GCC box-mediated gene expression positively or negatively as a respond to extracellular signals	Os02g0782700	Fujimoto *et al*., 2000
	TFmatrixID_0502, TF_motif_seq_0359	FAR1			**x**	**x**	**x**	TF activates the expression of *FHY1* and *FHL* genes involved in light responses.	AT3G22170	Mathelier *et al*., 2014
	TF_motif_seq_0010, TFmatrixID_0641, TFmatrixID_0045	HSF	**x**	**x**	**x**	**x**		TFs are heat stress transcription factors involved in heat stress responses that might be involved also in other environmental stress responses.	e.g AT2G26150, AT5G62020	Franco-Zorrilla *et al*., 2014
	TF_motif_seq_0032	LEA type 1			**x**	**x**	**x**	TFBS is found in barley (*Hordeum vulgare*) HVA1 gene encoding a class 3 late embryogenesis-abundant protein which is associated with tolerance for abiotic stresses	X78205	Straub *et al*., 1994; Xu *et al*., 1996; Busk & Pagès, 1998
	TF_motif_seq_0113	PsaH		**x**	**x**		**x**	TF has a possible role in the docking of the LHC I antenna complex to the core complex	X51911	Joshi 1987; De Pater *et al*., 1990
Hormonal regulation	TF_motif_seq_0439	Aux/IAA	**x**		**x**		**x**	Aux/IAA proteins are short-lived transcriptional factors that function as repressors of early auxin response genes at low auxin concentrations.	X68216	Ballas *et al*., 1993; Guilfoyle *et al*., 1998; Klinedinst *et al*., 2000
	TFmatrixID_0040, TFmatrixID_0156, TF_motif_seq_0335	B3;ARF	**x** **x** **x**	**x** **x**	**x** **x** **x**		**x** **x** **x**	TFBS is usually found in the promoters of primary/early auxin response genes.	e.g. AT1G19220, AT1G30330, AT5G60450	e.g. Ulmasov *et al*., 1999; Hagen & Guilfoyle, 2002
	TF_motif_seq_0422, TF_motif_seq_0446	LEA_5	**x**		**x**	**x**		TFBS plays important a role in ABA regulation. It is also binding site of *FUS3* which participates in controlling gene expression during late embryogenesis and seed development.	U22102	Hattori *et al*., 1995; Reidt *et al*., 2000; Curaba *et al*., 2004; Nag *et al*., 2005;

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