Annals of Botany **103**: 749–756, 2009
doi:10.1093/aob/mcn268, available online at www.aob.oxfordjournals.org

ANNALS OF BOTANY
Founded 1887

Novel localization of callose in the spores of *Physcomitrella patens* and phylogenomics of the callose synthase gene family

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Received: 12 October 2008 Returned for revision: 4 November 2008 Accepted: 25 November 2008 Published electronically: 20 January 2009

• *Background and Aims* Callose involvement in spore development is a plesiomorphic feature of land plants. Correlated light, fluorescence and immuno-electron microscopy was conducted on the developing spores of *Physcomitrella patens* to probe for callose. Using a bioinformatic approach, the callose synthase (*PpCalS*) genes were annotated and *PpCalS* and *AtCalS* gene families compared, testing the hypothesis that an exine development orthologue is present in *P. patens* based on deduced polypeptide similarity with *AtCalS5*, a known exine development gene.
• *Methods* Spores were stained with aniline blue fluorescent dye. Capsules were prepared for immuno-light and immuno-electron microscopy by gold labelling callose epitopes with monoclonal antibody. BLAST searches were conducted using the *AtCalS5* sequence as a query against the *P. patens* genome. Phylogenomic analysis of the *CalS* gene family was conducted using PAUP (v.4·1b10).
• *Key Results* Callose is briefly present in the aperture of developing *P. patens* spores. The *PpCalS* gene family consists of 12 copies that fall into three distinct clades with *AtCalS* genes. *PpCalS5* is an orthologue to *AtCalS5* with highly conserved domains and 64 % similarity of their deduced polypeptides.
• *Conclusions* This is the first study to identify the presence of callose in moss spores. *AtCalS5* was previously shown to be involved in pollen exine development, thus making *PpCalS5* a suspect gene involved in moss spore exine development.

Key words: Bryophyte, callose, callose synthase, exine development, moss, *Physcomitrella patens*, spores, sporogenesis.

INTRODUCTION

Spores are among the primary adaptations that enabled ancient land plants to survive in environmentally harsh landscapes (Graham, 1993; Shaw and Renzaglia, 2004; Blackmore, 2007; Renzaglia *et al.*, 2007). Early land plant radiation was coupled with elaboration from a single-celled zygote in the Charales to the multicellular sporophyte generation, which amplified spore production, but preceded seed evolution (Renzaglia *et al.*, 2007). Unicellular meiospores function as key perennating and dispersal units by producing complex walls that are resistant to microbial damage and decay. Within embryophytes, multilayered spore walls are systematically laid down in a prescribed manner. While it is generally accepted that bryophyte spores have an intine of cellulose that is surrounded by an exine impregnated with sporopollenin, precise identification of spore wall constituents is lacking.

As part of the Green Tree of Life Project to describe and compare the process of sporogenesis across bryophytes (mosses, liverworts, hornworts), ontogenetic changes in spore wall constituents were examined in the model moss *Physcomitrella patens*. A novel finding in this study was the discovery of callose in the aperture exine. While involvement of callose in sporogenesis of the green alga *Coleochaete* and liverworts is well-documented, to date this polysaccharide has not been detected in association with sporogenesis in any moss (Neidhart, 1979; Graham and Taylor, 1986; Brown and Lemmon, 1990).

Callose is a linear 1,3-β-glucan molecule that is widely distributed in the embryophyte cell wall. This transient polysaccharide is involved in fundamental biological processes and is considered to be evolutionarily important in land plants (Aspinall and Kessler, 1957; Graham *et al.*, 1991; Stone and Clarke, 1992; Garbary and Renzaglia, 1998; Hong *et al.*, 2001; Scherp *et al.*, 2001; Verma and Hong, 2001; Ligrone *et al.*, 2002). In angiosperms callose is necessary for proper pollen wall formation and patterning (Dong *et al.*, 2005; Nishikawa *et al.*, 2005), cell plate formation (Stone and Clarke, 1992; Hong *et al.*, 2001; Geisler-Lee *et al.*, 2002; Jacobs *et al.*, 2003), sieve plate production in phloem, megasporogenesis, microsporogenesis and wound healing (Scherp *et al.*, 2001; Verma and Hong, 2001; Evert and Eichhorn, 2006). Callose is reported to guide spore wall development in heterosporous lycopsids (Lugardon, 1990; Gabarayeva and Hemsley, 2006). In bryophytes, callose surrounds young spermatids during development and is associated with modification of plasmodesmata in nacreous leptoid pores (Gorska-Brylass, 1969; Stevenson, 1974; Graham *et al.*, 1991; Graham, 1993; Garbary and Renzaglia, 1998). Additionally, curdlans (callose-like molecules) are present in bacteria, and the genes responsible for the sucrose synthesis pathway necessary for callose

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synthesis are in cyanobacteria, suggesting this process is deeply conserved and is likely to have evolved specialized functions within the various groups of organisms (Scherp *et al.*, 2001; Verma and Hong, 2001; McIntosh *et al.*, 2005; Curatti *et al.*, 2006).

The availability of whole genome sequences affords the opportunity to explore the evolution of function and structure of genes across land plants. A number of *P. patens* gene families have been annotated and characterized including peptidoglycan biosynthesis (Machida *et al.*, 2006), inorganic phosphate (Pi) uptake (Wang *et al.*, 2008), aldehyde dehydrogenases (Wood and Duff, 2009) and cellulose synthase (Roberts and Bushoven, 2007). The production of callose via the callose synthase (*CalS* a.k.a. *GSL* 1,3-beta-glucan synthase EC 2·4·1·34) gene family has received much attention in *Arabidopsis thaliana* (Verma and Hong, 2001; Dong *et al.*, 2005; Enns *et al.*, 2005; Evert and Eichhorn, 2006). The 12 *Arabidopsis CalS* (*AtCalS*) genes exhibit multiple tissue expression patterns and functions. For example, *AtCalS1*, *AtCalS11* and *AtCalS12* are involved in cell plate formation, microspore separation, pollen tube growth, and prevention of pollen degeneration during early development (Hong *et al.*, 2001; Enns *et al.*, 2005). Among *AtCalS* genes, only *AtCalS5* has been implicated in exine development, with low expression in other tissues (Dong *et al.*, 2005; Nishikawa *et al.*, 2005).

Here the results of two complementary lines of enquiry are presented. First, through correlated light, fluorescence and immuno-gold electron microscopy, the occurrence of callose in the moss spore wall, specifically the aperture exine, is demonstrated. The second study is a bioinformatic analysis of the *CalS* gene family. The report of *AtCalS5* affecting pollen exine development in arabidopsis prompted the comparison of the *CalS* gene family between *Physcomitrella* and arabidopsis (Preuss *et al.*, 1994; Rhee and Somerville, 1998; Paxon-Sowders *et al.*, 2001; Ariizumi *et al.*, 2003; Dong *et al.*, 2005; Enns *et al.*, 2005; Nishikawa *et al.*, 2005). Annotation of 12 *PpCalS* genes in *P. patens* is provided and a phylogenomic analysis of the *CalS* gene family in moss and *Arabidopsis* is presented. Specifically, the hypothesis that an exine development orthologue is present in *P. patens* is tested based on amino acid sequence similarity with *AtCalS5*.

MATERIALS AND METHODS

Growing Physcomitrella patens

Physcomitrella patens Bruch & Schimp subsp. *patens* (Ashton and Cove, 1977) protonema was propagated vegetatively in 5·5-cm Petri dishes with BCD medium (Cove, 2000). Gametophores were transferred to sterile 30-mm-diameter peat pellets (Jiffy-7; Jiffy Products International) using tweezers. Pellets were prepared by soaking in distilled water until fully expanded, then autoclaved in Pyrex culture dishes. Water levels were maintained just below the upper surface of the cylindrical pellets. The inoculated peat pellets were cultured at 25 °C under 16-h light/8-h dark conditions for 6 weeks and then transferred to 15 °C under 8-h light/16-h dark conditions to induce gametangia development. Fertilization took place readily in the wet conditions of the peat pellets and capsules at all stages of development were ready for harvest 4 weeks after transfer.

Light microscopy

Capsules were broken open onto glass slides in 0·067 M sodium phosphate buffer, pH 8·5, as a control or stained with 1 % (w/v) aniline blue stain in the same buffer for 1 h each. Aniline blue binds 1,3-β-glucan that emits a yellow-green fluorescence when subjected to UV light. Callose fluorescence was observed using an excitation filter at 350 nm and an emission filter with a transmission cut-off at 460 nm. The slides were viewed on a Leica DM 5000B compound microscope equipped with UV fluorescence and a Q-Imaging Retiga 2000R digital camera.

Immuno-electron microscopy

For immuno-electron microscopy, capsules were fixed in 2·0 % glutaraldehyde in 0·05 M sodium phosphate buffer, pH 7·2, overnight at 4 °C. The fixed capsules were rinsed three times in buffer then serially dehydrated in ethanol. Specimens were slowly infiltrated with Spurr's or LR-White resin (Electron Microscopy Supply) and cured at 60 °C for 48 h (Spurr's) and 7 d (LR-White). The embedded capsules were cut with a diamond ultra knife to achieve sections of 100 nm (i.e. pale gold reflectance). The ultrathin sections were mounted on both 300- and 600-mesh uncoated nickel grids. The grids were placed on a slide warmer (45–50 °C) overnight to stick the sections to the grids. Immunogold preparation was conducted in a humid chamber for the three incubation steps and two washes: 10 μL of 1 % (w/v) bovine serum albumin (BSA) in phosphate-buffered saline (PBS), 30 min; 5 μL of primary monoclonal 1,3-β-glucan antibody (Biosupplies, Australia) diluted 1 : 20 with BSA–PBS, 4 h; followed by three exchanges of BSA–PBS, 5 μL of gold conjugated (size 15 nm) goat-anti-mouse secondary antibody (EMS, USA), 30 min; and four washes of PBS. Grids were then carefully rinsed with nano-pure water to remove residual chloride ions. The grids were air dried at room temperature and stained in 2 % aqueous uranyl acetate for 3 min and in Reynold's lead citrate for 30 s (Ligrone *et al.*, 2002). The grids were examined with a Hitachi 7650 transmission electron microscope and images were collected digitally.

Immuno-light microscopy

Sections of LR-White embedded capsules were cut with a diamond histoknife, mounted on BioBond (EMS, USA)-coated glass slides and labelled using the above procedure. The final staining step was replaced with incubation of the sections in a freshly prepared solution of Amersham IntenSE (Amersham Bioscience, UK) silver enhancement reagent. Negative controls were made for light and electron microscopy by omitting the incubation in the primary antibody.

Genome searches and bioinformatics

Previously identified *Arabidopsis thaliana* callose synthase sequences (Doblin *et al.*, 2001; Dong *et al.*, 2005, Enns

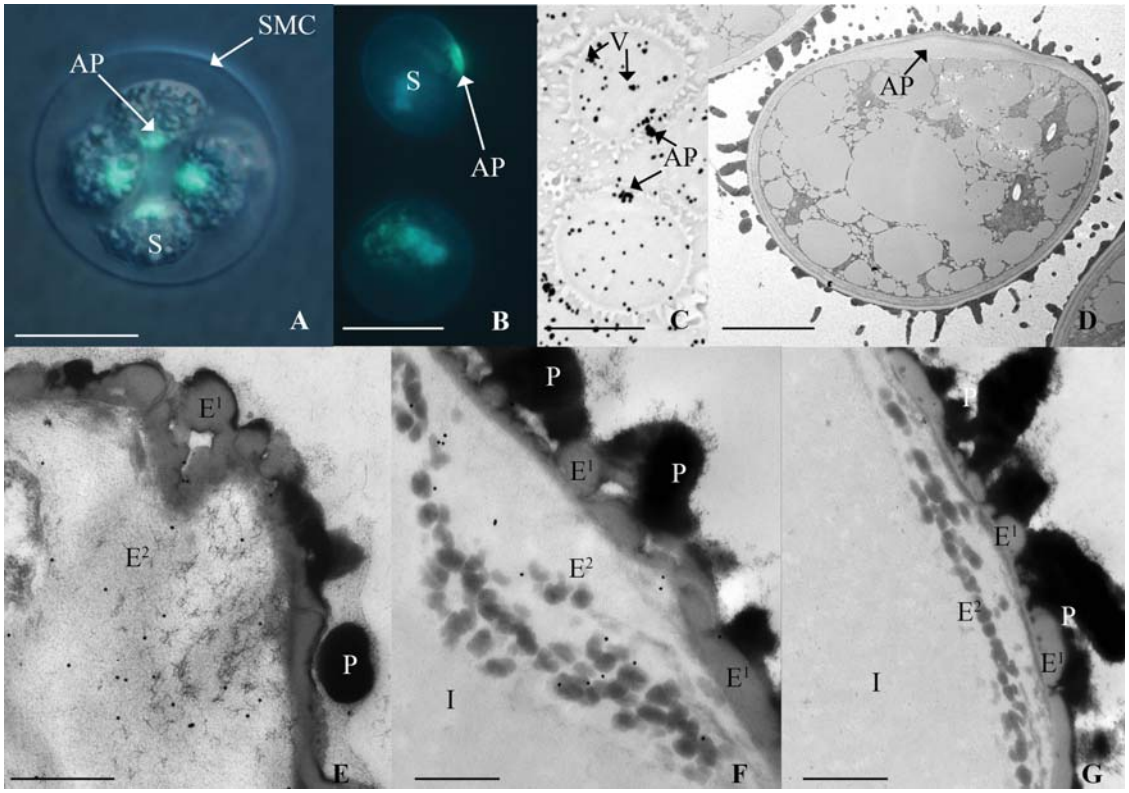

FIG. 1. Light and electron micrographs of spores from *Physcomitrella patens*. (A) Spores (S) in a tetrahedral tetrad inside the spore mother cell (SMC) wall stained with aniline blue; positive reaction for callose in aperture (AP) region of spores. (B) Spores liberated from SMC with wall in early developmental stages indicated by lack of spinose morphology stained with aniline blue; positive reaction in the aperture. (C) Positive reaction to silver enhanced anti-callose immunolabel in vacuoles (V) and the aperture of mature spores. (D) TEM of unlabelled spore with expanded aperture. (E–G) TEM of spore wall at the aperture region of proximal wall consists of perine (P), outer exine (E^1), inner exine (E^2) and intine (I): (E) oblique tangential section through aperture at early developmental stage indicated by relatively perine-free proximal wall has positive reaction to anti-callose immunogold label in the exine; (F) longitudinal section through aperture at later developmental stage indicated by the distinct presence of intine has notable positive reaction to anti-callose immunogold label associated with dark globules in (E^2); (G) longitudinal section through aperture labelled with only secondary antibody as a control. Scales bars: (A–C) = 15 μm; (D) = 5 μm; (E–G) = 500 nm.

et al., 2005; Nishikawa *et al.*, 2005) were retrieved from the NCBI database (http://www.ncbi.nlm.nih.gov). These sequences were used to search for *CalS* and *CalS-like* DNA sequences from the *Physcomitrella patens* ssp. *patens* (ver 1·1) genome (http://genome.jgi-psf.org/euk_home.html) using BLASTX, BLASTN and BLASTP (low complexity filter on, Blosum62 substitution matrix) (Altschul *et al.*, 1990, 1997). Protein motifs were queried using Pfam (Bateman *et al.*, 2004), PROSITE (Hulo *et al.*, 2006), CDD (Conserved Domain Database) or CDART (Conserved Domain Architecture Retrieval Tool) (Marchler-Bauer and Bryant, 2004; Marchler-Bauer *et al.*, 2005). Domains used for identification were Pfam PF02634 (glucan synthase), CDD 86706 (glucan synthase), IPR003440 (glycosyltransferase) and Pfam 04652 (DU605).

Sequence alignment and phylogenetic analysis

An initial alignment of 24 complete deduced plant *CalS* amino acid sequences was created in ClustalW (Chenna *et al.*, 2003) using the Gonnet protein weight matrix, pairwise gap opening/extension penalties of 10/0·1 and multiple alignment gap opening/extension penalties of 10/0·5. See Fig. 2 legend for accession numbers. This initial alignment was manually adjusted using GeneDoc (Nicholas *et al.*, 1997). Phylogenetic trees were generated by both neighbor-joining and parsimony methods using PAUP* (version 4.1b10; Sinauer Associates, Sunderland, MA, USA). Support for nodes on the estimated phylogeny was tested with 1000 bootstrap replicates using the parsimony method.

RESULTS

General morphology and immunocytochemistry

Aniline blue staining of tetrads surrounded by or released from the spore mother cell (SMC) walls revealed a bright yellow-green fluorescence restricted to the proximal side of each developing spore, i.e. the region where the four spores meet in a tetrad. (Fig. 1A, B). This area corresponds to the aperture, a specialized region of the spore wall that permits the protonemata to emerge from the spore coat during germination. In line with the result of aniline-blue staining, silver-enhanced immuno-labelling showed a positive reaction for callose in the aperture; more scattered silver precipitates were also visible in the vacuoles of maturing spores (Fig. 1C). No wall

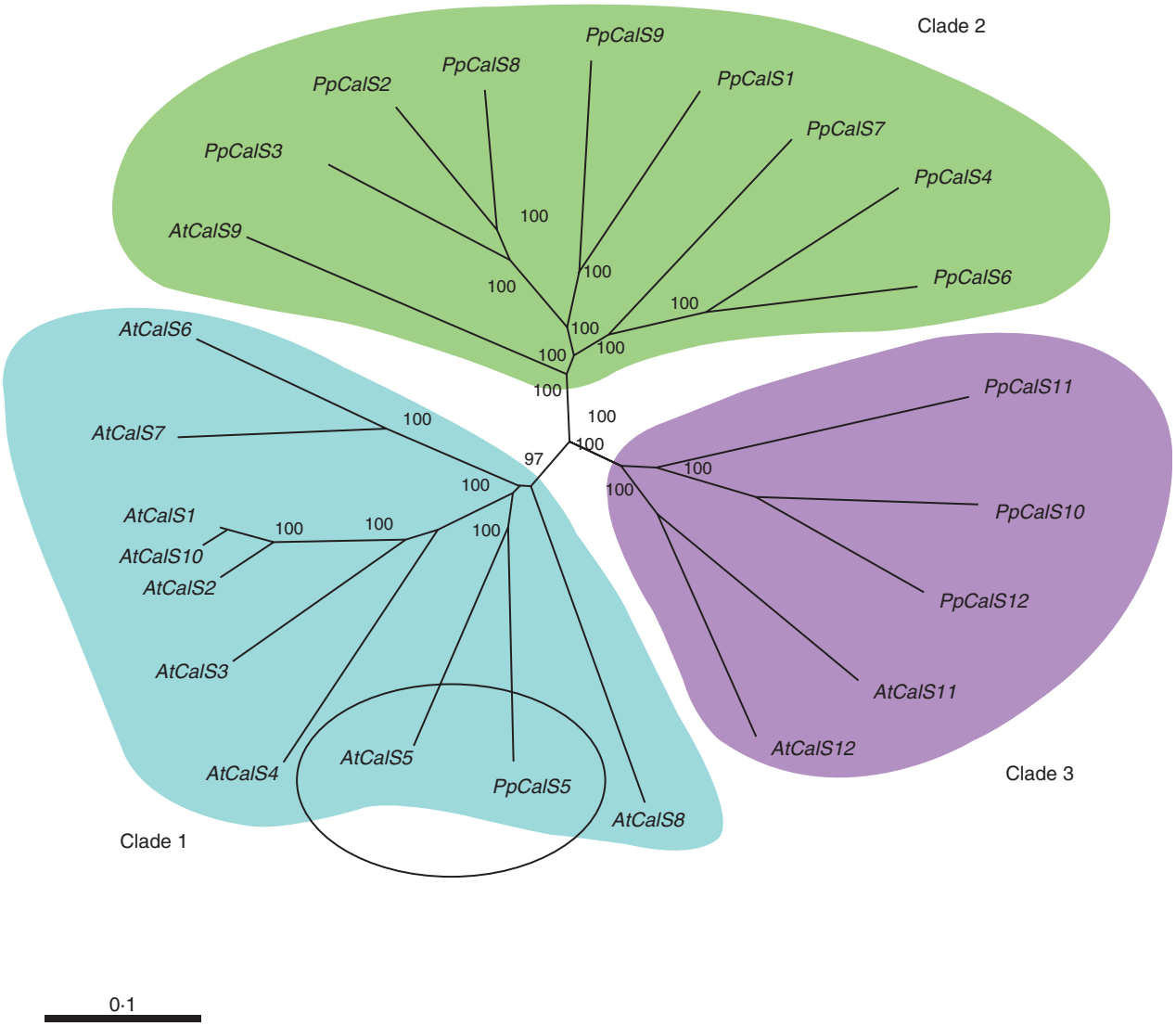

FIG. 2. Unrooted and bootstrapped gene tree of the *CalS* gene family from *Physcomitrella patens* and *Arabidopsis thaliana*. ClustalW (*) was used to generate an alignment that was manually curated with GeneDoc of the full-length deduced polypeptides. Bootstrap replicates = 10 000 with values reported as percentages for each of the nodes.

labelling was observed at any other stage of development and controls for all stages were completely negative.

Observed by electron microscopy in nearly mature spores, the aperture was found to consist of a local expansion of a loosely fibrillar intine layer underlying the exine (Fig. 1D). In the remaining wall the exine is uniform in thickness and consists of two layers, an inner fibrillar layer with scattered electron-opaque globules, and an outer thinner layer of homogeneous lightly opaque material which undulates and in part contributes to spore ornamentation (Fig. 1D–G). An opaque perine is deposited externally on the exine and is responsible for the spinose sculptoderm on the distal spore surface and globules over the proximal surface (Fig. 1D–G). In fully mature spores, perine deposition masks the outer exine layer. Immunogold labelling at the TEM level revealed that callose epitopes were restricted to the inner fibrillar exine layer of the aperture in developing and mature spores. Here the labelling was mainly associated with the dense globules (Fig. 1E and F), whilst virtually no background labelling was observed in other areas of the sections. Control samples labelled with only the secondary gold-conjugated antibody exhibited no labelling anywhere along the spore wall, including the aperture (Fig. 1G).

Sequence alignment and phylogenomic analysis

The assembled plant genome for *P. patens* (ver 1·1; Rensing *et al.*, 2008) was analysed for the presence of *CalS* and *CalS-like* DNA sequences. *Physcomitrella* genes were named based upon phylogenomic analysis (Fig. 2) and sequence similarity of the deduced polypeptide sequences to previously characterized *AtCalS* genes from arabidopsis (Table 1) (Verma and Hong, 2001; Dong *et al.*, 2005; Enns *et al.*, 2005; Evert and Eichhorn, 2006). The *P. patens* genome

TABLE 1. *Callose synthase (CalS) gene family in the* Physcomitrella patens *genome (v. 1·1)*

Gene annotation	Accession number	TM domains	Deduced polypeptide
Pp_CalS1	XP_001772017	14	1936
Pp_CalS2	XP_001759994	16	1930
Pp_CalS3	XP_001755638	17	1929
Pp_CalS4	XP_001764343	11	1966
Pp_CalS5	XP_001776005	15	1930
Pp_CalS6	XP_001773231	12	1934
Pp_CalS7	XP_001766872	13	1952
Pp_CalS8	XP_001764315	15	1942
Pp_CalS9	XP_001754415	12	1910
Pp_CalS10	XP_001783858	11	1759
Pp_CalS11	XP_001764182	13	1755
Pp_CalS12	XP_001754476	13	1769

contains 12 putative callose synthase genes (Table 1). The deduced *PpCalS* polypeptides range from 1755 to 1966 amino acid residues and are predicted to contain between 11 and 17 transmembrane domains. Nine deduced *P. patens* polypeptides (*PpCalS1–9*) have an average size of 1936 amino acids residues (+/– 16·0) and three deduced polypeptides (*PpCalS10–12*) have an average size of 1761 amino acids residues (+/– 7·0) (Table 1).

Phylogenomic analysis of the deduced polypeptides from *P. patens* and *A. thaliana* strongly supports the presence of three *CalS* clades (Fig. 2). Clade 1 contains nine *A. thaliana* sequences (*AtCalS1–8* and *AtCalS10*) and a single *P. patens* sequence (*PpCalS5*), Clade 2 contains one *A. thaliana* sequence (*AtCalS9*) and eight *P. patens* sequences (*PpCalS1–4* and *PpCalS6–9*). Clade 3 contains two *A. thaliana* sequences (*AtCalS11,12*) and three *P. patens* sequences (*PpCalS10–12*). *PpCalS10–12* are 170 amino acid residues shorter than *PpCalS1–9. PpCalS5* is nested within the *AtCalS* genes and is least similar to any of the *PpCalS* genes (Fig. 2). Comparison of the deduced polypeptide *PpCalS5* with *AtCalS5* reveals that both sequences share a number of protein motifs: DUF605 and Vta-like domains (pfam04652) within the N-terminus and four highly conserved regions in the inner loop (Fig. 3A). Structurally the *PpCalS5* consists of a 5' tail 483 amino acids in length, 15 transmembrane domains, and an inner loop 755 amino acids in length (Fig. 3B), while *AtCalS5* consists of a 5' tail, 16 transmembrane domains, and an inner loop of similar length.

DISCUSSION

This is the first report that callose is involved in sporogenesis in mosses. Although callose plays a role in tracheophyte spore and pollen development, liverworts were the only bryophytes conclusively demonstrated to involve callose in spore wall differentiation (Brown and Lemmon, 1987, 1988, 1990). Neidhart (1979) reported the existence of callose in the SMCs of hornworts but this has yet to be verified (Brown and Lemmon, 1990). Callose occurs in meiospore walls of the multicellular green alga *Coleochaete* (Graham and Taylor, 1986; Brown and Lemmon, 1990; Graham, 1993). In liverworts, callose is involved in pre- (SMC) and post-meiotic exine patterning which leads to the development of elaborate spore surface ornamentation (Brown and Lemmon, 1987). In contrast, in *P. patens* callose is absent from the SMCs but appears in developing spores and is localized in the expanded aperture region. As spores reach their final stages of development, the aperture collapses and callose is lost. This highly specific localization implies callose is involved in expansion of the aperture during wall development. In seed plants, callose serves to separate the developing microspores and provides a template for pollen exine development (Hong *et al.*, 2001; Dong *et al.*, 2005; Nishikawa *et al.*, 2005). Although this polysaccharide is localized in different spore/pollen wall regions and appears at different developmental stages across plants, the present demonstration of callose in *Physcomitrella* spores supports the notion that callose involvement in sporogenesis is a signature of embryophytes (Graham and Taylor, 1986; Brown and Lemmon, 1987, 1990; Graham, 1993).

Phylogenomic comparisons between *Physcomitrella* and *Arabidopsis* identify three clades of callose synthase genes based on deduced polypeptide sequences. Thus, at least two duplications of the *CalS* gene occurred prior to the divergence of mosses, some 400 million years ago (Pryer *et al.*, 2004; Newton *et al.*, 2007). Different amino acid sequences may reflect tissue- or development-specific gene expression. Similar interpretations were reported for genes responsible for root hair and rhizoid development (Menand *et al.*, 2007) as well as the cellulose synthase (Roberts and Bushoven, 2007) and β-expansin genes (Carey and Cosgrove, 2007). It is safe to assume that selective pressures specific to the moss versus flowering plant life cycle played a role in *CalS* gene evolution. Each of the three gene clades contains combinations of genes from both plants, but with varying degrees of gene duplication in the two plants. Like *Arabidopsis*, *Physcomitrella* has 12 *CalS* genes, but with the majority in clade 2. Within this clade a single *Arabidopsis* gene, *AtCalS9* (a.k.a. *AtGSL10*) shown to affect pollen development at the mitotic stage (Huang *et al.*, 2008), is sister to eight moss genes.

Another intriguing finding is that *PpCalS10–12* genes are more similar to each other and to *AtCalS11,12* (a.k.a. *GSL 1and5*) than to any other *PpCalS* gene. Double mutants of *AtCalS11,12* produce smaller flowers, short lateral bolts, shorter roots, and severely deformed pollen with unusual pore structures (Enns *et al.*, 2005). The latter phenotype was the result of failure to form the callose wall that separates the microspores while in the tetrads. Additionally, some of the deformed pollen grains could germinate but were infertile suggesting that *AtCalS11,12* genes have partially redundant roles in both the sporophyte and gametophyte (Enns *et al.*, 2005). The functions of orthologs *PpCalS10–12* as compared with *AtCalS11,12* remain to be determined but may be hypothesized to be expressed in vegetative cell walls. It is interesting to note that the intersporal walls of some liverworts, not mosses, contain callose that separates the spores in tetrads. With the *Marchantia* genome sequencing project in progress (http://www.jgi.doe.gov/sequencing/cspseqplans2008.html), callose synthase genes for this liverwort may be added soon to the analysis, and will shed light on sequence homology between intersporal wall callose in arabidopsis and liverworts.

With nearly 65 % identity of deduced polypeptide sequences to *AtCalS5*, the orthologous moss gene *PpCalS5*

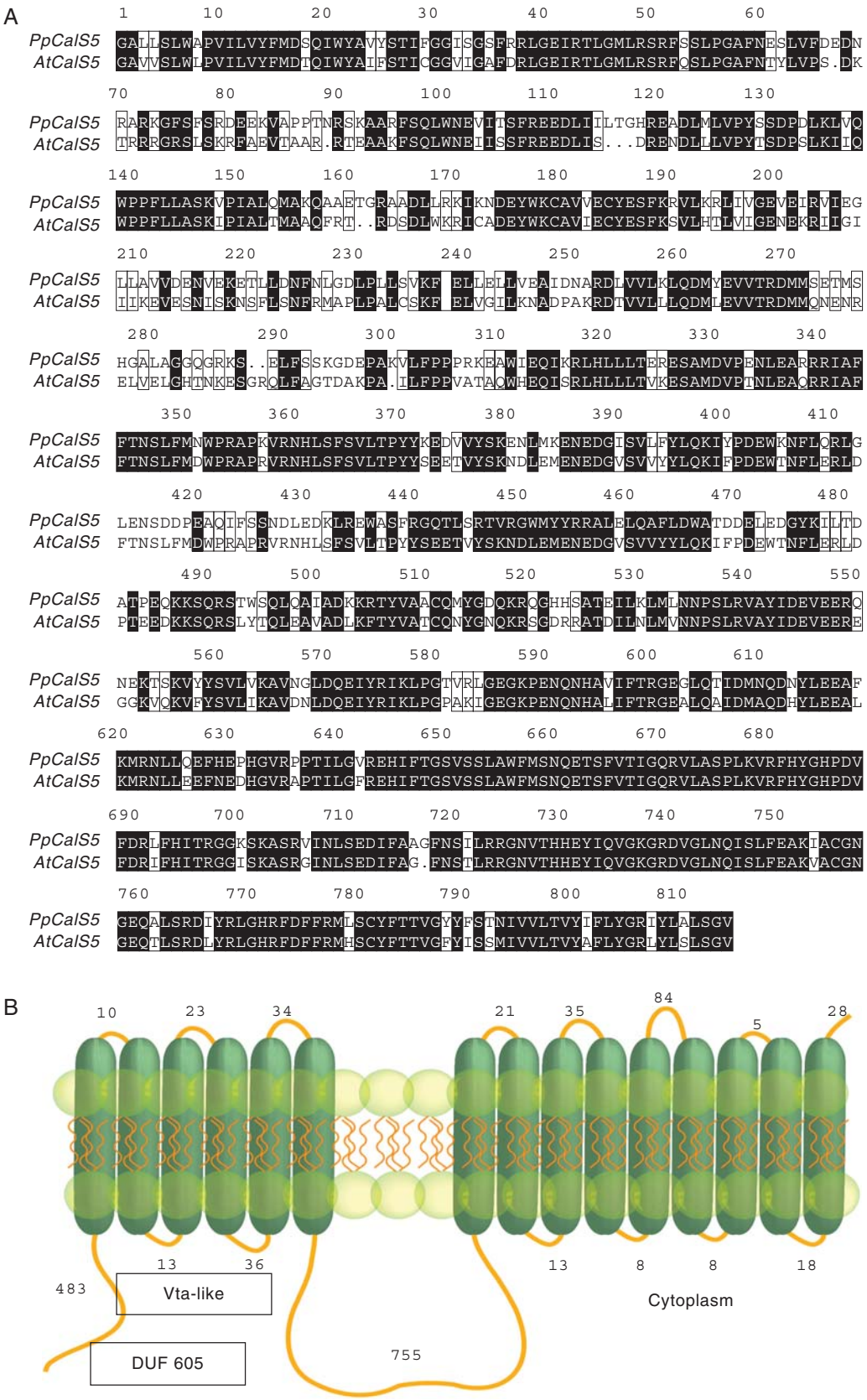

FIG. 3. Deduced polypeptide sequence alignment of *PpCalS5* and *AtCalS5*, and predicted membrane topology of *PpCalS5*. (A) Sequence alignment of inner loop of putative spore exine development callose synthase (*PpCalS5*) with known pollen exine development callose synthase (*AtCalS5*). Highly conserved domains shaded black. (B) Predicted protein structure of *PpCalS5* with putative conserved DUF 605 and Vta-like domains (pfam04652) in the N-terminus.

is a likely candidate gene involved in production of the exine callose localized in this study. *AtCalS5* has been shown through knockout mutants to exclusively affect pollen exine formation (Dong *et al.*, 2005). In support of this proposed function is that, in tobacco, barley and wheat, callose synthase expression is temporally and spatially correlated to specific tissues and organs (Doblin *et al.*, 2001; Wilson *et al.*, 2006; Voigt *et al.*, 2006). To address this postulate, studies are underway using GFP localization of *PpCalS5* in both generations of the life cycle of *Physcomitrella.*

Conspectus

Sporogenesis is a deeply conserved process and one of the key innovations that played a role in the origin and diversification of land plants (Renzaglia *et al.*, 2000, 2007; Shaw and Renzaglia, 2004; Blackmore, 2007). Although the location and timing of callose during sporogenesis in other plant groups is different from that observed in *P. patens*, the involvement of this polysaccharide in spore development is widespread in land plants. This raises the question whether similar callose synthase genes operate differently in different lineages of plants, or whether functionally divergent, species-specific callose synthase genes are responsible for differences in callose deposition. The answers to these questions will be elucidated as genomes become available from additional groups of plants.

The moss *Physcomitrella patens* is an important model system for understanding plant biology and evolution (Cove *et al.*, 1997; Wood *et al.*, 2000; Nishiyama *et al.*, 2003; Frank *et al.*, 2005; Lee *et al.*, 2005; Cove *et al.*, 2006; Quatrano *et al.*, 2007). *Physcomitrella* has an efficient and well-characterized gene-targeting system (i.e. homologous recombination; Schaefer and Zryd, 1997; Schaefer, 2002). Unparalleled among other model embryophytes, the dominant haploid phase of this moss ensures immediate and direct expression of transformed phenotypes (Lee *et al.*, 2005). An in depth understanding of the biology of *P. patens* is necessary to understand the genes responsible for transformed phenotypes. This is especially true for complex developmental processes such as spore wall development that require sophisticated labelling techniques and detailed microscopic observation. Indeed, with more plant genomes in the pipeline, the limiting factor in studies of functional and evolutionary genomics may be a lack of knowledge about the plants themselves. Studies, such as the present one, that couple structural development with genomic data, are necessary to generate and answer fundamental questions related to the evolution of development.

ACKNOWLEDGEMENTS

We are grateful for the assistance provided by Integrated Microscopy and Graphics Expertise staff and use of their electron microscope facilities at Southern Illinois University, Carbondale. The authors thank Tammy d'Artenay, Renee Lopez-Smith and Katayoun Mansouri for comments on an earlier version of the manuscript. This project was supported by two NSF Tree of Life initiatives (DEB-0228679, DEB-0531751).

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