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All type II classic MADS-box genes in the lycophyte *Selaginella moellendorffii* are broadly yet discretely expressed in vegetative and reproductive tissues

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Abstract

The MADS-box genes constitute a large transcription factor family that appear to have evolved by duplication and diversification of function. Two types of MADS-box genes are distinguished throughout eukaryotes, types I and II. Type II classic MADS-box genes, also known as MIKC-type, are key developmental regulators in flowering plants and are particularly well-studied for their role in floral organ specification. However, very little is known about the role that these genes might play outside of the flowering plants. We investigated the evolution of type II classic MADS-box genes across land plants by performing a maximum likelihood analysis with a particular focus on lycophytes. Here, we present the expression patterns of all three type II classic MADS-box homologs throughout plant development in the lycophyte *Selaginella moellendorffii*: *SmMADS1*, *SmMADS3*, and *SmMADS6*. We used scanning electron microscopy and histological analyses to define stages of sporangia development in *S. moellendorffii*. We performed phylogenetic analyses of this gene lineage across land plants and found that lycophyte sequences appeared before the multiple duplication events that gave rise to the major MADS-box gene lineages in seed plants. Our expression analyses by in situ hybridization show that all type II classic MADS-box genes in *S. moellendorffii* have broad but distinct patterns of expression in vegetative and reproductive tissues, where *SmMADS1* and *SmMADS6* only differ during late sporangia development. The broad expression during *S. moellendorffii* development suggests that MADS-box genes have undergone neofunctionalization and subfunctionalization after duplication events in seed plants.

KEYWORDS
lycophytes, root, SmMADS, sporangia, strobilus

1 | INTRODUCTION

MADS-box proteins are one of the most well-studied transcription factors in plant evolution and development and are characterized by the highly conserved DNA-binding MADS domain which has a length of 56–60 amino acids (Riechmann & Meyerowitz, 1997; Schwarz-Sommer et al., 1990; Yanofsky et al., 1990). The MADS-box family of proteins has been further classified into several different groups based on conserved protein motifs with two types of MADS-box genes distinguished throughout the eukaryotes, types I and II (Alvarez-Buylla et al., 2000). Type II MADS-box genes in plants are also known as MIKC-type genes based on the conserved domains where the MADS (M) domain is followed by an Intervening (I), a Keratin-like (K), and a C-terminal (C) domain (Alvarez-Buylla et al., 2000; Ma et al., 1991; Purugganan et al., 1995; Riechmann & Meyerowitz, 1997). Based on phylogenetic, structural features, and intron-exon structure, type I MADS-box genes are classified as Mα, Mβ, and Mγ, and type II are classified as $MIKC^C$ (or type II classic) and MIKC* (or type II star) (Alvarez-Buylla et al., 2000; Henschel et al., 2002; Pařenicová et al., 2003; Svensson et al., 2000). Type II star MADS-box genes play important roles in the gametophytic stage (Henschel et al., 2002; Svensson et al., 2000; Thangavel & Nayar, 2018) and only a few have been functionally characterized (reviewed in Gramzow & Theissen, 2010; Thangavel & Nayar, 2018). On the other hand, type II classic MADS-box genes have been broadly studied as their functions range from root development, floral transition, floral organ specification, and fruit development (reviewed in Gramzow & Theissen, 2010).

Type II classic MADS-box genes are particularly well known as many of them have roles in the appropriate development of the different floral organs and are divided into four functional classes: A, B, C, and E (Bowman et al., 1989, 1991; Coen & Meyerowitz, 1991; Pelaz et al., 2000; Theißen & Saedler, 2001). Generally, class A + E genes specify sepals, A + B + E specify petal identity, B + C + E specify stamen identity and class C + E genes specify carpel identity (Bowman et al., 1989, 1991; Coen & Meyerowitz, 1991; Pelaz et al., 2000). For the most part, the genes included in the ABCE model of floral organ identity belong to the superfamily of MADS-box transcription factors (reviewed in Gramzow & Theissen, 2010) with the exception of the A-class gene *APETALA2* (Jofuku et al., 1994). No functional characterization has been done in gymnosperms, but expression analyses suggest that type II classic MADS-box homologs are found in the ovules, in the fleshy integument of *Ginkgo biloba*, and in the fleshy envelopes surrounding the ovule in *Gnetum* (Becker et al., 2003; Lovisetto et al., 2012; Shindo et al., 1999).

Less is known about the role of MADS-box genes in the seedless vascular plant lineages: lycophytes and ferns (Figure 1a). Unlike the usually organ-specific expression found in seed plants, fern homologs have shown ubiquitous expression patterns, both in the haploid gametophytic and diploid sporophytic generations by northern blot analyses or reverse transcription-polymerase chain reaction (RT-PCR; Hasebe et al., 1998; Huang et al., 2014; Münster et al., 1997, 2002; Ruiz-Estévez et al., 2017). Lycophytes evolved over 400 million years (my) and occupy a key phylogenetic position as sister to all other vascular plant lineages (reviewed in Ambrose, 2013) (Figure 1a). Several key features of vascular plants evolved independently in lycophytes, ferns, and seed plants. This includes the evolution of leaves, heterospory, and endosporic development (Ambrose, 2013; Doyle, 2013), while certain features characterize lycophytes such as dichotomous (equal apical) branching and ligules (tongue-shaped structures) (Figure 1a,b). Heterospory is the production of two distinct types of spores: one producing the megagametophyte and eventually the female gamete (egg) and the other producing the male gametophyte that eventually produces the male gamete (sperm) (Figure 1b). Endosporic development is where the gametophyte develops within the spore. Heterospory and endosporic development are two features thought to be important for the evolution of seeds (Parihar, 1967). Similar to other vascular plants, lycophytes alternate between two multicellular generations: the dominant diploid sporophyte and haploid gametophyte (Figure 1b). Little is known about the molecular genetic network that built the diverse sporophyte body plan during the evolution of lycophytes, ferns, and seed plants.

Evolutionary studies of MADS-box proteins have shown that this large family has evolved through duplication and diversification (Alvarez-Buylla et al., 2000; Gramzow et al., 2012; Purugganan et al., 1995). Phylogenetic analyses of type II classic MADS-box genes include plants for which the whole genome is available, however, the vast majority of genome sequences available are from angiosperms and relatively few gymnosperms, lycophytes, and bryophytes (Michael & Jackson, 2013). These analyses on the MADS-box genes have led to the conclusion that some of the clades of floral developmental genes have homologs in gymnosperms but not in ferns, lycophytes or mosses (Gramzow et al., 2012, 2014; Hasebe et al., 1998; Münster et al., 1997; Tanabe et al., 2003).

In this context, where functional analyses of type II MADS-box genes outside angiosperms are restricted to only a few species, it is difficult to predict the functional

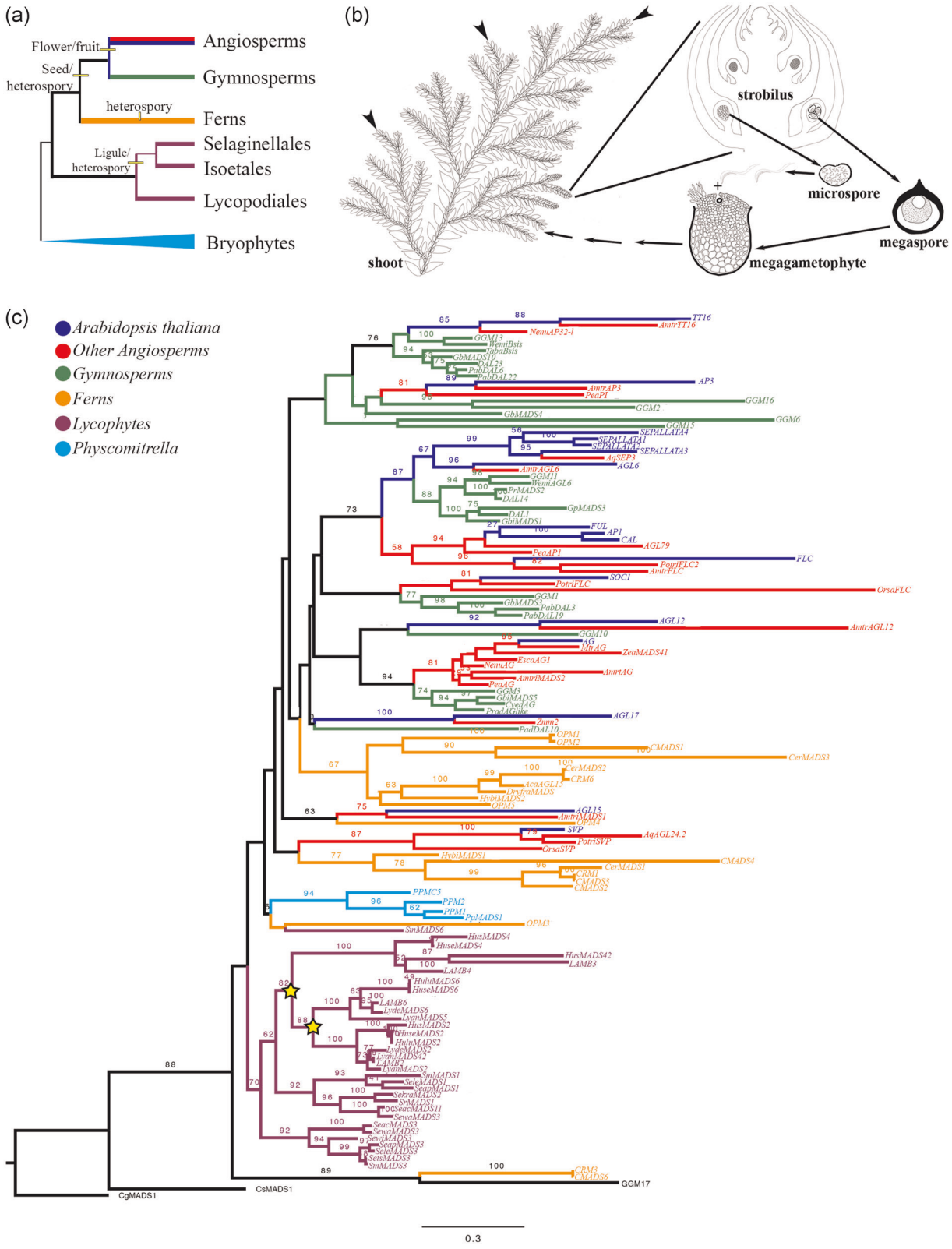

FIGURE 1 (See caption on next page)

evolution of this gene lineage. Thus, to fill the gaps in the understanding of this gene lineage we report here: (1) phylogenetic analysis of type II classic MADS-box homologs in land plants with an emphasis on lycophytes; (2) analysis of coding sequences across land plants to identify conserved regions across homologs that may help predict putative changes in protein function; (3) stages of sporangia development in the lycophyte *Selaginella moellendorffii* (Selaginellaceae); and (4) expression analyses of all type II classic MADS-box genes in *S. moellendorffii*. We chose to focus on *S. moellendorffii* for several reasons: first, because there is a genome available and the MADS-box genes have already been identified from it; second, because of its key evolutionary position as the sister lineage to all other vascular plants; and third, we have vegetative and reproductive tissues available.

2 | MATERIALS AND METHODS

2.1 | Phylogenetic analysis

To better understand the evolution of the three *S. moellendorffii* homologs, *SmMADS1, SmMADS3*, and *SmMADS6* (GenBank: XM_002977787.1, XM_002984875.1, and XM_002988269.1, respectively), we downloaded sequences of all major gene lineages of the MADS-box type II classic genes, and we aimed to include representatives of each clade from all major land plant lineages. The BLAST search was performed using public genome repositories such as GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Lycophyte sequences were obtained from the public transcriptome database OneKP (https://db.cngb.org/onekp/). Sequences were compiled with Aliview (Larsson, 2014) and manually edited to exclusively keep the open reading frame for all transcripts. Nucleotide sequences were subsequently aligned using the online version of MAFFT (http://mafft.cbrc.jp/alignment/software/; Katoh et al., 2002) with a gap open penalty of 3.0, offset value of 0.8 and all other default settings. Maximum likelihood (ML) phylogenetic analyses using the nucleotide sequences were performed with RaxML-HPC2 BlackBox (Stamatakis et al., 2008) through the CIPRES Science Gateway (https://www.phylo.org/; Miller et al., 2010). Bootstrapping was performed according to the default criteria in RaxML where the bootstrapping stopped after 200–600 replicates. Trees were observed and edited using FigTree v 1.4.2. (http://tree.bio.ed.ac.uk/software/figtree/). The ingroup consists of a total of 119 sequences from land plants. The outgroup consisted of algae sequences (Table S1).

To identify conserved motifs across the type II MADS-box classic genes, 63 complete sequences were selected representing all the major MADS-box subfamilies and with representatives from different plant lineages (24 angiosperms, 10 gymnosperms, 4 ferns, 23 lycophytes, and 2 from mosses). Sequences were permanently translated and uploaded as amino acids to the online MEME server (http://meme.nbcr.net; Bailey et al., 2006).

2.2 | Sporangia morphology in *S. moellendorffii*

S. moellendorffii tissue was collected at the New York Botanical Garden Nolen glasshouses (Accession No.: 159/2007), vouchered (NYBG-00698548), and immediately fixed in formalin-acetic acid-ethanol (FAA; 3.7% formaldehyde, 5% glacial acetic acid, 50% ethanol). For scanning electron microscopy (SEM), tissue was dehydrated through an alcohol series, critical point dried with a Denton Vacuum DCP-1, and coated with palladium and gold (Hummer 6.2; Anatech) and images were taken on a JEOL 5410LV (Peabody) SEM at an

FIGURE 1 Lycophytes in relation to land plants, lycophyte life cycle, and a MADS-box gene phylogeny. (a) Diagram illustrating the relationships among land plants with major morphological innovations mapped onto branches. (b) Overview of a general *Selaginella* life cycle with a prominent sporophyte phase (shoot) with some branches that terminate with a strobilus. Sporangia develop from the strobilus axis with more mature sporangia toward the base. *Selaginella* strobili produce two different types of sporangia (heterosporous): megasporangia and microsporangia. After meiosis, microspore tetrads or a megaspore tetrad are present in each sporangia. The haploid gametophyte phase develops within the spores (endosporic development). Microspores and megaspores are shed with the microgametophyte and megagametophyte, respectively, already developing. Sperm develop and are released from the microgametophyte and fuse with an egg present in the megagametophyte complete with rhizoids. After fertilization, the embryo develops within the megagametophyte and the young shoot continues to develop completing the life cycle. Dominant branches of the anisotomous shoot are indicated with arrowheads. Structures are not drawn to scale and are redrawn from Figure 2 or Parihar (1967). (c) Maximum Likelihood analysis for the type II classic MADS-box gene lineage in land plants. Each major plant lineage is color-coded: Angiosperms are in red and blue (*Arabidopsis thaliana* genes are blue), gymnosperms are green, ferns are orange, mosses are light blue, lycophytes are purple, charophycean algae outgroups are in black, and yellow stars indicate duplications

accelerating voltage of 10 kV. For light microscopy, fixed material was manually dehydrated through an alcohol-Histoclear series (National Diagnostics) and embedded in Paraplast X-tra (Fisher Healthcare). The samples were sectioned at 10μm with an MICROM HM355 (Thermo Fisher Scientific) rotary microtome. Sections were stained with Johansen's safranin, to identify lignification and presence of cuticle (Johansen, 1940), and 0.5% Astra Blue (Kraus et al., 1998) and mounted in Permount (Thermo Fisher Scientific). Sections were viewed and digitally photographed with a Zeiss Axioplan compound microscope equipped with a Nikon DXM1200C digital camera with ACT-1 software. Different stages of sporangia development were described using as reference anatomical descriptions reported from various lycophytes (reviewed in Foster & Gifford, 1959; Parihar, 1967).

2.3 | In situ hybridization of *S. moellendorffii*

Tissue was collected, processed, and sectioned as described above for light microscopy. Probes were generated by a two step PCR process. The first PCR reaction used *S. moellendorffii* cDNA to generate gene-specific fragments downstream of the well-conserved MADS domain; for a 606 bp *SmMADS1* gene-specific fragment (01SMADS1—5′-GCAC CACGACAGTGATTACTTC-3′ and 02SMADS1—5′-GAT CAATGGCTGCTGTCTGATG-3′), for a 486 bp *SmMADS3* gene-specific fragment (01SMADS3—5′-CGAGGGAAA CCATAACACCAG-3′ and 02SMADS3—5′-CGTTACCCC AAGTGCAGTGATG-3′), and for a 423 bp *SmMADS6* gene-specific fragment (01SMADS6—5′-CTCGATAACG ACTACTGGAATC-3′ and 02SMADS6—5′-TCACCGGA GTTGCAAAGAGGTG-3′). PCR fragments were cloned into pGEM T easy (Promega) and were verified by Sanger sequencing. Plasmids containing gene-specific fragments were subjected to a second round of PCR to add the T7 sequence to one end of the fragment; for antisense probes, the same forward primer was used and the reverse primer was a new primer that had the same sequence as the original reverse primer with an added T7 sequence at the 5′-end (CTAATACGACTCACTATAGGG). These fragments were purified using MinElute PCR purification columns (Qiagen) and eluted in RNase-free water. Digoxygenin-labeled gene specific probes were generated using DIG RNA labeling mix and T7 RNA polymerase according to manufacturer's protocol (Roche supplied by Sigma-Aldrich). In situ hybridization was performed as previously described (Ambrose et al., 2000). Images were viewed and captured as described above for the histological sections. Whole-mount in situ hybridization was performed in 15 mL falcon tubes, by fixing whole gemmae for 2 h in FAA, rehydrating to water, and then starting with the hydrochloric acid step of the in situ hybridization protocol as described in Ambrose et al. (2000). Whole-mount in situs were imaged on a Nikon SMZ1500 dissecting scope equipped with a Nikon DS-Ri1 digital camera.

3 | RESULTS

3.1 | The evolution of type II classic MADS-box lineage

To better understand the evolutionary history of lycophyte type II classic MADS-box genes, the phylogeny of the MADS-box genes, including sequences from mosses to angiosperms, with extended sampling in lycophytes, and using complete sequences of all homologs and parametric methods, is presented here (Figure 1c). The resulting phylogeny rescues the previous topology presented for the gene family (Alvarez-Buylla et al., 2000; Gramzow et al., 2012; Purugganan et al., 1995) where the major gene subfamilies (i.e., *TT16*, *AP3*, *SEP/AGL6*, *FUL/AP1*, *AG*, *AGL12*, and *AGL17*) are seed plant-specific with high bootstrap (BS) values (Figure 1c). Within the five lycophyte clades, three are Lycopodiales specific as the result of two duplication events before their diversification; the other two clades are specific to the Selaginellales, where *SmMADS1* and *SmMADS3* are separated into two clades. However, the position of *SmMADS6* is still unclear, being in a clade with a fern sequence (*OPM3*) and the *Physcomitrella* (*Physcomitrium*) sequences (Figure 1c). Our focus is to understand the type II classic MADS-box genes in lycophytes, and therefore, we only included a sampling of a few model species in ferns and seed plants. The lack of extensive sampling within these groups explains the low BS values in the main backbone of the topology as well as the unsupported placement of the clade containing the *Physcomitrella* sequences (Figure 1c).

To determine conserved motifs in type II classic MADS-box genes across land plants, we performed a Multiple EM for Motif Elicitation (MEME) analysis (Bailey et al., 2006). We were able to identify the canonical domains: MADS-box, the I-region, and the K-box and the highly variable C-region (Figures 1 and 2; Alvarez-Buylla et al., 2000; Kwantes et al., 2012; Ma et al., 1991; Purugganan et al., 1995; Riechmann & Meyerowitz, 1997; Vandenbussche et al., 1997). In addition to the well-characterized MIK-regions, we detected additional conserved motifs. The motif xxxLQ/RL/IG was previously described as highly conserved in AGAMOUS sequences, LAMB2, and PPM1 (Vandenbussche et al., 2003); here we show that it is also present in

SEPALLATA/AGL6, corresponding to Motif 11 in our analysis (Figures S1 and S2).

The fern sequences included in the analysis appear to have a more variable C-region compared to all the other proteins as these sequences lack motifs 4 and 6 that were detected in all the other proteins (Figure S1). To better understand their protein composition, more extended sampling among fern sequences is required. Interestingly, according to our MEME analysis, with the exception of SewaMADS3 and SewaMADS3-2, lycophyte sequences have the C-region relatively conserved, as they all share Motifs 7 and 8 (Figure S1). Motif 8 consists of xxxP/NxETREPP/VS/T and Motif 7 is located toward the C terminus of the protein and is rich in polar amino acids with a sequence consisting of xxL/SQTSLQ/HLG (Figures S1 and S2). SewaMADS3 and SewaMADS 3-2 sequences share the canonical M and I-regions with the other MADS-box genes, however, the rest of the protein is unique but highly conserved among them. According to our analyses, these two proteins share three long domains, which correspond to motifs 9, 10, and 13 (Figure S1).

3.2 | The morphology and development of *S. moellendorffii* sporangia

S. moellendorffii is an erect lycophyte with a dorsiventral shoot that branches dichotomously, however, one branch remains dominant over the other. Branch growth is therefore anisotomous (Figure 2a). The shoot has two ranks of dimorphic leaves: small appressed dorsal leaves and larger ventral leaves (Figure 2b,c). However, overall, both ranks of leaves are small or microphylls (Figure 2a,c). The leaves arise as flattened ridges from the shoot apical meristem although the apparent opposite leaf primordia do not occur simultaneously (Figure 2b). The leaves are ovate with a long tip and seta (hairs) along their margins and each has a tongue-like ligule attached to its adaxial surface (Figure 2b–d). When fertile, the branches terminate in a quadrangular strobilus (cone).

The strobilus is composed of four ranks of sporangia and each is associated with fertile leaves (sporophylls) (Figure 2e–h). However, the organs of the strobilus arise in a spiral phyllotaxy as in other *Selaginella* species (Mitchell, 1910), but the compact axis makes the organs appear opposite each other giving the characteristic quadrangular structure. The sporophylls are first apparent on the axis and grow out quickly as flattened ridges (Figure 2e,f). Sporangia develop after the sporophylls next to the shoot axis and are eventually covered by the developing sporophyll (Figure 2e). Like microphylls, each sporophyll bears a ligule (compare Figure 2b, 2d, and 2e–h). Ligules arise on the adaxial surface of the sporophyll and develop quickly. Sporangia continue to develop as globose structures with the youngest at the apex and the more mature sporangia toward the base (Figure 2e–h). Therefore, a developing strobilus is well structured to give a series of sporangia developmental stages in a single longitudinal section.

Selaginella is heterosporous as it produces two different kinds of spores—megasporangia which usually produce a single megaspore that will give rise to the female gametophyte and microsporangia, which produce microspores that will give rise to the male gametophyte. In many *Selaginella* species, the megasporangia appear at the base while microsporangia are produced toward the apex (Mitchell, 1910). *S. moellendorffii* has been described as having 1–3 megasporangia forming at the base of each strobilus and that *S. moellendorffii* megasporangia will abort under cultivation (Schulz et al., 2010), therefore, *S. moellendorffii* reproduction under cultivation, is not sexual. It proliferates vegetatively from bulbils on the rhizome and from gemmae that form at the tips of branches. These gemmae are composed of a shoot and rhizophore that fall to the ground to develop into a mature plant. The rhizophore arises from angle meristems at branch points and has features of shoots and roots, however, as soon as the rhizophore touches the ground it attains features of and functions as a root (Ambrose, 2013).

To better understand sporangia development, we performed histological analyses of developing strobili and defined the stages of sporangia development (Table 1 and Figure 2f–h). The first stage in sporangia development is the specification of the archesporial initial(s) and their first divisions (stage 1) (reviewed in Foster & Gifford, 1959; Parihar, 1967). Following several rounds of cell divisions, sporangia primordia appear as small protrusions on the flank of the strobilus close to the apex (stage 2) (Figure 2f). The sporangia rapidly grow as rounded structures and then appear elongated (stages 3 and 4) (Figure 2f). In stage 5, the epidermal cells appear darkly stained and ordered around the sporangia. The short stalk of the strobilus quickly differentiates and appears unstained at the base later in development (Figure 2f,g). At stage 6, periclinal divisions of the epidermal wall produce a two-layered sporangium wall (Figure 2f). At stage 7, another round of periclinal divisions produces a three-layered sporangium wall, and continued divisions in the center produce a mass of sporogenous cells (Figure 2g). By stage 8, the inner wall of the sporangia differentiates, stains a deep red, into the tapetum (Figure 2g). At stage 9, the tapetum wall is apparent and the sporocytes appear free and separate inside

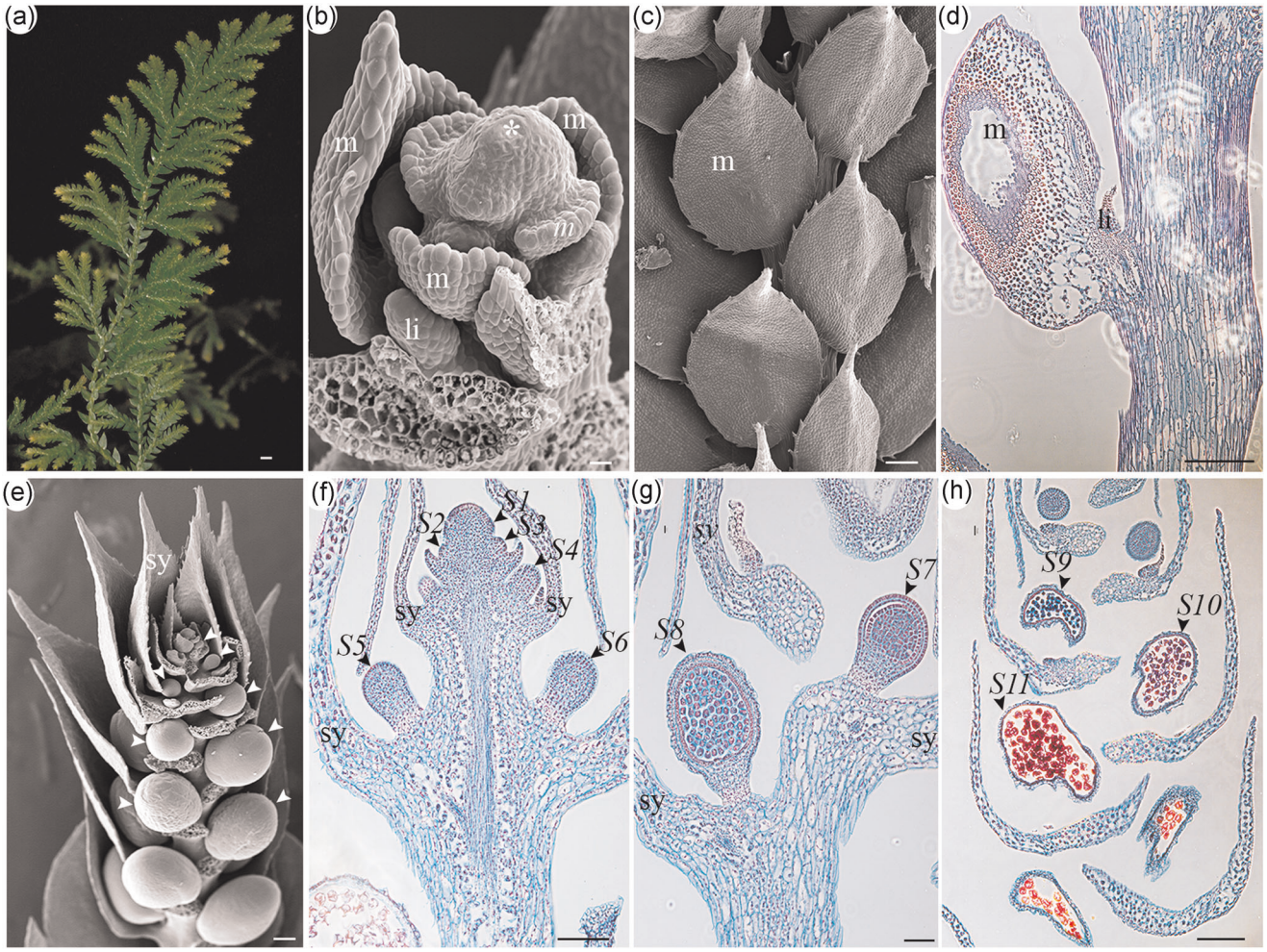

FIGURE 2 An overview of the morphology and anatomy of *Selaginella moellendorffii*. (a) Light micrograph of *S. moellendorffii* shoot illustrating the dichotomous branching at shoot tips with growth of one branch more dominant than the other (anisotomous). (b) SEM image of growing shoot tip with several leaves in the foreground removed. Leaves arise as flattened ridges opposite of each other although their initiation does not appear simultaneous. A ligule develops in the axil of each leaf. (c) SEM image shows a close-up of leaves from panel (a) showing smaller dorsal leaves and larger ventral leaves behind and oriented to the side. Leaves are acuminate with serrated margins. Note the dichotomy of the shoot axis at the top of the panel. (d) Histological stained longitudinal section through the shoot illustrating ligule in the leaf axil. Note typical vascular anatomy of *Selaginella* with trabeculae to connect vascular tissue to another shoot tissue across a cavity. (e) SEM of strobilus with meristem at the top and sporangia developing from meristem in two rows to give a four-ranked cone. Each sporangium is subtended by a leaf (sporophyll) with a ligule in the leaf axil. All sporangia shown here are microsporangia. (f) Histological stained longitudinal section through a developing strobilus illustrating that sporangia emerge on flanks of meristem quickly followed by sporophyll development. (g) Histological stained longitudinal section through a developing strobilus showing more mature sporangia than in panel (f) with well-differentiated layers in the sporangia. (h) Histological stained longitudinal section through a developing strobilus with nearly mature sporangia at the base with tetrads of spores apparent. Scale bar = 1 mm (a), scale bar = 10 μm (b), and scale bar = 100 μm (c–h). Arrowhead, sporangium; *, shoot apical meristem; li, ligule; m, microphyll; SEM, scanning electron microscopy; sy, sporophyll

the sporangium. At stage, 10, the sporocytes appear enlarged and begin to undergo meiosis (Figure 2h). At stage 11, meiosis is complete, and the microspore tetrad is apparent and the tapetum breaks down as the microspores mature (Figure 2h). All of the strobili that were sectioned for histological analyses were composed entirely of developing microsporangia, no megasporangia were observed.

3.3 | The temporal and spatial expression of type II classic MADS-box genes in *S. moellendorffii*

To better understand the role type II classic MADS-box genes may play in lycophyte development, we assessed the expression of all three MADS-box genes present in *S. moellendorffii* (Banks et al., 2011; Gramzow et al., 2012).

TABLE 1 Developmental landmarks for each stage identified during sporangium development

Stage	Developmental landmark
Stage 1	Archesporial initial(s) specified (Figure 2f)
Stage 2	Sporangia appear as small protrusions on the flank of the strobilus axis (Figure 2f)
Stage 3	Continued cell divisions produce globose structure (Figure 2f)
Stage 4	Continued cell divisions produce an elongated structure (Figure 2f)
Stage 5	Epidermal cells of sporangia appear more ordered; the stalk is apparent (Figure 2f)
Stage 6	Periclinal divisions produce two-layered sporangium wall (Figure 2f)
Stage 7	Periclinal divisions produce three-layered sporangium wall; sporogenous cells apparent (Figure 2g)
Stage 8	Tapetum differentiates; sporogenous cells proliferate (Figure 2g)
Stage 9	Tapetum apparent; sporocytes separate inside sporangium (Figure 2h)
Stage 10	Meiosis begins (Figure 2h)
Stage 11	Meiosis is complete and spore tetrads are apparent (Figure 2h)

By semiquantitative RT-PCR we found that all three genes are expressed at similar levels in the shoot, root, strobilus, and gemmae (data not shown). Therefore, we utilized in situ hybridization on sectioned and whole-mount tissue to investigate the temporal and spatial expression of each gene in the vegetative and reproductive development of *S. moellendorffii*.

Our results show that *SmMADS1* and *SmMADS6* have very similar expression patterns while *SmMADS3* shows different expressions compared to *SmMADS1* and *SmMADS6* (Figures 3–5). By whole-mount in situ hybridization, *SmMADS1* and *SmMADS6* expression is detected in the root tip of developing gemmae and scattered epidermal cells from the root (Figures 3a and 4a). In situ hybridization of sectioned tissue shows that *SmMADS1* and *SmMADS6* are not expressed in the shoot apical meristem but are expressed in emerging lateral primordia close to the shoot apex (Figures 3b and 4b). This expression is maintained in a portion of the adaxial region of the microphyll primordia in what will become the ligule and it is maintained when the ligule emerges from the adaxial region of the microphyll (Figures 3b and 4b). A low level of expression is detected in the body of the ligule as it differentiates into foot and body (Figures 3b and 4b). Expression of *SmMADS1* and *SmMADS6* expression is also detected in developing vasculature of the vegetative shoot (Figures 3b and 4b). No expression of *SmMADS1* or *SmMADS6* is maintained in the microphyll when it emerges as a flattened appendage or later as it fully expands (Figures 3b and 4b).

As during vegetative development, *SmMADS1* and *SmMADS6* have similar expression patterns in early strobilus development. *SmMADS1* and *SmMADS6* are expressed from the earliest stages of sporangia development (stage 1 and Table 1) but the expression is not found in the strobilus apical meristem (Figures 3c and 4c). However, *SmMADS1* and *SmMADS6* expression is detected in all emerging lateral strobilus primordia: sporophylls, ligules, and sporangia but this expression is not maintained in mature sporophylls (Figures 3c–e and 4c–e). The expression of both *SmMADS1* and *SmMADS6* is maintained throughout sporangia and their expression only diverges later in sporangia development (Figures 3c–g and 4c–g). In stages 1–5, expression of *SmMADS1* and *SmMADS6* expression is detected throughout the sporangia primordia as the rounded shape develops and the outer wall of the sporangia is discernible from the inner sporangia cells (Figures 3c and 4c). *SmMADS1* expression is maintained throughout the sporangia as all three cell layers of the sporangia form and the inner sporogenous cells proliferate (stage 7; Figure 3d). Although *SmMADS6* expression is detected in the proliferating sporogenous cells and the inner sporangia wall, the expression in the outer two walls of the sporangia is not as easily discerned (stage 7; Figure 4d). In stage 8, *SmMADS1* and *SmMADS6* expression is maintained in the sporogenous cells and inner cell wall or tapetum of the sporangia (Figures 3e and 4e). At stage 9, as the sporogenous cells continue to develop, *SmMADS1* and *SmMADS6* expression again diverges with *SmMADS1* expression detected in the sporogenous cells and *SmMADS6* expression restricted to the sporogenous cell membrane (Figures 3f and 4f). By stage 11, *SmMADS1* and *SmMADS6* expression is detected in the spore tetrad, however, *SmMADS1* expression is detected in its interior, and *SmMADS6* expression is detected on

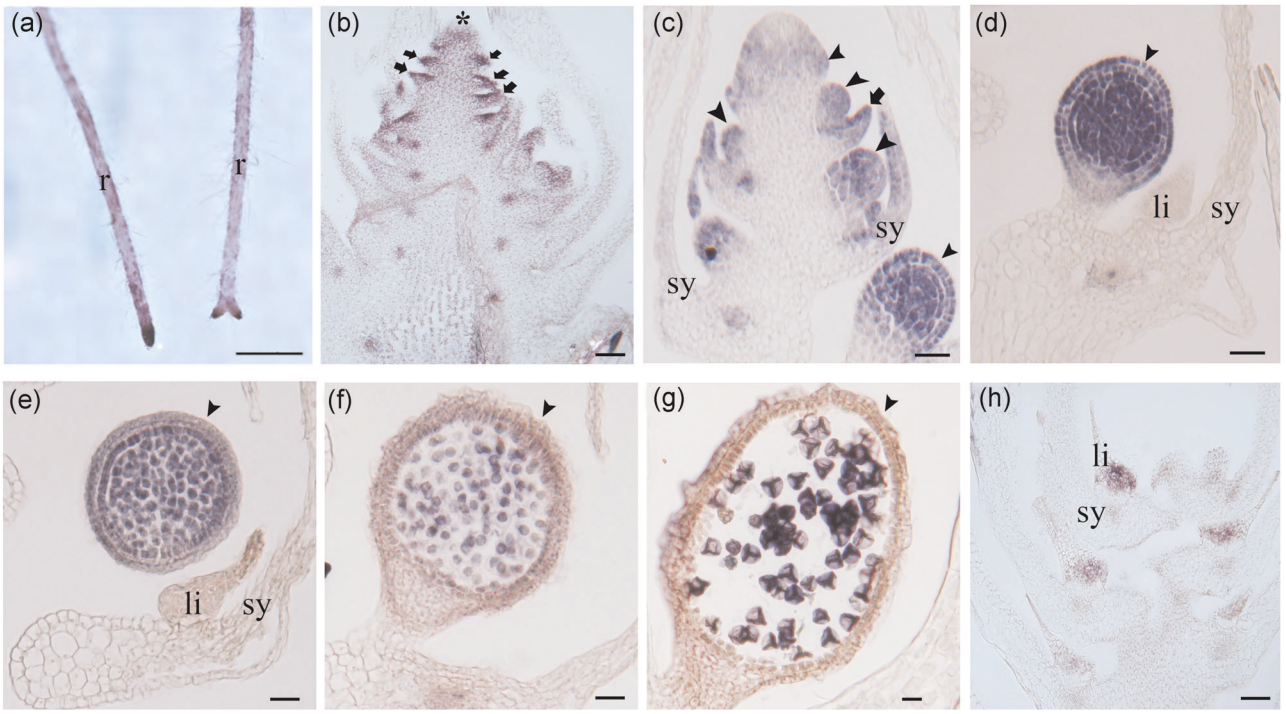

FIGURE 3 Expression of *SmMADS1* by in situ hybridization in the root (a), vegetative shoot (b), and reproductive (c–h) tissue. Whole-mount in situ hybridization (a) and in situs on sectioned tissue (b–h). (a) *SmMADS1* is expressed in the tips of unbranched and dichotomously branched roots of developing gemmae. (b) Longitudinal section of the vegetative shoot. *SmMADS1* expression is not detected in the shoot apical meristem but is detected in early emerging leaf primordia and later becomes restricted to the adaxial side of the leaf. However, expression in leaves is not maintained in more mature leaves. *SmMADS1* expression is also detected in the vasculature that appears as circles in this section. (c) Longitudinal section through strobilus. *SmMADS1* expression is not detected in the strobilus apical meristem but is detected in sporangia primordia before they are apparent and as they emerge from the flanks of the meristem. Expression of *SmMADS1* is detected throughout the sporangia and in subtending leaves, particularly the adaxial side. (d) *SmMADS1* expression is maintained throughout the sporangium as the wall layers and sporocytes become distinct. (e) *SmMADS1* expression can no longer be detected in the outer two cell layers of the sporangium but is detected in the tapetum and proliferating sporocytes. (f) *SmMADS1* expression can no longer be detected in the degenerating tapetum but is still detected in the sporocytes before meiosis. (g) *SmMADS1* expression is detected within the spore tetrad. (h) *SmMADS1* expression is detected in mature ligules. Scale bar = 100 μm (a, c–h); scale bar = 1 mm (b). *Indicates the shoot apical meristem; black arrow, young microphyll; black arrowhead, sporangium; li, ligule; m, microphyll; r, root; sy, sporophyll

the exterior of the tetrad (Figures 3g and 4g). The expression of *SmMADS1* is maintained in the ligule from the very early stages of its development (Figure 3b) until it is well-formed, and it differentiates into foot and body (Figure 3h). *SmMADS1* and *SmMADS6* expression is not detected in the stalk of the sporangia (Figures 3c,d,f,g and 4c–e).

Overall, *SmMADS3* has different expression patterns compared to either *SmMADS1* or *SmMADS6*. By whole-mount in situ hybridization, *SmMADS3* expression is not detected in the apex of the gemmae but low levels of expression are detected behind the branch point of developing roots (Figure 5a). In addition, low levels of *SmMADS3* expression is detected in a scattered pattern in the epidermis of developing roots (Figure 5a). *SmMADS3* is not expressed in the shoot apical meristem or in the developing microphylls, however, it is expressed at the base of the ligule when this is well developed (Figure 5d).

Similar to *SmMADS1* and *SmMADS6*, *SmMADS3* is not expressed in the strobilus apical meristem (Figure 5b). However, unlike *SmMADS1* and *6*, *SmMADS3* is not expressed in any emerging lateral primordia (Figure 5b,c). The expression of S*mMADS3* is first detected at stage 7, in the proliferating sporogenous cells after the three walls of the sporangia have clearly developed (Figure 5c–e). Expression of *SmMADS3* is maintained throughout the sporogenous cells before meiosis (stages 7–11) (Figure 5c–g). After meiosis at stage 11, *SmMADS3* expression is restricted to the interior of the spore tetrad (Figure 5g). In the vegetative parts of the plant, *SmMADS3* is expressed in the ligule when it is well developed, restricted to the body of the ligule, and in the differentiating vasculature of the sporophyll (Figure 5h).

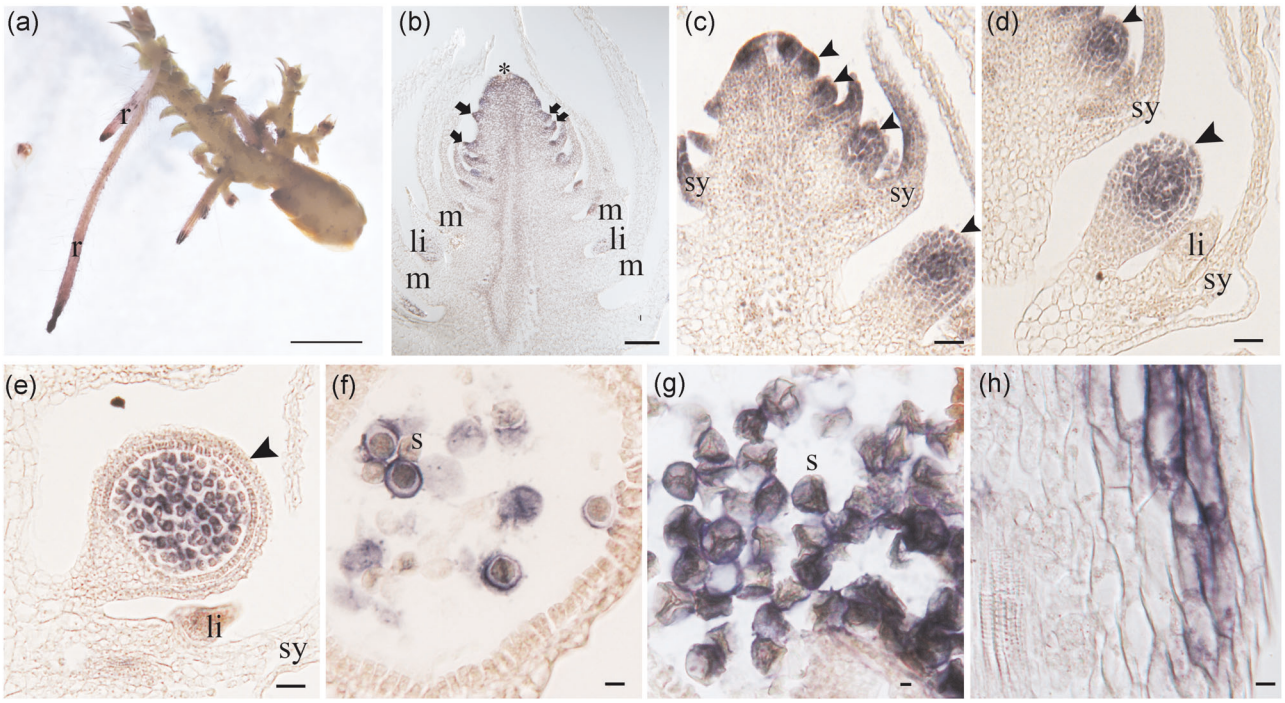

FIGURE 4 Expression of *SmMADS6* by in situ hybridization in vegetative (a, b, h) and reproductive (c–g) tissue. Whole-mount in situ hybridization (a) and in situs on sectioned tissue (b–h). (a) *SmMADS6* expression is detected in the tips of unbranched roots of developing gemmae. (b) Longitudinal section of the vegetative shoot. *SmMADS1* expression is not detected in the shoot apical meristem but is detected in early emerging leaf primordia and later becomes restricted to the adaxial side of the leaf. *SmMADS6* expression is also detected in ligule primordia. (c) Longitudinal section through strobilus. *SmMADS6* expression is not detected in the strobilus apical meristem but is detected in sporangia primordia before they are apparent and as they emerge from the flanks of the meristem. Expression of *SmMADS6* is detected throughout the developing sporangia primordia and in subtending leaves, particularly the adaxial side. (d) *SmMADS6* expression is detected in the inner wall and sporocytes as they become distinct. (e) *SmMADS6* expression is not detected in the outer 2 cell layers of the sporangium but there appears to be a low level of expression in the tapetum. *SmMADS6* expression is well expressed in the proliferating sporocytes. (f) *SmMADS6* expression is detected in the wall of the sporocyte. (g) *SmMADS6* expression is detected in the wall of the spore tetrad. (h) *SmMADS6* diffuse expression is detected in the stem. Scale bar = 100 μm (a, c–h); scale bar = 1 mm (b). *Indicates the shoot apical meristem; black arrow, young microphyll; black arrowhead, sporangium; li, ligule; m, microphyll; r, root; s, spores; sy, sporophyll

4 | DISCUSSION

MADS-box genes have been of particular interest to plant developmental biologists for many years, as it is a very diverse gene lineage with functions very well described throughout flowering plant development (e.g., Alvarez-Buylla et al., 2000; Gramzow & Theissen, 2010; Riechmann & Meyerowitz, 1997; Schwarz-Sommer et al., 1990; Thangavel & Nayar, 2018; Yanofsky et al., 1990). MADS-box genes are known to be present across land plants (Gramzow et al., 2012, 2014) but little is known about their function outside flowering plants. Given the morphological diversity among the major land plant lineages, we focused on a plant lineage key for understanding the evolution of land plants, the lycophytes. Lycophytes consist of three orders: Lycopodiales, Selaginellales, and Isoetales (PPG, 2016) with unique morphological features (Figures 1a,b and 2). Thus, this study has allowed filling gaps in understanding the functional evolution of this gene lineage and the putative function of MADS-box homologs in lycophytes.

4.1 | Evolutionary history of MADS-box genes across land plants reveals independent evolution in lycophytes

Our phylogenetic analysis shows all the major type II MADS-box lineages including representatives across land plants recover the same topology previously presented, with all the major MADS-box clades present across seed plants (Gramzow et al., 2012, 2014). However, only *AGL15* and *SVP* clades seem to be present in ferns. A clade of fern sequences was present before the multiple duplication events that gave rise to *SEP*, *FUL/AP1*, *FLC*, *SOC1*, *AG*, *AGL12*, and *AGL17* homologs (Figure 1).

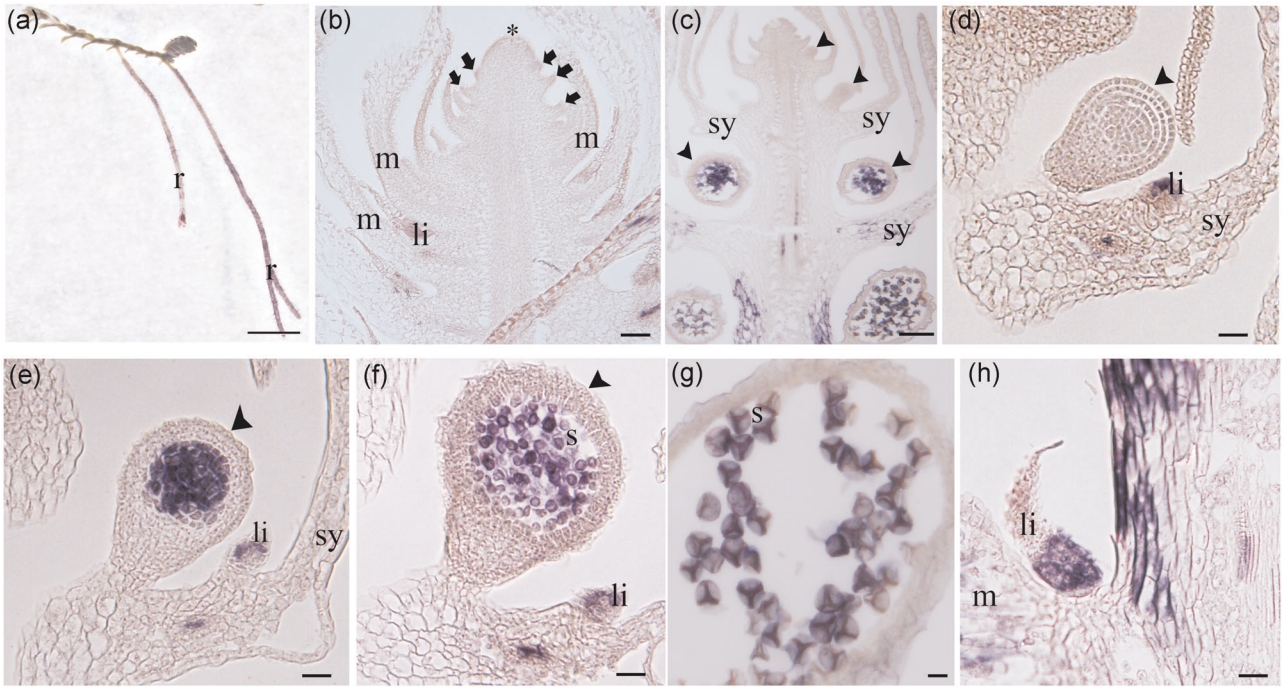

FIGURE 5 Expression of *SmMADS3* by in situ hybridization in vegetative (a, b, h) and reproductive (c-g) tissue. Whole-mount in situ hybridization (a) and in situs on sectioned tissue (b-h). (a) *SmMADS3* expression is not detected in the gemma body but diffuse and punctate expression is detected in the roots. (b) Longitudinal section of the vegetative shoot. *SmMADS3* expression is not detected in the shoot apical meristem or emerging lateral primordia. (c) Longitudinal section through strobilus. *SmMADS3* expression is not detected in the strobilus meristem or early emerging sporangia or sporophyll primordia. *SmMADS3* expression is detected later in sporangia development. (d) *SmMADS3* expression is not detected as the layers of the sporangia wall become distinct. (e) *SmMADS3* expression is first detected in the sporocytes as they proliferate during sporangia development. (f) *SmMADS3* expression is maintained during sporocyte proliferation before meiosis. (g) *SmMADS3* expression can be detected in spore tetrads. (h) *SmMADS3* expression is detected in the body of the ligule and in the shoot. Scale bar = 100 μm (a, c–h); scale bar = 1 mm (b). *Indicates the shoot apical meristem; black arrow, young microphyll; black arrowhead, sporangium; li, ligule; m, microphyll; r, root; s, spores; sy, sporophyll

In addition, our results show that the MADS-box genes underwent lycophyte-specific duplication events. Within the lycophytes, there are five MADS-box clades, the clade containing the Lycopodiales sequences have undergone two duplication events giving rise to three clades specific to this order (Figure 1c). The Lycopodiales are homosporous lycophytes and include one family and 16 genera (PPG, 2016). Although it is enticing to hypothesize that MADS-box gene duplications played a role in the diversification of the Lycopodiales, more extensive sampling in additional genera as well as expression analyses will be needed to better understand the role of MADS-box genes in this order. Two additional clades within lycophytes are exclusive to another lycophyte order, the Selaginellales (Figure 1c). The Selaginellales are comprised of a single family and single genus (PPG, 2016). Sequences from Isoetales, also composed of a single family and single genus, are not represented in our data set. Selaginellales and Isoetales are more closely related to each other than to the Lycopodiales, both are heterosporous lycophytes and both have ligules (reviewed in Ambrose, 2013). Sampling within Isoetales will be necessary to understand the evolution of MADS-box genes in the heterosporous lycophytes.

All lycophyte homologs form a well-supported clade (BS = 70) except for *SmMADS6* which is in a different clade with *Physcomitrella* homologs (Figure 1; Gramzow et al., 2012), making the position of *SmMADS6* still unclear, as the clade has low support (Figure 1). Here, we found that *S. moellendorffii* sequences, *SmMADS1* and *SmMADS3* are in different clades (BS = 92 for both clades; Figure 1). Our results support previous findings, which suggest that the common ancestor of land plants had a single type II classic MADS-box gene homolog and that by gene and genome duplications MADS-box genes increased over the course of the evolution of land plants (Gramzow et al., 2012; Tanabe et al., 2005; Zhao et al., 2017). Our results also indicate that the majority of lycophyte type II classic MADS-box genes underwent independent duplication events distinct from the euphyllophyte (ferns and seed plants) MADS-box genes.

To better understand the molecular evolution of the MADS-box genes across land plants, we analyzed the conservation of protein domains of the type II MADS-box sequences across land plants. Not surprisingly, our analyses found that the MADS, I, K regions are highly conserved in all analyzed sequences (Alvarez-Buylla et al., 2000; Ma et al., 1991; Purugganan et al., 1995; Riechmann & Meyerowitz, 1997; Vandenbussche et al., 1997) (Figure S1). This suggests that the DNA binding function, protein-protein interaction, and its specificity are maintained across land plants (Figures S1 and S2).

In flowering plants, MADS-box genes are known to form homodimers and heterodimers as well as exert their specific functions as tetramers (Theißen & Saedler, 2001). Further protein studies will be needed to assess protein-protein interactions of SmMADS proteins. It would not be surprising, given the similar expression patterns of SmMADS1 and SmMADS6 if these proteins interacted. We also found an extended N-terminal region similar to what is found in AGAMOUS from *Arabidopsis thaliana* in the CRM6 clade (CMADS1, CerMADS2, CerMADS3) from *Ceratopteris richardii*, in SmMADS1 of *S. moellendorffii*, and SrMADS1 of *Selaginella remotifolia* (Huang et al., 2014; Tanabe et al., 2003; Yanofsky et al., 1990). The C-region of the proteins is highly variable as has been found in other MADS-box proteins, but it may play a role in the formation of higher-order complexes (Kaufmann et al., 2005; Purugganan et al., 1995; Vandenbussche et al., 2003). However, short conserved motifs have been found in this region (Vandenbussche et al., 2003). We were able to identify motifs in the C-region exclusive to each clade which may confer a specific function to each group of genes (i.e., Motif 15 in AP1 and CAULIFLOWER; Motif 11 in SEPALLATA and AGL6). The lycophyte homologs analyzed have a conserved and unique C-region, sharing motifs 7 (L/SQTSLQ/HLG) and 8 (xxETREPP/TS/T; Figures S1 and S2). The high similarity in the protein sequence suggests that the putative function of these genes may be conserved in all lycophytes.

In summary, our evolutionary hypothesis of the type II MADS-box genes supports previous findings in terms of the molecular evolution of these sequences across land plants, with multiple duplication events in seed plants and lycophyte sequences as preduplication homologs (Alvarez-Buylla et al., 2000; Gramzow et al., 2012, 2014; Purugganan et al., 1995; Tanabe et al., 2005). This observation poses a new question regarding the conservation in their expression patterns and function after duplication events as well as the expression patterns and putative roles in preduplication genes.

4.2 | Sporangium development seems to be similar across lycophytes

Key features such as heterospory, endosporic development, leaves, and roots evolved independently during land plant evolution (Doyle, 2013) (Figure 1a). Several canonical leaf development genes have been found to be expressed during sporangia development in early-diverging land plants, thus indicating that developmental landmarks during lycophyte sporangia development will be important for understanding the role of particular genes during the development of these structures (Vasco et al., 2016; Zumajo-Cardona et al., 2019). Therefore, we presented a staged developmental series (Table 1 and Figure 2). We found that the development of *S. moellendorffii* sporangium is similar to what is known in other lycophytes (reviewed in Foster & Gifford, 1959; Mitchell, 1910; Parihar, 1967).

4.3 | The three *S. moellendorffii* MADS-box homologs, *SmMADS1*, *3*, and *6*, show discrete expression patterns throughout plant development

Here, we present the first comprehensive expression analyses for all the type II classic MADS-box genes known to exist in the *S. moellendorffii* (lycophyte) genome (Banks et al., 2011; Gramzow et al., 2012). *S. moellendorffii* homologs show broad but distinct temporal and spatial expression patterns during both vegetative and reproductive development (Figures 3–5). Although the sequences of *SmMADS1* and *SmMADS6* are more dissimilar, they have similar expression patterns in the root, vegetative axes, and in the reproductive axis or strobilus (Figures 3 and 4). Although the *SmMADS1* and *SmMADS3* sequences are more similar to each other, *SmMADS3* has a distinct expression pattern compared to the other two homologs (Figure 5). However, the expression of the three type II classic MADS-box genes do share some similarities: none are expressed in the shoot apical meristem (Figures 3b, 4b, and 5b), none are expressed in the strobilar apical meristem (Figures 3c, 4c, and 5c) and all are expressed during some stage of ligule development (Figures 3–5). There are no ligules in any other land plant groups and the interpretation of this structure is still debated (reviewed in Ambrose, 2013). Unlike *SmMADS1* and *SmMADS6*, *SmMADS3* is not expressed in emerging lateral primordia. *SmMADS3* is not detected in the provasculature of the vegetative shoot but is detected in the more mature differentiated vasculature of microphylls (Figure 5b–f,h). By whole-mount in situ hybridization, *SmMADS3* expression is not detected

in the apex of the gemmae but low levels of expression are detected behind the dichotomous branch point of developing roots (Figure 4a).

In the strobilus, *SmMADS1* and *SmMADS6* have similar expression patterns early in development (Figures 3c and 4c). The expression of both *SmMADS1* and *SmMADS6* is similar from stage 1 to 5 of sporangia development (Figures 3c–g and 4c–g; Table 1). Later during development and differentiation of the cell wall during stages 6 and 7, *SmMADS1* and *SmMADS6* expression diverges (Figures 3d,e and 4d,e). *SmMADS1* expression is maintained throughout the sporangia as all 3 cell layers of the sporangia form and the inner sporogenous cells proliferate (Figure 3c–e). Although *SmMADS6* expression is detected in the proliferating sporogenous cells and the inner sporangia wall, the expression in the outer two walls of the sporangia is not as easily discerned (Figure 4c–e). During stages 7–9, the sporogenous cells proliferate and develop, *SmMADS1* and *SmMADS6* expression is similarly maintained in the sporogenous cells and inner cell wall or tapetum of the sporangia (Figures 3c–e and 5c–e). In stages 9–10, *SmMADS1* and *SmMADS6* expression diverges again with *SmMADS1* expression detected in the sporogenous cells and *SmMADS6* expression restricted to the sporogenous cell membrane (Figures 3f and 4f). At stage 11, after meiosis, *SmMADS1* and *SmMADS6* expression is detected in the spore tetrad, however, *SmMADS1* expression is detected in the interior of the tetrad, and *SmMADS6* expression is detected on the exterior of the tetrad (Figures 3g and 4g). Unlike *SmMADS1* and *SmMADS6*, *SmMADS3* expression is not detected in any emerging lateral primordia of the strobilus (Figure 5c). *SmMADS3* expression is first detected at stage 7 and its expression in sporogenous cells is maintained through meiosis at stage 11 (Figure 5c–g).

In general, our results are similar to those previously described for the type II classic MADS-box genes in non-seed vascular plants. There are multiple copies of type II MADS-box genes that have been identified in ferns (Hasebe et al., 1998; Huang et al., 2014; Münster et al., 1997, 2002; Ruiz-Estévez et al., 2017). Northern analyses or RT-PCR analyses have shown that most of these MADS-box homologs have broad patterns of expression in vegetative or reproductive tissue. However, some fern MADS-box homologs do show more restricted patterns of expression. OPM4 expression was restricted to the sporophytic reproductive structure of *Ophioglosum pendunculosom* by RT-PCR (Münster et al., 2002). *CMADS4* had the highest level of expression in roots while *CMADS6* was only detected in hermaphroditic gametophytes of *Ceratopteris richardii* by Northern blots (Hasebe et al., 1998). The detailed expression pattern for a fern MADS-box gene has been reported for *CMADS1* in *Ceratopteris richardii* (Hasebe et al., 1998). *CMADS1* expression was detected in the shoot apical meristem, leaf primordia, vascular tissue, root apical meristems and throughout sporangia development by in situ hybridization. This pattern of expression is similar to what we found for *SmMADS1* and *SmMADS6* except that we did not find any *SmMADS* gene expressed in the shoot apical meristem.

Five type II classic MADS-box homologs, *LAMB2* to *LAMB6*, have been identified in another lycophyte, *Lycopodium annotinum* (Svensson & Engström, 2002; renamed *Spinulum annotinum*, PPG, 2016). *LAMB4*, *LAMB5*, and *LAMB6* have broad expression patterns in vegetative and reproductive sporophytic tissues by RT-PCR (Svensson & Engström, 2002). However, *LAMB2* expression is detected in vegetative tissues but not in the strobili (Svensson & Engström, 2002). The expression of one type II classic MADS-box gene has been studied in another *Selaginella* species, *S. remotifolia SrMADS1* is most closely related to *SmMADS1*, however, *SrMADS* was found to be expressed in all tissues except roots and rhizophores by RT-PCR (Tanabe et al., 2003). These results would suggest that these lycophyte homologs might have undergone subfunctionalization after the duplication events that specifically occurred in this plant lineage (Figures 1 and 3–5). However, it is possible that *SrMADS1* expression may be found in roots and rhizophores by more sensitive methods like in situ hybridization and that *SmMADS1* and *SrMADS1* have similar roles in *Selaginella* development.

Selaginella is a heterosporous lycophyte with endosporic development and therefore does not have a free-living female gametophyte stage (reviewed in Ambrose, 2013). Unfortunately, no female megasporangia developed in our study organism, therefore, we were unable to assess the expression of type II classic MADS-box in *S. moellendorffii* megasporangia development or female gametophytes. It will be necessary to assess expression and function of MADS-box genes in heterosporous lycophytes that produce both micro- and mega-sporangia. In addition, it would be expected that lycophyte MIKC* MADS-box genes would play a large role in the development of the gametophyte as has been shown for other land plants (Svensson et al., 2000).

Teasing apart the evolution of the MADS-box genes across land plants will be only possible with the knockouts of each gene in different lycophyte species. However, our results support the hypothesis suggesting that MADS-box genes outside of seed plants may be involved in a plethora of functions during plant development (Münster et al., 2002). MADS-box genes have undergone multiple duplication events in seed plants, suggesting that they became organ-specific in seed plants as the

result of neo- and subfunctionalization events. Additionally, multiple duplication events have occurred in the different plant lineages such as in ferns, lycophytes, and mosses, making it extremely difficult to tease out new plesiomorphic roles in plant development during plant evolution. Our results support previous findings that suggest that the common ancestor of land plants had a single type II classic MADS-box gene homolog and that the increase in the number of genes through gene and genome duplications and subsequent neo- and subfunctionalization has corresponded to the evolution of body organization in land plants (Gramzow et al., 2012; Harrison, 2017; Huang et al., 2014; Münster et al., 2002; Svensson et al., 2002; Tanabe et al., 2005; Zhao et al., 2017).

5 | CONCLUSIONS

MADS-box genes in *S. moellendoffii* show broad expression patterns throughout plant development. *SmMADS1* and *SmMADS6* show similar expression patterns, in the microphylls, in the root tips, and in the sporangium. On the other hand, *SmMADS3* is expressed in the sporangium and in the ligule. These expression patterns together with the evolutionary history of MADS-box genes across land plants suggest that the function of the MADS-box genes has become restricted to specific plant organs after duplication events that coincide with the diversification of seed plants, and before that, these genes had multiple roles throughout plant development.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Barbara A. Ambrose conceived of the study. Cecilia Zumajo-Cardona performed phylogenetic analyses. Tynisha L. Smalls cloned all sequences and generated probe fragments. Barbara A. Ambrose performed in situ hybridization. Barbara A. Ambrose and Cecilia Zumajo-Cardona arranged figures and wrote the manuscript. All authors read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in NCBI and are listed in Supplementary Table 1.

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SUPPORTING INFORMATION

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