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Class III HD-Zip activity coordinates leaf development in *Physcomitrella patens*

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ABSTRACT

Land plant bodies develop from meristems, groups of pluripotent stem cells, which may persist throughout the life of a plant or, alternatively, have a transitory existence. Early diverging land plants exhibit indeterminate (persistent) growth in their haploid gametophytic generation, whereas later diverging lineages exhibit indeterminate growth in their diploid sporophytic generation, raising the question of whether genetic machinery directing meristematic functions was co-opted between generations. Class III HD-Zip (C3HDZ) genes are required for the establishment and maintenance of shoot apical meristems in flowering plants. We demonstrate that in the moss *Physcomitrella patens*, C3HDZ genes are expressed in transitory meristems in both the gametophytic and sporophytic generations, but not in the persistent shoot meristem of the gametyphyte. Loss-of-function of *P. patens* C3HDZ was engineered using ectopic expression of miR166, an endogenous regulator of C3HDZ gene activity. Loss of C3HDZ gene function impaired the function of gametophytic transitory meristematic activity but did not compromise the functioning of the persistent shoot apical meristem during the gametophyte generation. These results argue against a wholesale co-option of meristematic gene regulatory networks from the gametophyte to the sporophyte during land plant evolution, instead suggesting that persistent meristems with a single apical cell in *P. patens* and persistent complex meristems in flowering plants are regulated by different genetic programs.

1. Introduction

Life cycles of land plants alternate between gamete-producing haploid (1N) gametophytes and spore-producing diploid (2N) sporophytes. The early diverging lineages of extant land plants, the liverworts, mosses, and hornworts collectively known as bryophytes, have a gametophyte dominant life cycle, with gametophytes growing from apical meristems and determinate sporophytes dependent upon the gametophyte body. In contrast, the lycophytes, ferns, and seed plants (collectively known as vascular plants), have a sporophyte dominant life cycle, with indeterminate sporophyte bodies generated from apical meristems and gametophytes being diminutive and either free living, or in the case of seed plants, determinate and dependent upon the sporophyte body. The ancestral land plants are thought to have had a dominant gametophyte generation, similar to the extant bryophytes (Bower, 1908; Kenrick and Crane, 1997; Graham et al., 2000). Thus, during land plant evolution there was a transition from a gametophyte-dominant life cycle to a sporophyte-dominant life cycle, in which the structural complexity of the sporophyte generation increased and that of the gametophyte generation decreased (Bower, 1908; Graham et al., 2000).

The indeterminate growth patterns of the complex bodies of bryophyte gametophytes and vascular plant sporophytes are produced by persistent apical meristems, whose activity continues for the life, or a good portion thereof, of the plant. In contrast, the determinate bryophyte sporophyte is produced through the action of non-apical (intercalary) meristematic activity or, in the case of mosses, transient apical growth. The origin of a persistent apical meristem was a significant innovation that was critical to the increasing complexity and dominance of the diploid generation (Kato and Akiyama, 2005; Ligrone et al., 2012). The regulation of sporophytic meristematic activities could be patterned by novel genes and developmental programs, or alternatively, be patterned

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by genes and developmental programs co-opted from existing gametophytic pathways. From a genomic perspective, most gene families known to control sporophyte development in flowering plants were also identified, albeit with fewer and less divergent paralogs, in the genome of the model moss *Physcomitrella patens* (Nishiyama et al., 2003; Floyd and Bowman, 2007; Rensing et al., 2008), suggesting that co-option of existing genetic programs rather than de novo evolution of new programs may have been important. However, functional analyses of specific genetic programs are required to provide insight into how plants with similar overall genetic toolkits can develop with substantially different life cycles.

One approach to address the question of whether the indeterminate meristem genetic programs have been co-opted between generations is to begin with genes known to be critical for the establishment and/or maintenance of the sporophyte meristem in flowering plants, where we have the most knowledge concerning meristem developmental genetics, and examine their functions in gametophyte-dominant lineages. This approach, tracing the evolutionary history of genes, has been taken with the Class I KNOX genes (KNOX1), which encode homeodomain proteins. In flowering plants, members of the KNOX1 gene family are required for the formation or maintenance of the sporophyte indeterminate shoot apical meristem (Vollbrecht et al., 1991; Barton and Poethig, 1993; Jackson et al., 1994; Long et al., 1996; Hay and Tsiantis, 2010). In all the major tracheophyte clades examined to date, KNOX1 gene expression is found in the persistent sporophyte shoot apical meristem (Sundas-Larsson et al., 1998; Harrison et al., 2005; Sano et al., 2005). While fragmentary transcripts from the three *P. patens* KNOX1 genes can be found in gametophytic tissues, full length transcripts have been identified only during the sporophyte generation (Sakakibara et al., 2008; Ortiz-Ramírez et al., 2016). The three *P. patens* KNOX1 genes function only in the sporophyte generation, in the transient apical cell, its derivatives and in the apical regions undergoing cell proliferation. No mutant phenotype is detected in the gametophytic shoot meristem, indicating the meristematic functions of KNOX1 genes have not been co-opted from the gametophytic stage to the sporophytic stage (Sakakibara et al., 2008).

Likewise, Class II KNOX (KNOX2) genes in the moss *P. patens* are also sporophyte-specific and functional data suggest that KNOX2 play a role in suppressing gametophyte developmental programs allowing the development of the morphologically distinct diploid phase (Sakakibara et al., 2013). In the flowering plant Arabidopsis loss-of-function KNOX2 alleles resemble gain-of-function KNOX1 alleles and vice versa, suggesting that they play antagonistic roles in the development of the sporophyte body in this species (Furumizu et al., 2015). The sole KNOX gene in *Chlamydomonas reinhardtii*, a chlorophycean green alga with a haplontic life cycle, functions to regulate zygotic gene expression following gamete fusion (Lee et al., 2008). Thus, the function of KNOX genes has been restricted to the diploid phase of the life cycle throughout plant evolution, and its role in persistent shoot meristems in vascular plants evolved from already existing functions in the sporophyte.

Another gene family required for persistent sporophyte shoot meristem activity in flowering plants is the Class III Homeodomain-Leucine Zipper (C3HDZ) gene family. The five gene family members in Arabidopsis [*AtHB8*, *CORONA/AtHB15* (*CNA*), *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), and *REVOLUTA* (*REV*)] are active in the sporophyte as regulators in the formation and function of the shoot apical meristem (SAM), the differentiation and patterning of vascular tissues, and the establishment of polarity in lateral organs (Talbert et al., 1995; McConnell and Barton, 1998; McConnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003; Prigge et al., 2005). *PHB, PHV, REV* and *CNA* are expressed during embryonic development and *rev phb phv* and *rev phb cna* plants lack an embryonic SAM (Emery et al., 2003; Prigge et al., 2005). *Arabidopsis* C3HDZ genes are also required for the patterning of vascular tissues and leaves. C3HDZ genes are expressed in the provasculature, the procambium, and differentiating xylem of vascular tissues, and the adaxial regions of leaves (McConnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003; Zhong and Ye, 2004; Prigge et al., 2005; Ilegems et al., 2010). C3HDZ activity is sporophyte specific in *Arabidopsis*, with a normal segregation ratio of the *Arabidopsis* multiple mutants indicating that C3HDZ gene activity is not required for *Arabidopsis* gametophyte development (Prigge et al., 2005; Izhaki and Bowman, 2007).

C3HDZ genes have been identified in all lineages of land plants and in *Chara*, a charophycean green algal relative, but are not present in chlorophycean green algae (Floyd and Bowman, 2004; Floyd et al., 2006; Prigge and Clark, 2006). All full-length land plant C3HDZ homologs contain a conserved microRNA binding site and are negatively regulated by miR165/166 (Emery et al., 2003; Floyd and Bowman, 2004; Mallory et al., 2004). In *Selaginella*, a lycophyte, C3HDZ gene expression is found in the apical cell of the persistent sporophyte shoot apical meristem and in developing vascular tissues, similar to what has been observed for flowering plants (Floyd and Bowman, 2006). However, in contrast to *Arabidopsis*, C3HDZ gene expression has been detected in both the gametophyte and sporophyte generations in the fern, *Ceratoptris*, and the hornwort, *Phaeoceros*, implying that expression in both generations of the land plant life cycle may have been the ancestral condition (Floyd et al., 2006). Furthermore, a *Chara* C3HDZ was cloned from mRNA derived from the haploid algal body suggesting that, in contrast to KNOX1 genes, C3HDZ may have functioned in the dominant haploid phase of the plant life cycle before the origin of the embryophytes and the multicellular sporophyte. This indicates that C3HDZ function in the sporophyte may have been co-opted from gametophyte developmental programs, and this it is possible that C3HDZ function in the sporophyte SAM may have been co-opted from gametophyte meristematic programs.

While C3HDZ genes are expressed in haploid-dominant algae and bryophytes, direct evidence for the function and tissue-level expression of C3HDZ transcription factors in haploid-dominant plants has not been demonstrated. In order to test the hypothesis that C3HDZ function in meristems was coopted from existing gametophyte developmental programs into the novel sporophyte during early land plant evolution, it is critical that functional analyses be undertaken in haploid dominant plants. In this study we present the first detailed analysis of the expression and function of C3HDZ genes in a land plant with a haploid dominant life cycles, *P. patens*.

2. Methods and materials

2.1. Sequence analysis and phylogenetic analysis

Sphagnum gametophores were collected from the conservatory at the University of California, Davis. *Dawsonia superba* gametophores and sporophytes were collected from wild populations near Mount Dona Buang, Victoria, Australia. RNA extraction, cDNA synthesis, degenerate PCR, RACE PCR, and clone screening were performed using degenerate primers and methods described in Floyd et al. (2006). *C. purpureus* EST sequence reads were retrieved from the GENBANK Sequence Read Archives (SRA) by BLAST similarity searches based on *P. patens* C3HDZs. Raw reads were assembled in Sequencher 5.1 (Genecodes Corporation). GENBANK numbers for the moss C3HDZ sequences are listed in Table 1. *Marchantia polymorpha*, *Phaeoceros carolinianus*, *Selaginella kraussiana*, and *Lycopodium lucidulum* C3HDZ mRNA sequences

Table 1
Accession numbers for moss C3HDZ sequences.

Physcomitrella patens	*PpC3HDZ1*	DQ385516
Physcomitrella patens	*PpC3HDZ2*	DQ385517
Physcomitrella patens	*PpC3HDZ3*	KU530548
Physcomitrella patens	*PpC3HDZ4*	KU530549
Physcomitrella patens	*PpHB10*	DQ385518
Dawsonia superba	*DsC3HDZ1*	KU530552
Dawsonia superba	*DsC3HDZ2*	KU530553
Dawsonia superba	*DsC3HDZ23*	KU530554
Ceratodon purpureus	*CpC3HDZ1*	KU530550
Ceratodon purpureus	*CpC3HDZ2*	KU530551
Sphagnum sp.	*SphC3HDZ1*	KU530555
Sphagnum sp.	*SphC3HDZ2*	KU530556
Sphagnum sp.	*SphC3HDZ3*	KU530557
Sphagnum sp.	*SphC3HDZ4*	KU530558

were previously described (Floyd et al., 2006) and deposited in GENBANK. The mRNA sequences for all C3HDZ genes included in this analysis are provided as a FASTA file in the Supplemental material.

For phylogenetic analysis nucleotide sequences were translated to amino acid sequences and aligned manually in Se-Al v2.0a11 for Macintosh (Rambaut, 1996). Ambiguously-aligned regions were excised and the remaining alignment, including 781 amino acid characters, was exported as a Nexus file. Bayesian phylogenetic analysis was performed using Mr. Bayes 3.1 (Huelsenbeck and Ronquist, 2001). The mixed model option (aamodelpr=mixed) was used to estimate the appropriate amino acid fixed rate model. The analysis was run for 500,000 generations, which was sufficient for the standard deviation of the split frequencies to drop below 0.01. To allow for the burn in phase, the first 500 trees (10% of the total number of saved trees) were discarded.

2.2. Plant materials and culture conditions

The Cove-NIBB strain of *P. patens* was used as the wild-type strain in the study. Wild-type and transformed *P. patens* lines were routinely subcultured for further analysis. Cultures were grown on BCDAT or BCD medium at 25 °C under continuous light (Nishiyama et al., 2000) to provide materials for *P. patens* transformation and protonema and gametophore observations. For spore germination, mature sporophytes were sterilized with 10% bleach washes, and then broken open in sterile water to make a spore solution. The spore solution was dispersed on BCDAT plates supplemented with 10 mM Calcium Chloride. To facilitate collection of protonemata for experimental procedures, medium plates overlaid with cellophane sheets were used. For observations of gametangia and sporophytes, protonemata were first cultured on sterile peat pellets at 25 °C under continuous light for at least one month and then transferred to 16 °C, 8 h light and 16 h dark conditions to induce the development of gametangia and sporophytes.

2.3. Histochemical detection of GUS activity

Histochemical assays of GUS activity were detected as described by Nishiyama et al. (2000) with modifications. Plant tissues were vacuumed in substrate solution for 30 min prior to incubation at 37 °C from 8 h to 4 days, depending on the tissue examined. The tissues were then fixed in 5% (v/v) formalin for 10 min. Gametangia and sporophyte samples were observed after the formalin fix. Fixed protonemata and gametophore tissues were dehydrated through an ethanol series before examination.

2.4. Microscopy

Wild type tissues were fixed in triple fix (1.5% (v/v) glutaraldehyde, 1% (v/v) paraformaldehyde, 2% (v/v) acrolein, 0.05% (v/v) Tween-20 and 84 mM Pipes buffer) and dehydrated through ethanol series. Tissues form functional mutants and GUS stained samples were fixed in 1% formalin and dehydrated through ethanol series. Fixed and dehydrated issues were then infiltrated and embedding into JB-4 plastic resin and subsequently sectioned at 5 μm. Sections, except GUS stained samples, were stained with 0.1% (w/v) toluidine blue prior to examination.

3. Results

3.1. Phylogenetic analyses of moss Class III HD-Zip genes

Previous phylogenetic analyses of C3HDZ indicated that multiple gene duplications occurred within the lineage leading to *P. patens*, a derived moss in the Funariadae (Floyd et al., 2006; Prigge and Clark, 2006). To determine the timing and phylogenetic distribution of gene duplications of C3HDZ genes within the moss clade, we isolated gene family members from a representative of two earlier-diverging moss lineages, *Sphagnum sp.* (Sphagnopsida; Fig. 1), and *Dawsonia superba*, (Polytrichopsida; Fig. 1). We also included C3HDZs from another bryopsid moss, *Ceratodon purpurea* (Fig. 1). Three gene family members from *D. superba* and 4 gene family members from *Sphagnum* were identified. We included in the analysis C3HDZ sequences previously identified for the alga *Chara*, the liverwort *M. polymorpha*, the hornwort *P. carolineanus*, the lycophytes *S. kraussiana* and *L. lucidulum*, and the gymnosperm *Ginkgo biloba*.

The analysis (Fig. S1) confirmed the monophylly of moss sequences inferred in previous analyses that included only *Physcomitrella* sequences (Floyd et al., 2006; Prigge and Clark, 2006). In all cases the resolution within the moss clade is consistent with hypotheses of moss phylogenetic relationships (Fig. 1), although it implies a complex series of duplications and losses. The moss sequences resolved into two subclades, A and B, (Fig. 1). Both subclades include sequences from *Sphagnum* and *Dawsonia*. All sequences from *Ceratodon* and *Physcomitrella* were resolved in subclade B.

The topology implies that the common ancestor of extant mosses likely inherited a single gene that duplicated prior to the divergence of the common ancestor of extant mosses, giving rise to subclades A and B (Fig. 1). Both ancient A and B paralogs were retained in the *Sphagnum* and *Dawsonia* lineages in which paralog A underwent additional, lineage-specific duplications whereas paralog A was lost in the bryopsid lineage after the divergence of the polytrichalean ancestor from that of the bryopsid mosses. Paralog B is still represented by a single gene in *Sphagnum* whereas there are two clades including *Ceratodon* and *Physcomitrella* (clades Ba and Bb, Fig. 1), one of which (Bb) also includes a *Dawsonia* sequence. In Clade Ba, a single *Ceratodon* gene (*CpC3HDZ1*) is resolved as sister to *PpC3HDZ1*. In Clade Bb, a single *Dawsonia* gene (*DsC3HDZ3*) is resolved as sister to a clade including the remaining four *Physcomitrella* genes and *Ceratodon CpC3HDZ2*. This single *Ceratodon* gene is resolved as the sister to *PpC3HDZ3* plus *PpHB10*. From the topology of Clade Bb we infer that a duplication of Paralog B occurred in a common ancestor of *Dawsonia* and the bryopsid mosses, after the divergence of the sphagnopsid lineage. One of these paralogs (Ba) was subsequently lost in the lineage leading to *Dawsonia*. The Bb paralog underwent additional duplications, one before the divergence of *Ceratodon* and *Physcomitrella*, and more recently within the *Physcomitrella* lineage.

In summary, C3HDZ phylogenetic analysis infers that the duplications giving rise to the five *Physcomitrella* C3HDZ genes occurred at three different points in history, one before the

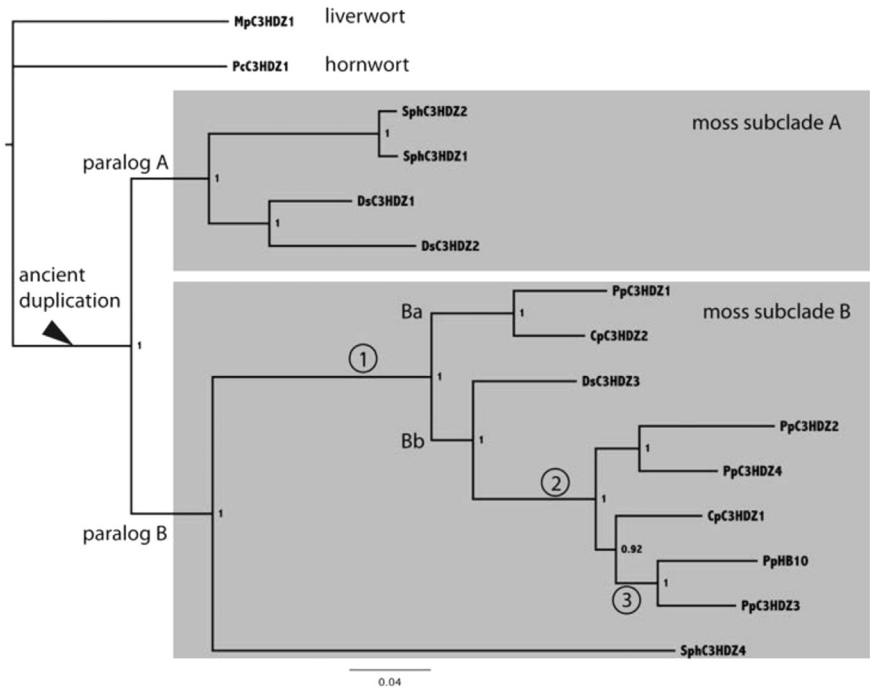

Fig. 1. Phylogeny of moss C3HDZ genes. Moss sequences were resolved as a monophyletic clade. An ancient duplication in the common ancestor of extant mosses is inferred that gave rise to ancient paralogs A and B. All *Physcomitrella* sequences are resolved in clade B, descended from one of the ancient paralogs whereas the other mosses included have paralogs in both clades A and B. Taxa abbreviations: Ds=*Dawsonia superba*; Cp=*Ceratodon purpurea*; Mp=*Marchantia polymorpha*; Pc=*Phaeoceros carolineanus*; Pp=*Physcomitrella patens*; Sph=*Sphagnum* ssp.

separation of Polytrichales and Bryopsida ancestors, one before the divergence of Funaridae and Dicranidae ancestors, and one after divergence of Funaridae and Dicranidae (Fig. 1). This analysis also indicates that one of two ancient C3HDZ paralogs, A, was lost in the bryopsid mosses whereas both paralogs A and B were retained in the earlier diverging moss lineages *Sphagnum* and *Dawsonia*.

3.2. Expression of P. patens C3HDZ genes in the gametophyte

We first examined PpC3HDZ expression patterns in wild-type *P. patens* by rtPCR to gain insight into which tissues and stages of the life cycle the genes are actively transcribed. Since all five PpC3HDZ genes are regulated by miR166, the detection of microRNA cleavage products was avoided by designing primer sets that amplify sequences across the microRNA cleavage site. Transcripts for all five PpC3HDZ genes were detected in both the protonema and gametophores of the haploid gametophyte, as well as the diploid sporophyte stages of the life cycle (Supplementary material Fig. S2).

To determine precise spatio-temporal expression patterns of PpC3HDZ genes, transgenes encoding PpC3HDZ-GUS fusion proteins were engineered at PpC3HDZ genomic loci via homologous recombination (Supplementary materials Fig. S3; Table S1). Spatio-temporal expression patterns of one representative from each PpC3HDZ clade (*PpC3HDZ1*, *PpC3HDZ3* and *PpC3HDZ4*) were examined in detail. The morphology of the *PpC3HDZ-GUS* insertion lines used in this study was indistinguishable from wild-type *P. patens*. The PpC3HDZ-GUS fusion proteins may be functional, or alternatively, may be non-functional and their compromised functions complemented by other PpC3HDZ proteins. In either case, GUS activity patterns of PpC3HDZ-GUS proteins should reflect endogenous PpC3HDZ gene expression patterns.

During vegetative gametophyte growth all three PpC3HDZ gene expression patterns are similar to each other (Fig. 2). Haploid spores germinate and develop into the juvenile gametophyte, protonema (Fig. 2A, C, and E), a uniseriate, branched filament developing by tip growth with two distinct cell types, chloronemata and caulonemata. Chloronemal cells develop first from germinating spores, and subsequent changes in the tip cells result in the production of caulonemal daughter cells (Christianson, 2000). Despite the positive results from semi-quantitative RT-PCR (Fig. S2), no PpC3HDZ-GUS expression was detected in the protonemal (either chloronemal or caulonemal cells) gametophyte development (Fig. 2A, C, and E). The discrepancy could reflect a sensitivity issue, with transcript levels detectable by RT-PCR but PpC3HDZ-GUS protein levels insufficient to produce detectable histochemical staining.

Side branch cells originating from sub-apical cells either grow into secondary protonemal filaments or differentiate into gametophore initials, with a higher frequency of the latter from caulonemal cells. The three-dimensional gametophore consists of an erect stem with spiral leaf arrangement. Divisions of the gametophore initial cell establish the apical-basal and medio-lateral growth axes as well as the tetrahedral apical initial cell with three cutting faces, which is the persistent shoot apical meristem of the gametophore (Harrison et al., 2009). The first few leaves that develop on gametophores are juvenile leaves, while subsequent leaves are adult leaves (Sakakibara et al., 2003). Juvenile leaves consist of a unistratose (single cell layer thick) leaf blade only, whereas adult leaves have a unistratose leaf blade and a multistratose midrib (Sakakibara et al., 2003). The adult leaf of *Physcomitrella* (and most mosses) initially emerges by activity of a two-sided apical cell (Fig. 2J) that is formed following divisions of each shoot apical cell derivative (Nägeli, 1845; Hoffmeister, 1862; Campbell 1905; Pottier, 1925). The leaf apical cell ceases activity and meristematic activity initiates at the base of the leaf resulting in further growth of the leaf across the entire base. The apical-cell derived tip then undergoes differentiation including pronounced elongation of some of the cells (Fig. 2N–P). Activity of the basal meristem produces a leaf base with cells in conspicuous parallel files (Fig. 2N). Bundles of conducting tissue are located at the center of the stem and in the midrib of adult leaves (Fig. 2N).

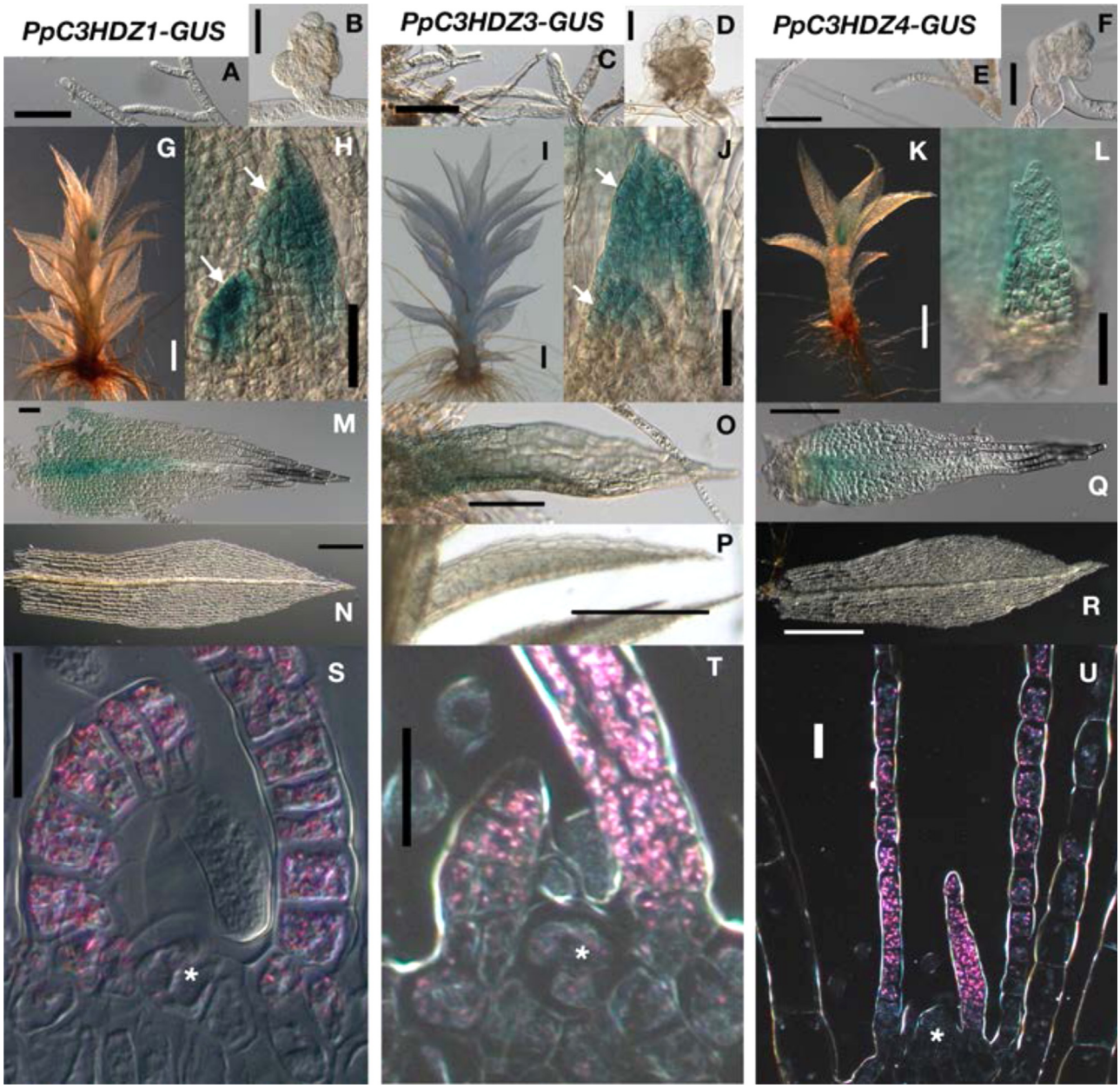

Fig. 2. Expression patterns of PpC3HDZ-GUS proteins in the gametophyte. (A–B, G–H, M–N, S) *PpC3HDZ1-GUS*; (C–D, I–J, O–P, T) *PpC3HDZ3-GUS*; (E–F, K–L, Q–R, U) *PpC3HDZ4-GUS*. (A, C, E) Protonemal tissues. (B, D, F) Young gametophore buds with developing juvenile leaves. (G, I, K) Well-established gametophores with adult leaves. (H, J, L) Close ups of gametophore apices with developing adult leaves (arrows, distal expression). (M, O, Q) Developing adult leaves with GUS signal at the leaf base. (N, P, R) Mature adult leaves with no detectable GUS signal. (S–U) Longitudinal sections of gametophore apices. Younger leaves locates closer to the center of the apex. Asterisks indicate the tetrahedral initial cells. Scale Bars = 1 mm (A, C, E, G, I), 500 μm (K, N, P, R), 100 μm (M, O, Q) or 50 μm (B, D, F, H, J, L, S–U).

No detectable staining is observed in initiating gametophores, during early bud growth, or juvenile leaves (Fig. 2B, D, and F), but PpC3HDZ-GUS signals become evident at the apices of well-established gametophores with adult leaves (Fig. 2G–L). Notably, little or no GUS expression is detected in the gametophore apical initial cell. (Fig. 2S–U). Only PpC3HDZ3-GUS was detected at low levels in the apical cell and in cells derived from it that become part of the stem and this was only observed by examining thin sections (Fig. 2T), not in whole mount stained tissue. PpC3HDZ-GUS expression mirrors the growth pattern of adult leaves. GUS activity is initially uniformly distributed in newly emerged adult leaves including the leaf apical cell. (Fig. 2G–L and S–U). After the leaf apical cell ceases activity, GUS activity diminishes in the apical part of the emerging leaf and remains in the basal region of the blade, and slightly more apically in the midrib (Fig. 2M, O, and Q). No GUS signal was detected in differentiated leaves (Fig. 2N, P, and R). In summary, PpC3HDZ expression correlates spatially and temporally with both apical and intercalary meristematic regions in developing leaves.

3.2.1. *Expression of PpC3HDZ genes in archegonia and antheridia*

As a monoicous moss, *P. patens* bears both sperm-producing antheridia and egg-producing archegonia at the apical regions of a single gametophore. The three PpC3HDZ genes are expressed in similar patterns in developing gametangia (Fig. 3; Supplementary material Table S2). PpC3HDZ-GUS activity was detected throughout young antheridia (Fig. 3A, K, and U) but not in mature antheridia or the sperm cells (Fig. 3B, L, and V). PpC3HDZ-GUS was expressed throughout young developing archegonia (Fig. 3C, M, and W). When archegonia were mature with an opened neck and

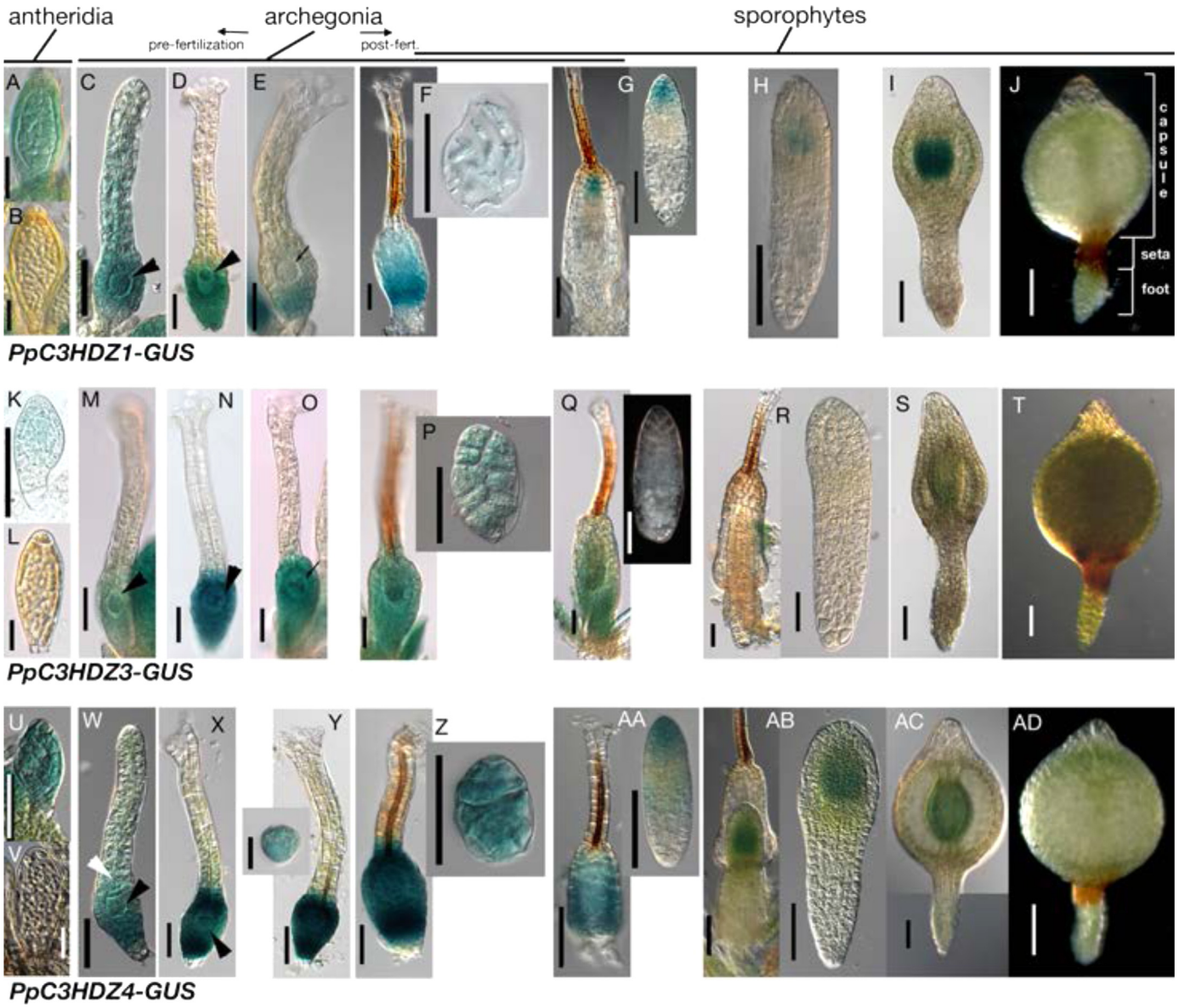

Fig. 3. Expression patterns of PpC3HDZ-GUS proteins during sexual reproduction. (A–J) *PpC3HDZ1-GUS*, (K–T) *PpC3HDZ3-GUS*, (U-AD) *PpC3HDZ4-GUS*. (A, K, U) Developing antheridia. (B, L, V) Mature antheridia. (C, M, W) Developing archegonia. (D, N, X) Mature archegonia with open neck and egg cell in the venter. GUS activity was detected in the ventral canal cell (white arrowhead in W) and the egg cell (black arrowheads in C–D, M–N, W–X). (E, O) Archegonia just prior to or just after fertilization. (F, P, Y–Z) Archegonia with fertilized egg cell. GUS activity was detected in the zygote (arrows and inset in Y). Inset in Y shows the isolated diploid zygote. (F–G, P–Q, Z-AA) Young sporophyte developing within the archegonia. Isolated sporophytes are shown in the insets on the right. (H, R, AB) Isolated sporophyte with periclinal cell divisions evident in the upper half of sporophyte. Archegonia enclosed sporophytes are shown on the left (R, AB). (I, S, AC) Young sporophyte with differentiating capsule. (J, T, AD) Sporophyte with spores. Scale bars = 50 μm (A–F, K–P, U–Z), 100 μm (G–I, Q–S, AA–AC), 200 μm (J, T, AD).

an isolated egg cell, PpC3HDZ-GUS expression was higher at the venter and the egg cell (Fig. 3D, N, and X).

Upon fertilization, the neck of a fertilized archegonium senesces, causing it to twist and turn brown. The archegonial wall grows along with the young sporophyte embryo as a protective structure during early embryonic stages. In post-fertilization archegonia, GUS signals in *PpC3HDZ1-GUS* and *PpC3HDZ3-GUS* gradually receded to basal regions of the venter (Fig. 3E–F, O–Q). High expression levels of *PpC3HDZ4-GUS* remained throughout the venter until the detachment of archegonial wall (Fig. 3Y-AA). Thus, the three examined PpC3HDZ genes are active in the meristematic tissues of the archegonia both before and after fertilization (Supplementary material Table S2). Signal for all three expression transformants was detected in the unfertilized egg cell (Fig. 3D, N, ans X).

3.2.2. *Expression of Physcomitrella Class III HD-Zip genes in sporophytes*

While the expression of the three PpC3HDZ genes in gametophytes was similar prior to fertilization, the expression patterns following fertilization varied among the PpC3HDZ genes, both in the diploid sporophyte and in the post-fertilization archegonia (Fig. 3E–I, O–S, Y-AC; Supplementary material Tables S2 and S3).

Prior to fertilization, *PpC3HDZ3-GUS* and *PpC3HDZ4-GUS* signals were present in the egg cell, and upon fertilization signal was also detected in the zygote and the young embryo (Fig. 3O–P, Y–Z). In contrast, despite a positive signal in the unfertilized egg cell, *PpC3HDZ1-GUS* expression in the diploid zygote decreased dramatically and no expression was detected in the young embryo (Fig. 3E and F). At later stages the initial uniform expression of *PpC3HDZ4-GUS* expression became restricted to the apical meristem (Fig. 3Y–AA). In contrast, *PpC3HDZ3-GUS* expression was

detected only during the early stages of sporophyte development (Fig. 3P–Q) and *PpC3HDZ1-GUS* expression was detected only during the later stages of apical meristem activity (Fig. 3F–G).

The first division of the zygote produces a basal cell and an apical cell. The basal cell of the zygote undergoes a few divisions and differentiates into the foot. The apical cell of the zygote undergoes oblique divisions producing a two-sided sporophyte apical cell (Kreulen, 1975; Sakakibara et al., 2008) and subsequent divisions establish the longitudinal axis of the sporophyte [Kofuji et al. (2009); Fig. 3F, P, and Z]. Rapid activity of the apical cell and periclinal division and anticlinal divisions of the derivatives results in an embryo with tiers of four inner cells and four outer cells (Campbell, 1905; Smith, 1955). These divisions define the outer part of the sporophyte, known as the amphithecium and the inner part, the endothecium. When the embryo has about about eight tiers it exhibits obvious morphological/anatomical zonation (Fig.). The lower tiers enlarge and elongate parallel to the long axis of the sporophyte forming beginning of the seta. Above the zone of elongation, the cells of one or two tiers divide by anticlinal cell divisions forming an intercalary seta meristem zone that contributes cells basally to the elongating seta. Above this intercalary seta meristem the cells also divide anticlinally and periclinally but remain small and undifferentiated, producing a transition zone between the elongating seta and relatively quiet apical region.

PpC3HDZ1-GUS expression was weak in the young embryo (Fig. 3F). In embryos with clearly defined regions, *PpC3HDZ1-GUS* expression was strong and restricted to the apical region (Figs. 3G, 4A-B). At this stage the, apical region included the apical cell, its most recent derivative with a single periclinal derivative, and four clearly defined tiers with amphithecium and endothecium. The amphithecium was partly two-layered due to single periclinal divisions of the outer cells of each tier proceeding acropetally. In embryos in which the amphithecium was two cells deep, *PpC3HDZ1-GUS* expression had declined in the apical cell and the amphithecium but remained strong in the three tiers of endothecium (Fig. 4C and D). In later stages it was apparent that the apical cell underwent two or three more divisions to produce additional tiers after which it enlarged and stop dividing (Fig. 4E and F). *PpHDZ1-GUS* was not expressed in the newly formed layers and appeared at this time to be positioned centrally in the apical part of the sporophyte (Fig. 3H and I; Fig. 4E and F). *PpC3HDZ1-GUS* expression remained strong in the same three endothecial tiers (present in earlier stages) which subsequently underwent coordinated divisions to produce the collumella, an inner core of four cells across, surrounded by 16 cells (Fig. 4G). The 16 outer cells then underwent two successive radial (periclinal) divisions to produce three layers, the inner sporogenous layer surrounded by two tapetal layers (Fig. 3H and I; Fig. 4H–J). During subsequent expansion of the capsule the cells of the collumella enlarge and become vacuolated without dividing whereas the cells of the sporogenous and tapetal layers also undergo anticlinal divisions in a longitudinal plane producing layers of relatively small, densely cytoplasmic cells (Fig. 4H–J). The endothecium splits apart from the amphithecium creating a cylindrical cavity (Fig. 4I). The cells of the sporogenous layer underwent one further nuclear division with the plane of division perpendicular to the axis of the sporophyte. Following this division new walls form around the daughter nuclei, which are the sporogenous cells that will undergo meiosis. At this point *PpC3HDZ-GUS* activity was still present, although diminished, in the cells of the collumella and tapetal layers but remained strong in the small, densely cytoplasmic sporogenous cells (Fig. 4J). No signal was detected following meiosis (Fig. 3J).

PpC3HDZ3-GUS signal was strong in zygotes and early embryos but was no longer detectable in sporophytes at the end of the rapid apical growth phase in which zonation into subapical, transition, and apical regions was clear (Fig. 3Q) or any subsequent stages of sporophyte development (Fig. 3R–T).

PpC3HDZ4-GUS expression (present in the zygote) remained detectable throughout the embryo during through the early phase of rapid apical growth (Fig. 3Y-AA). By the end of the rapid apical growth phase, *PpC3HDZ4-GUS* expression was present in the seta meristem region and in the upper tiers and apical cell but was eliminated from the transition zone (Fig. 3AA). In sporophytes in which the seta had completed development, *PpC3HDZ4-GUS* expression was no longer detected below the transition zone (Fig. 3AB). Expression during the remaining development of the apical region was similar to *PpC3HDZ1-GUS. PpC3HDZ4-GUS* signal disappeared from the amphithecial layers and did not persist in the apical cell for the last few divisions so that the signal was centrally located in the endothecial layers that form the sporogenous tissue and collumella (Fig. 3AB and AC). No signal was detected in sporophytes after meiosis has taken place (Fig. 3AD).

In summary, PpC3HDZ genes are expressed in the apical meristem, the seta meristem, and sporogenous tissue and associated collumella of the developing sporophyte (Suppl. Table 3). *PpC3HDZ4* expression is detected in all three sporophyte meristems, whereas *PpC3HDZ1* and is expressed in the apical and sporogenous meristems and *PpC3HDZ3* in the zygote and young embryo. Only one of the three genes (*PpC3HDZ4*) was expressed in the seta meristem.

3.3. C3HDZ expression in Dawsonia superba

As an approach to test whether the expression patterns we observed in *P. patens* may be generalized amongst peristomate mosses, we examined C3HDZ expression in *D. superba*, a member of the Polytrichidae (Fig. 5A). RT-PCR indicated that all three *Dawsonia* C3HDZ genes are expressed in both gametophytic and sporophytic (in both the capsule and seta) stages of the life cycle, as shown for *DsC3HDZ1* (Fig. 5B). Analysis via in situ hybridization of *DsC3HDZ1* in the gametophore apex detected expression in the leaves, but not the region of the apical cell (Fig. 5C and D). Unlike *Physcomitrella*, some expression extended down from the point of the attachment of the leaves on the apex into the center of the gametophore in the enlarging hydroid cell. These cells are derived from the same derivatives of the apical cell (merophytes) and are thus developmentally related to the leaves. Within the leaves, expression is most conspicuous in the developing adaxial lamellae, which are parallel uniseriate sheets of brick-like cells, specialized for photosynthesis (Fig. 5D and E). Development of the lamellae appears to entail tightly coordinated cell divisions.

3.4. Experiments to compromise C3HDZ function

3.4.1. Single gene knockout lines

Stable gene replacement transformants were isolated for each of the five PpC3HDZ genes and insertion positions were confirmed by PCR genotyping (except for *PpC3HDZ1KO*; Supplementary material Fig. S3; Table S4). No abnormal phenotypes were observed during growth of the gametophyte, including both protonemal growth and gametophore development (Supplementary material Fig. S4). Since each of the three genes for which detailed expression data were obtained exhibit nearly indistinguishable expression patterns during gametophyte development, the lack of gametophyte phenotypic defects in the single gene deletion lines could be a result of genetic redundancy, with the functions of individual genes compensated by the activity of other PpC3HDZ genes.

3.4.2. Constitutive expression of miR166

To compromise the function of all PpC3HDZ genes simultaneously, we constitutively expressed a gene encoding miR166,

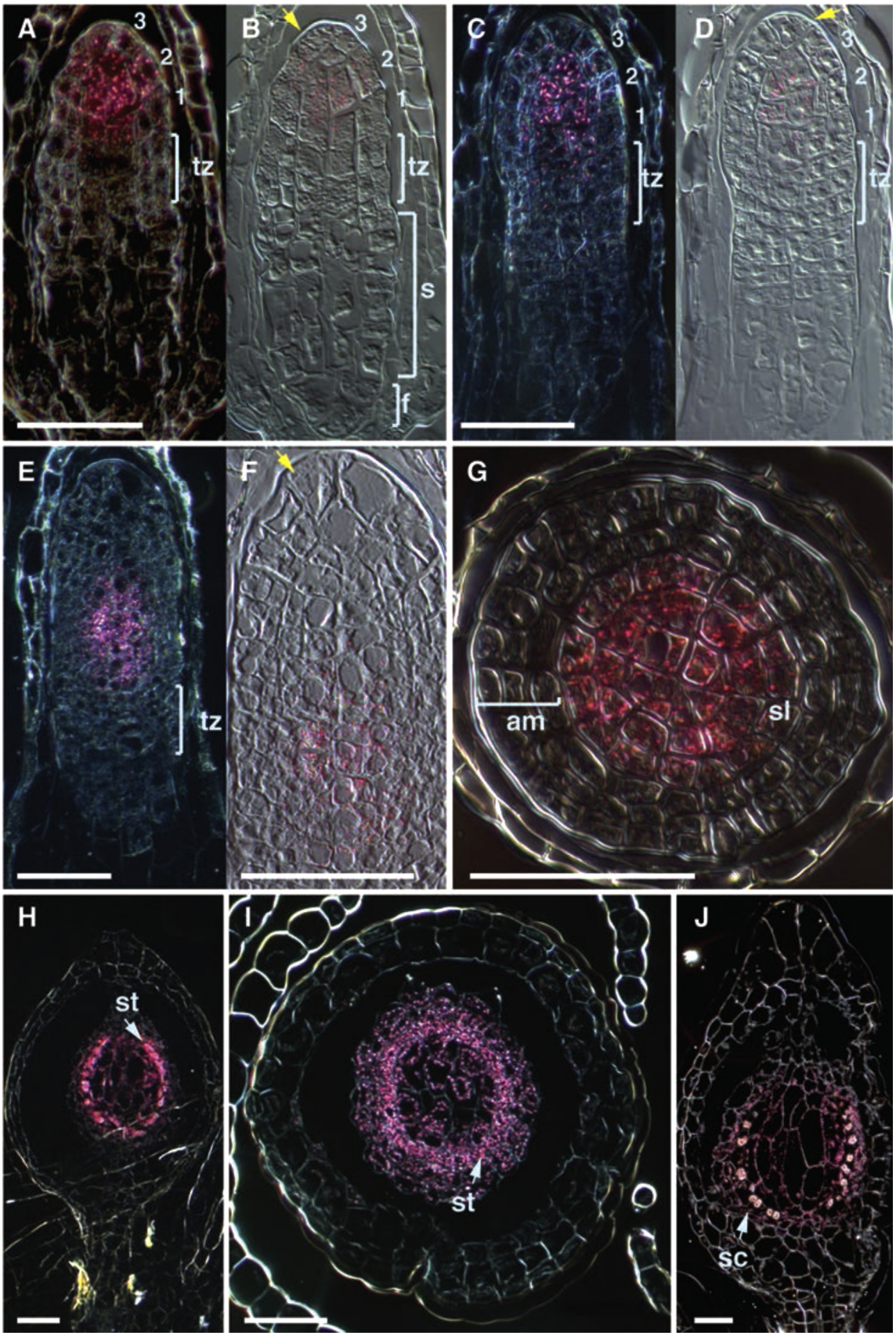

Fig. 4. Histological sectioning of developing sporophytes expressing *PpC3HDZ1-GUS*. (A) Dark field (DF) and (B) differential interference contrast (DIC) views of young sporophyte exhibiting foot, seta, transition zone and apical zone. GUS staining throughout three tiers above transition zone including apical cell (yellow arrow) (longitudinal section, LS). (C) DF and (D) DIC views of later sporophyte with two-layered amphithecium. GUS staining mostly confined to the endothecium of the three tiers above the tz, absent in apical cell. (E) DF and (F) DIC views of later sporophyte. GUS staining confined to region derived from original three tiers. Apical cell (yellow arrow) has produced additional tiers above zone of GUS expression. (G) DF cross section (XS) of sporophyte at similar stage to F; GUS staining restricted to endothecium including the inner core which will become the collumella and the sporogenous layer (sl). The amphithecium (am) is three cells thick. (H) LS and (I) XS DF views of sporophyte in which outer layer of endothecium as seen in F has produced two new layers. GUS staining is strongest in the innermost layer, which is the sporogenous tissue, but is still present in outer tapetal layers and inner collumella (st). Amphithecium and endothecium have separated forming a cylindrical cavity. (J) LS DF view showing cells of the sporogenous layer have undergone final division to produce sporogenous cells (sc) that will undergo meiosis. GUS staining still present but diminished in all endothecial layers, strongest in sporogenous cells. Scale bars = 50 μm.

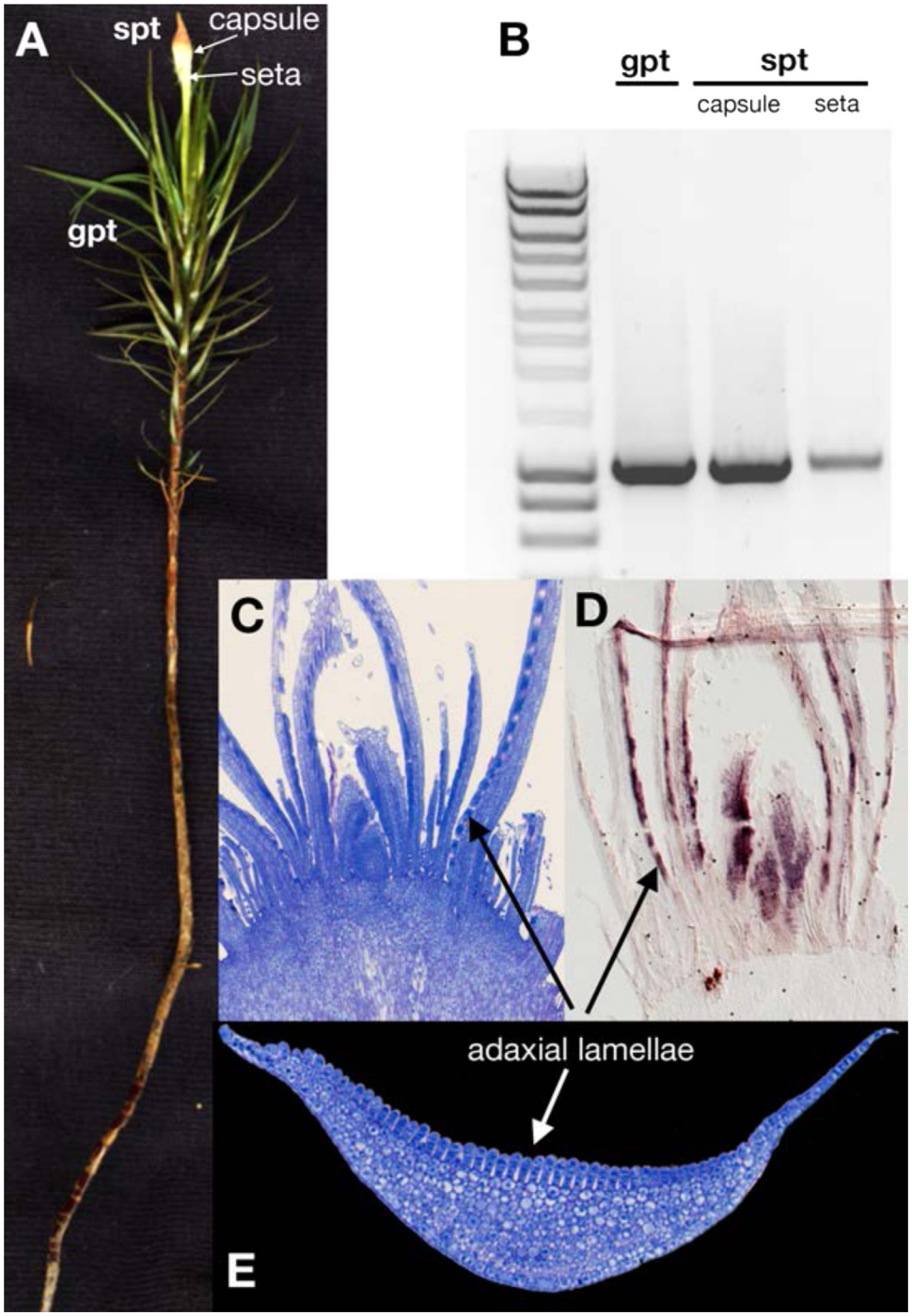

Fig. 5. C3HDZ expression in *Dawsonia superba*. (A) *Dawsonia superba*. (B) Expression of *DsC3HDZ1* as assayed by rtPCR. (C) Longitudinal section through apex of gametophyte. (D) Expression of *DsC3HDZ1* as assayed by in situ hybridization. (E) Cross section of leaf showing detail of adaxial lamellae. gpt, gametophyte; spt, sporophyte.

SkmiR166 (derived from *Selaginella kraussiana*), which targets C3HDZ gene transcripts throughout land plants (Floyd and Bowman, 2004). Activity of the cauliflower mosaic virus 35S (CaMV 35S) promoter has been shown to be relatively constitutive in the cells and tissues of the gametophyte (Saidi et al., 2009) and activity of the double CaMV 35S promoter (2X35S) has been shown to be 6-times more active than single 35S promoter in haploid protoplasts (Horstmann et al., 2004).

The *2X35S:SkmiR166* cassette was inserted into the genome of both wild-type and transgenic *PpC3HDZ4-GUS-1* lines. Because of the higher gene targeting efficiency of the *PpC3HDZ2* locus in previous experiments, this locus was used as the transformation platform for the miR166 cassette (Supplementary material Fig. S3). Thus, in a *PpC3HDZ4-GUS-1* background, if the *2X35S:SkmiR166* transgene is targeted to the *PpC3HDZ2* locus, *PpC3HDZ2* should be inactivated due to the insertion of the transgene, while transcripts of *PpHB10*, *PpC3HDZ1*, and *PpC3HDZ3*, as well as *PpC3HDZ4-GUS* should be targeted by the constitutively expressed miR166. Alternatively, if the transgene was not targeted to the PpC3HDZ locus, all PpC3HDZ transcripts should still be targeted by miR166. Stable transformants harboring the *2X35S:SkmiR166* transgene had a range of phenotypes. Three stable transformants, two in an otherwise wild-type background (*PpC3HDZ-miR166-14* and *PpC3HDZ-miR166-17*) and one from the *PpC3HDZ4-GUS*

background (*PpC3HDZ4G-miR166-8*), exhibited the most extreme phenotypic effects and were analyzed in more detail.

Since *PpC3HDZ4-GUS* transcripts are also targeted by miR166, expression levels of PpC3HDZ4-GUS protein, monitored by histochemical staining, provides a visual assay of the efficiency of the *miR166* cassette in the *PpC3HDZ4G-miR166-8* line (Fig. 6A–F). Most gametophores that developed in the *PpC3HDZ4G-miR166-8* line did not show any GUS activity under standard histological assay conditions (8 hours of substrate incubation) or even after a prolonged 4-day incubation period, indicating the *2X35S:SkmiR166* cassette reduced the transcript level of *PpC3HDZ4-GUS*, and by inference, transcripts of the other PpC3HDZ genes.

3.4.3. *PpC3HDZ-miR166 gametophores have short leaves that resemble the distal portion of wild-type leaves*

Development of protonemata, including the morphology of chloronemal cells and caulonemal cells, and bud initial differentiation in the *PpC3HDZ-miR166-14 and -17* and *PpC3HDZ4G-8* lines (collectively the *PpC3HDZ-miR166* lines) was variable and in some cases, such as in *PpC3HDZ-miR166-17* lines, appeared to deviate from that of wild type (Supplementary material Fig. S4A–C). In contrast, the gametophores in PpC3HDZ-miR166-transformant colonies displayed a consistent phenotype including a shorter stature and altered leaf development, and due to these defects the gametophores are not as conspicuous within the protonemata as are wild-type gametophores during their early development (Supplementary material Fig. S4D–F). The effects of the *2X35S:SkmiR166* transgene varied within a single clonal colony with different gametophores displaying varying degrees of phenotypic defects. The level of GUS activity in *PpC3HDZ4G-miR166-8* gametophores was correlated with the severity of phenotypic defects observed. While most *PpC3HDZ4G-miR166-8* gametophores did not exhibit any detectable GUS activity, gametophores with a wild-type phenotype occasionally developed, and in these, the GUS expression pattern in developing leaves resembled that of the parental *PpC3HDZ4-GUS* line. *PpC3HDZ4G-miR166-8* gametophores with no GUS staining under prolonged substrate incubation exhibited the most severe phenotype (Fig. 6E and F), and their leaf morphology resembled those found in *PpC3HDZ-miR-14* and *PpC3HDZ-miR-17* gametophores (Fig. 6M, S). *PpC3HDZ4G-miR166-8* apices with weak GUS signal (Fig. 6C and D) developed leaves that exhibited phenotypes intermediate (Fig. 6D) between those of wild type (Fig. 6H) and those of the most severely affected *PpC3HDZ-miR166* gametophores (Fig. 6M and S).

A gametophore apical initial cell was present in the apices of the loss-of-function PpC3HDZ-miR166 transformants (Fig. 6J, O, arrow). PpC3HDZ-miR166 stems were shorter than those of wild type (Fig. 6G, L, and R), and varied in their girth, with lack of conducting tissue in some stems (Fig. 6N).

Leaf shape and size were severely altered in PpC3HDZ-miR166 gametophores. The shapes and organization of cells of PpC3HDZ-miR166 leaves (Fig. 6M and S) resembled those in the distal half of a wild-type leaf (Fig. 6H), with exaggerated serrations along the leaf margin. The elongated box-shaped proximal cells of wild-type leaves (Fig. 6H, bottom inset) were not found in PpC3HDZ-miR166 leaves. The average leaf cell sizes were comparable in PpC3HDZ-miR166 and wild-type leaves (Table 2). The diminutive leaf size in PpC3HDZ-miR166 lines was the result of a reduced number of cells, especially along the proximal-distal axis, where cell number was about one-half that of a wild-type leaf. In extreme cases, conducting tissue was absent in the midrib of PpC3HDZ-miR166 leaves (Fig. 6P).

Two transformants, *PpC3HDZ-miR-14* and *-17*, were induced to undergo sexual reproduction and gametangia developing on the apices of mutant gametophores were examined in further detail. PpC3HDZ-miR166 gametangia were elongated compared to those of the wild type (Fig. 6K and Q). Some PpC3HDZ-miR166 archegonia had an extended venter base, and some had an elongated venter (Fig. 6Q). In wild-type plants, only one egg cell is present inside the venter when archegonia are mature with an open neck. However, in some cases more than one cell was present in PpC3HDZ-miR166 venters when the neck was open for fertilization.

4. Discussion

Plant growth is characterized by meristems, self-renewing groups of cells that produce cells for tissue differentiation. The two daughter cells of a meristematic cell can have one of two different fates: continue in the role of a meristematic cell, or alternatively, follow a path of differentiation and contribute to the development of an organ. Land plant meristems can be categorized into two types: persistent and transient meristems. Persistent meristems typically remain active throughout the plant lifetime, or a good portion thereof, and are usually located at the growing tips of the plant body. The *P. patens* gametophyte has two types of persistent meristems: the apical protonemal cell and the tetrahedral gametophore apical initial. On the other hand, transient meristems function only ephemerally and can be situated at either terminal or intercalary positions—in *P. patens,* the intercalary meristems at the gametophyte leaf bases and the apical meristem and intercalary seta meristem of the sporophyte.

4.1. *C3HDZ activity is not required for persistent meristematic growth of the gametophyte*

While we could detect C3HDZ expression via rtPCR, no *PpC3HDZ-GUS* expression was detected during the protonemal gametophyte growth, and in some PpC3HDZ-miR166 lines subtle differences in protonemal growth were observed. No PpC3HDZ-GUS expression was detected in bud initials or any tissues of young gametophores, including the gametophore apical initial. *DsC3HDZ1* expression was also not detected in the gametophore apical cells in *Dawsonia superba*. Gametophore apical initial cells were functional in PpC3HDZ-miR166 lines, suggesting PpC3HDZ genes are not required for the maintenance of the gametophyte persistent apical meristem. Thus, unlike *Arabidopsis* where these genes are required for persistent shoot apical meristem function in the sporophyte, PpC3HDZ genes are not required for the establishment or maintenance of the persistent apical meristem of the gametophore in either of the mosses examined in this study. However, the gametophore axes of the more severely affected PpC3HDZ-miR166 lines were shorter, had fewer cells in cross section, and lacked conducting cells, indicating that PpC3HDZ activity is required for the growth of these tissues once they are formed from the apex.

Our results are reminiscent of those reported for KNOX1 genes, whose activity is required for the persistent shoot apical meristem of the sporophyte in flowering plants, but whose activity in *P. patens* is not required for the persistent apical meristem of the gametophyte (Barton and Poethig, 1993; Sakakibara et al., 2008). Collectively, these data argue against any large-scale co-option of gene function from an ancestral gametophyte apical meristem to function in a similar manner in a sporophyte apical meristem.

Unlike the KNOX1 genes, whose function is restricted to the sporophyte (diploid) generation in all plants in which they have been investigated, in mosses C3HDZ activity is required in gametophytes and, based on expression data, is likely required in sporophytes (Sakakibara et al., 2008; Ortiz-Ramírez et al., 2016). Since C3HDZ expression is detected in the gametophytic generations of the alga *Chara*, bryophytes, and ferns (Floyd et al., 2006), the lack of C3HDZ activity in either the male or female gametophytes in *Arabidopsis* (Prigge et al., 2005) represents a loss on the

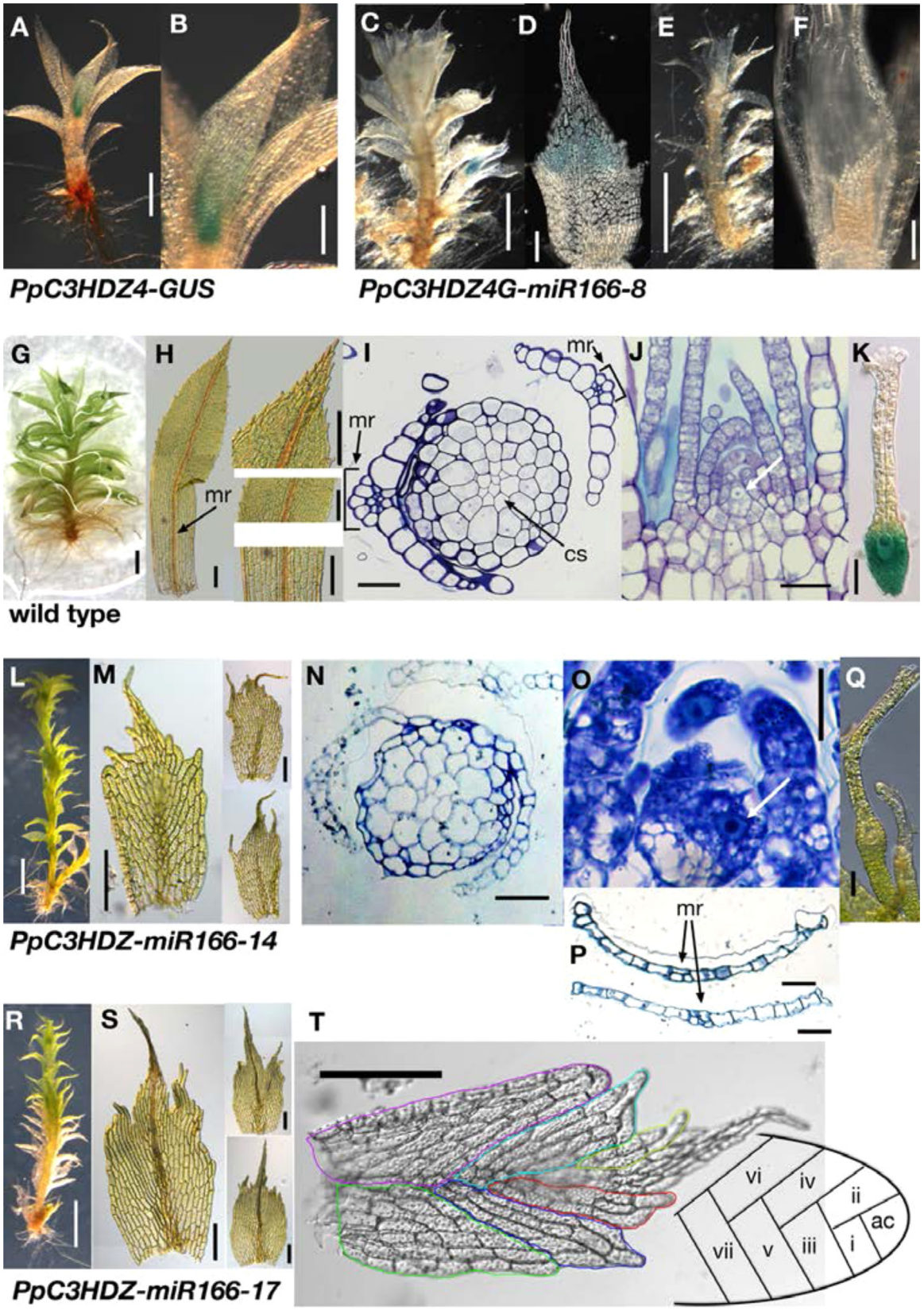

Fig. 6. Gametophores of PpC3HDZ-miR166 transformants. (A–F) Histochemical GUS staining of *PpC3HDZ4-GUS* background *PpC3HDZ4G-miR166* transformant gametophores. (A–B) *PpC3HDZ4-GUS* after 8 h of substrate incubation. (C–F) *PpC3HDZ4G-miR166-8* after 4 days of substrate incubation. Wild type (G–K), *PpC3HDZ-miR-14* (L–Q, T), *PpC3HDZ-miR-17* (R-S). Wild-type gametophore (G) compared to PpC3HDZ-miR166-transformant gametophores (L, R). Mature adult leaves (H, M, S–T). Close ups of wild-type leaf regions are shown in inset of (H). Cross sections (I, N) and longitudinal sections of gametophore apices of wild type (J) and PpC3HDZ-miR166-transformant (O) gametophores. Arrows point to the initial cells. Cross-sections of a wild-type leaf (I) and PpC3HDZ-miR166-transformant leaves (P). Archegonia with opened necks (K, Q). Putative merophytes are outlined in the *PpC3HDZ-miR166-14* leaf in T (same as left leaf in M). The diagram depicts the normal pattern of merophyte production (i, youngest; vii, oldest); ac = leaf apical cell. mr = leaf midrib in H, I, and P; cs = central strand in I. Scale bars = 20 μm (K, O, R), 50 μm (L, M, S, T, Y), 100 μm (B, F, I, N, P, W), 200 μm (D, H, J, O, Q, V, X), 500 μm (A), 1 mm (C, E, G, U).

Table 2
Leaf measurements of wild type and PpC3HDZ-miR166 transformants.

Leaf sample		*PpC3HDZ-miR-14* ($n=4$)	*PpC3HDZ-miR-17* ($n=4$)	WT ($n=3$)
Length	**Average number of cells**	15.0 ± 1.8	15.0 ± 0.8	31.7 ± 6.1
	Average length (μm)	989.5 ± 165.6	1372.0 ± 56.5	2627.0 ± 138.7
	Average length (μm) per cell	71.1 ± 10.1	91.7 ± 6.3	85.6 ± 20.3
Width	**Average number of cells**	15.0 ± 1.5	27.3 ± 1.5	32.0 ± 2.0
	Average width (μm)	360.7 ± 78.6	608.7 ± 30.3	593.4 ± 33.8
	Average width (μm) per cell	24.2 ± 5.6	22.4 ± 2.0	18.6 ± 1.8

lineage leading to flowering plants, paralleling the reduction of this generation during land plant evolution (Bower, 1908).

4.2. C3HDZ activity is required for leaf patterning and growth in P. patens

In contrast to their dispensability for apical meristem functioning, expression patterns and loss-of-function phenotypes of PpC3HDZ genes indicate a role for these genes in transient meristematic growth from intercalary meristems in the gametophyte. During the development of an adult leaf, two transient meristems, the apex of an initiating leaf and the intercalary meristems at the leaf base, are activated at different stages. The expression patterns of at least three PpC3HDZ genes mirror the spatial-temporal patterns of the two leaf transient meristems, and the development of leaf transient meristems was hindered in PpC3HDZ-miR166 mutant leaves. In wild-type *P. patens*, the distal leaf region reaches maturity before the rest of the leaf, whereas the leaf base remains meristematic and gives rise to the proximal portion of the leaf. While PpC3HDZ expression is initially throughout the emerging leaf, expression becomes restricted to the leaf base as the leaf apical cell ceases to divide and the leaf apex differentiates, a pattern that parallels the proximo-distal pattern in cell cycling (Harrison et al., 2009). PpC3HDZ-miR166 leaves resemble poorly formed distal tips of wild-type leaves and lack the basal portion of the leaf normally formed by the intercalary meristem. One interpretation is PpC3HDZ genes promote or maintain cell proliferation, and in PpC3HDZ-miR166 lines premature termination of cell proliferation results in a lack of cells for proximal leaf development. Such observations are concordant with the findings of (Harrison et al., 2009) where intrinsic autonomous cues were proposed to be important in *P. patens* leaf development. The fact that PpC3HDZ-miR166 leaves undergo limited apical growth similar to that of wild-type leaves may indicate that PpC3HDZ genes are not required for the establishment of the leaf apex meristem, but are required for the establishment and/or the maintenance of the intercalary meristem at the leaf base.

Conducting tissues were sometimes missing from PpC3HDZ-miR166-transformant leaves and stems. While PpC3HDZ gene expression was detected in the midribs of developing adult leaves, the loss of conducting tissues could either be due to a specific effect of PpC3HDZ activity in the differentiation of conducting tissues or alternatively, a consequence of the defect in cell proliferation within the leaves.

4.3. C3HDZ function is associated with tissue level meristematic activity

In the *Physcomitrella* gametophore, C3HDZ activity is most important for leaf meristematic activity and less important for the leaf apical meristem. The gametophore apical cell in mosses has reported to be quiescent, dividing only infrequently, whereas cells of the leaf primordium divide much more rapidly (Gifford, 1983). This is also the case for *Physcomitrella*, where a single division of the gametophore apical cell occurred within a 20 hour observation period, while three-four divisions of the early leaf apical cell occurred within a similar time period (Harrison et al., 2009). Each derivative of the gametophore apical cell undergoes an asymmetrical division. The uppermost of these undergoes a second asymmetrical division (Campbell, 1905; Smith, 1955). Subsequently, each of the three daughter cells develops autonomously, contributing cells to distinct parts of the shoot and one leaf. The leaf apical cell produces derivatives on two alternating sides, and these derivatives then undergo coordinated rounds of cell division in specific planes contributing to the growth and shape of the leaf (Harrison et al., 2009). For example, more vertical divisions occur in the middle regions than in basal or apical segments that cause the leaf to have be wider, giving the oblanceolate shape (Harrison et al., 2009). Cells at the base of the leaf ultimately divide in one plane only, elongating the entire leaf.

In *P. patens* the leaf apical cell alternately produces approximately four lateral derivatives from each cutting face, which then divide producing significant sectors of the leaf [i–vii in Fig. 6T; see Fig. 4 of (Harrison et al., 2009)]. The first two segments give rise to up to half of the leaf length, comprising the entire base. Each of these first derivatives undergoes two successive vertical divisions followed by rounds of horizontal divisions within each resulting daughter cell. It must be inferred that some of the cells undergo at least one more round of vertical divisions since the mature moss leaves are more than six cells in width. Otherwise cell divisions must occur primarily in the horizontal plane producing the parallel files of cells and the leaf base with parallel margins. Remaining derivatives of the leaf apical cell also undergo waves of vertical and horizontal divisions so that each derivative gives rise to a sector of the apical portion of the leaf. The mitotic events and cell expansion appear to be coordinated and non-cell autonomous so that growth of the leaf is symmetrical and the ultimate derivative elongate leaf cells become arranged in vertical files with their long axes parallel to the long axis of the leaf.

One feature of PpC3HDZ-miR166 leaves is that the descendents of successive derivatives of the leaf apical cell do not form a coherent tissue as they do in wild-type leaves. In wild-type leaves the descendents of each the primary derivatives (i–vii in Fig. 6T) form a seamless cohesive tissue producing leaves with smooth margins (Fig. 6H). One interpretation of PpC3HDZ-miR166 leaves is that each of the primary derivatives from the leaf apical cell recapitulates the development observed from the apical cell itself, with leaf merophytes, as outlined in Fig. 6T, behaving independently and resulting in bifurcating leaves. This suggests that C3HDZ activity is most important for stimulating coordinated, tissue level meristematic activity in the moss gametophyte and less important for stimulating cell-autonomous divisions. Given the association between C3HDZ function and auxin synthesis and transport in flowering plants (Izhaki and Bowman, 2007; Ilegems et al., 2010; Brandt et al., 2012; Reinhart et al., 2013), the two defects in leaf development—reduction in intercalary meristem

function and loss of tissue continuity—may be linked to PpC3HDZ gene activity influencing auxin synthesis and transport within the leaf. Protein localization of PIN auxin efflux carriers in *P. patens* leaves, and a narrow leaf phenotype in PpPIN loss-of-function alleles in consistent with the C3HDZ loss-of-function phenotype being at least partially mediated through alterations in auxin biology (Bennett et al., 2014; Viaene et al., 2014).

While moss sporophytes do not have a persistent meristem, three transient meristems contribute to the moss sporophyte body (Campbell, 1905; Bower, 1935; Smith, 1955; Wardlaw, 1955; Sakakibara et al., 2008). First, an apical cell that divides in a self-renewing manner is active in the early embryo producing a spindle-shaped embryo with three defined regions that give rise to the foot, seta and capsule. Second, in mosses (excepting Sphagnopsida and *Andreaea*), a centrally located unifacial seta meristem produces derivatives basally that elongate and differentiate into the seta. Third, the tissue layers of the capsule arise from successive periclinal divisions of both amphithecial and endothecial initials. The transient meristematic activity of the processes forming the walls of the capsule, the sporogenous tissue, and the columella are all associated with C3HDZ expression, suggesting that C3HDZ functions in meristems and cell proliferation are not generation specific.

4.4. Evolution of C3HDZ genes and shoot meristems in land plants

We initiated this study to assess whether C3HDZ genes are active in the *P. patens* gametophyte persistent shoot apical meristem in a manner similar to their essential action in the *Arabidopsis* sporophyte persistent shoot apical meristem (Emery et al., 2003; Prigge et al., 2005). The answer to this would seem to be 'no', which brings up the question as to the relationship between C3HDZ genes and the evolution of shoot meristems in land plants. Land plants evolved from an ancestral charophycean freshwater alga, however, it is an open question whether the alga possessed a focal meristem, as found in extant *Chara*, or growth occurred more diffusely, as in extant *Coleochaete*. Thus, while the ancestral land plant diploid generation consisted of a single cell, the morphology and anatomy of the ancestral land plant gametophyte generation, while likely multicellular, is enigmatic. Within this framework, we present scenarios for the evolution of land plant meristems based on C3HDZ and KNOX1 expression patterns.

Despite the data in moss for KNOX1 (Sakakibara et al., 2008) and C3HDZ genes arguing against a transfer, it is possible that some meristem functions were co-opted from the gametophyte to the act in the sporophyte. While the gametophore apical cell of *P. patens* or *D. superba* are unlikely to require C3HDZ activity, may be these meristems are somehow unique as compared to other persistent gametophyte meristems which produce a thalloid (e.g. liverworts, hornworts, ferns) rather than a radial bodyplan (as in mosses). Thus, to conclusively rule out an ancestral role for C3HDZ genes in the gametophyte meristem, their functions should be investigated in both a bryophyte taxon whose continuing growth habit produces a thallus and a multicellular charophycean alga. The *P. patens* C3HDZ genes do play a role in meristematic development of leaves, which develop directly from derivatives of the apical cell, and a similar role can be proposed in the moss *D. superba*. It is possible that a more general role stimulating meristematic activity in the gametophyte was co-opted into the sporophyte developmental program.

Regardless of whether gametophytic genetic programs were co-opted, the persistent sporophyte shoot apical meristem of vascular plants may have had antecedents in the sporophytes of their bryophyte-like ancestors, and that may be evident in the sporophytes of extant bryophytes. At least three different hypotheses concerning the antecedent for the vascular plant persistent SAM have been proposed. In one view, its origins lie in a transient seta meristem of a bryophyte-like ancestor (Mishler and Churchill, 1984). This would require both an apical displacement of the meristem and a change in directionality of derivatives produced, in addition to a transition from transience to persistence. In a second view, the vascular plant SAM originated with a continuation of an embryonic apical meristem, such as that observed in extant mosses (Albert, 1999). This scenario has been argued against due to the lack of such a transient meristem in hornwort embryos. Thirdly, it has been proposed that the vascular plant SAM arose de novo, as an entity interpolated into the life cycle of a bryophyte-like ancestor (Kato and Akiyama, 2005). That PpC3HDZ genes are expressed in the apical cell, as well as the apical region, of young *P. patens* embryos in a pattern reminiscent of members of another gene family, KNOX1 (Sakakibara et al., 2008), lends support for the first two hypotheses. While not dismissing a transient seta meristem ancestry, Occam's razor would favor an origin in a transient apical meristem as both its position and displacement of derivatives parallel those in the vascular plant SAM. It is tempting to speculate that a prolongation of the initial expression of members of the KNOX and C3HDZ gene families, perhaps due to the addition of genetic factors to establish a feedback loop maintaining the apical cell indefinitely, and thus a long-lived persistent shoot meristem, contributed to Bower's (1908) 'extension of the first office of the sporophyte' during land plant evolution.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2016.01.012.

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