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*Cellulose synthase (CESA) gene expression profiling of  
Physcomitrella patens*

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**Cellulose synthase (CESA) gene expression profiling of  
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**Title:**

*Cellulose synthase (CESA) gene expression profiling of Physcomitrella patens*

**Short title:**

Cellulose synthase expression in *Physcomitrella patens*

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**Abbreviations:**

*GUS*:  $\beta$ -glucuronidase

*CESA*: cellulose synthase A

*Pro*: promoter

*CSC*: cellulose synthase complex

*irx*: irregular xylem

RT-qPCR: reverse transcriptase quantitative polymer chain reaction

*vH+PP*: v-Type  $H^+$  translocating pyrophosphatase

*ACT*: actin

**Abstract:**

The *Cellulose Synthase (CESA)* gene family of seed plants comprises six clades that encode isoforms with conserved expression patterns and distinct functions in cellulose synthesis complex (CSC) formation and primary and secondary cell wall synthesis. In mosses, which have rosette CSCs like those of seed plants but lack lignified secondary cell walls, the *CESA* gene family diversified independently and includes no members of the six functionally distinct seed plant clades. There are seven *CESA* isoforms encoded in the genome of the moss *Physcomitrella patens*. However, only *PpCESA5* has been characterized functionally and little information is available on the expression of other *PpCESA* family members. We have profiled *PpCESA* expression through quantitative RT-PCR, analysis of promoter-reporter lines, and cluster analysis of public microarray data in an effort to identify expression and co-expression patterns that could help reveal the functions of *PpCESA* isoforms in protein complex formation and development of specific tissues. In contrast to the tissue-specific expression observed for seed plant *CESAs*, each of the *PpCESAs* was broadly expressed throughout most developing tissues. Although a few statistically significant differences in expression of *PpCESAs* were noted when some tissues and hormonal treatments were compared, no strong co-expression patterns were observed. Along with *CESA* phylogenies and lack of single *PpCESA* mutant phenotypes reported elsewhere, broad overlapping expression of the *PpCESAs* indicates a high degree of interchangeability and is consistent with a different pattern of functional specialization in the evolution of the seed plant and moss *CESA* families.

**Introduction:**

Cellulose synthases (CESAs) are key proteins in the synthesis of cellulose in plants (Delmer, 1999). Multiple CESA isoforms are encoded by the genomes of vascular and nonvascular plants (Roberts and Bushoven, 2007; Somerville, 2006), and in both cases the CESAs form rosette structures known as cellulose synthase complexes (CSCs) (Kimura et al., 1999; Reiss et al., 1984). Arabidopsis has two distinct types of CSCs that are specific for either primary or secondary cell wall formation, and each is obligately hetero-oligomeric, consisting of three different CESA isoforms that are required for assembly of the complex. As a result, *CESA* null mutations have strong phenotypes in Arabidopsis, causing either collapsed xylem in the case of secondary *CESAs* or embryo lethality in the case of two of the primary *CESAs* (Desprez et al., 2007; Persson et al., 2007; Taylor et al., 2003). The six functionally distinct CESA types that form these Arabidopsis CSCs diversified before the divergence of gymnosperms and angiosperms, and are widely shared among seed plants (Yin et al., 2014).

Along with phenotype analysis of *CESA* mutants, analysis of *CESA* co-expression patterns has provided critical information for understanding the composition of the hetero-oligomeric seed plant CSCs. For example, expression analysis has contributed to functional characterization of the CESAs of various plants including poplar (Djerbi et al., 2004), pine (Nairn and Haselkorn, 2005), barley (Burton et al., 2004), maize (Appenzeller et al., 2004), rice (Wang et al., 2010) and *Brachypodium distachyon* (Handakumbura et al., 2013), and dissection of the distinct roles of the ~~partially-redundant~~-6-like AtCESAs, which occupy the same position in primary cell wall specific CSCs, but have different mutant phenotypes (Persson et al., 2007). The secondary cell wall specific *CESAs* are strongly co-expressed and other secondary cell wall associated genes have been identified based on co-regulation with these *CESAs* (Brown et al.,

2005; Hirano et al., 2013; Persson et al., 2005; Ruprecht et al., 2011). Genes co-regulated with primary cell wall specific *CESAs* have also been identified using this approach (Persson et al., 2005)

The moss *Physcomitrella patens* (Hedw.) Bruch & Schimp has multiple *CESA* isoforms and rosette CSCs, but it lacks lignified secondary cell walls characteristic of vascular plants (Roberts et al., 2012). The seven *CESA* genes in *P. patens* have diversified independently from seed plant *CESAs* (Roberts and Bushoven, 2007) and their individual functions are still unknown (Roberts et al., 2012). Understanding how the functions of PpCESAs differ from those of seed plant *CESAs* may provide insight into CSC evolution and the roles of the distinct *CESA* isoforms in CSC assembly and function.

The ease of genetic manipulation in *P. patens* facilitates investigation of gene function (Schaefer, 2002). Mutation analysis has shown that PpCESA5 is required for gametophore formation (Goss et al., 2012) and that a double *PpCESA6/7* knockout has shorter gametophores (Wise et al., 2011). With the exception of an analysis of EST abundances in various *P. patens* cDNA libraries (Nishiyama et al., 2003; Roberts and Bushoven, 2007) and a few focused studies (Goss et al., 2012; Wise et al., 2011), *PpCESA* expression patterns have not been characterized.

We have examined *PpCESA* expression through analysis of lines transformed with *promoter::β-glucuronidase (GUS)* reporters, relative quantitative RT-PCR, and hierarchical cluster analysis of public microarray data to determine whether specific *PpCESAs* are co-expressed as in seed plants and determine whether patterns of expression suggest potential *PpCESA* functions and interactions.



## Materials and Methods:

### Vector construction

Genomic sequences were downloaded from <http://www.cosmoss.org/> and primers with flanking attB1 and attB5r sites for Gateway Multisite cloning (Invitrogen, Grand Island, NY, USA) were designed to amplify approximately 2 kb of nucleotides upstream of the start of each coding sequence (Table S1). Promoters were amplified from 4  $\mu$ L of genomic DNA (Roberts et al., 2011) in 50  $\mu$ L reactions using Phusion High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA) with a 30 s denaturation at 98°C; 35 cycles of 7 s at 98°C, 7 s at 58-68°C (Table S1), and 1 min at 72°C; and a final 5 min extension at 72°C. Using the same PCR conditions, the *GUS* gene was amplified from pRITA (Genbank FB507484.1) and the *PpCESA5* coding sequence was amplified from pdp24095 (RIKEN BRC, Ibaraki, Japan) with primers flanked by attB5 and attB2 sites (Table S1). PpCESA promoters were cloned into pDONR P1-P5r and *GUS* and *PpCESA5* coding sequences were cloned into pDONR P5-P2 using BP Clonase II (Invitrogen).

To construct a destination vector, the si3pTH plasmid containing a hygromycin resistance cassette flanked by 5' and 3' segments of the 108 targeting locus (gift of Pierre-Francois Perroud) was digested with *Sall*, targeting a site between the hygromycin<sup>R</sup> gene and the 3' 108 locus. A Gateway cassette (Invitrogen) was amplified with primers flanked with *XhoI* sites (Table S1) using Platinum Taq (Invitrogen) with a 2 min denaturation at 95°C; 35 cycles of 30 s at 95°C, 30 s at 64°C, and 1 min at 72°C; and a final 5 min extension at 72°C, cloned into PCR TOPO 2.1 (Invitrogen) and sequence verified. The *XhoI* fragment containing the

Gateway cassette was ligated into the *Sall* site of si3 pTH and the Gateway enabled vector (si3-pTH-GW) was propagated in cccb cells (Invitrogen).

To construct the *PpCESApro::GUS* vectors, entry clones containing the *PpCESA* promoters and *GUS* gene were inserted into the si3-pTH-GW destination vector using LR Clonase II Plus (Invitrogen). Entry clones containing the *PpCESA5* promoter and *PpCESA5* coding sequence were inserted in si3-pTH-GW for the rescue vector. All vectors were linearized with *SwaI*, except *PpCESA8pro::GUS*, which was linearized with *PvuII*.

#### Construction and analysis of *promoter::GUS* lines

Wild type *P. patens* strain Gransden 2011 (Rensing et al., 2008) was transformed as described previously (Roberts et al., 2011) and 3 to 7 stable lines from each transformation were analyzed for GUS activity. Homogenized tissue was subcultured at low density on solid BCDAT medium for 6 d to generate protonemal colonies. For analysis of bud and gametophore development, tissue clumps were cultured on solid BCD medium for 1 to 3 weeks (Roberts et al., 2011). Tissue was stained for GUS activity as described previously (Wang et al., 2008) and dissected with fine forceps. Images were captured using a Leica M165FC stereomicroscope with a Leica DFC310FX camera (Leica Microsystems Inc., Buffalo Grove, IL, USA).

#### Primer design for quantitative PCR

*PpCESA* coding sequences were aligned using Clustal with Geneious software (Biomatters Ltd., Auckland, New Zealand). Potential primers (18-23 bp,  $T_m=60^\circ\text{C}$ , 50-400 bp amplicon) were screened for specificity using the NCBI/Primer-Blast tool (Ye et al., 2012). Specificity was tested by PCR against plasmids containing cDNA clones of each of the seven *PpCESAs* using Taq polymerase (New England Biolabs) in 25  $\mu\text{L}$  reactions with 1  $\mu\text{M}$  primers and 1 ng of template with a 2 min denaturation at  $95^\circ\text{C}$ ; 32 cycles of 30 s at  $95^\circ\text{C}$ , 30s at  $60^\circ\text{C}$ ,

and 30 s at 72°C; and a final 5 min extension at 72°C and analysis by gel electrophoresis, and verified by amplifying reverse transcribed *P. patens* RNA (see below) and sequencing the products. Primer concentrations were optimized and tested for efficiency. Primers that amplify actin (*ACT*) and v-Type H<sup>+</sup> translocating pyrophosphatase (*vH+PP*) genes were used as references for all samples (Le Bail et al., 2013).

#### RNA extraction and cDNA synthesis

RNA was extracted from approximately 100 mg of squeeze dried 6 d-old protonemal tissue using an RNeasy Plant Mini Kit (QIAGEN Inc., Venlo, Limburg, Netherlands) or approximately 20 developing gametophores collected with micro dissecting scissors (Electron Microscopy Sciences, Hatfield, PA, USA) from 3 week-old cultures using an RNeasy Micro kit (QIAGEN Inc.). Tissue was frozen in liquid nitrogen with 700 µL of RLT buffer and 100 mg of garnet beads (BioSpec Products, Bartlesville, OK, USA) and disrupted with a Tissue Lyzer (QIAGEN Inc.) with a frequency of 30 Hz for 10 min. DNA was removed on column using RNase-Free DNase (QIAGEN Inc.). For hormone treatments, RNA was extracted from tissue grown from protoplasts on PRMB for 3 d (Roberts et al., 2011) and transferred to BCD, BCDAT, BCD + 3 µM benzylaminopurine (cytokinin) or BCD + 1 µM naphthaleneacetic acid (axuin) for 7 d. All samples were collected in biological replicates of 3.

RNA quality was tested using a Bioanalyzer with RNA 6000 Nano chip (Agilent Technologies, Santa Clara, CA, USA). RNA with RIN quality scores of >7 was reverse transcribed using M-MuLV transcriptase (New England Biolabs) according to manufacturer's instructions and diluted 1:1 with nuclease-free water.

#### Quantitative PCR

cDNA samples were tested in duplicate with no template controls in every run and a no reverse transcriptase control for each sample. The 20  $\mu$ L qPCR reactions with SYBR Green I Master Mix (Hoffmann-La Roche AG, Basel, Switzerland), 25 ng of cDNA, and primers at optimized concentrations (Table S2) were analyzed on a Roche Lightcycler 480 Multiwell Plate 96. Reactions were denatured for 10 min at 95°C and subjected to 32 cycles of 10 s at 95°C, 20 s at 60°C, and 20 s at 72°C for quantification, followed by 5 s denaturation at 95°C, 1 min annealing at 65°C, and ramping at 2.2°C/s to 97°C for melting curve analysis. Target/average reference cross point ratios were calculated for each sample and standard errors were calculated for biological replicates. For statistical analysis, data were log transformed to meet normal distribution and homogeneity and subjected to one-way ANOVA and Tukey-Kramer T test. Protonema and hormone treatment experiments were repeated with similar results.

#### Microarray analysis

*PpCESA* expression data from public microarray experiments (Hiss et al., 2014) were analyzed using the hierarchical clustering tool for anatomy, development, and perturbations in Genevestigator (Zimmermann et al., 2004). The *PpCESAs* are represented on the arrays as Phypa\_105213 (*PpCESA5*), Phypa\_233978 (*PpCESA8*), Phypa\_202222 (*PpCESA3*), Phypa\_213586 (*PpCESA4*), Phypa\_192909/ Phypa\_192906 (*PpCESA6/7*), and Phypa\_169568 (*PpCESA10*).

## **Results:**

#### Promoter::GUS localization

All lines stably transformed with *promoter::GUS* reporter vectors were examined for consistency and two were used for detailed analysis of each promoter. Protonemal filaments

grown on BCDAT were sampled daily from day 4 to day 7 after plating. No differences in *PpCESA* expression patterns were seen during this 4 d time course. However, 6 d-old cultures provided the most complete representation of primary and branching filaments with few senescing filaments. GUS activity shows that all seven *PpCESA* promoters were active in protonema (Figure 1A-H).

*PpCESA* expression throughout gametophore development was examined in early buds and buds with leaves sampled at 1 week, young gametophores with 6-10 leaves sampled at 2 weeks, and mature gametophores that had stopped producing new leaves sampled at 3 weeks. All *PpCESA* promoters except *PpCESA4pro* and *PpCESA10pro* were active in early buds (Figure 2A-H) and the apical meristems of buds with leaves as shown for *PpCESA3pro* (Figure 2I). *PpCESA4pro* and *PpCESA10pro* activity was detected only in the axillary hairs of buds with 2 to 3 leaves as shown for *PpCESA4pro* (Figure 2J). In gametophores with 6 to 10 leaves, promoters for *PpCESA3*, *PpCESA5*, *PpCESA6*, *PpCESA7* and *PpCESA8* remained active in meristems and were also active in stems and rhizoids (Figure 1I-P). *PpCESA6* was strongly expressed at the base of the stem and throughout the rhizoids (Figure 1L). Lines transformed with *PpCESA4pro::GUS* and *PpCESA10pro::GUS* revealed little or no GUS activity in gametophore meristems, stems or rhizoids (Figures 1J,O), although *PpCESA10pro* was active in leaf margins (Figure 1O). Mature gametophores were examined, but no promoter activity was detected (not shown).

Young to fully expanded leaves with prominent midribs were dissected from gametophores with 6 to 10 leaves and examined with a compound microscope. *PpCESA3*, *PpCESA5*, and *PpCESA6* promoters were active in emerging leaves (Figure 1I,K,L) and near the base of expanding leaves with some staining in developing midribs (Figure 1Q,S,T). In lines

transformed with *PpCESA7pro::GUS*, faint staining was seen at the base of young and expanding leaves and in midribs of expanding leaves (Figure 1M,U). *PpCESA8pro::GUS* lines showed relatively strong staining in young gametophore leaves (Fig. 1N) and midribs of expanding leaves (Figure 1V). *PpCESA10pro* was active in the margins of young leaves (Fig. 1O) and bases of expanding leaves (Figure 1W). No staining was seen in the gametophore leaves in the *PpCESA4pro::GUS* lines (Figure 1J, 1R). No *PpCESA* promoters were active in the midribs of mature leaves (not shown).

The functionality of the cloned *PpCESA5* promoter was confirmed by complementing the *ppcesa5KO* mutant, which lacks normal gametophores (Goss et al., 2012), with the *PpCESA5pro::PpCESA5* vector (Figure 1Y,Z). The other promoters could not be tested because other single *PpCESA* knockouts produced no obvious morphological phenotypes (Wise et al. (2011) and unpublished data).

#### *PpCESA* expression levels measured by RT-qPCR

Testing of primer specificity by PCR showed that all primer pairs amplified fragments of the expected size when paired with their corresponding cDNA plasmid template and no amplification was seen when primers were paired with other *PpCESA* cDNA templates or in no template control reactions (Figure S1). All primer pairs had efficiencies of 90% to 110% (Table S1). Despite repeated attempts, we were unable to design efficient primers that specifically amplified *PpCESA6*, which is nearly identical to *PpCESA7* throughout the CDS and UTR sequences (Wise et al., 2011).

RT-qPCR performed on RNA isolated from cultured protonemal tissue and leafy gametophores isolated by dissection show that *PpCESA10* is more highly expressed in the protonemal tissue ( $P < 0.0001$ ) and *PpCESA3*, *PpCESA5*, and *PpCESA7* are more highly

expressed in gametophores ( $P < 0.005$ ) (Figure 3A). To test whether differences in *PpCESA* expression extrapolated from analysis of EST abundances (Nishiyama et al., 2003; Roberts and Bushoven, 2007) are valid, *PpCESA* expression was measured by RT-qPCR in tissues that had been induced to differentiate on media containing different nitrogen sources (ammonium and nitrate (BCDAT), which stimulates protonemal growth, or only nitrate (BCD), which promotes gametophore development) and hormone supplements (cytokinin, which promotes over-production gametophores, or auxin, which promotes over-production of rhizoids) (Cove and Quatrano, 2004; Nishiyama et al., 2003). *PpCESA8* is up-regulated in tissue cultured on BCD vs. BCDAT medium, whereas all other *PpCESAs* are expressed at equal levels on both media (Figure 3B). The auxin treatment resulted in significant up-regulation of *PpCESA3*, *PpCESA4*, *PpCESA7* and *PpCESA8* compared to BCDAT controls, whereas only *PpCESA7* and *PpCESA8* were up-regulated compared to BCD controls. The expression of *PpCESA5* and *PpCESA10* was not significantly different in cultures with and without auxin (Figure 3B). The cytokinin treatment resulted in significant up-regulation of all *PpCESAs*, except *PpCESA10*, compared to both BCDAT and BCD controls.

#### Microarray analysis of *PpCESA* expression

Microarray expression profiles of the 7 *PpCESAs* (including profiles of *PpCESA6* and *PpCESA7* combined) analyzed relative to anatomy and development showed high expression of *PpCESA10* and *PpCESA5* in protonema and in association with protonemal development, including germination of protoplasts and spores (Figures S2, S3). In contrast, all *PpCESAs* except *PpCESA10* were expressed at moderate to high levels in gametophores and in association with gametophore growth and development. Although phyllids (i.e. leaves) are part of the gametophore, the phyllid experiments included in the array data involved

dedifferentiation of excised phyllids and initiation of protonemal growth (Hiss et al., 2014). Thus, expression of *PpCESA10* in these samples confirms association with protonemal development. Many of the experimental perturbations tested had little effect on *PpCESA* expression (Figure S4). However, up-regulation of *PpCESA10* and *PpCESA8* was detected at most time points in the phyllid dedifferentiation experiment, whereas *PpCESA5* was highly up-regulated in the first two hours after leaf excision and down-regulated at later time points. *PpCESA8* and *PpCESA4* were down-regulated by high light and dehydration/rehydration treatments. *PpCESA3*, *PpCESA5* and *PpCESA8* were down-regulated in the dark.

#### **Discussion:**

Numerous rosette CSCs were visible with freeze fracture electron microscopy in protonemal tips of *P. patens* and the related species *Funaria hygrometrica* (Reiss et al., 1984; Roberts et al., 2012) indicating the importance of cellulose synthesis in protonemal development. High expression detected by RT-qPCR shows that *PpCESA10* is associated with protonemal cellulose synthesis. However, promoter-GUS analysis indicates that all *PpCESAs* may participate in deposition of the protonemal cell wall. This is consistent with microarray data (Hiss et al., 2014) analyzed through Genevestigator (Nebion AG), which show high *PpCESA10* and low to moderate expression of other *PpCESAs* in protonema and in association with their development (Figures S2,S3).

Cell wall synthesis in gametophores appears to involve multiple *PpCESAs*, with expression of isoforms changing throughout development. All *PpCESAs* except *PpCESA10* are expressed at moderate to high levels during gametophore development based on analysis of microarray data (Figures S2, S3). RT-qPCR corroborated this and revealed that *PpCESA3*,



*PpCESA5*, and *PpCESA7* are more highly expressed in gametophores compared to protonema (Figure 3A). RT-qPCR also showed up-regulation of all but *PpCESA10* in cytokinin-treated cultures that overproduced gametophores (Figure 3B), consistent in general with analysis of ESTs (Nishiyama et al., 2003; Roberts and Bushoven, 2007). Expression of PpCESA3, PpCESA5, PpCESA6, PpCESA7, and PpCESA8 is associated with cell division in the basal regions of developing leaves (Harrison et al., 2009), gametophore buds, and meristems (Figures 1 and 2). Expression of *PpCESA3*, *PpCESA6*, *PpCESA7*, and *PpCESA8* in midribs may indicate participation in thickening of midrib cell walls (Figure 1). *PpCESA3* co-expression with NAC transcription factor *PpVN7* (Xu et al., 2014) is also consistent with a role in this process. *PpCESA10* is expressed transiently in the margins of leaves that are just beginning to expand, consistent with a specific role in gametophore development.

PpCESA6, PpCESA7 and PpCESA8 appear to be the main contributors to cellulose synthesis in rhizoids based on up-regulation of *PpCESA7* and *PpCESA8* by auxin compared to BCD (Figure 3B) and over representation of *PpCESA6* and *PpCESA8* ESTs in libraries from auxin-treated tissue (Roberts and Bushoven, 2007). Strong *PpCESA6pro* activity (Figure 1L) and PpCESA6-GFP localization in rhizoids (Wise et al., 2011), also suggest that PpCESA6 is involved in rhizoid development. However, analysis of *ppcesa6KO*, *ppcesa7KO*, (Wise et al., 2011) and *ppcesa8KO* (unpublished data) have shown no defects in rhizoid development indicating possible redundant function in rhizoid development (Wise et al., 2011).

We anticipated that expression profiling would indicate which PpCESAs interact within hetero-oligomeric CSCs, as was the case with *Arabidopsis* (Brown et al., 2005; Persson et al., 2005). However, no strong co-expression patterns were detected. Whereas *PpCESA4* and *PpCESA10* are down-regulated in gametophore buds and meristems, only *PpCESA10* is

significantly up-regulated in protonema, and cluster analysis of microarray data does not show co-expression. *PpCESA5* is expressed in gametophore meristems as expected based on the mutant phenotype (Goss et al., 2012). Although *PpCESA3*, *PpCESA6*, *PpCESA7*, and *PpCESA8* are also expressed in gametophore meristems, knockout mutants of *PpCESA6* and *PpCESA7* (Wise et al., 2011) and *PpCESA3* and *PpCESA8* (unpublished data) do not phenocopy *ppcesa5KO*. Thus, *P. patens* differs from *Arabidopsis*, in which null mutations in any of the *CESAs* expressed in secondary cell wall synthesis (i.e. *AtCESA4*, *AtCESA7* and *AtCESA8*) result in the same collapsed xylem phenotype and null mutations in either of two *CESAs* expressed in primary cell wall synthesis (i.e. *AtCESA1* and *AtCESA3*) are embryo lethal (Persson et al., 2007; Taylor et al., 2003). Instead, overlapping *CESA* expression and lack of strong phenotypes for most single *PpCESA*KOs are consistent with functional interchangeability of *PpCESAs*. Phylogenetic analysis of *CESAs* indicates that *PpCESAs* diversified and evolved specialized functions independently from seed plant *CESAs* and that the common ancestor of mosses and seed plants had a single *CESA* gene, and therefore homo-oligomeric CSCs (Roberts and Bushoven, 2007). This implies that the evolution of seed plant and moss *CESA* families involved different patterns of functional specialization and that obligate hetero-oligomeric CSCs have not evolved in the moss lineage.

**Supporting information:**

Supplemental File 1. Supplemental tables

Supplemental File 2. Primer specificity test

Supplemental File 3. Cluster analysis of public microarray data

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**Figure Legends:****Figure 1. *PpCESA* expression analyzed by histochemical staining. (A-G)**

All *PpCESApro::GUS* lines grown on BCDAT medium for 6 d and stained for 6 h show expression in protonema. **(I-O)** *PpCESApro::GUS* lines grown on BCD for 2 weeks and stained for 16 h show expression in **(I,K,M,N)** gametophore meristems, leaves, and young rhizoids **(L)** throughout gametophore stems and older rhizoids, and also in leaves or **(O)** only within leaves or **(J)** no expression. **(Q-W)** Leaves from gametophores with 6-10 leaves from *PpCESApro::GUS* lines sampled at 2 weeks and stained for 16 h show **(Q,S,T,V)** strong staining in the leaf bases, **(U)** weak staining in the leaf bases, or **(Q,T,U,V)** weak staining in developing veins. **(H,P,X)** No staining in negative control line transformed with a vector containing *GUS* with no promoter. **(Y)** *cesa5KO* rescued with *PpCESA5pro::PpCESA5* showing gametophores and **(Z)** negative control *cesa5KO* transformed with empty si3pTH vector without gametophores. Bars shown in A for A-H, I for I-P, Q for Q-X and Y for Y-Z.

**Figure 2. *PpCESA* expression in buds analyzed by histochemical staining.**

*PpCESApro::GUS* lines grown on BCD for 1 week and stained for 6 h show expression throughout young buds in **(A)** *PpCESA3pro::GUS*, **(C)** *PpCESA5pro::GUS*, **(D)** *PpCESA6pro::GUS*, **(E)** *PpCESA7pro::GUS*, and **(F)** *PpCESA8pro::GUS*. No staining was seen within the buds in **(B)** *PpCESA4pro::GUS*, **(G)** *PpCESA10pro::GUS*, and **(H)** negative control *GUS* lines. Expression in **(I)** the apical meristem of older buds with leaves in *PpCESA3pro::GUS* and **(J)** axillary hairs (arrows) in *PpCESA4pro::GUS*.

Figure 3. **RT qPCR analysis of CESA expression in *P. patens*.** *PpCESA* expression levels relative to *PpVhpp* and *PpACT* in **(A)** 6-day-old protonemal cultures and developing gametophores dissected from 3-week-old cultures of *P. patens*, *PpCESA10* is more highly expressed in protonemal tissue and *PpCESA3*, *PpCESA5*, and *PpCESA7* are more highly expressed in gametophores, and **(B)** cultures grown on BCDAT, BCD, BCD+auxin, and BCD+cytokinin for 7 days. *PpCESA8* has higher expression on BCD medium, which induces gametophore development, while all other *PpCESAs* have equivalent expression on both BCD and BCDAT, which induces protonemal growth. *PpCESA3*, *PpCESA4*, *PpCESA7*, and *PpCESA8* were up-regulated in auxin-treated tissues that over-produce rhizoids and *PpCESA3*, *PpCESA4*, *PpCESA5*, *PpCESA7*, and *PpCESA8* were up-regulated in cytokinin-treated tissues that over-produce gametophores.

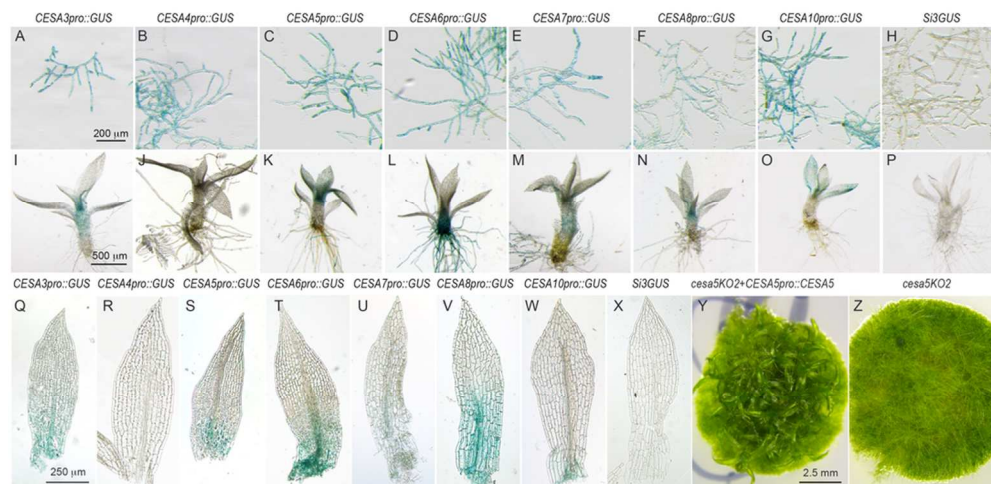


Figure 1. PpCESA expression analyzed by histochemical staining. (A-G) All PpCESApro::GUS lines grown on BCDAT medium for 6 d and stained for 6 h show expression in protonema. (I-O) PpCESApro::GUS lines grown on BCD for 2 weeks and stained for 16 h show expression in (I,K,M,N) gametophore meristems, leaves, and young rhizoids (L) throughout gametophore stems and older rhizoids, and also in leaves or (O) only within leaves or (J) no expression. (Q-W) Leaves from gametophores with 6-10 leaves from PpCESApro::GUS lines sampled at 2 weeks and stained for 16 h show (Q,S,T,V) strong staining in the leaf bases, (U) weak staining in the leaf bases, or (Q,T,U,V) weak staining in developing veins. (H,P,X) No staining in negative control line transformed with a vector containing GUS with no promoter. (Y) *cesa5KO* rescued with PpCESA5pro::PpCESA5 showing gametophores and (Z) negative control *cesa5KO* transformed with empty si3pTH vector without gametophores. Bars shown in A for A-H, I for I-P, Q for Q-X and Y for Y-Z. 92x45mm (300 x 300 DPI)

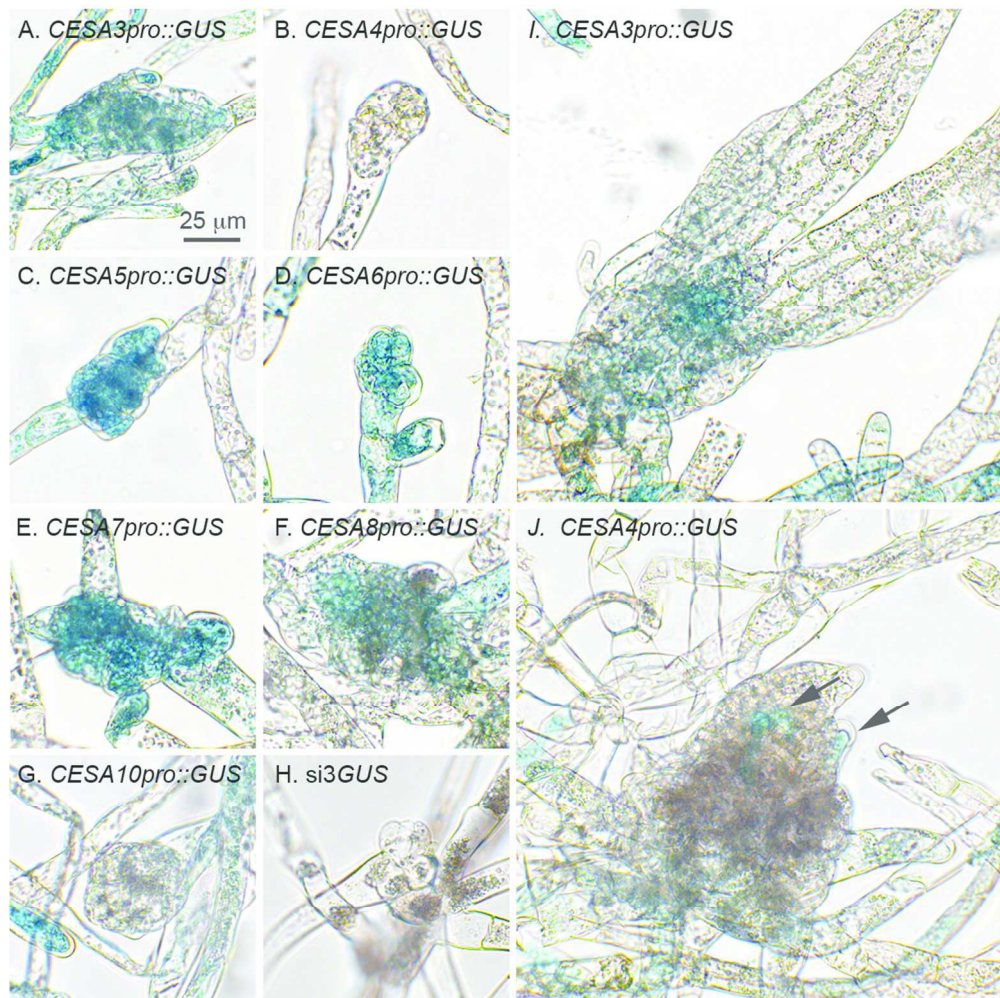


Figure 2. PpCESA expression in buds analyzed by histochemical staining. PpCESApro::GUS lines grown on BCD for 1 week and stained for 6 h show expression throughout young buds in (A) PpCESA3pro::GUS, (C) PpCESA5pro::GUS, (D) PpCESA6pro::GUS, (E) PpCESA7pro::GUS, and (F) PpCESA8pro::GUS. No staining was seen within the buds in (B) PpCESA4pro::GUS, (G) PpCESA10pro::GUS, and (H) negative control GUS lines. Expression in (I) the apical meristem of older buds with leaves in PpCESA3pro::GUS and (J) axillary hairs (arrows) in PpCESA4pro::GUS.

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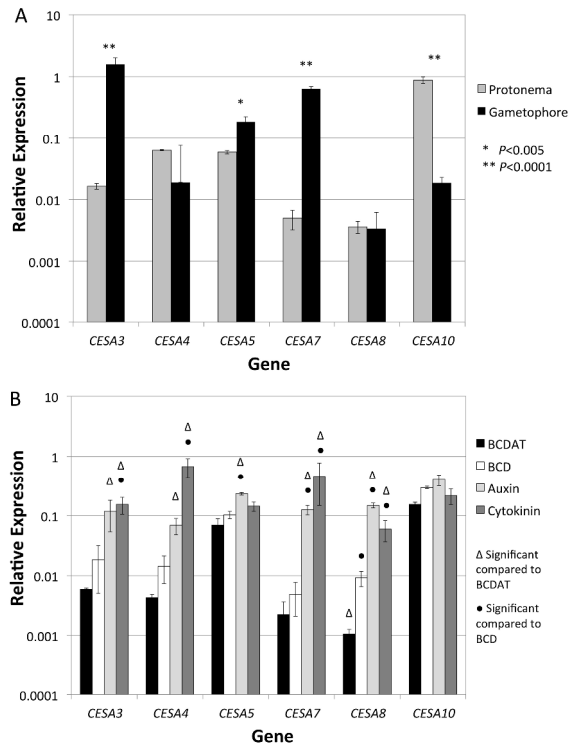


Figure 3. RT qPCR analysis of CESA expression in *P. patens*. PpCESA expression levels relative to PpVhpp and PpACT in (A) 6-day-old protonemal cultures and developing gametophores dissected from 3-week-old cultures of *P. patens*, PpCESA10 is more highly expressed in protonemal tissue and PpCESA3, PpCESA5, and PpCESA7 are more highly expressed in gametophores, and (B) cultures grown on BCDAT, BCD, BCD+auxin, and BCD+cytokinin for 7 days. PpCESA8 has higher expression on BCD medium, which induces gametophore development, while all other PpCESAs have equivalent expression on both BCD and BCDAT, which induces protonemal growth. PpCESA3, PpCESA4, PpCESA7, and PpCESA8 were up-regulated in auxin-treated tissues that over-produce rhizoids and PpCESA3, PpCESA4, PpCESA5, PpCESA7, and PpCESA8 were up-regulated in cytokinin-treated tissues that over-produce gametophores.

609x1219mm (300 x 300 DPI)

Table S1: Primers used for *PpCESApromoter::GUS* vector construction. Sequence ID numbers are from [www.cosmoss.org](http://www.cosmoss.org) except as indicated.

Primer Name	Sequence ID	Sequence	Annealing temp.
CesA3PROattB1	Pp1s8_137V6	GGGGACAAGTTTGTACAAAAAAGCAGGCTAATACACACACAGCGTCCCAAT	58°C
CesA3PROattB5r		GGGGACAACCTTTGTATACAAAAGTTGTGCTGCAACGCCACTCCGCT	
CesA4PROattB1	Pp1s90_244V6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGATTGATTGTTGTGCGTGAT	59°C
CesA4PROattB5r		GGGGACAACCTTTGTATACAAAAGTTGGATGCAAGAATTTCTTTTCC	
CesA5PROattB1	Pp1s30_48V6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGCCTTGTCTTATCGACTGC	64°C
CesA5PROattB5r		GGGGACAACCTTTGTATACAAAAGTTGCGCTCACCGGCGCTGCAACA	
CesA6PROattB1	Pp1s189_96V6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTGAGGGATCCATTCCAGTT	64°C
CesA6PROattB5r		GGGGACAACCTTTGTATACAAAAGTTGGCTTCCCTAACTCCACCACT	
CesA7PROattB1	Pp1s189_92V6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTCGTGTACAATATCGCATCAT	64°C
CesA7PROattB5r		GGGGACAACCTTTGTATACAAAAGTTGGCCGCAAACCACCTTC	
CesA8PROattB1	Pp1s112_75V6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTTTGAGCTTTGAGCAATGTTGG	62°C
CesA8PROattB5r		GGGGACAACCTTTGTATACAAAAGTTGTGCAATACGACGCCGCTAGC	
CesA10PROattB1	Pp1s213_4V6	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTTACCTCGCTGTCTGTC	64°C
CesA10PROattB5r		GGGGACAACCTTTGTATACAAAAGTTGGCTGCCGAAATCCCTCCCTC	
GUSattB5	Genbank	TATCATCTCGAGATTACTGCAGGTCGAGCCCACTGG	62°C
GUSattB2	FB507484.1	ATCACAAGTTTGTACAAAAAAGCTGAACGCTCGAGTGAGCTG	
CesA5attB5	Pp1s30_48V6	GGGGACAACCTTTGTATACAAAAGTTGCGATGGAGGCTAATGCAGGCCTTAT	68°C
CesA5attB2		GGGGACCACTTTGTACAAGAAAGCTGGGTACTAACAGCTAAGCCCGCACTCGAC	

Table S2: Primers used for RT-qPCR.

Description		Sequence	Amplicon size	Primer concentrations ( $\mu\text{M}$ )	Annealing temp.
<i>PpCESA3</i>	cesa3f2	TCTAATTGAGCCGAGGGCACC	172	0.50	86°C
	cesa3r	ATCCTCCGGCACCTTCATTG			
<i>PpCESA4</i>	cesa4f2	CGGTCAATTTGGACAACCATG	102	0.40	80°C
	cesa4r2	GCGTTGCAGATAGCATCACT			
<i>PpCESA5</i>	cesa5df	TGCAGGCTCACACAATCGTA	129	0.75	85°C
	cesa5cR	GTC AACCGTGACTCCCACAT			
<i>PpCESA7</i>	cesa7aF	GCGAATGCAGGGCTGCTG	92	0.50	86°C
	cesa7bR	ACATTACTCAACGGCCTCGG			
<i>PpCESA8</i>	cesa8F5	AATTCACGGGCCACGGCCTGA	135	0.60	83°C
	cesa8R4	GCAAGTGCGACAACTGGAAAGG			
<i>PpCESA10</i>	cesa10f2	GGAGATTGACTCATGCCACCT	194	0.85	85°C
	cesa10r2	AACCTCCCTCTCCACTTGCT			

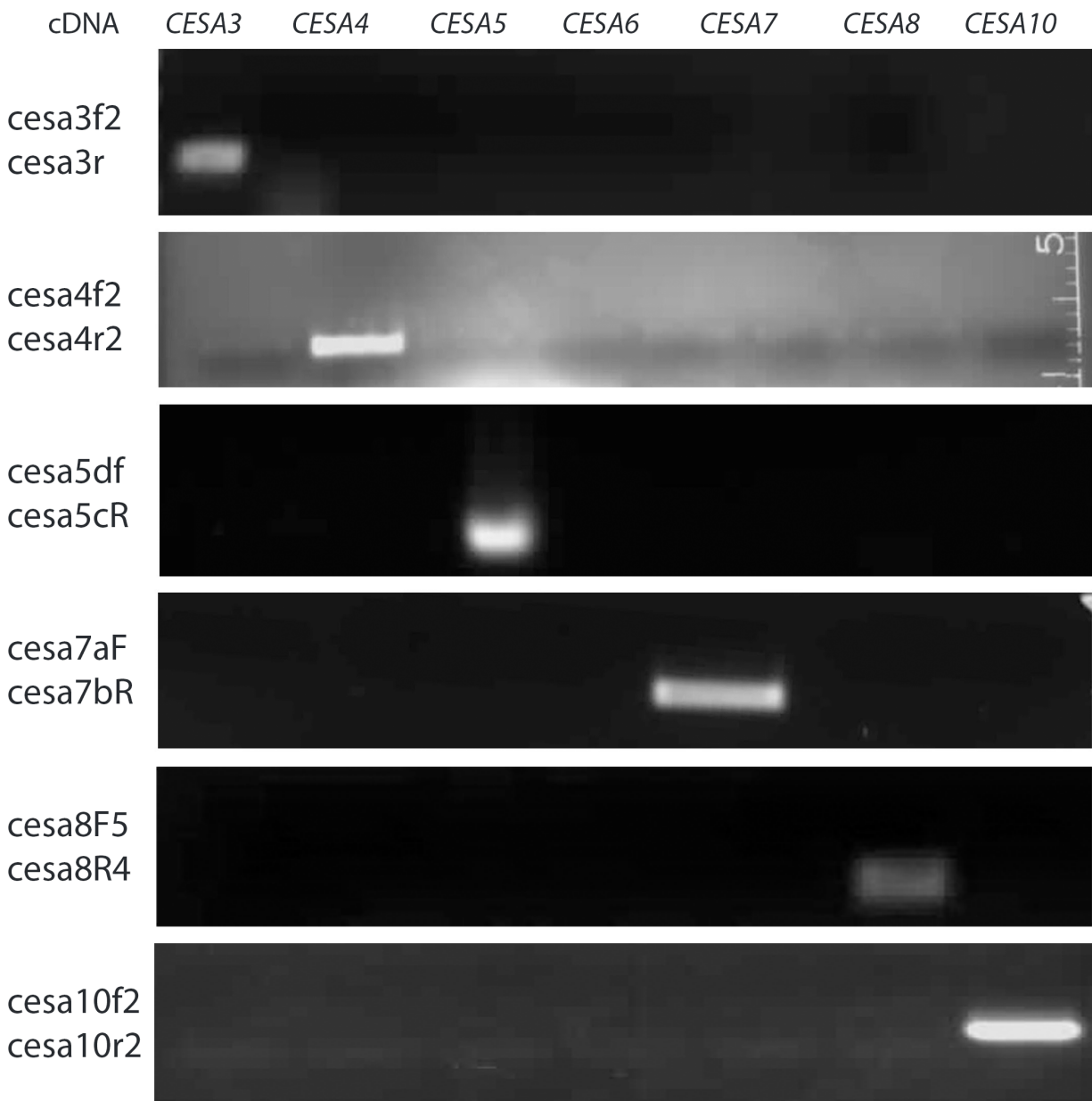


Figure S1. Primer specificity test. Primer pairs (left) were tested for specificity by PCR using plasmids containing full-length *PpCESA* cDNA clones (top) as templates in 25  $\mu$ L PCR reaction. 10  $\mu$ L of each PCR reaction was run on a 2% agarose gel at 100V in 1x TAE buffer. Primers amplified only the expected targets. All visible bands on the gels are shown.





**Dataset:** 3 developmental stages (sample selection: PP-SAMPLES-0)  
6 genes (gene selection: PP-GENES-0)

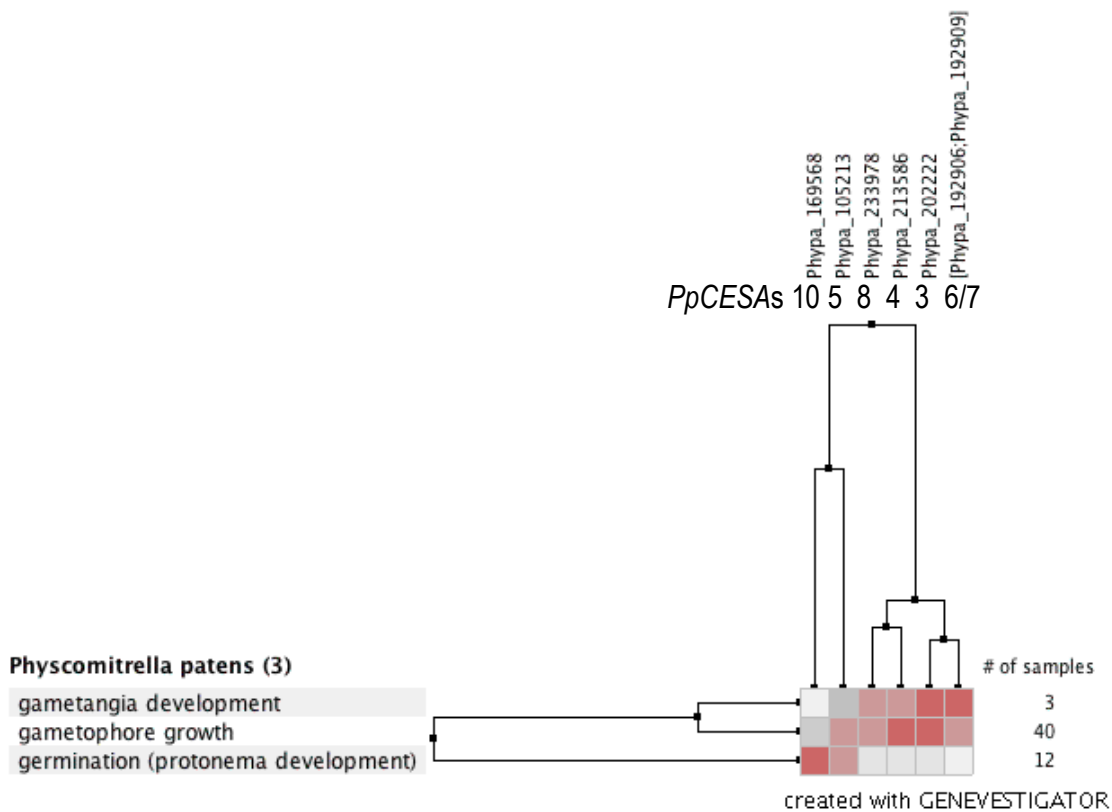


Figure S3. Expression matrix of *PpCESAs* clustered by gene and by developmental process.

**Dataset:** 26 perturbations (sample selection: PP-SAMPLES-0)  
 6 genes (gene selection: PP-GENES-0)

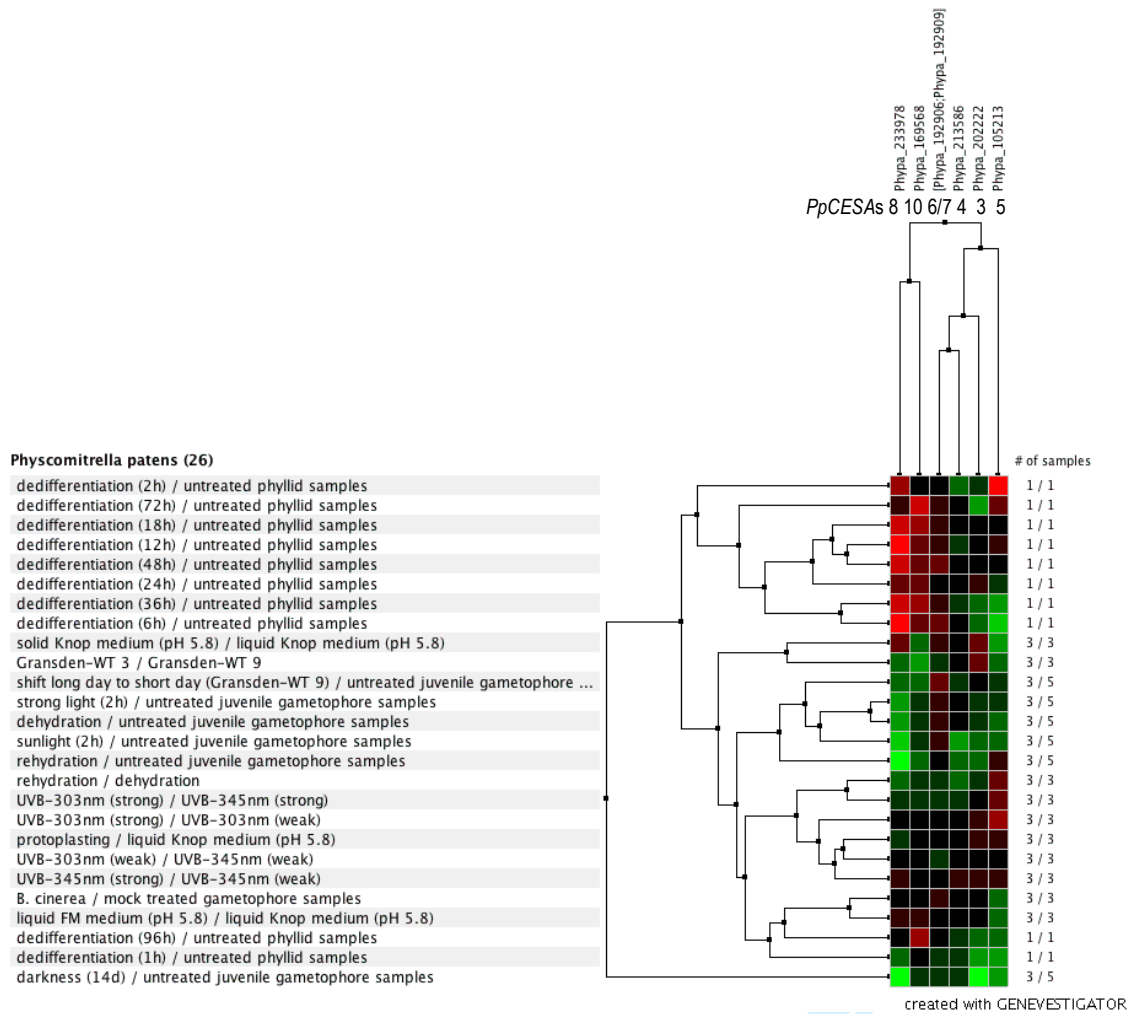
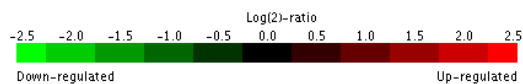


Figure S4. Expression matrix of *PpCESAs* clustered by gene and by experimental perturbation.