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A Complementation Assay for in Vivo Protein Structure/Function Analysis in *Physcomitrella patens* (Funariaceae)

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Protocol Note

A complementation assay for in vivo protein structure/function analysis in *Physcomitrella patens* (Funariaceae)[1]

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- *Premise of the study:* A method for rapid in vivo functional analysis of engineered proteins was developed using *Physcomitrella patens.*
- *Methods and Results:* A complementation assay was designed for testing structure/function relationships in cellulose synthase (CESA) proteins. The components of the assay include (1) construction of test vectors that drive expression of epitope-tagged PpCESA5 carrying engineered mutations, (2) transformation of a *ppcesa5* knockout line that fails to produce gametophores with test and control vectors, (3) scoring the stable transformants for gametophore production, (4) statistical analysis comparing complementation rates for test vectors to positive and negative control vectors, and (5) analysis of transgenic protein expression by Western blotting. The assay distinguished mutations that generate fully functional, nonfunctional, and partially functional proteins.
- *Conclusions:* Compared with existing methods for in vivo testing of protein function, this complementation assay provides a rapid method for investigating protein structure/function relationships in plants.

Key words: cellulose synthase; complementation assay; gametophore development; *Physcomitrella patens*; protein structure/function relationships.

Protein structure/function relationships can be investigated using a structural model to generate hypotheses and an in vitro reconstitution assay for functional analysis of protein variants. However, this approach is only available for some proteins. For example, elucidation of the structure/function relationships for bacterial cellulose synthases has progressed due to two recent technical advances—solving of the crystal structure of *Rhodobacter sphaeroides* (van Niel 1944) Imhoff et al. 1984 cellulose synthase (Morgan et al., 2013) and in vitro reconstitution of functional cellulose synthase from purified *R. sphaeroides* proteins (Omadjela et al., 2013). A computational model for the cytoplasmic region of a plant cellulose synthase (designated CESA) from *Gossypium hirsutum* L. (Sethaphong et al., 2013) has been used to postulate functions of the structural features that distinguish CESAs from bacterial cellulose synthases (Slabaugh et al., 2014a). However, methods for routine in vitro assay of CESA activity are not available for functional testing (Guerriero et al., 2010).

In vivo complementation assays provide alternatives to in vitro assays for protein functional analysis. This approach was used effectively to test an engineered point mutation in an *Arabidopsis thaliana* (L.) Heynh. *CESA* that was predicted to impair protein function based on computational modeling (Slabaugh et al., 2014b). However, due to the time and space required for genetic transformation and backcrossing, *A. thaliana* is not ideal for screening large numbers of mutations. As in all mosses, the protonema and gametophores of *Physcomitrella patens* (Hedw.) Bruch & Schimp. are haploid. Thus, mutant phenotypes can be detected immediately without the need for backcrossing to produce homozygous diploid lines. This makes *P. patens* a faster alternative to *A. thaliana* for assays that involve genetic transformation (Cove, 2005; Cove et al., 2006).

Physcomitrella patens cell walls contain the same classes of polysaccharides as vascular plant cell walls, with some differences in side-chain structure (Roberts et al., 2012). Also, *P. patens* has rosette cellulose synthesis complexes (Roberts et al., 2012) and seven *CESA* genes (Roberts and Bushoven, 2007). However, consistent with the absence of lignified vascular tissue in mosses, the *PpCESA* genes are not orthologous to the *CESA* genes that synthesize primary and secondary cell walls in seed plants (Roberts and Bushoven, 2007). We have previously generated *ppcesa5* knockout lines that are deficient in gametophore production. In these lines, the gametophore buds typically produce irregular clumps of tissue, but occasionally form aberrant gametophores consisting of a few small deformed leaves with irregular phyllotaxy (Goss et al., 2012). When transformed with a vector that drives constitutive expression of

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the wild-type PpCESA5 protein, these lines produces normal gametophores (Goss et al., 2012). This provides a platform for developing a complementation assay to test CESA proteins that have been modified by site-directed mutagenesis with the extent of rescue of the mutant phenotype providing a measure of protein function.

Here we describe a complementation assay in *P. patens* for analysis of structure/function relationships in plant CESA proteins that is rapid and has modest space requirements. Although designed for analysis of CESA proteins, this assay could be adapted to study structure/function relationships of other proteins where the knockout phenotype can be easily scored.

METHODS AND RESULTS

***Vector construction and* ppcesa5*KO-2 transformation*—**Expression vectors carrying a wild-type (positive control) or a mutated (test) *PpCESA5* coding sequence fused at the N-terminus to a triple hemagglutinin (3XHA) epitope tag were constructed using PCR fusion and MultiSite Gateway cloning (Atanassov et al., 2009). To create Gateway entry clones carrying point mutations, gene fragments were amplified from wild-type *PpCESA5* cDNA clone pdp24095 (RIKEN BioResource Center, http://www.brc.riken.jp/lab/epd/Eng/) using primer pairs that added an *att* site at one end and introduced a mutation at the other (Appendix 1; Appendix 2, Fig. A1, A2). The fragments were fused in a single overlap extension reaction (Appendix 2) and cloned into pDONR 221 P5-P2 according to the manufacturer's instructions (Life Technologies, Grand Island, New York, USA). *CESA5* entry clones were sequence verified using primers listed in Appendix 3. To create the *3XHA* entry clone, two oligonucleotides (Appendix 1) were fused, cloned into pDONR 221 P1-P5r (Life Technologies), and sequence verified using primer M13 Forward (−20) (Life Technologies). Each *CESA5* entry clone was transferred along with a *3XHA* entry clone to the pTHAct1Gate destination vector using LR Clonase II Plus as described by the manufacturer (Life Technologies). The pTHAct1Gate vector contains an *Act1* promoter from rice, which drives constitutive expression in *P. patens*, and sequences that target the vector to the *P. patens* 108 locus, which can be disrupted without producing a phenotype (Perroud and Quatrano, 2006).

Protoplasts prepared from mutant *P. patens* line *ppcesa5*KO-2 (Goss et al., 2012) were divided into five or six samples (6×10^5 protoplasts each) and each sample was transformed with a test (R453K, R453D, or R453G), positive wild-type CESA5 expression, or empty pTHAct1 negative control vector (Table 1), following the detailed protocol of Roberts et al. (2011). Regenerated protoplasts were subjected to two rounds of selection (Roberts et al., 2011), which typically resulted in 10–40 vigorously growing, stably transformed lines for each vector (Appendix 2, Fig. A3). Results from an experiment (i.e., five to six transformations using the same batch of protoplasts) were excluded from the statistical analysis when fewer than 10 lines were obtained for the positive or negative control transformations. In cases where one or more test vectors from an otherwise successful experiment yielded fewer than 10 lines, results from only those test vectors were excluded. Isolated colonies, each comprising an independent stably transformed line, were arrayed on solid BCDAT plates (Roberts et al., 2011) and incubated in a growth chamber at 25°C with fluorescent lights at a photon flux density of 60 μM $m^{-2}s^{-1}$ for 7 d.

***Complementation analysis*—**To make the assay as rapid as possible, we evaluated phenotypes using primary colonies derived from independently transformed protoplasts and selected for stable antibiotic resistance. In contrast to tissue subcultured from wild-type and mutant lines, the positive and negative control transformations produced multiple colonies, each representing an independent stably transformed line, that could be directly and quantitatively compared to colonies derived from test transformations. Comparisons were based on the percentage of stably transformed lines that produced gametophores and on gametophore morphology. A dissecting microscope (Meiji EMZ-TR, Meiji Techno Co. Ltd., Tokyo, Japan) was used at 30× to count the number of lines with and without gametophores. Colonies and individual gametophores were photographed using a stereomicroscope (Leica M165 FC with Leica BFC310 FX camera, Leica Microsystems, Buffalo Grove, Illinois, USA), and individual leaves were photographed using a compound microscope (Olympus BX51, Olympus Corporation, Lake Success, New York, USA) with a Spot Flex camera (Diagnostic Instruments, Sterling Heights, Michigan, USA). The positive control *CESA5* vector complemented the *ppcesa5*KO-2 phenotype, with 78% ± 3.8% of lines (average for seven independent transformations: 138 stably transformed lines) producing gametophores that generally resembled wild-type gametophores (Fig. 1A). When *ppcesa5*KO-2 was transformed with the empty negative control vector, only 13% ± 4.9% of lines (average for seven independent transformations: 182 stably transformed lines) produced gametophores. These were distinctly abnormal with fewer than 10 small misshapen leaves and abnormal phyllotaxy (Fig. 1A) and resembled those that are produced at low frequency by the untransformed *ppcesa5*KO-2 line (Goss et al., 2012). Fewer than 10% of stable lines produced gametophores in a typical negative control transformation, and even when the percentage was higher the gametophores were never normal in appearance. To ensure consistency, colonies were scored for presence and absence of gametophores without considering gametophore morphology. Statistical analysis (see below) confirmed that this was adequate for distinguishing test vectors that complemented or failed to complement the mutant phenotype. However, deviations from expected morphologies were recorded, and this information was used to identify vectors that partially complemented the mutant phenotype (see below).

We tested the complementation assay with expression vectors carrying the *PpCESA5* coding sequence with three different point mutations at R453 (Table 1). The R453K mutation is homologous to *fra6*, a recessive mutation in *A. thaliana* (*AtCESA8*, R362K) that results in decreased fiber wall thickness and cellulose content, but has a less severe phenotype compared to other *atcesa8* mutant alleles (Zhong et al., 2003). Although *AtCESA8* expression is correlated with secondary cell wall deposition and *fra6* is defective in fiber development, the affected residue is conserved (Zhong et al., 2003). Thus, the phenotypes for homologous mutations in different *CESA* genes would be expected to depend on the developmental function of the mutated CESA. When the *ppcesa5*KO-2 line was transformed with the R453K expression vector, the percentage of stable lines producing gametophores was intermediate between the positive and negative control and gametophore morphology was similar to wild type (Fig. 1). When the basic polar R453 residue was instead replaced with an acidic polar D residue (R453D), all stable lines lacked gametophores (Fig. 1). Finally, replacement of R453 with a nonpolar G residue (R453G) yielded results similar to the positive control for both percentage of stable lines with gametophores and gametophore morphology (Fig. 1).

***Statistics and experimental analysis*—**A two-tailed Fisher's exact test of independence (Sokal and Rohlf, 1981) was used for statistical analysis of complementation. Statistics were calculated using the 2 × 2 contingency table at http://vassarstats.net/tab2x2.html and sample calculations were verified manually. We first confirmed that the numbers of colonies with and without gametophores were significantly different for the CESA5 positive control and the empty negative control vectors ($P < 0.0001$, pooled results from seven experiments). Comparison of each test vector to the positive and negative controls using counts pooled from two or three transformations (Fig. 1B) showed that R453K and R453D differed significantly from the positive control ($P < 0.0001$), but not the empty negative control vector ($P = 0.27$ and $P = 0.50$, respectively). In contrast, R453G differed significantly from the negative control ($P < 0.0001$), but not the CESA5 positive control vector ($P = 0.14$). Although R453K did not differ significantly from the negative control vector in the numbers of colonies with and without gametophores, the gametophores that were produced resembled those observed following transformation with the CESA5 positive control vector (Fig. 1A), indicating that the encoded protein may be partially functional. The vectors tested fall into three classes (1) R453G fully complements, (2) R453D does not complement, and (3) R453K partially complements the *cesa5*KO-2 phenotype. Thus, this relatively rapid assay allows us to screen mutations and tentatively identify those that generate functional, nonfunctional, and partially functional proteins. Because these three classes of mutations could be distinguished based on statistical comparison to positive and negative controls, we were able to streamline the assay by not genotyping the

Table 1. Expression vectors used to transform *Physcomitrella patens*.

Name	Expression cassette	Comments
CESA5	*Act1::3XHA-PpCESA5*	Positive control
Empty	None	Negative control
R453K	*Act1::3XHA-ppcesa5*-R453K	Homologous to *fra6*[a]
R453G	*Act1::3XHA-ppcesa5*-R453G	Variant of R453K
R453D	*Act1::3XHA-ppcesa5*-R453D	Variant of R453K

[a]Zhong et al., 2003.

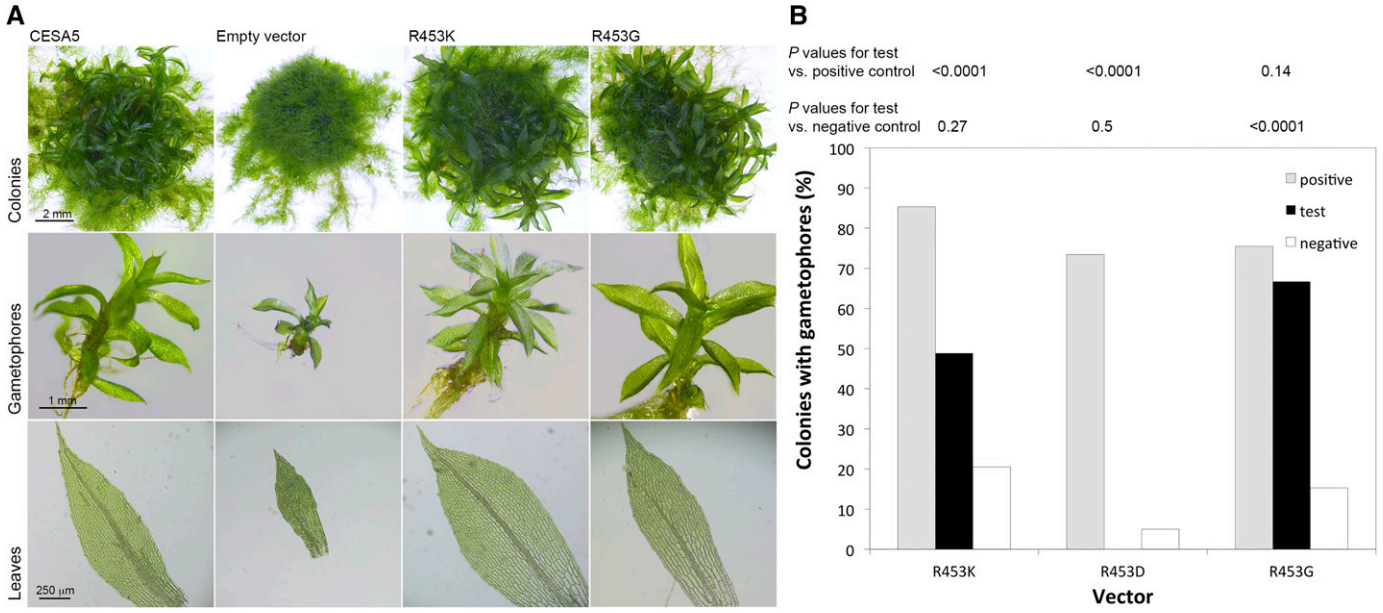

Fig. 1. Functional analysis of engineered CESA proteins by complementation in *Physcomitrella patens*. A. Colonies, gametophores, and gametophore leaves from lines resulting from stable transformation of *ppcesa5*KO-2 protoplasts transformed with a vector driving expression of wild-type PpCESA5 (positive control), an empty vector control or vectors driving expression of PpCESA5 carrying engineered point mutations (R453K and R453G). Scale bars shown in the first column apply to all images in the row. B. *ppcesa5*KO-2 complementation rates for vectors driving expression of PpCESA5 carrying engineered point mutations compared to positive and negative control vectors from the same experiment. Means for two (R453K and R453D) or three (R453G) experiments are shown with *P* values determined using a two-tailed Fisher's exact test of independence.

stable transformants. The failure of gametophore production in 12–39% of the stable lines from transformation with the positive control CESA5 vector can potentially be explained by (1) disruption of the promoter or coding sequence of the vector during integration, which would result in lack of transgene expression, or (2) disruption of another locus that is necessary for gametophore development due to mistargeting of the vector, which would explain lack of complementation when protein expression is detected (see below). This is supported by the observation that transformation of *P. patens* with a gene disruption library resulted in a high proportion of lines with gametophore defects (Egener et al., 2002). These mistargeting events are stochastic (Kamisugi et al., 2006), and statistical analysis confirms that they do not interfere with the ability to distinguish complementing from noncomplementing vectors and partially complementing vectors.

***Protein determination*—**Before concluding that a specific mutation impairs the function of an encoded protein, it is necessary to rule out lack of expression of the full-length transgenic protein as an explanation for failure of the vector to complement the mutant phenotype. This was tested by Western blot analysis of microsomal proteins isolated from arbitrarily selected stable lines representing a small proportion of lines tested for complementation (see Appendix 2 for protein extraction methods). Equal masses of protein extracted from 13 stable CESA5 positive control lines, or 12 stable lines transformed with a particular test vector along with one CESA5 positive control line, were separated by polyacrylamide gel electrophoresis (PAGE) and transferred to membranes for total protein detection with Ponceau S and immuno-detection of hemagglutinin (HA)–tagged protein as described in Appendix 2. As shown in Fig. 2, we tested different protein loadings and found that 7 µg per lane was sufficient for detecting expression of the transgenic protein. Of 13 CESA5 positive control lines tested, 11 produced an HA-tagged protein with the expected molecular mass of 122 kDa (Fig. 2). The two lines in which the transgenic protein was not detected failed to produce gametophores (Fig. 2). However, two lines that expressed the full-length protein also failed to produce gametophores, possibly due to mistargeting of the expression vector to a separate locus required for gametophore development. These data also show that successful complementation occurs over a range of protein expression levels. Full-length transgenic protein was detected in 11 of the 12 lines transformed with R453G or R453K. As noted for CESA5, transgenic protein was undetectable in some, but not all, of the lines that failed to produce gametophores (Fig. 2). Although none of the stable lines transformed with R453D produced gametophores, all of the 12 lines tested produced a full-length transgenic protein (Fig. 2). These results rule out lack of expression as an explanation for the failure of the R453D protein to complement the mutant phenotype. Thus, it can be concluded that, in contrast to the wild-type CESA5 and R453G proteins, the R453D protein is nonfunctional. Because (1) transgenic protein is strongly expressed in a high proportion of stably transformed lines and (2) the wild-type CESA5 protein complements the mutant phenotype over a range of protein expression levels, detailed quantitative analysis of protein expression is not required for detection of mutations that impair protein function.

***Comparison to other methods*—**Complementation assays provide means to test hypotheses about the functions of specific proteins and protein modifications and are particularly useful when a robust in vitro assay is not available, as is the case for CESA proteins (Guerriero et al., 2010). *Arabidopsis thaliana* has been used previously for genetic analysis of engineered CESA variants (Wang et al., 2006; Chen et al., 2010; Bischoff et al., 2011; Slabaugh et al., 2014b). However, *P. patens* has the advantages that transformation, selection, and scoring can be completed in 33 d and less space is required to maintain cultures. In *A. thaliana*, production of transformants requires three months (Zhang et al., 2006), with additional time needed for backcrossing to obtain homozygous lines. Because the dominant phase of the *P. patens* life cycle is haploid, backcrossing in unnecessary.

In *P. patens*, an assay has been developed for using image analysis to screen for mutants that affect protonemal growth and to test for transient complementation of those phenotypes (Bibeau and Vidali, 2014). In contrast to this highly sensitive assay for detecting protonemal growth defects, the assay reported here is suitable for analysis of proteins involved in other aspects of *P. patens* development. It is common practice to use complementation analysis to verify knockout phenotypes in *P. patens*, and cross-species complementation has been used to test for functional conservation of vascular plant and *P. patens* proteins (e.g., Bravo-Garcia et al., 2009). Here we have described a systematic approach for quantitative analysis of complementation based on comparison to positive and negative controls that can be used for functional testing of heterologous genes, as well as engineered mutations.

An effective complementation assay is based on a mutant with a measurable phenotype that is easy to score. The absence of normal gametophores in the *ppcesa5*KO mutants (Goss et al., 2012) is readily observed with a dissecting

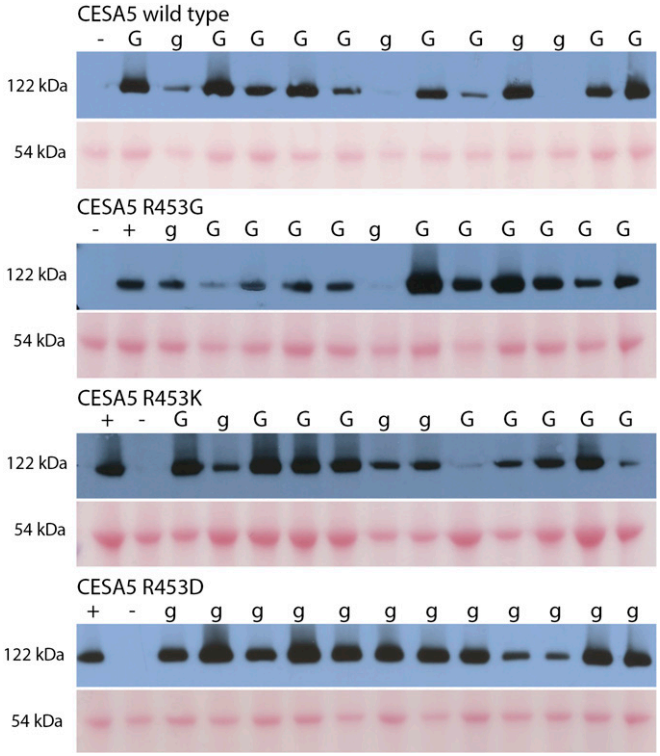

Fig. 2. Western blot analysis of protein expression for *P. patens* lines derived from transformation of *ppcesa5*KO-2 protoplasts with vectors driving expression of wild-type PpCESA5 (positive control) or PpCESA5 carrying engineered point mutation (R453K, R453G, and R453D). Western blots probed with anti-HA are shown above the same blot stained with Ponceau S as a loading control. Protein loading per lane was 7.1 µg (PpCESA5), 9.9 µg (R453G), 33 µg (R453K), or 7.4 µg (R453D). Lines that produced gametophores are indicated by "G," and those producing no gametophores are indicated by "g." Positive (+) and negative (-) control lines are included with lines from each test transformation.

microscope. Scoring for presence or absence of gametophores, combined with statistical analysis comparing test vectors to a positive control vector that drives expression of the wild-type gene and an empty negative control vector, enabled us to discriminate between test vectors that complement and fail to complement the mutant phenotype. The assay also allows detection of mutations that generate partially functional proteins as exemplified by *ppcesa5R453K*. The homologous *fra6* mutation in *A. thaliana* is a partial loss-of-function mutation (Zhong et al., 2003), indicating that results from *P. patens* can be translated to vascular plants. Gene variants that exhibit partial complementation may be of special interest and can be further characterized in *P. patens* or *A. thaliana*. The use of PCR fusion combined with Gateway cloning (Life Technologies) enables rapid construction of vectors that drive expression of proteins with point mutations, insertions, deletions, or chimeric sequences without added recombination or restriction sites (Atanassov et al., 2009). The ability to test expression and molecular mass of the transgenic protein by Western blotting is an essential component of the assay that was facilitated by epitope tagging. However, detailed analysis of protein expression levels is unnecessary due to consistently high transgenic protein expression along with successful complementation over a range of wild-type CESA5 protein expression levels. Although we designed this assay for functional testing of genes that encode CESA proteins, the approach could be adapted for any *P. patens* gene with a mutant phenotype that can be easily scored.

CONCLUSIONS

In summary, the strengths of this complementation assay include (1) a clear phenotype that can be easily scored, (2) inclusion of positive and negative controls and statistical analysis allowing classification of engineered proteins as either fully functional, partially functional, or nonfunctional, (3) engineering of genes through PCR fusion without introduction of restriction or recombination sites, (4) epitope-tagging to facilitate testing for expression of full-length transgenic protein through Western blotting, and (5) efficiency due to rapid development of *P. patens* and no requirements for backcrossing or genotyping. Thus, *P. patens* is well suited for rapid identification of mutations, which can be further investigated in other organisms, if desired.

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Appendix 1. Primers used in vector construction. The *att* sites are shown in yellow, start codons are shown in green, stop codon is shown in red, and engineered mutations are shown in blue.

Primer	Sequence
CESA5-attB5	5′-GGGGACAACTTTGTATACAAAAGTTGCGATGGAGGCTAATGCAGGCCTTAT-3′
CESA5-attB2	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTACTAACAGCTAAGCCCGCACTCGAC-3′
R453G-SF1	5′-GTGAAGGAGCGCGGGGCTATGAAGCGA-3′
R453G-SR1	5′-TCGCTTCATAGCCCCGCGCTCCTTCAC-3′
R453K-SF1	5′-GTGAAGGAGCGCAAGGCTATGAAGCGA-3′
R453K-SR1	5′-TCGCTTCATAGCCTTGCGCTCCTTCAC-3′
R453F-SF1	5′-GTGAAGGAGCGCGATGCTATGAAGCGA-3′
R453F-SR1	5′-TCGCTTCATAGCATCGCGCTCCTTCAC-3′
3XHA-attB1	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACCATGGCCTACCCCTACGATGTGCCCGATTACGCCTACCCATACGATGTGCCCGATTACGCT-3′
3XHA-attB5r	5′-GGGGACAACTTTTGTATACAAAGTTGTGGCGTAATCGGGCACATCGTAGGGGTAAGCGTAATCGGGCACATCGTATGGGTAGGCGTAATCGGGCACATC-3′

Appendix 2. Benchtop protocol for the *Physcomitrella patens* complementation assay.

Vector construction

PCR fusion for Gateway: This protocol was modified from Atanassov et al. (2009).

1. Design attB5 and attB2 primers that include the *att* sites along with sequences from your gene of interest, including the start and stop codons, and SF1 and SR1 primers that include an engineered mutation with about 12 nucleotides upstream and downstream of the mutation (Appendix 1, Fig. A1).
2. Amplify fragments F1 and F2 (Fig. A1) in 50-µL reactions using Phusion DNA polymerase (M0530S, New England Biolabs, Ipswich, Massachusetts, USA) and recommended cycling conditions. We used a 25-s annealing step at 68°C, but this may need to be adjusted depending on the primers used.

F1 PCR mix:	F2 PCR mix:
10 µL HF Phusion buffer	10 µL HF Phusion buffer
0.5 µL Phusion enzyme	0.5 µL Phusion enzyme
1 µL dNTP (10 µM)	1 µL dNTP (10 µM)
2 µL *CESA* cDNA (1–10 ng/µL)	2 µL *CESA* cDNA (1–10 ng/µL)
1 µL attB5 primer (25 µM)	1 µL attB2 primer (25 µM)
1 µL SR1 primer (25 µM)	1 µL SF1 primer (25 µM)
34.5 µL purified water	34.5 µL purified water

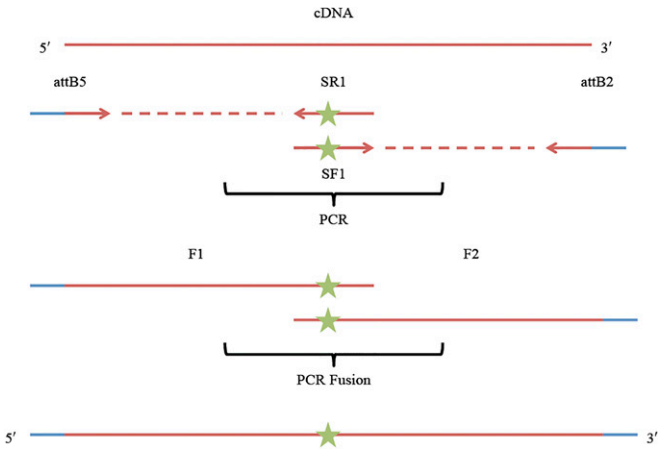

Fig. A1. A schematic of site-directed mutagenesis through PCR fusion. Fragments F1 and F2 of the gene of interest are amplified using the primers attB5 and SR1 and attB2 and SF1, respectively. Fragments F1 and F2 are combined in a single-cycle PCR fusion reaction to create the mutated gene of interest, which is cloned into pDONR P5-P2 for MultiSite Gateway cloning. Primers are depicted as arrows, *attB* sites appear in blue, coding sequence is in red, and the point mutation appears as a green star.

3. Run 1 µL of each PCR reaction on a gel with a calibrated ladder (Fig. A2) to verify fragment size and estimate DNA concentration.
4. If correct, proceed to PCR fusion with the following caveats:
   - Total DNA mass must not exceed 800 ng.
   - Add fragments in equimolar amounts. Example: F1 is ~2500 bp and F2 is ~1000 bp, thus 480 ng of F1 and 320 ng of F2 were used (Fig. A2).

PCR Fusion mix:	Thermocycler conditions:
6 µL HF Phusion Buffer	Hot Start: 98°C
0.3 µL Phusion enzyme	Denaturation: 98°C, 30 s
0.3 µL dNTP (10 µM)	Annealing: 60°C, 1 min
F1 and F2 PCR reactions up to 800 ng	Extension: 72°C, 7 min
Purified water to 30 µL	

5. PEG purify the PCR fusion product using 30% PEG/$MgCl_2$ included in the Invitrogen MultiSite Gateway Pro (12537-102) or BP Clonase II (11789-020) kit according to the manufacturer's instructions (Life Technologies, Grand Island, New York, USA) and resuspend the pellet in 7 µL of TE buffer.

Expression vector construction:

1. Clone the PCR fusion product (3.5 µL) into pDONR P5-P2 in a BP reaction using Invitrogen MultiSite Gateway Pro according to the manufacturer's instructions (Life Technologies).
2. Transform competent *E. coli* with 2 µL of the BP reaction and incubate on the appropriate selective medium according to the manufacturer's instructions (e.g., Invitrogen TOP10, Life Technologies).
3. Isolate plasmid DNA from four overnight cultures inoculated with single colonies using a commercial kit according the manufacturer's instructions (e.g., QIAprep Spin Miniprep Kit, QIAGEN, Venlo, The Netherlands).
4. Design and perform restriction digests to identify plasmids containing the expected insert. For the *CESA5* entry clones, 2.5 units each of EcoRI-HF and EcoRV-HF were used in 20-µL reactions according to the manufacturer's instructions (New England Biolabs).

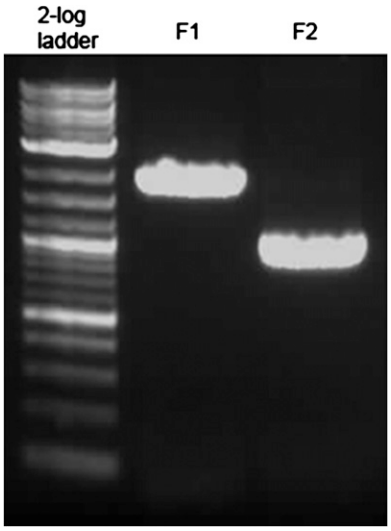

Fig. A2. PCR products separated on a 1% agarose gel (100 V, 25 min). Concentrations of F1 and F2 were both estimated at ~240 ng/μL. However, F1 is ~2500 bp and F2 is ~1000 bp. Thus, 2 μL of PCR reaction F1 was combined with 1.33 μL of PCR reaction F2 for the PCR fusion reaction.

5. Sequence a plasmid with the expected restriction fragment pattern to verify the mutation and the absence of PCR errors. The primers used to sequence the *CESA5* entry clones are listed in Appendix 3.
6. To create an expression vector, clone the sequence-verified plasmid along with a pDONR P1-P5r entry clone containing an epitope tag (available from the author) into pTHAct1Gate (available from Pierre-Francois Perroud, Washington University, St. Louis, Missouri, USA) using LR Clonase II Plus as described by the manufacturer (Life Technologies).
7. Transform competent *E. coli* with the LR reaction and incubate on the appropriate selective medium as in step 2.
8. Isolate plasmid DNA from four overnight cultures inoculated with single colonies as in step 3.
9. Digest 1 μL of each plasmid prep with 2.5 units of *Swa*I in a 20-μL reaction according the manufacturer's instructions (New England Biolabs) to screen for the expected restriction fragment pattern (2519-bp backbone plus the expression vector of approximately 10,000 bp).
10. Sequence a plasmid with the expected restriction fragment pattern to confirm proper integration of both entry clones into the destination vector. Primer P395 (Appendix 3) was used for expression vectors containing *CESA5* entry clones.

Expression vector preparation:

1. Isolate at least 300 μg of plasmid DNA using a commercial midiprep kit (e.g., GenElute HP Plasmid Midiprep Kit [Sigma-Aldrich, St. Louis, Missouri, USA] or Nucleobond Xtra Midi [Machery-Nagel, Düren, Germany]) or another method.
2. Linearize 100 μg of plasmid DNA with 100 units of *Swa*I in a 200-μL reaction according the manufacturer's instructions (New England Biolabs).
3. Ethanol precipitate DNA using sterile technique as described previously (Roberts et al., 2011).

Protoplast isolation and transformation

1. Prepare protoplasts from two plates according to the detailed protocol provided in Roberts et al. (2011) through step 3.3.11 using a *P. patens* line in which your gene of interest is knocked out. We have achieved higher regeneration rates when protoplasts were isolated separately from two plates and combined in the final wash step.
2. After resuspension in 3M medium (step 3.3.11), transfer 6×10^5 protoplasts to each of 5–6 tubes containing 50 μg of DNA (one positive control, one negative control, and up to four test vectors) and proceed with transformation and selection as described (Roberts et al., 2011).

Complementation assay

1. After the second round of selection, array stably transformed lines (Fig. A3) on plates containing BCDAT medium (Roberts et al., 2011).
2. Incubate at 25°C with fluorescent lights at a photon flux density of 85 μM $m^{-2}s^{-1}$ for 7 d.
3. To score the *cesa5*KO phenotype, use a dissecting microscope at 30× to count the number of lines with gametophores. To test complementation of a different gene, a scoring method can be developed that is appropriate for the mutant phenotype.
4. If necessary, use a compound microscope to further analyze the morphology of specific tissues (protonemal, gametophores, etc.).

Statistics

In the case of the *ppcesaR453* mutants, Fisher's two-tailed exact test (Sokal and Rohlf, 1981), calculated using the 2×2 contingency table at http://vassarstats.net/tab2x2.html, was used to test the significance of test rescue rates vs. rescue rates of the positive and negative controls. However, another test may be more appropriate depending on how the phenotype is scored.

Microsomal protein extraction

This protocol was modified from Hutton et al. (1998).

1. Collect colonies for protein extraction when they are 0.75–1 cm in diameter (2–3 wk after they are arrayed on BCDAT, Fig. A3D).
   - Remove traces of agar and blot colonies firmly with tissue to remove surface water.
   - Place each colony in a preweighed 1.5-mL centrifuge tube on ice.

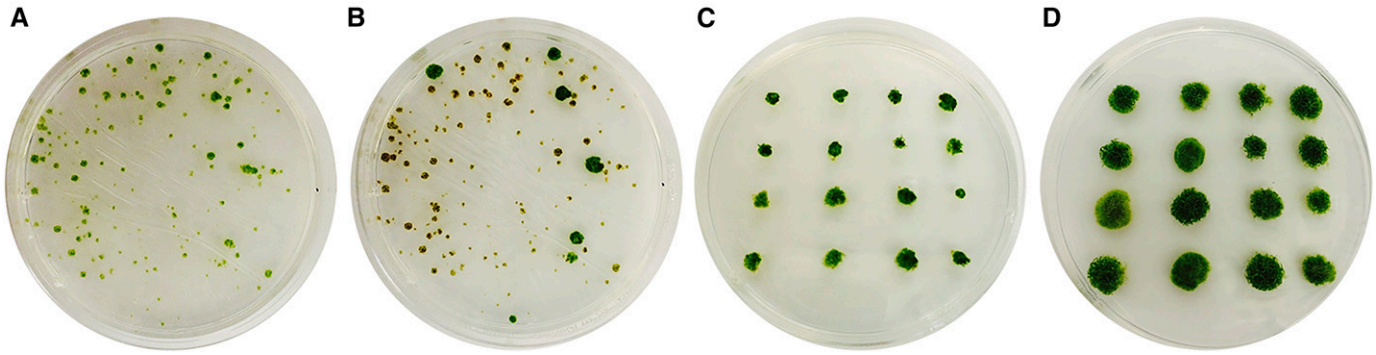

Fig. A3. Selection of stable transformants. A. Colonies derived from *ppcesa5*KO-2 protoplasts that survived the first round of antibiotic selection following transformation with a positive control vector that drives constitutive expression of wild-type PpCESA5; stable and unstable transformants are present. B. The plate shown in A after the second round of antibiotic selection showing vigorously growing stable transformants among dead colonies. C. Stable transformants from the plate shown in B after being arrayed with colonies from two other plates from the same transformation. D. Colonies ready to be harvested for protein extraction.

- Reweigh tubes to determine tissue weight (expected range 8–12 mg).
- Tissue may be stored at −80°C prior to protein extraction.
- Samples and reagents must remain on ice for the remainder of this protocol.

2. Add 300 µL of extraction buffer to each tube (50 mM HEPES [pH 7.6], 0.1 mM EDTA, 500 mM sucrose, 1 : 100 protease inhibitor cocktail for plant cell lysate, P9599 [Sigma-Aldrich]).
3. Homogenize tissue using a motor-driven pellet pestle (Z359971, Sigma-Aldrich) until no visible clumps remain.
4. Centrifuge at 10,000 × *g*, 10 min, 4°C.
5. Collect supernatant and centrifuge at 10,000 × *g*, 15 min, 4°C.
6. Transfer supernatant to an ultracentrifuge tube and centrifuge at 107,400 × *g*, 30 min, 4°C.
7. Remove supernatant and resuspend pellet in 32 µL of sample buffer (30 mM Tris [pH 6.8], 2% SDS, 0.00002% bromphenol blue, 10% glycerol).

Protein quantification and normalization

Determine the protein concentrations of microsomal extracts using the Pierce BCA Protein Assay kit (23225, Thermo Scientific Inc., Rockford, Illinois, USA) according to the manufacturer's instructions with the following modifications:

- Centrifuge samples and standards for 30 s, 13,000 × *g*.
- Test 7-µL samples of microsomal extracts (in duplicate) and each standard (expect 500 µg/mL or higher for microsomal extracts).

Western blot

1. Normalize samples to the lowest concentration by adjusting loading volume or adding sample buffer to adjust concentration.
2. Add 10% β-mercaptoethanol to each sample.
3. Run samples (at least 5 µg, but ideally 7–10 µg, total protein) on an SDS-PAGE gel and transfer proteins to a PVDF membrane.
4. Stain membranes with Ponceau S to confirm consistent protein loading (Romero-Calvo et al., 2010). Additional washing with TBST (50 mM Tris [pH 7.5], 150 mM NaCl, 0.05% Tween 20) may be required to remove stain.
5. Probe membrane with an antibody that detects the transgenic protein. Our method follows, but other methods could be used.
   - Block membrane in 5% (w/v) milk protein in TBST, 80 rpm, room temperature (RT), 1 h.
   - Rinse membrane with TBST.
   - Incubate membrane with 1 : 1000 anti-HA (HA.11 Clone 16B120 [BioLegend, Dedham, Massachusetts, USA]) in 5% (w/v) blocking agent in 1× TBST + 0.02% sodium azide, 80 rpm, 4°C, 12–16 h.
   - Wash membrane in TBST, 80 rpm, 4 min. Repeat three times.
   - Incubate membrane in 1 : 4000 HRP-conjugated antimouse (A4416, Sigma-Aldrich), 80 rpm, RT, 1 h.
   - Wash membrane in TBST, 80 rpm, 4 min. Repeat three times.
   - Develop membrane using Pierce ECL Western Blotting Substrate kit (32106, Life Technologies) according to the manufacturer's instructions.

Appendix 3. Primers used for sequencing *CESA5* entry clones.

Primer	Sequence
P99	5′-CCCGTTTTTGAAAGGTCTGA-3′
P117	5′-CCTTATTGCAGGCTCACACA-3′
P118	5′-CGTACATCAACGCCACAATC-3′
P143	5′-TTTGGACGATGACTCTCACG-3′
P153	5′-GATTTTGGATCAGTTCCCGA-3′
P395	5′-GAGCTATGGTGGCAATTACGAC-3′
P403	5′-CTTTCTCTCGATTTTCGTGACC-3′