Genetic Regulation of the 2D to 3D Growth Transition in the Moss *Physcomitrella patens*

**Highlights**
- *NO GAMETOPHORES 1 (PpNOG1)* regulates the 2D to 3D growth transition in *P. patens*
- PpNOG1 acts upstream of 3D-promoting PpAPB transcription factors
- PpNOG1 is required for the formation of apical initial cells specified for 3D growth
- *NOG1* genes are found only in land plants and thus evolved coincident with 3D growth

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**In Brief**
Moody et al. use forward genetics, somatic hybridization, and genome sequencing to identify *NO GAMETOPHORES 1 (PpNOG1)*, a gene that regulates the two-dimensional (2D) to three-dimensional (3D) growth transition in the moss *Physcomitrella patens*. PpNOG1 promotes the formation of apical initials that are required for the establishment of 3D growth.
Genetic Regulation of the 2D to 3D Growth Transition in the Moss *Physcomitrella patens*

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**SUMMARY**

One of the most important events in the history of life on earth was the colonization of land by plants; this transition coincided with and was most likely enabled by the evolution of 3-dimensional (3D) growth. Today, the diverse morphologies exhibited across the terrestrial biosphere arise from the differential regulation of 3D growth processes during development. In many plants, 3D growth is initiated during the first few divisions of the zygote, and therefore, the genetic basis cannot be dissected because mutants do not survive. However, in mosses, which are representatives of the earliest land plants, 3D shoot growth is preceded by a 2D filamentous phase that can be maintained indefinitely. Here, we used the moss *Physcomitrella patens* to identify genetic regulators of the 2D to 3D transition. Mutant screens yielded individuals that could only grow in 2D, and through an innovative strategy that combined somatic hybridization with bulk segregant analysis and genome sequencing, the causative mutation was identified in one of them. The NO GAMETOPORES 1 (NOG1) gene, which encodes a ubiquitin-associated protein, is present only in land plant genomes. In mutants that lack PpNOG1 function, transcripts encoding 3D-promoting PpABP transcription factors [1] are significantly reduced, and apical initial cells specified for 3D growth are not formed. PpNOG1 acts at the earliest identified stage of the 2D to 3D transition, possibly through degradation of proteins that suppress 3D growth. The acquisition of NOG1 function in land plants could thus have enabled the evolution and development of 3D morphology.

**RESULTS AND DISCUSSION**

To discover novel regulators of 3D growth, we designed a forward genetic screen to identify mutants of the moss *Physcomitrella patens* that could grow as 2D filaments, but not as 3D shoots. In wild-type (WT) *P. patens*, haploid spores germinate to produce apical initials that divide in a single plane to generate filaments of chloronemal and then caulonemal cells that are collectively referred to as protonema [2–4] (Figure 1A). Caulonemal cells divide either in a single plane to extend the filaments or in two planes to form side-branch initials. Most side-branch initials develop into secondary caulonemal filaments (Figure 1A), but ~5% are specified to cleave in three planes, producing 3D leafy shoots known as gametophores (Figure 1B). In a primary screen of 9,000 UV-mutagenized lines of the Villersexel (Vx) strain [5] of *P. patens*, 26 mutants were identified that developed normal 2D filaments but were unable to form 3D gametophores. A secondary screen discarded 12 of those mutants because plants entirely comprised chloronemal filaments and were therefore likely to be defective in the chloronemal-to-caulonemal rather than the 2D to 3D growth transition. Because gametophores are induced by cytokinin and auxin in wild-type *P. patens* (Figure S1), and mutants defective in the respective signaling pathways fail to form gametophores [6–8], a tertiary screen was carried out to further eliminate mutants in the cytokinin biosynthesis pathway. Another 12 mutants that formed normal gametophores when exposed to the cytokinin analog 6-benzylaminopurine (BAP) were discarded at this stage. One of the remaining mutants, *P. patens no gametoophores 1* - Reference (Ppnog1-R), exhibited normal 2D growth, with no detectable defects in protonemal tip growth or in branching (Figures 1C and 1D), but the formation of gametophores was completely abolished, even in the presence of cytokinin and/or auxin (Figures 1E and S1). Ppnog1-R mutants are thus unable to establish 3D growth.

Because the Ppnog1-R mutant does not produce gametophores, egg-producing archegonia and sperm-producing antheridia cannot develop, and thus the causative mutation could not be mapped by conventional genetic crosses. A novel strategy was therefore designed for gene discovery. As a first step, a previously published technique [9] was used to generate somatic hybrids between the infertile Ppnog1-R mutant and fertile lines of the Gransden (Gd) strain of *P. patens*. The presumed diploid hybrids produced phenotypically normal gametophores that generated sporophytes after fertilization (Figures 2A–2C). Spores obtained from three independent Ppnog1-R/Gd hybrid sporophytes exhibited phenotypic segregation ratios consistent with meiosis from a tetraploid, confirming that the original hybrids (and the generated spores) were diploid (Figures 2A, 2B, and 2D). Genomic DNA was extracted from diploid segregants (120 mutant, no 3D growth; 120 wild-type, 3D growth) and sequenced in two separate pools alongside both Vx and Gd parental lines. When allele frequencies for the 120 mutant individuals were plotted across all 27 chromosomes in the *P. patens* genome assembly, a single allele...
frequency peak of 1 was revealed on chromosome 1, identifying the genetic interval containing the Ppnog1-R locus (Figures 2C and 2E). The allele frequency plot for the 120 wild-type individuals revealed a mutant allele frequency of 0.4 at the corresponding location, representing the expected frequency from a mixture of wild-type homozygous and heterozygous individuals within the pool (Figures 2D and 2F). Interrogation for SNPs and other deviations from the annotated genome sequence (P. patens V3.3) in the genetic interval that contained the Ppnog1-R locus, revealed mutations in the coding or regulatory regions of four annotated genes (Figure 2E). Of the four mutations identified, we postulated that the Ppnog1-R mutant phenotype was caused by a C > T transition that generated a premature termination codon (R76Ter) in a gene (#32970008) that encodes a ubiquitin-associated protein (Figure 2E).

To validate that mutations in gene #32970008 caused the Ppnog1-R phenotype, we carried out complementation experiments. Three splice variants of the transcript were detected in the Ppnog1-R mutant, and all resulted in the introduction of a premature termination codon into the coding sequence (Figures 3A and 3B). The mutant phenotype was complemented when a full-length coding sequence was introduced into the genome (Figures 3C–3F and S3A; Data S1A), and thus the gene was named PpNOG1. Given the likely presence of...
additional UV-induced mutations in the Ppno1-R mutant, further validation was obtained by generating disruptant lines in which the PpNOG1 genomic locus in the Vx strain was disrupted with a PpNOG1 transcript containing a premature termination codon (R76Ter; Figures 3B and S3B–S3E; Data S1B). Two disruptant mutants were obtained (Ppno1-D1 and Ppno1-D2), in which there was no transition from 2D to 3D growth (Figures 3G and 3H). Intriguingly, the recombination event in the Ppno1-D1 line was such that both the mutant and native PpNOG1 sequences could be transcribed (Figures 3B–3E), suggesting that the truncated protein had a dominant-negative effect. However, this effect is not seen in the diploid Ppno1-R/Gd hybrid (Figure 2C) or in the complemented Ppno1-R lines (Figure 3F), where gametophores form normally. As such, the dominant negative effect most likely reflects the relative levels of mutant versus native transcript in each case. In disruptant, diploid, and rescue lines, the mutant transcript is driven by the endogenous PpNOG1 promoter, but the native transcript is driven by either a relatively short PpNOG1 promoter sequence introduced by the transformation construct (disruptant) (Figure S3E), the endogenous PpNOG1 promoter (diploid) or the actin promoter (rescue). Therefore, when the native transcript can accumulate to the same level as the mutant transcript, the mutant allele behaves as a recessive. Together, these results confirmed that a premature termination codon in the PpNOG1 gene prevents 3D growth.

Although gametophore development is completely abolished in Ppno1-D1, D1 and D2 mutants, bulbous side branch cells characteristic of gametophore initiation were occasionally observed (at ~10% frequency of wild-type) (Figures 4A and 4E). Unlike in wild-type (Figures 4B and 4C), however, the first division plane in these “bud” initials was not oblique (Figure 4F) and cell plates were positioned randomly during subsequent divisions (Figures 4G–4L). Similar misplacement of cell division planes is seen when the TONNEAU gene is mutated in P. patens, but in Ppton1 mutants (which cannot form prophase bands), recognizable but distorted 3D leafy shoots are never formed in Ppno1 mutants, and with few exceptions development arrests at the five-cell stage. Although the arrested phenotype is similar to that seen when the calpain protease PpDEK1 is mutated [11, 12], there are important distinctions between the two mutant phenotypes. The first is that supernumerary bud initials form on Ppdek1 mutant caulonema [11], indicative of opposing roles during the initiation of 3D growth, with PpDEK1 acting to repress the 2D to 3D transition and PpNOG1 acting to promote it. The second is that the oblique cell division that distinguishes 3D-forming bud initials from 2D branch initials is positioned correctly in Ppdek1 mutants [11], suggesting that PpNOG1 acts earlier than PpDEK1 during 3D specification. Together, the substantially reduced number of bud initials, the misplaced oblique cell division plane in the bud initials that do form, and the complete absence of gametophores in Ppno1 mutants suggest that PpNOG1 plays a critical role in promoting the transition from 2D to 3D growth in P. patens.

Four PpAPB genes that encode AP2-like transcription factors are both necessary and sufficient in P. patens for the formation of bud initials that can develop into gametophores [1], and as such it is important to understand the relationship between PpAPB and PpNOG1 function. PpAPB [1] and PpNOG1 (Figure 4M) transcript levels are both induced by auxin, with PpAPB genes showing the more dramatic response. In contrast, PpNOG1 transcript levels show no consistent response to cytokinin (Figure 4M), suggesting a role in specification of 3D growth per se as opposed to a role in the cytokinin response pathway. Notably, the accumulation of PpAPB transcripts was significantly reduced in both Ppno1-D1 and Ppno1-D2 disruptants (Figures 4N and 4O). Collectively these results suggest that the genes act in the same pathway and that at the level of transcription, PpNOG1 acts upstream of PpAPB genes. However, interactions are unlikely to be direct. The presence of a ubiquitin associating domain in PpNOG1 may indicate a role in protein degradation, particularly given that at least one ortholog in flowering plants (Figure S4) is known to bind ubiquitin [13]. With PpDEK1
Figure 4. PpNOG1 Function

(A–L) Propidium iodide stained Vx (A–D) and Ppnog1-R (E–L) side-branch cells (A and E) and buds at 2- (B and F), 3- (C, G, and H), 4- (D, I, and J), and 5- (K and L) cell stages. Scale bars, 10 μm. ●, gametophore initial.

(M–O) Relative transcript levels in protonemata: PpNOG1 in wild-type ± BAP and/or NAA (M) and PpAPB1-4 in Vx and the Ppnog1-D1 and Ppnog1-D2 disruptants (N and O). ANOVA, ***p < 0.05.

(P and Q) Model for PpNOG1 function: PpDEK1 and PpNOG1 act antagonistically during side-branch initiation to regulate PpAPB transcription (P) and then act together to enable divisions that produce the gametophore initial (Q).

See Figure S4 for NOG1 phylogeny.
encoding a membrane-bound protease [14], and mutants in both proteasome [15] and N-end rule [16] degradation pathways perturbing (but not abolishing) gametophore development. Post-translational regulation is emerging as a common theme in the transition from 2D to 3D growth. In this context, we propose that PpNOG1 regulates the transition to 3D growth by first inducing the degradation of protein(s) that repress PpAPB activity (and hence opposing PpDEK1 activity by inducing calonema to produce bud initials) (Figure 4P), and then by acting within the bud initial (alongside PpDEK1) to ensure that cell divisions are correctly orientated to produce the earliest regulatory module associated with 3D growth. In this context, PpNOG1 regulates the transition to 3D growth. Post-translational regulation is emerging as a common theme in the transition from 2D to 3D growth. In this context, we propose that PpNOG1 regulates the transition to 3D growth by first inducing the degradation of protein(s) that repress PpAPB activity (and hence opposing PpDEK1 activity by inducing calonema to produce bud initials) (Figure 4P), and then by acting within the bud initial (alongside PpDEK1) to ensure that cell divisions are correctly orientated to produce an apical initial that can cleave in three planes and self-renew (Figure 4Q). Given that NOG1 sequences are absent from all non-plant genomes (Figure S4), it is plausible that this role evolved to facilitate 3D growth in plants and thus that the antagonistic relationship between NOG1 and DEK1 likely represents the earliest regulatory module associated with 3D growth in both developmental and evolutionary terms.

STAR METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and four data files and can be found with this article online at [https://doi.org/10.1016/j.cub.2017.12.052](https://doi.org/10.1016/j.cub.2017.12.052).

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AUTHOR CONTRIBUTIONS

L.A.M. performed all of the experimental work, with technical assistance from E.R.; L.A.M., S.K., and J.A.L. conceived and designed the study; S.K. carried out the bioinformatics; and L.A.M. and J.A.L. wrote the manuscript, with editorial input from S.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Anti Digoxygenin AP Fab Fragments | Sigma-Aldrich | Cat#11093274910 |
| **Bacterial and Virus Strains** |        |            |
| E. coli strain DH5a | Widely distributed | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| 6-Benzylaminopurine (BAP) | Sigma-Aldrich | Cat#B3408 |
| 1-Naphthaleneacetic acid (NAA) | Sigma-Aldrich | Cat#N0640 |
| Propidium Iodide (PI) | Sigma-Aldrich | Cat#P4864 |
| G418 disulphate | Melford | Cat#G0175 |
| Hygromycin B | Melford | Cat#H7502 |
| Zeocin | ThermoFisher | Cat#R25001 |
| Driselase Basidiomycetes sp. | Sigma-Aldrich | Cat#D8037 |
| Poly(ethylene glycol) BioUltra 6,000 (PEG) | Sigma-Aldrich | Cat#81255 |
| **Critical Commercial Assays** |        |            |
| GoTaq G2 DNA Polymerase | Promega | Cat#M7841 |
| Phusion High-Fidelity DNA Polymerase | ThermoFisher | Cat#F5305 |
| TURBO DNA-free Kit | Ambion | Cat#AM1907 |
| Superscript III Reverse Transcriptase | Invitrogen | Cat#18080093 |
| SYBR Green PCR Master Mix | Applied Biosystems | Cat#4309155 |
| **Experimental Models: Organisms/Strains** |        |            |
| Physcomitrella patens subsp. patens | Widely distributed | N/A |
| Physcomitrella patens Vx::mCherry | Pierre-François Perroud | [5] |
| Physcomitrella patens Gd::GFP | Pierre-François Perroud | [5] |
| Ppnog1-R | This paper | N/A |
| Ppnog1/Gd-2 | This paper | N/A |
| Ppnog1/Gd-3 | This paper | N/A |
| Ppnog1/Gd-8 | This paper | N/A |
| Ppnog1-R+pAct::PpNOG1:GFP-4 | This paper | N/A |
| Ppnog1-R+pAct::PpNOG1:GFP-7 | This paper | N/A |
| Ppnog1-R+pAct::PpNOG1:GFP-73 | This paper | N/A |
| Ppnog1-R+pAct::PpNOG1:GFP-77 | This paper | N/A |
| Ppnog1-R+pAct::PpNOG1:GFP-80 | This paper | N/A |
| Ppnog1-D1 | This paper | N/A |
| Ppnog1-D2 | This paper | N/A |
| **Oligonucleotides** |        |            |
| See Data S2 | N/A | N/A |
| **Recombinant DNA** |        |            |
| pZAG1 | Yuji Hiwatashi (gift) | N/A |
| pAct::PpNOG1-mGFP | This paper | N/A |
| pNOG1::PpNOG1*-mGFPMutNPTII-3' | This paper | N/A |
| pGFPMutNPTII plasmid | Yuji Hiwatashi | [17] |
| **Software and Algorithms** |        |            |
| BWA-MEM | Sourceforge | [18] |
| OrthoFinder | GitHub | [19] |
| GATK | Broad Institute | [20] |
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jane Langdale (jane.langdale@plants.ox.ac.uk). Please note that the transfer of transgenic *P. patens* lines will be governed by an MTA and will be dependent on appropriate import permits being acquired by the receiver.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plants

Vx::mCherry and Gd::GFP marker lines were obtained from Pierre-Francois Perroud [5]. The Gransden strain *Physcomitrella patens* subsp. *patens* [21], Vx::mCherry and Gd::GFP marker lines, and somatic hybrids were grown and maintained under sterile conditions on BCD or BCDAT medium. BCD medium contained 250 mg/L MgSO4·7H2O, 250 mg/L KH2PO4 (pH 6.5), 1010 mg/L KNO3, 12.5 mg/L FeSO4·7H2O, 0.001% Trace Element Solution (TES – 0.614 mg/L H3BO3, 0.055 mg/L AlK(SO4)2·12H2O, 0.055 mg/L CuSO4·5H2O, 0.028 mg/L KBr, 0.028 mg/L LiCl, 0.389 mg/L MnCl2·4H2O, 0.055 mg/L CoCl2·6H2O, 0.055 mg/L ZnSO4·7H2O, 0.028 mg/L KI and 0.028 mg/L SnCl2·2H2O) and 8% agar, supplemented with 1 mM ammonium tartrate [22]. Plants were grown at 24°C with a 16 h: 8 h, light (300 µmol m⁻² s⁻¹): dark cycle. Sporophytes were induced as described previously [23] and harvested after approximately 3-6 months. Sporangia were sterilized in 20% Parozone bleach for 15 min at room temperature and then washed three times in sterile water. Spores were released using a sterile pipette tip and plated onto cellophane-overlaid BCDAT medium.

METHOD DETAILS

UV mutagenesis and screening

1% Driselase was prepared in 8% mannitol, incubated at room temperature for 15 min with gentle agitation, centrifuged for 3 min at 3,300 xg and then filter-sterilized (pellet discarded). 7-day old Vx::mCherry protonemata were harvested from cellophane-overlaid BCDAT medium and added to 1% Driselase and incubated, with gentle agitation, for 40 min. The cell suspension was then filtered through a sterile 50 µm filter into a round bottomed tube. The solution was centrifuged for 3 min at 120 xg at room temperature with no braking. Cells were then washed twice using 6 mL 8% mannitol and then resuspended in 6 mL 8% mannitol and cell density determined using a haemocytometer [24]. Protoplasts were plated at a density of 5x10⁴ cells mL⁻¹, onto cellophane overlaid BCDAT supplemented with 10 mM CaCl₂, 6% mannitol and 0.5% glucose (BCDATG medium). Protoplasts were then exposed to a 75000 µM dose of UV radiation using a Stratalinker UV Crosslinker to achieve an 85%-90% kill rate (this will vary according to the equipment utilized and must be calibrated prior to screening). UV-treated protoplasts were incubated at 24°C in the dark for 48 h prior to growth under standard conditions for one week. Cellophane discs were then transferred to medium containing BCDAT medium and grown under standard conditions for a further week. Regenerating protoplasts were transferred to individual wells of 24-well plates containing BCD medium to induce gametophores. Regenerating mutagenized lines that did not produce gametophores after a period of two months were selected for further validation.

To determine whether 3D-defective mutants were cytokinin-responsive, protonemata were cultured on cellophane-overlaid BCDAT medium for four days under standard growth conditions. Cellophane discs were then transferred to BCDAT medium containing 5 µM benzylaminopurine (BAP) and cultivated for 3 d.

Microscopy

Fluorescence microscopy was carried out with a Zeiss LSM510 META confocal microscope. A 40x water immersive lens (C-Apochromat 40x/1.20 W) was used for all imaging. Propidium iodide (PI) was excited at 488 nm with 15% laser power and detected with a 665-615 bandpass filter. Tissues were carefully removed from cellophane-overlaid BCD plates, submerged in 10 µg/mL PI for 1 min and then mounted on slides in water. Other images were captured using either a Leica DMRB microscope or a Leica M165C microscope, equipped with a QImaging Micropublishing 5.0 RTV camera.

Somatic hybridization and segregation analysis

Protoplasts were isolated from *nog1-R* and Gd::GFP as described above. The cell suspensions derived from each strain were adjusted to a density of 1x10⁶ cells mL⁻¹ in 8% mannitol. 1 mL of each strain were combined, mixed gently and then centrifuged for 3 min at 120xg at room temperature with no braking. Protoplasts were resuspended in 250 µL PW solution (10 mM CaCl₂ and 8.5% mannitol). Somatic hybridization was initiated by adding 750 µL PEG/F (5 mM CaCl₂ and 50% PEG 6000 (w/v)) and mixed gently. After 40 min, 1.5 mL of PW solution was added. After 50 min, 10 mL of PW solution was added. After 60 min, 10 mL of PW solution was added. Following each addition of PW solution, protoplasts were mixed gently. After 70 min, protoplasts were centrifuged at 120 xg for 3 min at room temperature with no braking. The pellet was resuspended in 4 mL 8% mannitol and then 1 mL each plated onto cellophane-overlaid BCDATG medium [25]. Plates were kept in the dark for 48 h and then transferred to standard growth conditions. After one week, cellophane discs were removed and then transferred to BCDAT medium supplemented with both 50 µg/mL G418 and 20 µg/mL hygromycin B, to select for stable *Pnog1-R/Gd::GFP* diploid hybrids.
Sporophytes were induced as above and the resulting spore progeny allowed to grow on BCDAT for two weeks. Segregation analysis was carried out by transferring sporelings to individual wells of a 24-well tissue culture plate containing BCD medium for two months, and then scoring phenotypes.

**Isolation of genomic DNA and sequencing**

Total genomic DNA was isolated from one week old protonemata maintained on cellophane-overlaid BCDAT medium using the CTAB method [26]. Essentially, tissue was first ground in liquid nitrogen, and suspended in 500 µL CTAB extraction solution (1.5% CTAB, 1.05 M NaCl, 75 mM Tris-HCl, 15 mM EDTA pH8.0). After incubation at 65°C for 10 min, an equal volume of chloroform:isoamylalcohol (24:1) was added, samples were mixed by vortexing and then centrifuged at 13,000 rpm for 10 min. The upper aqueous layer of each sample was transferred to a fresh tube. DNA was precipitated by adding 0.7 volumes of isopropanol, and centrifuged at 13,000 rpm for 10 min. Pellets were washed with 500 µL 70% ethanol, and air-dried. DNA samples were dissolved in 50 µL H2O and stored at −20°C. Genomic DNA extracted from individual protonemal cultures was pooled in equimolar amounts to construct separate mutant (120 individuals; 1.7 µg total) and wild-type (120 individuals; 3.5 µg total) pools. Genomic DNA was extracted from both Vx::mCherry (2.6 µg total) and Gd::GFP (3.5 µg total) parental lines to identify SNPs from both genetic backgrounds for use in bulk segregant analysis. DNA samples were sequenced using an Illumina HiSeq4000 platform (150 bp PE read lengths) at the Wellcome Trust Centre for Human Genetics, University of Oxford.

**Read processing and variant calling**

Raw reads were subject to quality filtering using Trimmomatic [27] to remove low quality bases, read-pairs and contaminating adaptor sequences prior to read mapping and variant calling. Sequences were searched for all common Illumina adaptors and the settings used for read processing by Trimmomatic were LEADING:20 TRAILING:20 SLIDINGWINDOW:5:20 MINLEN:50. Each of the trimmed quality filtered paired-end reads libraries were mapped using BWA-MEM [18] to the *P. patens* genome (Version Ppatens_318_v3) obtained from Phytozome V11. Duplicate mapped reads were removed, mapped reads were realigned around indels, and variants called according to best practice guidelines from GATK using GATK v3.6 [20].

**Candidate gene discovery through bulk segregant analysis**

Comparison of the sequence data from the Gd::GFP and Vx::mCherry parental strains identified a set of 2,255,671 single nucleotide variants that distinguished the two strains. To conduct the bulk segregant analysis, the reads from the wild-type and mutant pools were mapped to the reference genome and the allele frequency of the strain-distinguishing variants in both pools was evaluated. Strain-distinguishing variants were used for analysis if: 1) the coverage depth in both the wild-type and mutant pools was > 0.5 and < 2 times the mean coverage depth and; 2) the variant quality was > 500. These allele frequencies were used to identify the chromosomal region linked to the mutant allele. To confirm the presence of the premature termination codon in the mutant, the full-length transcript was amplified using the primers 32970008(exon)_GSPFSalI and 32970008(exon)_GSPRHindIII (Data S2), and sequenced.

**Physcomitrella patens transformation**

2g polyethylene glycol 6000 (previously autoclaved in flat-bottomed autoclavable vial) was melted using a microwave. 5 mL mannitol/Ca(NO3)2 solution (0.8% mannitol, 0.1 M Ca(NO3)2, 10 mM Tris pH8.0) was then added to the molten PEG and incubated for 2-3 hours at room temperature. Protoplasts were isolated from both nog1-R and Vx::mCherry as described above. However, following cell density determination, protoplasts were resuspended in MMM (0.5 M mannitol, 0.15 M MgCl2, 0.1% MES pH5.6) to achieve a final cell density of 1.5x10^6 cells mL⁻¹. 10 µg of the linearized construct was added to a round bottomed tube. 300 µL of protoplast suspension and 300 µL PEG solution were then added to the tube in drops and mixed gently. The samples were heat-shocked for 5 min at 45°C and incubated for an additional 5 min at room temperature. 300 µL 8% mannitol was added to each tube, 5 times at 3 min intervals and tilted gently to mix after each addition. 1 mL 8% mannitol was then added to each tube, 5 times at 3 min intervals and tilted gently to mix after each addition. Cells were centrifuged for 4 min at 120 xg, supernatants discarded and pellets resuspended in 3 mL 8% mannitol. 1 mL was plated onto each of three cellophane-overlaid BCDATG plates, which were subsequently sealed and kept in the dark for 48 h. Plates were transferred to standard growth conditions for a further 5 days [24]. Cellophane discs were then removed and transferred to BCDAT medium supplemented with either 50 µg/mL G418 or 100 µg/mL Zeocin.

After two weeks, plants were transferred back to non-selective BCDAT medium for another week and then stable transformants were selected by plating on selective medium again for a further week. 10 µg plasmid DNA was transformed into protoplasts. Stable transgenic lines were analyzed by PCR (using primers in Data S2) and DNA gel blot analysis (see below).

**Generation of nog1-R complementation lines**

The full-length *PpNOG1* coding sequence (excluding the stop codon) was amplified from *P. patens* Gransden wild-type protonemata cDNA using the primers 32970008(exon)_GSPFSall and 32970008(exon)_GSPRHindIII (Data S3) and ligated into Sall/HindIII cut pZAG1 plasmid (a gift from Yuji Hiwatashi). The resultant construct, pAct:*PpNOG1*-mGFP (Data S1A), was linearized using KpnI and transformed into protoplasts isolated from *Pp nog1-R* mutants. Stable transformants were selected using 100 µg/mL Zeocin.
Generation of a nog1 disruptant mutant
A genomic DNA fragment from -827bp upstream of the start codon up to but excluding the start codon of the PpNOG1 sequence was PCR-amplified from 32970008_KI.5FKpnI_revised and 32970008_KI.5RXhol primers (Data S2) and ligated into KpnI/Xhol cut pGFPmutNPTII plasmid [17] to create pNOG1-mGFPmutNPTII. A PpNOG1 genomic DNA fragment including the stop codon up to 1420bp downstream of the stop codon was PCR-amplified using 32970008_KI.3FNotI and 32970008_KI.3RSaclI primers (Data S2) and ligated into NotI/SaclI cut pNOG1-mGFPmutNPTII to create pNOG1-mGFPmutNPTII-3'. The PpNOG1 coding sequence was PCR-amplified from cDNA prepared from the nog1-R mutant using 32970008(exon)_GSPFSall and 32970008(exon)_GSPFHindIII primers (Data S2) and then ligated into SalI/HindIII cut pNOG1-mGFPmutNPTII-3' to create pNOG1: PpNOG1*-mGFPmutNPTII-3' [Data S1B]. pNOG1: PpNOG1*-mGFPmutNPTII-3' was linearized using KpnI before being transformed into protoplasts isolated from Vx::mCherry. Stable transformants were selected using 50 µg/mL G418.

DNA gel blot analysis
15 µg genomic DNA was digested with HindIII for 3 h at 37°C, according to the manufacturer’s instructions (NEB), and then electrophoresed on a 1% agarose gel. The gel was treated with depuration solution (0.25 M HCl) for 20 min, denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 30 min, neutralization solution (1M Tris.Cl pH8.0, 1.5 M NaCl) for 30 min and then soaked in 2xSSC before being incorporated into the blotting apparatus and the DNA transferred to N+ Hybond membrane (Amersham). The nylon membrane was rinsed in 2xSSC and then UV-crosslinked using a Stratallinker.

The pNOG1 fragment for hybridization was PCR amplified from pNOG1: PpNOG1*-mGFPmutNPTII-3' using GoTag Flexi DNA Polymerase (Promega), but with the addition of 0.25 mM Digoxygenin-11-dUTP (Roche) (Data S2).

Membranes were incubated in DIG Easy HybTM (Roche) for 2 h at 58°C. 20 µg sheared salmon sperm and 30 ng purified probe were added to 50 µL water (per 15 mL of DIG Easy HybTM solution), boiled at 100°C for 5 min and then allowed to cool before adding to the DIG Easy HybTM solution. Blots were hybridized overnight at 58°C and then washed twice for 5 min in 2xSSC, 0.1% SDS at room temperature, and twice for 30 min in 0.5xSSC, 0.1% SDS at 58°C. Membranes were incubated in 1xMaleic Acid Buffer (0.1 M Maleic Acid, 0.15 M NaCl) for 3 min and subsequently in blocking buffer (5% skimmed milk powder in 1xMaleic Acid Buffer) for 2 h at room temperature. Blocking buffer was then replaced with 50 mL blocking buffer containing 3.75 U Anti Digoxygenin AP Fab Fragments (Roche) and blots incubated for 30 min. Blots were washed with 1xWashing Buffer (0.1xMaleic Acid Buffer + 0.3% Tween 20) three times for 15 min and once in 1xDetection Buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH9.5) for 3 min. Membranes were incubated in the presence of 5:1000 dilution of CDP-Star Detection reagent (manufacturer) in a sheet protector for 5 min and then exposed to film in an autoradiography cassette.

Quantitative RT-PCR
Total RNA was isolated using the RNeasy Mini kit (QIAGEN) and DNase treated (Turbo DNase, Ambion). cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen) and 500 ng DNase-treated RNA. For quantitative RT-PCR, primer pairs were designed to amplify ~150bp fragment of each of the genes of interest. Amplification was detected using SYBR Green (Life Technologies) on a StepOneTM Real-Time PCR System (Applied Biosystems). Cycling conditions were 95°C for 1 min. To test for genomic DNA contamination, no RT controls were included. To test responsiveness of PpNOG1 to cytokinin and auxin, 7 old wild-type Gd protonemata were transferred to BCD medium supplemented with 1 µM BAP, 1 µM NAA or 1 µM BAP/1 µM NAA (plus controls), and grown for an additional 3 d. To quantify PpAPB transcripts, RNA was isolated from 14 d old protonemata (Vx::mCherry and PpNOG1-D1) grown on cellophane-overlaid BCD medium.

Phylogenetic analysis
The complete set of predicted proteomes for all species in Phytozome version 10 were subject to orthogroup inference using OrthoFinder [19]. The orthogroup containing the NOG1 gene was identified and the constituent sequences subject to multiple sequence alignment using MergeAlign [28]. It was apparent from manual inspection of the multiple sequence alignment that several gene models were likely incorrect (Spirodela polyriza, Vitis vinifera, Eucalyptus gradis, Setaria italica, Gossypium raimondii) or potentially absent (Selaginella moellendorfii). Gene models were corrected or predicted manually and added to the multiple sequence alignment. Further sequences were added to the alignment after a manual BLAST search of Phytozome version 12 (Data S3A). After empirical testing of the multiple sequence alignment for maximum likelihood phylogenetic tree inference using IQTREE [29], a subset of sequences was selected, retaining representatives of genera in all phyla and evidence of gene duplications where present, while eliminating sequences that are likely mis-annotated (Data S3B). The best-fitting model parameters (JTT+G4) were estimated from the revised alignment (Data S4) and a consensus phylogenetic tree was estimated from 1000 bootstrap replicates. The data were imported into ITOL [30] to generate the pictorial representation.

QUANTIFICATION AND STATISTICAL ANALYSES
Experimental design, sampling and statistical methods
The number of gametophores per culture was quantified by counting the number of gametophores derived from a homogenized culture of protonemata. Cultures were normalized to a uniform OD so that direct comparisons could be made between wild-type
and transgenic lines. For all experiments, at least ten individuals representing more than two independent transgenic lines were evaluated. For all counts, the Standard Error of the Mean was determined and a t test was performed using Excel.

qRT-PCR experiments were carried out using three technical replicates for each of three independent biological samples, alongside water controls. Ct values were calculated from raw amplification data using the Real-time PCR Miner software (http://www.ewindup.info/miner/). The mean Ct value between the three technical replicates was then calculated. Fold changes in gene expression were calculated relative to controls using the 2^{-ΔΔCt} method [31]. Two or three genes were used as constitutive controls: EF1alpha (Pp1s84_186V6), DNAJ (Pp1s123_116V6), ACT7 (Pp1s198_154V6). An ANOVA test was performed to compare whether relative change was statistically significant.

A phenotypic segregation analysis was performed on the progeny derived from three independent Ppnog1-R/Gd hybrid sporophytes. At least 570 individuals were scored per line and a Chi-square test was performed to check for conformity to a 1:4:1 segregation ratio expected from meiosis from a tetraploid.

**DATA AND SOFTWARE AVAILABILITY**

The accession number for the raw genome sequence reads reported in this paper is EBI ArrayExpress: E-MTAB-5096. The accession numbers for the sequences of full length PpNOG1 cDNA plus the three splice variants in nog1-R mutants reported in this paper are NCBI: MG280833, MG280834, MG280835, and MG280836, respectively.