CLAVATA Was a Genetic Novelty for the Morphological Innovation of 3D Growth in Land Plants

Highlights

- CLAVATA originated in the last common ancestor of land plants
- CLAVATA regulates cell proliferation, fate, and growth in Physcomitrella
- CLAVATA orients cell division planes in Physcomitrella and Arabidopsis
- CLEs act via receptors that are conserved between Physcomitrella and Arabidopsis

Authors

Chris D. Whitewoods, Joseph Cammarata, Zoe Nemec Venza, ..., Adrienne H.K. Roeder, Michael J. Scanlon, C. Jill Harrison

Correspondence

jill.harrison@bristol.ac.uk

In Brief

Whitewoods, Cammarata, et al. show that a conserved CLAVATA (CLV) pathway arose in the last common ancestor of land plants. CLV regulates cell division plane orientation during the 2D to 3D growth transition in a moss, and roles for CLV are shared between mosses and flowering plants, suggesting that CLV enabled 3D growth to arise in land plants.
CLAVATA Was a Genetic Novelty for the Morphological Innovation of 3D Growth in Land Plants

Chris D. Whitewoods,1,6,12 Joseph Cammarata,1,2,3,12 Zoe Nemec Venza,4 Stephanie Sang,4,10 Ashley D. Crook,5 Tsuyoshi Aoyama,1,4,11 Xiao Y. Wang,1 Manuel Waller,6 Yasuko Kamisugi,7 Andrew C. Cuming,7 Péter Szővényi,6 Zachary L. Nimchuk,5,8 Adrienne H.K. Roeder,2,3 Michael J. Scanlon,2 and C. Jill Harrison1,4,13,*

1Plant Sciences Department, Cambridge University, Downing Street, Cambridge CB2 3EA, UK
2Plant Biology Section, School of Integrative Plant Science, Cornell University, Tower Road, Ithaca, NY 14853, USA
3Well Institute for Cell and Molecular Biology, Cornell University, Ithaca, NY 14853, USA
4School of Biological Sciences, University of Bristol, 24 Tyndall Avenue, Bristol BS8 1TQ, UK
5Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
6Department of Systematic and Evolutionary Botany, University of Zurich, Zollikerstrasse 107, 8008 Zurich, Switzerland
7Centre for Plant Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK
8Curriculum in Genetics and Molecular Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA
9Present address: John Innes Centre, Norwich Research Park, Colney Lane, Norwich NR4 7UH, UK
10Present address: Department of Organismal Biology and Anatomy, The University of Chicago, 1027 E. 57th Street, Chicago, IL 60637, USA
11Present address: National Institute for Basic Biology, Nishigonaka 38, Myodaiji, Okazaki, 444-8585 Aichi, Japan
12These authors contributed equally
13Lead Contact
*Correspondence: jill.harrison@bristol.ac.uk
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SUMMARY

How genes shape diverse plant and animal body forms is a key question in biology. Unlike animal cells, plant cells are confined by rigid cell walls, and cell division plane orientation and growth rather than cell movement determine overall body form. The emergence of plants on land coincided with a new capacity to rotate stem cell divisions through multiple planes, and this enabled three-dimensional (3D) forms to arise from ancestral forms constrained to 2D growth. The genes involved in this evolutionary innovation are largely unknown. The evolution of 3D growth is recapitulated during the development of modern mosses when leafy shoots arise from a filamentous (2D) precursor tissue. Here, we show that a conserved, CLAVATA peptide and receptor-like kinase pathway originated with land plants and orients stem cell division planes during the transition from 2D to 3D growth in a moss, Physcomitrella. We find that this newly identified role for CLAVATA in regulating cell division plane orientation is shared between Physcomitrella and Arabidopsis. We report that roles for CLAVATA in regulating cell proliferation and cell fate are also shared and that CLAVATA-like peptides act via conserved receptor components in Physcomitrella. Our results suggest that CLAVATA was a genetic novelty enabling the morphological innovation of 3D growth in land plants.

INTRODUCTION

The conquest of land was enabled by a series of innovations that allowed plant forms to radiate and occupy new volumes of space in the sub-aerial environment [1]. Among these, the innovation of shooting systems with organs positioned radially around an upright stem stands out as a primer for massively increased plant productivity and diversity [1]. Such three-dimensional (3D) growth forms first arose as a consequence of a novel stem cell function gained by land plants, namely the capacity to rotate stem cell divisions through multiple planes and are therefore generally constrained to smaller filamentous or mat-like (two-dimensional [2D]) growth forms (Figure 1A) [1, 3]. The evolutionary transition from 2D to 3D growth is recapitulated during the development of modern mosses when a branching, filamentous (protonemal) precursor tissue (2D) gives rise to 3D gametophytes (3D), whereas loss of APB activity marks filament fate (2D) [6, 7]. A strongly oblique cell division is the first reliable morphological marker of gametophore development [6, 7]. This is followed by a second oblique apical cell division, which is approximately perpendicular to the first, after which division planes rotate during two successive rounds of division to establish a tetrahedral apical stem cell [6]. The tetrahedral apical cell divides in spiraling

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planes to replace itself and produce daughter cells that generate the 3D gametophore axis and leaves [6]. The mechanisms regulating such novel and rotating stem cell division plane orientations during evolutionary and developmental transitions to 3D growth are unknown.

In Arabidopsis, the CLAVATA (CLV) and WUSCHEL (WUS) pathways act in a feedback loop to regulate many aspects of stem cell function, including cell fate [8, 9], proliferation [9–11], and growth [12]. CLV3 encodes a small, secreted peptide that is expressed in the upper cell layers of the central zone and can move throughout the meristem [13–15]. CLV1 is expressed in the underlying cell layers of the central zone and encodes a receptor-like kinase that acts as a receptor for CLV3 [11, 16] in conjunction with CLV2, CORYNE (CRN), RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), and BARELY ANY MERISTEM (BAM) [17, 18]. WUS activity promotes meristem cell proliferation [19], and CLV signaling restricts the size of the WUS expression domain [13]. WUS acts non-cell autonomously, moving from the organizing center to the uppermost meristem cell layers, where it promotes CLV3 expression [20], thereby closing the feedback loop that maintains meristem size.

RESULTS

The CLAVATA Pathway Originated in the Last Common Ancestor of Land Plants

To determine how the CLV pathway evolved and identify potential roles for CLV in Physcomitrella stem cell function, we first queried publicly accessible genome and transcriptome databases from a wide range of green algae and land plants for CLV3-like (CLE), CLV1/BAM, RPK2, CLV2, and CRN homologs (Figure 1B; Table S1). We found no CLV pathway homologs in the chlorophyte or charophyte algae sampled but found at least one CLE homolog and one CLV1/BAM homolog in each early-diverging bryophyte lineage and all other land plants, suggesting that the core CLV signaling module comprises at least one CLE peptide and a CLV/BAM receptor-like kinase. RPK2 homologs were present in all land plants sampled except the hornwort, Anthoceros agrestis. In Physcomitrella, we identified seven genes with a conserved CLE domain encoding a 12-amino-acid peptide motif similar to CLV3, but sequences outside the conserved CLE domain were divergent (Figure 1; Table S1). The genome encodes four CLV3-like peptides: PpCLE1, 2, and 3 encode the peptide motif RMVPTGPNPLHN; PpCLE4 encodes the motif RMVPSGPNPLHN; PpCLE5 and 6 encode the motif RLVPPTGPNPLHN; and PpCLE7 encodes the motif RLVPTGPNPLHN. Concomitantly with 3D Growth

(A) Phylogenetic relationships among land plants and their freshwater algal sister lineages redrawn from [4] and [5], respectively. Although chlorophytes and charophytes undergo stem cell divisions in a single orientation (2D growth), land plants undergo stem cell divisions in multiple orientations to generate elaborate three-dimensional forms (3D growth).

(B) The number of CLV pathway homologs was determined by BLAST against genome or draft genome (G) and transcriptome (T) databases as described in STAR Methods. Cha, charophytes; Chl, chlorophytes; H, hornworts; L, liverworts; M, mosses; VP, vascular plants.

See also Figures S1, S2, and S3 and Table S1.

Figure 1. The CLV Pathway Originated in the Last Common Ancestor of Land Plants, Concomitantly with 3D Growth

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in day 10 filamentous tissues (Figure S4). These results were broadly consistent with reports from transcriptome data (Figure S5)\[ 23, 24\]. We also constructed promoter::NLSGFPGUS (promoter::NGG) fusion lines for PpCLE1, PpCLE2, PpCLE7, PpCLV1a, PpCLV1b, and PpRPK2 as RT-PCR showed that these 6 genes were upregulated at around the time of gametophore initiation (see Strategy for generation of promoter::NLSGFP reporter lines in Methods S1; Figure 2). In 3-week-old spot cultures (Figures 2A–2F), PpCLE1::NGG, PpCLE2::NGG, PpCLE7::NGG, and PpCLV1a::NGG lines accumulated local signal in various protonemal cell types around the buds (Figures 2G–2J and 2M–2P; arrows indicate signal in protonemata), PpCLV1b::NGG and PpRPK2::NGG signal accumulated mainly in the apical region of buds (Q and R). At two later stages of gametophore development (S–X and Y–J), all promoters were active in gametophores, although the patterns and intensity of activity varied between reporters and by developmental stage. PpCLE1::NGG lines stained most strongly in leaves (S, Y, and E'), PpCLE2::NGG lines most strongly in leaves and gametophore bases (T, Z, and F'), and PpCLE7::NGG lines accumulated stain in rhizoid tips (arrow in U), leaf bases (arrow in A'), and hairs around the apex and the gametophore axis (G'). PpCLV1a::NGG lines did not stain intensely at early stages of gametophore development (P and V) but accumulated signal in gametophore axes and leaves at later stages (B' and H'). In contrast, PpCLV1b::NGG and PpRPK2::NGG lines accumulated signal in gametophore axes and leaves from early stages of development (W and X), and strong signal was detected in branches initiating at gametophore bases (arrows in X, C', and D'). All tissues in (A)–(D') were stained in a solution containing 0.5 mM FeCN for times specified in (A)–(F), and gametophores in (E')–(J') were stained three times longer in a solution containing 2 mM FeCN.

The scale bars in (A)–(F) represent 1 mm, the scale bars in (M)–(R) represent 100 μm, and insets in (G)–(L) indicate position of buds in (M)–(R). The scale bars in (S)–(J') represent 1 mm. See also Methods S1 and Table S4.

Figure 2. CLV Pathway Components Are Expressed in Physcomitrella Protonemata and Gametophores

(A–J') GUS staining of PpCLE1::NGG (A, G, M, S, Y, and E'), PpCLE2::NGG (B, H, N, T, Z, and F'), PpCLE7::NGG (C, I, O, U, A', and G'), PpCLV1a::NGG (D, J, P, V, B', and H'), PpCLV1b::NGG (E, K, Q, W, C', and I'), and PpRPK2::NGG (F, L, R, X, D', and J') lines revealed complex expression dynamics. Although PpCLE::NGG and PpCLV1a::NGG signal accumulated in protonemal tissues close to buds (G–J and M–P; arrows indicate signal in protonemata), PpCLV1b::NGG and PpRPK2::NGG signal accumulated mainly in the apical region of buds (Q and R). At two later stages of gametophore development (S–X and Y–J), all promoters were active in gametophores, although the patterns and intensity of activity varied between reporters and by developmental stage. PpCLE1::NGG lines stained most strongly in leaves (S, Y, and E'), PpCLE2::NGG lines most strongly in leaves and gametophore bases (T, Z, and F'), and PpCLE7::NGG lines accumulated signal in rhizoid tips (arrow in U), leaf bases (arrow in A'), and hairs around the apex and the gametophore axis (G'). PpCLV1a::NGG lines did not stain intensely at early stages of gametophore development (P and V) but accumulated signal in gametophore axes and leaves at later stages (B' and H'). In contrast, PpCLV1b::NGG and PpRPK2::NGG lines accumulated signal in gametophore axes and leaves from early stages of development (W and X), and strong signal was detected in branches initiating at gametophore bases (arrows in X, C', and D'). All tissues in (A)–(D') were stained in a solution containing 0.5 mM FeCN for times specified in (A)–(F), and gametophores in (E')–(J') were stained three times longer in a solution containing 2 mM FeCN.

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A WT  
B PpcleAmiR1-3  
C PpcleAmiR4-7  
D Ppclv1a1b  
E Pprpk2

F 2 cell stage  
G  
H  
I  
J

K 4 cell stage  
L  
M  
N  
O

P 6-10 cell stage  
Q  
R  
S  
T

U  
V Col-0  
W clv1, bam1, bam2, bam3  
3.2±0.36

(legend on next page)
division planes that were frequently parallel rather than perpendicular round of cell division from the apical cell also had misset

(Figures 2M–2X). These beta-glucuronidase (GUS) accumulation patterns suggested highly dynamic foci of expression for PpCLEs 1, 2, and 7 and PpCLV1a, PpCLV1b, and PpRPK2 in Physcomitrella, prompting us to investigate roles for CLV pathway components in gametophore initiation and development, i.e., during the transition to 3D growth.

Physcomitrella Mutants Lacking CLAVATA Function Have a Defective 2D to 3D Growth Transition

To identify the functions of CLV pathway components, we used artificial microRNAs (AmiRNAs) to silence expression of PpCLEs 1, 2, and 3 and PpCLEs 4, 5, 6, and 7 (see Strategy for generating PpcleAmiR lines in Methods S1). We used a CRISPR-Cas9 approach to disrupt the function of PpCLV1 paralogs (see CRISPR/Cas9 strategy for generating PpcleAmiR mutants in Methods S1), and gene targeting was used to abrogate PpRPK2 function (see Strategy for generating Pprpk2 KO lines in Methods S1). PpcleAmiR1-3, PpcleAmiR4-7, Ppclv1a1b, and Pprpk2 lines were able to form dense protonephal tissues and thus had a relatively normal 2D growth phase (Figures 3A–3E). However, all four mutant classes had defective development during the 3D growth phase, with a reduction in the overall number of mature gametophores and defects in gametophore development (Figures 3A–3E and 3U). Further examination revealed many more gametophore buds with 1 or fewer leaves in PpcleAmiR1-3, PpcleAmiR4-7, and Pprpk2 mutants than in wild-type (WT) plants (Figure 3U), and Ppclv1a1b mutants had many small gametophores arrested at a later stage of development (Figure 3U). These data suggested early defects in gametophore development with potential feedback onto the gametophore initiation process. To determine how WT and mutant phenotypes diverged during development, we imaged gametophore buds at 2-cell, 4-cell, and a later stage of bud development [6] (Figures 3F–3T). Although WT gametophores initiated normally and showed characteristic oblique cell division plane orientations, the plane of the first division was strongly disrupted in PpcleAmiR1-3 and PpcleAmiR4-7 mutants, and it was set at a shallow angle relative to the main growth axis (compare Figure 3F to Figures 3G and 3H). A second round of cell division from the apical cell also had misset division planes that were frequently parallel rather than perpen-
dicular to the first division plane, and a subset of gametophores therefore formed finger-like projections in place of gametophores (compare Figure 3K to Figures 3L and 3M). At developmental stages where the tetrahedral shape of the apical cell is normally established [6], mutants also had defects indicating problems with growth and cell fate specification, appearing to reiterate divisions normally characteristic of the first gametophore initial (compare Figure 3P to Figures 3Q and 3R). Ppclv1a1b mutant phenotypes diverged from WT after the 2-cell stage, subsequently showing a similar pattern of division to PpcleAmiR1-3 and PpcleAmiR4-7 mutants (Figures 3K–3N and 3P–3S), and some cells reverted to filament identity (Figure 3S). Pprpk2 mutant defects were less severe than Ppce and Ppclv1a1b defects at the earliest developmental stages, and at later stages, swollen cell shapes suggested growth defects as well as division plane defects (Figure 3T). The mutant phenotypes above suggest key roles for the Physcomitrella CLV pathway in modulating cell division planes, cell fate, growth, and proliferation during the 2D–3D developmental transition. The formation of long projections of swollen cells in Ppce mutants (e.g., Figures 3L and 3M) suggests that gametophore identity is attained normally, as cell swelling is a characteristic of gametophore rather than filament initials. The manifestation of plane orientation defects in the first division suggests that WT and mutant gametophore development diverge at the single-celled stage, after cell fate is specified.

Roles for CLAVATA in Regulating Cell Division Plane Orientation Are Conserved between Physcomitrella and Arabidopsis

As roles for CLV in cell division plane orientation were previously unreported, we sought to identify conservation of function with Arabidopsis. To this end, we examined Arabidopsis clv1/bam1/bam2/bam3 quadruple mutant meristems, in which the function of the entire CLV/BAM gene clade is lost [25]. Whereas division plane orientations are normally stereotypic in root meristems, we detected strongly disordered planes in the stem cell niche and ground tissue layers of clv1/bam1/bam2/bam3 mutant roots (Figures 3V, 3W, and S6). Thus, a newly identified role for CLV in cell division plane orientation is conserved between Physcomitrella and Arabidopsis.

Figure 3. The CLV Pathway Regulates Cell Division Plane Orientations during 3D Growth in Physcomitrella and Arabidopsis

(A–E) Although WT plants (A) developed many normal gametophores, PpcleAmiR1-3 (B), PpcleAmiR4-7 (C), Ppclv1a1b (D), and Pprpk2 (E) mutants had no obvious gametophores. The scale bar represents 0.35 cm.

(F–T) PpcleAmiR1-3, PpcleAmiR4-7, Ppclv1a1b, and Pprpk2 mutants have cell division plane defects at the onset of 3D morphogenesis. (F–J) The first division of each bud is indicated by a yellow arrow and is set at a strongly oblique angle in WT (F), Ppclv1a1b (I), and Pprpk2 (J) plants, but is weakly oblique in PpcleAmiR1-3 (G) and PpcleAmiR4-7 (H) mutants.

(K–O) Whereas (K) the second division (blue arrow) from the apical cell (asterisk) is normally oblique and roughly perpendicular to the first, in PpcleAmiR1-3 (L), PpcleAmiR4-7 (M) and Ppclv1a1b (N) mutants, it is roughly parallel to the first. Pprpk2 (O) mutants look normal at this stage.

(P–T) The stereotypical divisions that normally generate the tetrahedral shape of the gametophore apical cell at the 6- to 10-celled stage of development (P) are misset in PpcleAmiR1-3 (Q), PpcleAmiR4-7 (R), and Ppclv1a1b (S), and Pprpk2 (T) mutants. The scale bar represents 30 μm.

(U) Bar chart and boxplot showing that gametophore initiation was disrupted in PpcleAmiR1-3, PpcleAmiR4-7, PpCLV1a1b, and Pprpk2 mutants. The number of gametophores with >3 leaves was counted in 5 WT and mutant plants from a single line representing each mutant class. Gametophore buds with <1 leaf were counted from a 5-mm² area in 3 WT and mutant plants from a single line representing each mutant class. ANOVA, Tukey’s Honest Significant Difference (HSD) test; p < 0.005.

(V and W) Confocal micrographs of WT (Col-0) (V) and clv1/bam1/bam2/bam3 mutant (W) root tips showing disordered cell division plane orientations in the meristem and ground tissue layers. The box in (W) indicates the meristem, and arrowheads indicate the developmental onset of abnormal periclinal division plane orientations in the cortex layer (shaded green). The scale bar represents 20 μm. See also Figure S6, Methods S1, and Table S4.
Physcomitrella Mutants with Disrupted CLV Function Have Defective Gametophore Development

In Arabidopsis and other flowering plants, the CLV pathway is known for its role in maintaining the size of the meristematic stem cell pool [26], and increases in the number of stem cells lead to highly enlarged meristems in both stem cell pool [26], and increases in the number of stem cells known for its role in maintaining the size of the meristematic 

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In Arabidopsis and other flowering plants, the CLV pathway is known for its role in maintaining the size of the meristematic stem cell pool [26], and increases in the number of stem cells lead to highly enlarged meristems in both clv1 and clv3 (cle) mutants. However, Physcomitrella does not fit the Arabidopsis paradigm of meristem function because the shoot apex comprises a single apical stem cell. The apical cell cleaves merophyte daughter cells in a spiral pattern, and merophytes subsequently divide to generate leaf initials and stem tissues [6]. To investigate whether roles for CLV in regulating stem cell function are conserved between Physcomitrella and Arabidopsis, we imaged one of the largest gametophores from 1-month-old WT and mutant plants using light and confocal microscopy and found that mutant gametophores were reduced in height and had developmental defects (Figure 4). Although PpclleAmiR1-3, PpclleAmiR4-7, and Pprpk2 mutants were most severely reduced in height (Figures 4B, 4C, and 4G), Ppcllv1a and Ppcllv1b mutants had milder defects (Figures 4D and 4E). PpclleAmiR1-3, PpclleAmiR4-7, Ppcllv1a1b, and Pprpk2 mutants had defective leaf development, and Ppcllv1b, Ppcllv1a1b, and Pprpk2 mutants also had strong cell fate and/or proliferation defects, developing a callus-like mass at the gametophore base (Figures 4L–4N). Closer inspection revealed that these masses arose by the activity of many ectopic apical cells at the gametophore base (Figure 5). These loss-of-function data suggest that CLV has roles in regulating stem cell function that are conserved between Physcomitrella and Arabidopsis.

CLE Peptides Can Suppress Cell Proliferation in Physcomitrella Gametophores

To further assay conservation in CLV function, we undertook a gain-of-function approach by applying synthetic CLE peptides to growing plants (Figures 6 and S7). After 4 weeks of growth, we found that treatment with a 1-$\mu$M concentration of CLE had no appreciable effect on plant spread or the number of gametophores initiating, indicating that protoneal development is normal (Figure S7). However, although solute controls, a randomized peptide and Arabidopsis CLE41 (a TDIF CLE) have no appreciable effect on gametophore development, Arabidopsis CLV3 and all of the Physcomitrella CLEs cause gametophore dwarling and a strong reduction in leaf size correlating with a reduction in leaf cell number (Figure 6). Although this phenotype superficially resembles the stunted gametophore phenotypes of PpclleAmiR1-3 and PpclleAmiR4-7 mutants (Figures 4B and 4C), we found no evidence of developmental arrest or meristematic overproliferation following CLE application and no difference in the number of gametophores initiating was detected following CLE treatment (data not shown). These data show that CLEs act through a conserved signaling module to regulate cell proliferation specifically during the 3D growth phase in Physcomitrella.

CLE Peptides Can Act through Receptor Components that Are Conserved between Physcomitrella and Arabidopsis

Previous studies in Arabidopsis have shown that application of CLV3-like, but not TDIF-like, CLEs to roots can arrest meristem function [27]. To assay conservation in peptide function, we germinated Arabidopsis seeds on Murashige and Skoog (MS) medium plates containing soluble or peptides at a 1 $\mu$M concentration. Although solute controls, a randomized peptide, and CLE41 caused no arrest of root development, CLV3 and all of the Physcomitrella CLEs caused a significant reduction in root length in Arabidopsis resulting from collapse of the root meristem (Figures 7A–7C, 7E, and 7F). Physcomitrella CLEs therefore regulate growth and proliferation in a similar manner to CLV3 in Arabidopsis. To confirm that PpCLEs can act through a conserved receptor machinery, we used peptide treatment assays on Arabidopsis and...
Physcomitrella rpk2 mutants (Figure 7). Whereas treatment of WT Arabidopsis plants with CLV3-like peptides strongly inhibited root growth, rpk2 mutants showed less growth inhibition when treated with Arabidopsis and Physcomitrella peptides (Figures 7A–7C, 7E, and 7F). These data are in line with previously published results showing that CLV signaling in Arabidopsis [17] and show that Physcomitrella CLEs can also act via RPK2 in Arabidopsis. To determine whether Physcomitrella CLEs act via PpRPK2, we performed similar experiments in WT, Ppcle, and Pprpk2 mutant backgrounds. Ppcle mutant gametophores are roughly the same size as Pprpk2 mutant gametophores, and we reasoned that, if PpCLEs act via PpRPK2, we should detect a response in Ppcle mutants, but not Pprpk2 mutants. As in previous experiments, we found strong inhibition of gametophore development in WT plants (Figure 7D). Potentially due to lack of positional information, treatment of Ppcle mutants with CLE peptides did not rescue developmental defects but nevertheless induced a gametophore dwarfing response, consistent with an intact receptor machinery (Figures 7D and 7G–7I). In contrast, Pprpk2 mutants showed no morphological

Figure 5. Overproliferation Phenotypes in PpAmiRcle, Ppclv1, and Pprpk2 Mutants
(A–E) Light micrographs of mutant gametophore morphology showing that gametophores (B) arrest, (C and E) develop multiple axes (pink arrows), and (C–E) develop swollen bases relative to (A) WT plants. The scale bar represents 200 μm.
(F and G) Confocal micrographs showing (F) overall gametophore morphology and (G) a branch initiating in a leaf axil in WT plants.
(H) Schematic showing Physcomitrella gametophore apex organization with an apical cell (pale yellow) and rotating division plane orientations.
(I–T) Confocal micrographs showing (I–K) PpcleAmiR1-3 mutant gametophore morphologies, with (I) overproliferation at the gametophore base and (J and K) disorganized growth with ectopic meristems.
(L–N) PpcleAmiR4-7 mutant gametophore morphologies with (L) split leaf phenotypes and (M and N) meristem overproliferation and termination.
(O–Q) Ppclv1a1b mutant gametophore morphology (O), with multiple growth axes and multiple meristems at the gametophore base (P and Q).
(R–T) Pprpk2 mutant gametophore morphology with multiple growth axes (R) and multiple meristems at the gametophore base (S and T).
Yellow arrowheads indicate regions of overproliferation or ectopic meristems. Yellow boxes show regions magnified from (J), (M), (P), and (S) to (K), (N), (Q), and (T). The scale bars represent 50 μm.
response to CLE application, suggesting that PpCLEs act via PpRPK2 in regulating 3D growth (Figures 7D and 7G–7J).

**DISCUSSION**

How Might CLV Pattern Cell Division Plane Orientation?

We propose that the CLV pathway regulates the 2D to 3D developmental transition in *Physcomitrella* by orienting gametophore cell division planes and regulating growth and fate. How ligands and receptors act together to do this is not yet clear. One possibility is that CLE ligands diffuse to create a concentration gradient that division planes are patterned against. A similar mechanism involving CLEs patterns cambial meristems in *Arabidopsis* [28], where CLE41 is synthesized in the phloem and diffuses to bind PXY receptors in neighboring procambial cells, thereby imparting spatial information for periclinal division [28]. Constitutive or ectopic expression of *CLE41* disrupts this positional information, resulting in disordered cambial division planes [28]. In *Physcomitrella*, similar patterning could be achieved by sub-cellular localization of receptors to create a graded CLV response in bud initials, or at later stages of development, patterning could be provided by receptor expression in different portions of buds.

It is also possible that CLV signaling does not directly modulate cell division planes but that CLV influences cell division planes via hormone signaling, cell geometry, and/or cell mechanics. Auxin signaling and the activity of microtubule-interacting proteins, such as CLIP-associated proteins (CLASPs), are known to specify cell division planes in *Arabidopsis* embryos [29], and auxin signaling modulates the activity of previously identified factors necessary for correct division plane orientation in *Physcomitrella* buds, including *DEK1* and *NOG1* [30, 31]. There appears to be a complex interplay between auxin and cytokinin in *Physcomitrella* [32–34], and several phenotypes suggest that this interplay is disrupted in *Ppcle*, *Ppclv*, and *Pprpk2* mutants. For instance, cell fate and proliferation at the gametophore base are perturbed (Figures 4 and 5) and leaf cell proliferation is perturbed in plants treated with CLEs (Figure 6), and these aspects of development are auxin and cytokinin regulated [33, 34]. Linking CLV signaling to the hormone pathways regulating growth and fate will be important in unravelling mechanisms of cell division plane specification during 3D growth.

**CLAVATA-Regulated Stem Cell Function Is an Ancestral Feature of Land Plants**

The data we present are important in two evolutionary contexts. First, they show that the CLV pathway originated with land plants and that CLV-regulated stem cell proliferation and function is
likely to be an ancestral feature of land plants. The acquired capacity of land plants to orient stem cell divisions in multiple planes enabled diversification by permitting plants to develop upright axes with organs arranged in multiple orientations, a crucial step in shoot evolution [1]. Stem cell division plane defects in *Ppcle* mutants specifically affect the transition to 3D growth and the 3D growth phase, and morphological responses to peptide application are also specific to the 3D growth phase. Thus, in an ancient land plant group, CLV regulates a developmental transition that mirrors an evolutionary transition. The data suggest that CLV was a genetic novelty for a key morphological innovation of land plants.

**CLAVATA-Regulated Meristem Functions Originated prior to WOX- and KNOX-Regulated Meristem Functions**

Second, the data are important in the context of evolving gene regulatory networks for land plant meristem function. Whereas the first land plant meristems comprised a single gametophytic stem cell, the multicellular sporophyte meristems of vascular plants combine stem cell and more generally proliferative capacities [1]. Class I KNOX genes regulate meristemetic proliferation in vascular plants [35, 36], but these roles are not shared between bryophytes and vascular plants. Moss KNOX (*MKN*) genes are primarily expressed in sporophyte tissues [24, 37], and although loss-of-function *mkn2* mutants have elongation...
defects in sporophytes, they have normal gametophytes [37]. WOX genes are key regulators of stem cell proliferation in Arabidopsis [19]. However, this function was acquired by the recently derived WUS gene clade [38, 39], and the downstream pathways regulated by CLV in Physcomitrella are likely to be distinct from those in Arabidopsis as Ppwox13L mutant gametophores develop normally [40]. Thus, class I KNOX- and WOX-regulated meristem functions were both acquired after the bryophyte-vascular plant divergence. CLV was important in the origin of plant meristem functions in the gametophyte stage of the life cycle, and we speculate that CLV was recruited to regulate stem cell function in the sporophyte stage of the life cycle prior to the origin of KNOX- and WOX- regulated meristem functions.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, four tables, three data files, and supplemental text and is available online at https://doi.org/10.1016/j.cub.2018.05.068.

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## STAR★METHODS

### KEY RESOURCES TABLE

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(Continued on next page)
**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, Jill Harrison (jill.harrison@bristol.ac.uk). Please note that the transfer of transgenic materials will be subject to MTA and any relevant import permits.
EXPERIMENTAL MODELS AND SUBJECT DETAILS

Arabidopsis plant growth

Columbia (Col-0), rpk2-4 (clt) or clv1/bam1/bam2/bam3 mutants [17, 25] were used for Arabidopsis experiments. Homozygous rpk2-4 mutants were confirmed using a BamHI dCAPs screen with a PCR fragment amplified using primers AtRPK2-BamHIF and AtRPK2-BamHIR (see primer list). Seeds were surface sterilized in 5% (v/v) sodium hypochlorite for 10 min and washed three times with sterile de-ionised water. They were then stratified at 4°C in darkness for 48 hr and sown on 0.5 X MS plates containing 0.8% agar [53]. Plants were grown vertically for 7 days at 25°C in a 16 hr light/ 8 hr dark cycle prior to observation (rpk2 experiments) or at 22°C under continuous light (clv1/bam1/bam2/bam3 experiments).

Physcomitrella plant growth

The Gransden strain of Physcomitrella patens [54] was used for all experiments. Plants were grown in sterile culture on BCDAT plates at 23°C in continuous light at 30-50 μmols⁻¹ in Sanyo MLR-351 growth cabinets. BCDAT medium comprises 250mg/L MgSO₄·7H₂O, 250mg/L KH₂PO₄ (pH6.5), 1010mg/L KNO₃, 12.5mg/L FeSO₄·7H₂O, 0.001% Trace Element Solution (0.614mg/L H₃BO₃, 0.055mg/L AlK(SO₄)₂·12H₂O, 0.055mg/L CuSO₄·5H₂O, 0.028mg/L KBr, 0.028mg/L LiCl, 0.389mg/L MnCl₂·4H₂O, 0.055mg/L CoCl₂·6H₂O, 0.055mg/L ZnSO₄·7H₂O, 0.028mg/L KI and 0.028mg/L SnCl₂·2H₂O), 0.92 g/L C₆H₁₂N₂O₆ and 8g/L agar with CaCl₂ added to a 1mM concentration after autoclaving. Protonemal cultures for transformation were grown on BCDAT plates overlaid with autoclaved cellophane disks and molecular and phenotypic analyses were undertaken using 1 mm spot cultures unless otherwise stated.

METHOD DETAILS

Sequence retrieval

CLE genes

Previously described Arabidopsis thaliana and Oryza sativa CLE sequences were respectively retrieved from TAIR and RAP-DB [55]. Selaginella moellendorffii [56], Glycine max [57, 58] and Picea abies [59] CLEs were retrieved from NCBI. To extend taxon sampling within land plants and identify previously unknown CLEs, the CLE domains of Arabidopsis thaliana CLV3 and CLE41 were used as tBLASTn queries with an e-value cutoff of e⁻100 to screen transcriptome or draft genome assemblies of a basal angiosperm (Amborella trichopoda), a fern (Diploziaum trichopoda), a hornwort (Anthoceros agrestis), a moss (Physcomitrella patens v1.6 [60]) and a liverwort (Marchantia polymorpha). Positive hits were used in reciprocal BLASTs until no new sequences were retrieved. All sequences retrieved were checked for the presence of a signal peptide [61] using SignalP [50, 62]. Newly identified CLE sequences were named with a two-letter prefix denoting the genus and species and numbered (Table S1). A list of taxa searched is given in Table S2.

CLV1/RPK2 genes

Arabidopsis CLV1 and RPK2 sequences were used to query the databases listed above using tBLASTn searches with an e-value cutoff of e⁻1000. As the LRR-Receptor kinase family is large, only sequences that retrieved CLV1/RPK2 genes were used to query the databases listed above using tBLASTn searches with an e-value cutoff of e⁻1000. As the LRR-Receptor kinase family is large, only sequences that retrieved CLV1/RPK2 genes were used to query the databases listed above using tBLASTn searches with an e-value cutoff of e⁻1000. As the LRR-Receptor kinase family is large, only sequences that retrieved CLV1/RPK2 genes were used to query the databases listed above using tBLASTn searches with an e-value cutoff of e⁻1000. As the LRR-Receptor kinase family is large, only sequences that retrieved CLV1/RPK2 genes were used to query the databases listed above using tBLASTn searches with an e-value cutoff of e⁻1000.

Phylogenetic reconstruction

To infer CLE relationships, the conserved 12 amino acid CLE motif from 193 CLEs was used in neighbor joining reconstructions compiled with the JTT model in MEGA7.0.26 [51] (Figure S1; Data S1). This approach was taken because there is little conservation in CLE structure outside the CLE motif and some characters can only yield limited phylogenetic signal (see [63]). To infer CLV/BAM relationships, 525 conserved amino acid residues from 36 genes were used in maximum likelihood reconstructions with the JTT model in MEGA7.0.26 [51] (Figure S2; Data S2). To infer RPK2 relationships, 782 conserved amino acid residues from 18 genes were used in maximum likelihood reconstructions with the JTT model in MEGA7.0.26 [51] (Figure S3; Data S3). For all analyses 100 bootstrap replicates were performed and support values over 50% (CLE tree) or 70% (CLV1/BAM and RPK2 trees) are represented above the branches.

Molecular biology

RT-PCR

Total RNA was isolated from 4 day-old protonemal cultures and 10, 21 or 28 day old spot cultures using the QIAGEN RNeasy method. RNA was DNase treated prior to reverse transcription with SuperScript II following manufacturer’s guidelines. Semiquantitative...
RT-PCR was undertaken using UBIQUITIN (Pp1s56_52V6.1) as a loading control. Where possible, primers were designed to span introns to detect genomic contamination, and sequences are listed in Table S4.

### Genomic DNA extraction

Genomic DNA was extracted from protonemal cultures using a CTAB (Hexadecyltrimethylammonium bromide) protocol. Snap-frozen tissue was ground in liquid nitrogen and transferred to tubes containing prewarmed extraction buffer (2% CTAB, 1.4 M NaCl, 100 mM Tris pH8.0, 20 mM EDTA pH8.0, 2% PVP and 1 mg/mL RNaseA), with no more than 100 mg of tissue per mL of buffer. Samples were incubated for 10 min at 65°C and an equal volume of 24:1 chloroform:isoamyl alcohol was added and mixed with each sample to form an emulsion. The tubes were centrifuged at high speed (> 10,000 rpm) for 10 min, and the aqueous phase was transferred to a fresh tube prior to DNA precipitation with an equal volume of isopropanol and repeated centrifugation. DNA was washed with 70% ethanol and dissolved in water, 10 mM Tris pH 8.0 or 10 mM Tris pH 8.0 with 1 mM Na2EDTA.

### Generation of promoter::NGG constructs

Promoter sequences from *PpCLE1* (2.1 kbp), *PpCLE2* (2.1 kbp), *PpCLE7* (2 kbp), *PpCLV1a* (2 kbp), *PpCLV1b* (2.8 kbp) and *PpRPK2* (1.4 kbp) were PCR amplified using a proof-reading Taq polymerase and primers listed in Table S4 and cloned directly or via pGEMT Easy into the Smal site of PIG1NGGII [41] or derivatives with alternative selection cassettes and sequenced prior to linearization and transformation as illustrated in Methods S1.

### Generation of AmiR constructs

To generate *PpcleAmiR1-3* and *PpcleAmiR4-7* constructs, resistance cassettes from pGREEN [45] were first inserted into a blunt-ended HindII site of pBJ36 [44]. A soybean UBIQUITIN promoter from pBRACT211 [45] was inserted into the Smal site to drive AmiRNA expression and the resultant plasmids were named pJH125 (KanR) and pJH131 (HygR). AmiRNAs were designed according to [42], generated by degenerate PCR using a proof-reading Taq polymerase and the pRS300 plasmid as a template, cloned into pGEMT-EASY and transferred as Xmal/BamHI fragments into pJH125 or pJH131. Silencing constructs were checked by sequencing and digested with SacI for transformation as illustrated in Methods S1.

### Generation of CRISPR constructs

Small cassettes containing two BsaI restriction sites and sgRNAs [66] driven by the Physcomitrella U3 or U6 promoter and flanked by attB sites were synthesized and cloned into pDONR201. sgRNA sequences were selected and screened for off target hits in the *Physcomitrella* V3 genome using http://crispor.tefor.net/. To clone guide RNAs into expression cassettes, two primers consisting of guide sequences with overhangs for U3 and U6 promoters were annealed and ligated into U3 or U6 expression vectors pre-digested with BsaI. Constructs were checked by sequencing and co-transformed with pACT::Cas9 [46] to engineer mutants as illustrated in Methods S1.

### Generation of RPK2 KO construct

5' and 3' flanking regions were PCR amplified with a proof-reading Taq polymerase and cloned sequentially into pGEMT-EASY using primers listed in Table S4. The resultant plasmid was digested with Pmel and Ascl, and the AphIV cassette from pBHRF-108 [48] was ligated between *PpRPK2* flanking regions. This plasmid was checked by sequencing and linearized for transformation as illustrated in Methods S1.

### Transgenic line generation and phenotype analyses

#### Moss transformation and line authentication

For gene targeting and AmiR approaches, 10-20 μg of plasmid DNA was isolated using the QIAGEN Plasmid Plus Midi system and linearized as illustrated in Methods S1. For CRISPR approaches, 5-7 μg of Cas9 and pNRF, and 2-3 μg of each gRNA-expressing construct were purified and pooled for transformation [46] at a concentration of at least 1 μg per μL. All solutions for the transformation procedure were prepared prior to commencing transformation [67]. First, a polyethylene glycol (PEG) solution was prepared by adding 10 mL of mannitol/CaNO3 solution (8% mannitol, 0.1 M Ca(NO3), 10 mM Tris pH7.2) to 2 g of molten PEG 6000, and the tube containing the solution was left in a water bath at 45°C. To isolate protoplasts, homogenous protonemal cultures were grown for 5 days to a week post passage. A 1% driselase solution was prepared in 25 mL 8% mannitol, and the supernatant was removed and filter sterilized into to a clean 50 mL falcon tube following centrifugation. Tissue from 4-6 plates was transferred into the driselase solution and the tissue suspension was left for 30-40 min with intermittent mixing to allow cell wall digestion. The mixture was then transferred into a fresh tube through a 50 μm filter to remove cell and cell wall debris. Protoplasts were sedimented by centrifugation for 3 min at 120 g, resuspended and washed three times in 10 mL of 8.0% mannitol prior to counting with a hemocytometer. Protoplasts were then sedimented and resuspended to a density of 1.2 x 10^6 per mL in MMM solution (0.5 M mannitol, 0.15 M MgCl2 and 0.1% MES pH5.6). 300 μL aliquots of protoplasts were dispensed into Falcon tubes prior to addition of DNA and 300 μL PEG solution, and cells were then heat shocked for 5 min at 45°C. Transformation mixtures were progressively diluted with 1 mL of 8% mannitol solution and washed. Protoplasts were then sedimented by centrifugation as above and washed four more times. After the final wash and spin, protoplasts were resuspended in 5 mL liquid BCD medium (constituents as specified above but without ammonium tartrate or agar) with 8% mannitol, 10 mM CaCl2 and 0.5% glucose, wrapped in aluminum foil and left at 23°C overnight. The next day, the protoplast suspension was plated onto BCDAT plates overlain with cellophane and containing 8% mannitol and 5 g/L glucose, using c.1 mL per plate. Plants were grown under standard conditions until regenerants comprised 10-20 cells. Cellophane discs were then transferred onto BCDAT plates containing antibiotics for selection (25 μg/mL Hyg, 50 μg/mL G418, 100 μg/mL BSD). Plants were grown for 2 weeks on selection plates prior to transfer onto BCDAT plates lacking antibiotic for 2 weeks and then back on to selection.
plates for a further 2 weeks. All lines were screened by PCR, RT-PCR, Southern analysis or sequencing as illustrated in Methods S1. PCR conditions were standard and primer sequences are listed in Table S4.

**Southern hybridization**

For *P. palustris* Southern blots, 10-15 μg genomic DNA was digested with EcoRV and fractionated on 0.8% agarose in a pre-hybridization solution (3 X SSC, 1% SDS, 0.1% sodium pyrophosphate, 5 X Denhardt’s and 200 μg per mL sheared salmon sperm DNA). The probe template was excised with EcoRV and BamHI from the *P. palustris* construct and the probe was synthesized using an Amersham Rediprime II DNA labeling kit as per manufacturer’s instructions. Hybridization was undertaken in a 3 X SSC buffer at 58°C and this was followed by two 20 min washes at 58°C in 3 X SSC and 2 X SSC buffers respectively. Membranes were wrapped in Saran Wrap and used to expose X-ray film, and film was then developed using a film processor. For promoter::NGG and *P. prp* Southern blots, 2.5-3 μg genomic DNA was digested as illustrated in Methods S1. Probe templates comprising *PIG1* flanking sequence, *PpRPK2* coding sequence or a hygromycin resistance cassette were PCR amplified and labeled using the Roche DIG High Prime system. Hybridization was undertaken overnight at 42°C using the Roche DIG Easy Hyb system. Washing and detection were performed using the manufacturer’s protocol from the Roche DIG High Prime DNA labeling and Detection Starter kit II.

**Physcomitrella plant imaging**

To assess whole plant and gametophore phenotypes, 4 to 5 week-old spot cultures were imaged using a Keyence VHX-1000E digital microscope with a 20-50 X or 50-200 X objective. To analyze bud phenotypes, confocal imaging was undertaken on tissue stained with 0.5 mg/ml propidium iodide using a Leica TCS SP5 microscope with excitation from the 488 or 514 laser line and emission collected at 600-650 nm or using a Zeiss 710 LSM with excitation from a 514 laser line and emission collected at 566-650 nm. To analyze leaf phenotypes, leaves were removed from gametophores, arranged in heteroblastic series, cleared in 1% chloral hydrate overnight, washed in deionised water and treated with 2M NaOH for 2 hr. They were then washed with water and stained with 0.05% toluidine blue for 2 min before destaining for 10 min in water. The stained leaves were then mounted on a slide under a coverslip and imaged to visualize cell outlines. Adobe Illustrator was used to trace leaf outlines to produce silhouettes for illustration purposes (Figure 6). Quantitative analyses of leaf size were performed using ImageJ, and cell numbers were evaluated using the ‘analyze particles’ option [68]. Leaf size comparisons were undertaken using leaves from the same point in the heteroblastic leaf series [33] as stipulated in figure legends.

**Arabidopsis plant imaging**

Root length was scored from scanned images of plants grown on ½ X MS plates using ImageJ [68]. To visualize rpk2 meristems, roots were stained with Lugol’s stain, cleared, and imaged using a 20 X objective on a Leica DMRXA microscope with DIC [69]. clv1/bam1/bam2/bam3 roots were stained with 15 mM propidium iodide and imaged using a C-Apochromat 40 X/1.20 W Korr objective on a Zeiss LSM710 microscope. Excitation and emission windows for propidium iodide were 560 nm and 566-719 nm respectively. Confocal images were analyzed and processed using ImageJ and Adobe Photoshop.

**GUS staining and imaging**

*Physcomitrella* plants grown on BCDAT were cut out of plates with agar and incubated at 37°C in a 100 mM phosphate buffer with 10 mM Tris pH8.0, 1 mM EDTA pH8.0, 0.05% Triton X-100, 1 mg/mL X-Gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucuronide) and potassium ferri/ferrocyanide using concentrations and times indicated in Figure 2 and legend. Plants were bleached in 70% ethanol and dissected and mounted in 0.3% low melting point agarose prior to imaging with a Keyence VHX-1000 digital microscope with a 0-50 X or a 50-200 X objective.

**CLE peptide application**

Synthetic CLE peptides (Genecust, >95% purity) were dissolved in phosphate buffer (50 μM, pH6.8) to stock concentrations of 1 mM and 10 mM. Plants were grown on BCDAT plates containing peptides diluted to concentrations specified in the main text.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Quantification and statistical analyses were undertaken as stipulated in main text and SI figures and figure legends.

**DATA AND SOFTWARE AVAILABILITY**

Genome and transcriptome data were searched as described in Method Details and details of data repositories are listed in Table S2.