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A prion-like protein regulates the 2-dimensional to 3-dimensional growth transition in the moss *Physcomitrium patens*

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SUMMARY STATEMENT

The transition to 3D growth is negatively regulated by a prion-like protein that both alters cytokinin signaling and has been implicated in liquid-liquid phase separation (LLPS).

ABSTRACT

The colonization of the land by plants coincided with the evolution of 3-dimensional (3D) growth; the acquisition of apical cells with the capacity to rotate the plane of cell division. The moss *Physcomitrium patens* has recently been developed as a model to dissect the genetic basis of 3D growth, an invariable and unifying feature of all land plants. The cytokinin-unresponsive *Ppnog1-R* mutant incorrectly orients division planes in developing buds and thus fails to make the transition to 3D growth. To reveal the genetic interactors of the *PpNOG1* gene, which encodes a protein with a C-terminal UBA domain, we performed a screen and identified the *suppressor of nog1a* (*snog1a*) mutant. We have mapped the causative mutation to a gene that encodes a prion-like protein related to FLOE2/3 and demonstrated that the mutant phenotypes observed in both a *nog1* disruptant mutant (*nog1dis*) and *snog1a* can be attributed to changes in cytokinin perception. We present a revised model for 3D growth and suggest that the 2D-to-3D growth transition is regulated, at least in part, by liquid-liquid phase separation (LLPS).

INTRODUCTION

In the absence of cell movement, plants rely on cell growth processes, combined with asymmetric and precisely orientated cell divisions, to generate new morphologies and diverse cell types with specialised functions. The main driving force behind cellular diversity is the formation of apical cells that can divide to self-renew and generate new cell types, and it is the geometry of an apical cell and the way it divides that can greatly influence the pattern of growth and development that follows. Thus, diversification of plant form can largely be attributed to altered division processes in apical cells, which occurred prior to the transition from water to land approximately 470 million years ago (Kenrick & Crane, 1997).

Charophycean green algae, the sister lineage to the land plants, can develop apical cells but these only have the capacity for either 1-dimensional (1D) growth (one cutting face) or 2-dimensional (2D) growth (two cutting faces). As a result, the morphologies represented within the multicellular charophytes are typically filamentous (e.g., *Chara braunii*) or disc-like (e.g., *Coleochaete orbicularis*) (Kenrick and Crane, 1997; Wickett et al., 2014; Delwiche and Cooper, 2015; Harrison, 2017). 3-dimensional (3D) growth, the development of apical cells with three or more cutting faces, is a unifying and unvarying feature of all land plants. It was

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69   likely the emergence of 3D growth processes, along with the development of a multicellular

70   sporophyte and the acquisition of vegetative desiccation tolerance, that enabled successful

71   terrestrialization (Delwiche & Cooper, 2015; Harrison, 2017; Moody, 2020).

72   The gametophyte of the moss *Physcomitrium patens* is well suited to studies of 3D growth.

73   This is because an extensive 2D filamentous growth phase precedes the transition to 3D

74   growth, and thus the 2D to 3D growth transition can be studied without causing lethality, as

75   the 2D growth phase can be vegetatively propagated in perpetuity (Moody et al., 2018;

76   Moody et al., 2018b; Moody et al., 2021). The gametophyte phase of the *P. patens* life cycle

77   begins with the germination of a haploid spore, which gives rise to a 2D branching

78   filamentous network known as the protonema, which extends by tip growth. The protonema

79   consists of two cell types; the first to emerge are the chloroplast-dense chloronemal cells,

80   and subsequently caulonemal cells that form because of an auxin-mediated reprogramming

81   of a chloronemal apical cell into a caulonemal apical cell (Jang and Dolan, 2011; Jaeger and

82   Moody, 2021). Caulonemal cells can undergo a lateral division to produce side branch

83   initials, most of which go on to form secondary protonema (2D fate, approximately 95%) but

84   some acquire 3D fate (gametophore initials) and give rise to gametophores (approximately

85   5%) (Aoyama et al., 2012). The fate of a side branch initial is likely determined by highly

86   localized cues within a caulonemal cell prior to side branch emergence, as a single

87   caulonemal cell can simultaneously divide to give rise to both a filament and a gametophore

88   (Harrison et al., 2009). Elegant work by Aoyama and colleagues has demonstrated that

89   persistent expression of a group of AP2-type transcription factors (*PpAPB1-4*) is required to

90   commit a side branch to 3D cell fate. Loss of *PpAPB1-4* expression corresponds to the

91   maintenance of 2D cell fate, and consequently, mutants lacking all four genes fail to make

92   the transition from 2D to 3D growth (Aoyama et al., 2012).

93   A filament initial is readily distinguishable from a gametophore initial: (i) the angle of the

94   lateral "transition" division can dictate whether a caulonemal cell gives rise to a filament or a

95   gametophore (Tang et al., 2020); and (ii) gametophore initials swell diffusely and divide in a

96   characteristically oblique manner during the specification of 3D growth, at the first, second

97   and third divisions of the developing gametophore. Successive rotating divisions then lead to

98   the establishment of a tetrahedral apical cell at the apex of the shoot. The apical cell

99   continuously self-renews and divides in three planes to form phyllid initials that go on to form

100   the leaf-like phyllids that are organized around the central axes of developing gametophores

101   in a spiral phyllotaxy (Harrison et al., 2009). Mature gametophores bear both archegonia and

102   antheridia, which house eggs and sperm respectively. Following fertilization, a mitotic

103   programme initiates, leading to the formation of a multicellular diploid sporophyte, which

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104 undergoes meiosis to produce haploid spores to restart the life cycle (Cove and Knight,
105 1993).

106 It has long been known that cytokinin induces the formation of gametophore initials but is
107 insufficient to maintain 3D growth, as treatment with high levels of cytokinin produces buds
108 that develop into callus-like tissue, rather than structurally organized gametophores
109 (Brandes and Kende, 1968; Ashton et al., 1979). In a variety of developmental contexts,
110 auxin has been consistently implicated as the signal required to 'break symmetry' in plants
111 (Petricka et al., 2009; Shao and Dong, 2016). Perhaps unsurprisingly, the formation of
112 gametophore initial cells and the specification and maintenance of 3D growth also relies
113 upon auxin signaling, although the application of high levels of auxin can antagonize
114 cytokinin and reverse the effects of cytokinin treatment (Brandes and Kende, 1968).
115 Nevertheless, auxin levels are notably high during the specification of 3D growth but
116 diminish once a tetrahedral apical cell has been established (Thelander *et al.*, 2018). It is
117 likely that auxin acts to promote cell differentiation and that cytokinin acts to continually
118 maintain apical cell proliferation (Hata and Kyozuka, 2021). Thus, it appears that a highly
119 regulated balancing act between auxin and cytokinin is required for both the specification
120 and maintenance of 3D growth.

121 In recent years, functional studies have demonstrated that the transition to 3D growth is
122 complex and regulated at many levels; both epigenetically (Mosquna et al., 2009; Okano et
123 al., 2009; Raquid et al., 2023) and transcriptionally (Aoyama et al., 2012), and an ever-
124 expanding number of studies have begun to connect complex cell signaling pathways to
125 post-translational regulation (Girod et al., 1999; Perroud et al., 2014; Demko et al., 2014;
126 Johansen et al., 2016; Hoernstein et al., 2016; Schuessele et al., 2016; Whitewoods et al.,
127 2018; Moody et al., 2018; Perroud et al., 2020; Moody et al., 2021; Cammarata et al., 2022).

128 Using a forward genetics approach, we previously demonstrated that the *NO*
129 *GAMETOPHORES 1* (*PpNOG1*) gene is essential for the transition to 3D growth in *P.*
130 *patens*. Notably, the *PpNOG1* gene encodes a protein with a prominent C-terminal ubiquitin-
131 associated (UBA) domain, which has been shown to be associated with protein degradation
132 processes (Hofman and Bucher, 1996; Su and Lau, 2009; Moody et al., 2018). Mutants
133 lacking a functional copy of *PpNOG1* (the '*no gametophores 1 – Reference'* mutant;
134 *Ppnog1-R*) produce significantly fewer gametophore initials than wild type, even in the
135 presence of cytokinin. Those gametophore initial cells that do form cannot correctly orient
136 the characteristically oblique plane of the first cell division. Cell division planes are then
137 misplaced thereafter, leading to the formation of defective gametophores that undergo very
138 early developmental arrest (Moody et al., 2018). Since the *PpAPB* genes are downregulated
139 in the *Ppnog1R* mutant, we previously proposed that PpNOG1 may positively regulate 3D

140 growth by degrading a repressor of *PpAPB* transcriptional activation, although the identity of
141 the target(s) remains unknown (Aoyama et al., 2012; Moody et al., 2018). To build on our
142 understanding of the role played by *PpNOG1* in both the initiation and specification of 3D
143 growth, we generated a *Ppnog1* disruption mutant (*Ppnog1dis*) and then performed a
144 suppressor screen to identify mutations that alleviated the *Ppnog1dis* mutant phenotype (i.e.,
145 reversion to 3D growth). Here, we describe the screen, along with the detailed
146 characterization of the *suppressor of nog1a* (*snog1a*) mutant and the identification of the
147 causative mutation within a gene encoding a prion-like protein.

148

149 **RESULTS**

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151 **Disruption of the *PpNOG1* locus recapitulates the *Ppnog1-R* mutant phenotype.**

152 Previously, we performed a UV-mediated forward genetic screen that led to the identification
153 of the *PpNOG1* gene (Moody et al., 2018). To explore the *PpNOG1* genetic interaction
154 network underpinning the 2D to 3D growth transition, we designed a suppressor screen to
155 identify mutations that alleviated the 3D-defective phenotype caused by loss of *PpNOG1*
156 function (i.e., the reacquisition of 3D growth). A preliminary suppressor screen of
157 approximately 3,000 UV-mutagenized lines of the original *Ppnog1-R* mutant, generated in
158 our original forward genetic screen, identified eight mutants that exhibited a complete
159 reversion to 3D growth. In each of these mutants, the phenotype was caused by correction
160 of the previously described mutation; a T>C transition that converted the premature
161 termination codon back to an arginine residue (data not shown). We therefore decided to
162 disrupt the *PpNOG1* locus in such a way that would prevent repair simply through the
163 introduction of UV-induced SNPs. To that end, we set out to generate a *nog1* knockout
164 mutant in which the entire coding sequence of *PpNOG1* had been replaced with a
165 hygromycin resistance cassette. Several attempts were made to obtain a line in which the
166 entire *PpNOG1* sequence had been removed. However, as was the case in previous
167 attempts (Moody et al., 2018), we could achieve disruption of the *PpNOG1* locus but
168 unfortunately not a full deletion. Nevertheless, a *nog1* null mutant was generated in which a
169 portion of the *PpNOG1* promoter had been excised following recombination (Fig. S1A).
170 Furthermore, we were consistently unable to detect a *PpNOG1* transcript in this line, and
171 thus had confirmed that we had generated a bona fide *PpNOG1* disruptant mutant (*nog1dis*)
172 (Fig. S1B). Consistent with the original *Ppnog1-R* mutant, the *nog1dis* mutant completely
173 and consistently failed to make the transition to 3D growth (Fig. 1). Thus, disruption of the
174 *PpNOG1* locus recapitulated the original *Ppnog1-R* mutant phenotype and generated a
175 suitable line for mutagenesis.

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The *suppressor of nog1a* (*snog1a*) mutant can specify 3D growth and responds to cytokinin.

A suppressor screen of 2,864 UV-mutagenized lines of the *nog1dis* mutant yielded two '*suppressor of nog1*' (*snog1*) mutants that exhibited a restoration of 3D growth. In one of these mutants, *suppressor of nog1a* (*snog1a*), the formation of gametophores was partially restored to approximately 45% of the frequency of wild type (Fig. 2A,B), although these were somewhat stunted and emerged later than those formed in the wild type (Fig. 2C,D; Fig. S2).

In contrast to the *nog1dis* mutant, in which responses to cytokinin are impaired in a similar manner to that of the *Ppnog1-R* mutant, buds can be induced by cytokinin treatment in the *snog1a* mutant, although to a lesser extent than in wild type (Fig. 3). Thus, the mutant phenotypes observed in both the *nog1dis* and *snog1a* mutants can be attributed to changes in cytokinin perception.

To determine whether the cell division orientation defects had been repaired in the *snog1a* mutant, we obtained z-stack projections of developing buds stained with propidium iodide. In wild-type *P. patens*, the first division of the gametophore initial cell was invariably oblique and yielded an apical and a basal cell (Fig. 3A). Two additional oblique divisions of the apical and basal cells then occurred rather synchronously. Although the angle of the division plane was consistent in each case, the order in which the cells divided was inconsistent; we generally observed that the apical cell divided before the basal cell as often as the basal cell divided before the apical cell (Fig. 3B,C). Successive rotating divisions specified a gametophore apical cell with a characteristic tetrahedral shape, which self-renewed and divided to give rise to the phyllids, which wrap around the central axis of the gametophore in a spiral phyllotaxy (Fig. 3D) (Harrison et al., 2009). In the *nog1dis* mutant, similarly to the previously described *Ppnog1-R* mutant, significantly fewer gametophore initial cells formed, and the first division plane of the gametophore initial cell was not characteristically oblique. In most cases the initial division pattern followed that of a filament initial cell, in which the division plane was positioned roughly parallel to the parental cell from which it was derived (Fig. 3E). Cell plates were then positioned randomly during subsequent divisions, which prevented the specification and maintenance of a tetrahedral apical cell. In some cases, the gametophore initial cells swelled and elongated excessively but could then divide in a somewhat oblique manner at the first and second divisions (Fig. 3F). However, cell plates were invariably misplaced from the onset of the third division (Fig. 3G). Most developing gametophores arrested early in development, but occasionally callus-like buds appeared because of uncontrolled proliferation (Fig. 3H). Moreover, bifurcation events were

212 occasionally observed, which were a result of confused cell fate acquisition, and often
213 associated with supernumerary apical cell formation. However, none of these apical cells
214 were successfully maintained long enough to produce a gametophore. In the *snog1a* mutant,
215 the formation of gametophore initial cells was partially restored, and these largely followed a
216 wild-type pattern of development, with some exceptions. In some cases, we observed that
217 the angle of the first division plane had been corrected to some extent, but not fully, and in
218 these instances a mature gametophore was not formed (Fig. 3I). However, in most cases,
219 the first division plane was characteristically oblique and thus the orientation of the division
220 plane had been fully corrected (Fig. 3J). This was followed by two further correctly oriented
221 oblique divisions (Fig. 3K), which formed the prerequisites for the specification of a
222 conspicuous tetrahedral apical cell at the apex of developing gametophores (Fig. 3L). Thus,
223 restoration of gametophore initial cell formation in the *snog1a* mutant was accompanied by
224 the reversion of the cell division orientation defects observed in the *nog1dis* mutant.

225 Notably, the phenotype observed in the *snog1a* mutant only constituted a partial restoration
226 of the *nog1dis* phenotype. Nevertheless, a tetrahedral apical cell was established and
227 maintained in the *snog1a* mutant, and the gametophores formed were fully viable.

228

229 **The causative mutation of *snog1a* resides in a gene that encodes a prion-like protein.**

230 To identify the causative mutation in the reproductively viable *snog1a* mutant, we obtained
231 phenotypically segregating populations by performing a cross between the *snog1a* mutant
232 and the highly fertile non-mutagenized Reute::mCherry strain (Perroud et al., 2020).
233 Because a conventional cross between two different haploid strains was carried out, and at
234 least two genetic loci were mutated (the mutation within the *PpNOG1* gene, and the
235 unknown mutation that caused the *snog1a* phenotype), we expected one quarter of the
236 progeny to exhibit the original *nog1dis* mutant phenotype and the remainder to exhibit
237 varying capacities for 3D growth (Fig. S3A). The frequencies that we observed were
238 generally consistent with the mutation of a single genetic locus in the *snog1a* mutant,
239 although this is not statistically significant (Fig. 5A). One possible explanation for the
240 reduced *nog1dis* mutant phenotype frequency observed was that disruption of the *PpNOG1*
241 gene affects haploid spore germination and/or the viability of young sporelings.
242 Nevertheless, the frequencies observed did not support the idea that causative mutations
243 resided in two or more genetic loci. We therefore prepared genomic DNA from 80 individuals
244 that exhibited the *nog1dis* phenotype and pooled these in equimolar amounts, and this
245 became the 'wild-type pool' (all individuals resembled the non-mutagenized parental line,
246 *nog1dis*). We also prepared genomic DNA from 98 individuals with the capacity for 3D
247 growth, showing a preference for those that most closely resembled the *snog1a* mutant, and

248   this became the 'mutant pool' (Supplementary Figure S3A). These two pools were

249   sequenced at 44X coverage alongside both parental lines; the *nog1dis* mutant (generated in

250   the Villersexel wild-type strain) and the Reute::mCherry line. To identify the genomic region

251   containing the *snog1a* mutation, bulk segregant analysis was performed. Single nucleotide

252   polymorphisms (SNPs) that differed between the two parental lines were identified as

253   markers and the frequency of each SNP variant was mapped across the chromosomes to

254   show the parental origin of each region. For regions not associated with the phenotypes in

255   the two pools, the expected SNP frequency was around 0.5, showing equal contribution from

256   both parents. The expected *snog1a* mutant allele frequency was 0 in the wild-type pool and

257   0.67 in the mutant pool (Fig. S3B,C). When the allele frequencies for the mutant individuals

258   were plotted across all 27 chromosomes in the *P. patens* genome assembly, a peak of the

259   expected allele frequency was revealed on chromosome 8, which provided a region of the

260   genome to interrogate for causative mutations (Fig. S3D). Our analysis revealed that two C

261   > T transitions generated two distinct in-frame termination codons (Gln^{335}Ter and Gln^{374}Ter)

262   in a single gene (Pp3c8_19720) (Fig. 5B,C). We cloned and sequenced the coding

263   sequence of this gene and confirmed that both point mutations were present in the *snog1a*

264   mutant, but absent from both the *nog1dis* mutant and the Reute::mCherry line

265   (Supplementary Data S1). In addition, sequencing of the corresponding transcript confirmed

266   the presence of the point mutation but did not reveal any splice variants in the *snog1a*

267   mutant, a phenomenon observed in both previously described *Ppnog1-R* and *Ppnog2-R*

268   mutants (Supplementary Data S2) (Moody et al., 2018; Moody et al., 2021).

269   The gene mutated in the *snog1a* mutant encodes a protein with a C-terminal UBA, and thus

270   the protein domain architecture resembles that of the PpNOG1 protein (Moody et al., 2018).

271   To infer phylogenetic relationships for the putative SUPPRESSOR OF NOG1A protein, we

272   set out to retrieve orthologous sequences from the genomes of representatives of the

273   chlorophytes, charophytes, bryophytes, lycophytes, monilophytes, gymnosperms and

274   angiosperms (Supplementary Data S3). Remarkably, Pp3c8_19720 shares homology with

275   an Arabidopsis prion-like protein (AtFLOE1) that can undergo hydration-dependent phase

276   separation (PS) to regulate seed germination, and related proteins (AtFLOE2 and AtFLOE3)

277   that have been shown to undergo PS in a transient expression system (Dorone et al., 2021).

278   As with the analyses conducted by Dorone *et al.* (2021), our own phylogenetic analyses,

279   revealed the presence of two discrete clades, a FLOE1-like (FLOE1L) and a FLOE2-like

280   (FLOE2L) clade. However, while it was previously stated that the FLOE1L clade was

281   restricted to seed plants, we found FLOE1L representatives in the seed plants in addition to

282   the monilophytes. Conversely, the FLOE2L clade included Pp3c8_19720, as well as three

283   additional *P. patens* paralogues, and homologues in most land plants except for hornworts,

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284 where they appear to have been lost. FLOE2L homologues were also identified in some

285 green algal lineages (Fig. S4, Supplementary Data S3). Given the significant homology

286 between Pp3c8_19720 and representatives within the FLOE2L clade, we hereafter refer to

287 Pp3c8_19720 as PpFLOE2L-1.

288 The AtFLOE1, AtFLOE2 and AtFLOE3 proteins contain a predicted folded domain (known

289 as the nucleation domain), a coiled-coil domain (CC), a UBA domain at the C-terminus and

290 two disordered regions; one enriched for aspartic acid and serine (DS-rich domain.) and one

291 enriched for glutamine, proline, and serine (QPS-rich domain) (Dorone et al., 2021).

292 Alignment of PpFLOE2L-1 with AtFLOE1, 2 and 3 confirmed that these protein domains

293 were conserved in PpFLOE2L-1 (Fig. 5D, Fig. S5). As a result of the nonsense mutations

294 introduced into *PpFLOE2L-1*, a truncated protein was formed in *snog1a*, which was missing

295 most of the QPS-rich domain in addition to the UBA (Fig. 5D; Supplementary Data S2).

296 To confirm that we had correctly identified the causative mutation, we used the rice actin

297 promoter to drive the expression of a wild-type version of *PpFLOE2L-1* in the *snog1a*

298 mutant. The resulting line exhibited a reversion to the 3D-defective phenotype seen in the

299 *nog1dis* mutant (Fig. 6A,B and Fig. S6). We also generated two independent lines in which

300 *PpFLOE2L-1* was deleted in the *nog1dis* mutant. Each line exhibited a restoration of 3D

301 growth, and thus the *snog1a* mutant phenotype had successfully been recapitulated (Fig.

302 6C-E, Fig. S7). Thus, the causative mutation of *snog1a* resides in a gene that encodes a

303 prion-like protein.

304

DISCUSSION

306 The acquisition of apical cells with the capacity for 3D growth occurred in the last common

307 ancestor of land plants, and enabled the diverse morphologies seen across the planet today

308 (Kenrick and Crane, 1997; Delwiche and Cooper, 2015). We previously showed that the *NO*

309 *GAMETOPHORES 1* (*PpNOG1*) gene is required for 3D growth in *P. patens*, an extant

310 representative of the bryophytes. Notably, in mutants lacking *PpNOG1* function (*Ppnog1-R*),

311 gametophore initial cell formation is significantly reduced, even in the presence of cytokinin.

312 Furthermore, cell division planes are misoriented in emerging gametophores, which

313 subsequently undergo premature developmental arrest. Thus, *PpNOG1* promotes the

314 cytokinin-mediated transition from 2D to 3D growth and positively regulates the orientation of

315 cell divisions required to establish a tetrahedral apical cell (Moody et al., 2018).

316 To reveal new insights into the genetic interaction network underpinning the transition from

317 2D to 3D growth, we first recapitulated the *Ppnog1-R* mutant phenotype in an independent

318   *Ppnog1* disruption mutant (*nog1dis*), in which *PpNOG1* was not expressed (Fig. 1). The

319   *nog1dis* mutant was generated using a targeted approach, and thus lacked the background

320   SNPs that were introduced into the *Ppnog1-R* mutant by UV-based mutagenesis. We then

321   performed a UV-mediated suppressor screen to identify mutations that alleviated the

322   *nog1dis* mutant phenotype to permit the reversion to 3D growth. The primary focus of this

323   paper is the characterisation of the '*suppressor of nog1a*' (*snog1a*) mutant identified in this

324   screen.

325   Similarly to the original *Ppnog1-R* mutant, the *nog1dis* mutant was unable to form

326   gametophores even in the presence of cytokinin (Fig. 1,3). Mutants in which all four *PpAPB*

327   genes have been disrupted exhibit a similar level of cytokinin-unresponsiveness to both

328   *Ppnog1-R* and *nog1dis* mutants and fail to make the 3D growth transition (Aoyama et al.,

329   2012). Furthermore, we previously observed that *PpAPB* genes are downregulated when

330   *PpNOG1* is absent (Moody et al., 2018). Thus, *PpNOG1*, along with the *PpAPB* genes are

331   integral regulators of cytokinin perception during the switch from 2D to 3D growth. Since the

332   *snog1a* mutant exhibits responsiveness to cytokinin, this suggests that those cytokinin

333   signaling components, ordinarily repressed in *nog1dis*, have been reactivated in the *snog1a*

334   mutant. This demonstrates that the phenotypes observed in both *nog1dis* and *snog1a* can

335   be attributed to alterations in cytokinin perception. Thus, this study has begun to shed light

336   on the potential mechanism underlying cytokinin-mediated 3D growth initiation, a topic that

337   has fascinated biologists for several decades (Brandes and Kende, 1968; Ashton et al.,

338   1979; Reski and Abel, 1985; Schulz et al., 2000; Schulz et al., 2001; von Schwartzenberg et

339   al., 2007; von Schwartzenberg et al., 2016; Cammarata et al., 2022).

340   Although the formation of gametophore initial cells was not fully restored in the *snog1a*

341   mutant, those that formed usually followed a wild-type pattern of development to achieve the

342   establishment of a tetrahedral apical cell (Fig. 4). It has previously been shown that the

343   positioning of cell division planes in emerging gametophores is dependent on microtubules

344   (Kosetsu et al., 2017; Kozgunova et al., 2022). Thus, it is likely that the cell division

345   orientation defects observed in the *nog1dis* mutant are due to aberrant organisation of the

346   microtubule cytoskeleton. Since the correction of division plane orientation observed in the

347   *snog1a* mutant is accompanied by the restoration of a cytokinin response, we speculate that

348   that microtubule organisation that occurs during the 3D growth transition is dependent on

349   cytokinin. This is consistent with other reports in the literature (Montesinos et al., 2020).

350   There is an increasing volume of literature that describes the regulatory role of cuticle-

351   related genes in the 3D growth transition (Renault et al., 2017; Lee et al., 2020;

352   Kreigshauser et al., 2021; Moody et al., 2021). These mutants invariably exhibit

353   perturbations in the frequencies of gametophore initial cells that form, and those that do form

354   exhibit division orientation defects that arrest gametophore development. Furthermore, the

355   expression of these genes has been shown to be induced by cytokinin (Moody et al., 2021).

356   Although we understand that a cuticle is absent in the protonema but observed in

357   gametophores, the developmental stage at which cuticle biosynthesis initiates remains

358   unclear (Renault et al., 2017; Lee et al., 2020; Kreigshauser et al., 2021). In several of the

359   3D-defective mutants described in the literature so far, including the *Ppnog1-R* mutant, the

360   cuticle is invariably absent (Aoyama et al., 2012; Renault et al., 2017; Moody et al., 2018;

361   Kreigshauser et al., 2021; Zhang et al., 2024). Thus, it is possible that the acquisition of the

362   cuticle was a pre-requisite for 3D growth, and that the mechanical constraints imposed by

363   the cuticle alter the division properties of cells formed in the steps toward the establishment

364   of a tetrahedral apical cell. To support this hypothesis, the cuticle is notably absent in the

365   *nog1dis* mutant but has been partially restored in the *snog1a* mutant, in which the partial

366   reversion to 3D growth has occurred.

367   Our mapping approach and phylogenetic analyses revealed that the gene mutated in the

368   *snog1a* mutant (Pp3c8_19720) was a homologue of the previously characterized FLOE

369   genes in Arabidopsis; AtFLOE1, AtFLOE2 and AtFLOE3 (Fig. S4) (Dorone et al., 2021).

370   Dorone et al. have demonstrated that AtFLOE1 undergoes reversible hydration-dependent

371   liquid-liquid phase separation (LLPS) in the embryo, and that this process is dependent on

372   the presence of a QPS-rich disordered domain. LLPS drives the formation of AtFLOE1

373   condensates when a seed is hydrated, but AtFLOE1 remains dispersed in desiccated seeds.

374   Since loss of AtFLOE1 function can promote germination during drought or salt stress, it is

375   thought that AtFLOE1 functions to inhibit seed germination in unfavourable conditions. The

376   authors also demonstrated that AtFLOE2 and AtFLOE3, in addition to representatives from

377   algae and bryophytes, undergo LLPS in a transient expression system (Dorone et al., 2021).

378   AtFLOE1 is a member of the FLOE1-like (FLOE1L) clade, which contains only those

379   representatives from the monilophytes and seed plants. On the other hand, AtFLOE2 and

380   AtFLOE3 reside within the FLOE2-like (FLOE2L) clade, which contains representatives from

381   algal species as well as all land plant lineages. This includes Pp3c8_19720 (PpFLOE2L-1),

382   in addition to three additional homologues in *P. patens*. We were able to complement the

383   *snog1a* mutant phenotype with a full-length version of the wild-type coding sequence and

384   recapitulate the phenotype by generating two independent double disruptant mutants (Fig.

385   6). The confirmation of gene identity enabled us to amend the name of *Pp3c8_19720* to

386   PpFLOE2L-1. The presence of three additional *FLOE2L* homologues in the *P. patens*

387   genome suggests that these genes may function redundantly. Thus, we hypothesise that

388   higher order mutants of these genes will exhibit a progressive restoration of 3D growth, and

389 indeed cuticle formation. FLOE2L genes were notably absent from hornworts, which
390 suggests that the genetic toolkit underpinning 3D growth processes in hornworts is
391 somewhat distinct.

392 Liquid-liquid phase separation (LLPS) is a phenomenon that has been increasingly linked to
393 important developmental processes in plants (Fang et al., 2019; Huang et al., 2021; Cao et
394 al., 2023). Furthermore, there have been reports that LLPS enables ubiquitin-binding shuttle
395 proteins (i.e., those containing UBAs) to degrade ubiquitinated substrates (Dao and
396 Castaneda, 2020). Similarly, to the three other *FLOE2-like* genes identified in *P. patens*,
397 *PpFLOE2L-1* encodes a protein that contains a disordered domain enriched in glutamine,
398 proline, and serine (QPS-rich), previously shown to serve as a prerequisite for LLPS; coiled-
399 coil (CC) and nucleation domains; a disordered domain enriched in aspartic acid and serine
400 (DS-rich); and a UBA. The presence of the UBA suggests that PpFLOE2L-1 plays a role in
401 protein degradation, which is curiously reminiscent of the PpNOG1 protein. Due to the
402 presence of two in-frame premature stop codons in the *PpFLOE2L-1* transcript, the *snog1a*
403 mutant lacks most of the QPS-rich disordered domain as well as the UBA (Fig. 5). Thus, we
404 hypothesise that PpFLOE2L-1 undergoes cell-type dependent LLPS to compartmentalize
405 the cellular components required to degrade a ubiquitinated repressor of the 2D to 3D
406 growth transition. In support of this notion, it has been reported that E3 ligases involved in
407 protein degradation processes are able to function within condensates. Thus, we propose
408 LLPS as a mechanism by which the induction of 3D growth can be rapidly triggered in
409 response to both intrinsic and extrinsic cues, at the correct stage of development.

410

411 **SUMMARY**

412 Since the loss of *PpFLOE2L-1* function can reverse the 3D-defective phenotype of the
413 *nog1dis* mutant phenotype, we have demonstrated that *PpFLOE2L-1* acts as a negative
414 regulator of 3D growth. Thus, we propose that PpNOG1 acts upstream of, and represses the
415 activity of PpFLOE2L-1, which in turn induces the degradation of protein(s) that repress
416 *PpAPB* gene transcription. We speculate that the decision to induce 2D versus 3D growth is
417 dependent on the biophysical state of PpFLOE2L-1, and that these changes dynamically
418 alter in a spatial and temporal manner; condensed when a side branch acquires 2D fate and
419 dispersed when a side branch acquires 3D fate. We also propose that the transcriptional
420 targets of the PpAPBs trigger the cytokinin response required to initiate the 3D growth
421 transition. In the presence of cytokinin, cuticle-related genes are induced, which we propose
422 subsequently triggers the induction of a CLAVATA-dependent auxin response to diminish
423 the cytokinin response and prevent the inappropriate induction of 3D growth (Fig. 7).

12

424

MATERIALS AND METHODS

Physcomitrium patens growth conditions

To encourage bud development, *P. patens* was grown on cellophane-overlaid BCD medium (1 mM $MgSO_4$, 1.84 mM KH_2PO_4 (pH 6.5), 10 mM KNO_3, 45 µM $FeSO_4$; 1 mM $CaCl_2$; 0.1% Trace Elements Solution: 116 µM $AlK(SO_4)_2$, 220 µM $CuSO_4$, 10 mM H_3BO_4, 235 µM KBr, 660 µM LiCl, 230 µM $CoCl_2$, 190 µM $ZnSO_4$, 2 mM $MnCl_2$, 170 µM KI, 124 µM $SnCl_2$) containing 0.8% agar. For routine propagation, and to stimulate filamentous growth, *P. patens* was grown on BCD medium supplemented with 5 mM ammonium tartrate (BCDAT). To enable propagation, tissues were harvested and homogenized in sterile water using an IKA T-25 digital ULTRA-TURRAX®. Tissues were then pipetted onto cellophane-overlaid BCDAT plates in a laminar flow hood. Plates were then placed in a growth cabinet at 24°C with a 16 h:8 h light (300 µmol m^{-2} s^{-1}): dark cycle. Protoplasts were regenerated on cellophane-overlaid Protoplast Regeneration Medium (PRMB; BCDAT supplemented with 10 mM $CaCl_2$, 0.5% glucose and 6% (w/v) D-mannitol) (Cove et al., 2009).

439

Generation of the *nog1dis* 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 using NOG1.5FKpnI and NOG1.5RXhoI primers (Table 1) and ligated into KpnI/XhoI cut pAHG1 (a kind gift from Yasuko Kamisugi and Andrew Cuming) to create pAHG1-*NOG1*-5'. A *PpNOG1* genomic DNA fragment including the stop codon up to 1420bp downstream of the *PpNOG1* stop codon was PCR-amplified using NOG1.3FNotI and NOG1.3RNotI primers and ligated into NotI cut pAHG1-*NOG1*-5' to create *pNOG1*delH. *pNOG1*delH was linearized using KpnI prior to transformation into protoplasts isolated from the Villersexel wild type strain. Stable transformants were selected using 15 $mgmL^{-1}$ Hygromycin B (Sigma-Aldrich cat. no. H9773).

450

RT-PCR to detect absence of *NOG1* in the *nog1dis* mutant

Total RNA was isolated from two-week-old Villersexel wild type and the *nog1dis* mutant using the RNeasy kit (Qiagen) according to the manufacturer's instructions. DNase treatment was carried out using TURBO DNase (Ambion) and cDNA synthesis performed using Superscript[TM] III (ThermoFisher Scientific), as specified by the manufacturers. The *tubulin* transcript was amplified using PptubF and PptubR, and the *PpNOG1* transcript was amplified using 32970008(exon)_GSP.F and 32970008(exon)_GSP.R (Table 1).

458

UV mutagenesis and screening

One-week old protonemata from the *nog1dis* mutant was digested for 1 h in 1% Driselase dissolved in 8% mannitol. The resulting cell suspension was passed through a 40 μm cell strainer and centrifuged for 3 min at 120 xg without braking. Protoplasts were subsequently washed twice in 8% mannitol, with repeated centrifugation steps in between washes. Cells were resuspended in 6 ml 8% mannitol, counted using a haemocytometer, and then plated at a density of 50,000 cells per plate onto cellophane overlaid PRMB. Protoplasts were immediately exposed to a 75,000 mJ dose of UV light using a Stratalinker UV Crosslinker and this was performed with the petri dish lids removed. Following irradiation, the lids were quickly replaced, and the plates were wrapped with micropore tape. Plates were incubated at 24°C in the dark for 24 h, to prevent photoactivatable DNA damage repair, before being transferred to standard growth conditions for a further 2-3 weeks. When visible, regenerating protoplasts were transferred into individual wells of 24 well plates containing BCD medium. After approximately one month of growth in standard conditions, the mutagenized plants were screened for reversion of the *nog1dis* phenotype (i.e., restoration of gametophore development).

Imaging

To stain cell walls, tissues were submerged in propidium iodide (10 μgmL^{-1}) for around 10 min and then mounted on slides in water. Images were acquired using a Leica SP5 scanning confocal microscope with a 40x water immersive lens. A 488 nm laser was used to excite the propidium iodide with 30% laser power and fluorescence was detected at 600-630 nm.

Plant phenotyping

To assay the number of gametophores produced by different *P. patens* lines, tissues were grown on cellophane-overlaid BCDAT plates for one week and then the tissues were harvested into separate 50 ml tubes containing 10 ml sterile water. The tissues were each homogenized with an IKA disperser for 20 s, and then 1 ml of homogenate was removed to measure optical density (OD600) in a spectrophotometer. The density of the homogenate was normalized by adding the required amount of water to each tube. 50 μl of homogenate was pipetted onto BCD medium in small petri dishes, with 5 repeats for each line. These plates were grown for two months under standard conditions, so that mature gametophores had sufficient time to develop. The number of gametophores formed on each plate were counted. To measure gametophore height, a representative and intact sample from each line

493   was extracted from the tissue, placed in a petri dish and photographed. The heights were

494   measured in ImageJ, by drawing a line from the apex to the base. To determine the cytokinin

495   responsiveness of each line, tissues were grown on BCD medium for one week and then the

496   cellophanes were cut and transferred to BCD medium containing 1 μM BAP for 3 days (or

497   control medium) before being examined and images captured using a Leica M165C

498   stereomicroscope equipped with a QImaging Micropublishing 5.1 RTV camera.

499   **Plant crossing and bulk segregant analysis**

500   Both the *snog1a* mutant and the Reute::mCherry line (Perroud et al., 2019) were grown in

501   magenta pots on BCD medium in standard growth conditions for 2-3 months. Pots were

502   subsequently transferred to sporophyte induction conditions (16 h dark: 8 h light, 16°C). After

503   approximately three weeks, gametophores were examined for the presence of gametangia

504   (archegonia and antheridia). When gametangia were present, the Reute::mCherry line was

505   submerged under water to make a sperm suspension, which was subsequently added to the

506   *Ppsnog1a* mutant to allow outcrossing. After approximately 4-6 weeks, the resulting

507   sporangia were removed carefully using forceps and examined under a Leica M165C

508   fluorescence stereomicroscope to detect mCherry expression. This demonstrated a

509   successful outcrossing event. Sporangia were sterilized in 70% ethanol for 4 min, washed 3

510   times with sterile water and then incubated at 4°C in the dark for one week. In a laminar flow

511   hood, the sporangia were transferred to a 15 ml tube containing 10 ml of sterile water and

512   then the sporangia ruptured with a pipette tip. 1 ml of spore suspension was plated onto

513   each of ten cellophane-overlaid BCDAT plates and then grown in standard conditions to

514   permit germination and subsequent growth. Individual sporelings were then transferred to

515   wells of 24-well plates containing BCD medium and grown for a further 4-6 weeks. The

516   progeny were screened for the presence or absence of gametophores, and sorted into two

517   populations; *nog1dis*-like (wild-type pool, 80 individuals) and wild type or *snog1a*-like (mutant

518   pool, 98 individuals). Genomic DNA was then extracted from one-week old protonemal

519   tissues prepared from each individual line.

520

521   **Isolation of genomic DNA**

522   Immediately before use, 0.07% (v/v) 2-mercaptoethanol and 0.1% (w/v) ascorbic acid was

523   added to aliquots of extraction buffer (100 mM Tris-HCl pH 8.0, 1.42 M NaCl, 2 % CTAB, 20

524   mM EDTA, 2% PVP-40). The aliquots of extraction buffer were pre-warmed at 65°C in a

525   water bath. One plate of 1-week-old protonemal tissues, grown on cellophane-overlaid

526   BCDAT plates, was harvested and then blotted on filter paper to remove excess water.

527   Tissues were then placed in a 2 ml microcentrifuge tube and frozen in liquid nitrogen. A

528   miniature pestle was used to grind the tissue into powder, and then 500 μl of pre-warmed

529 extraction buffer was added to the powder while frozen and further homogenised. An

530 additional 200 µl of extraction buffer was added to the tube, along with 7 µl 10 mgmL^{-1}

531 RNase A, and then incubated at 65°C for 10 min. 600 µl of chloroform-isoamyl alcohol (24:1)

532 was added and the tube, shaken and centrifuged at 13,000 rpm for 10 min. Then the upper

533 aqueous phase of the mixture was transferred to a fresh tube, 0.7 volumes of isopropanol

534 were added, the tube shaken and spun again immediately at 13,000 rpm for 10 min. The

535 pellet was washed in 70% ethanol, allowed to air-dry, and then resuspended in 30 µl

536 nuclease-free water. A NanoDrop™ spectrophotometer was used to assess the quantity and

537 quality of the extracted DNA.

538

539 **Preparation of genomic DNA samples for Whole Genome Sequencing**

540 In total, four genomic DNA samples were prepared for sequencing; both parental lines

541 (*nog1dis* and Reute::mCherry) and two pooled samples (a *snog1a*-like 'mutant pool' and a

542 *nog1*-like 'wild-type pool'). The pooled samples were prepared by pooling 1 µg genomic

543 DNA extracted from all individuals in that population (80 individuals in the 'WT pool' and 98

544 individuals in the 'mutant pool').

545

546 **Candidate identification through bulk segregant analysis**

547 Whole genome was performed using a NovoSeq X Plus sequencing platform (150 bp PE

548 read lengths, 44X coverage) at Novogene. Bioinformatic analysis was performed as

549 described previously (Moody et al., 2018; Moody et al., 2021). The presence of the

550 premature termination codons in Pp3c8_19720 was confirmed by sequencing PCR products,

551 amplified from both wild type and snog1a derived genomic DNA, using the primers

552 Pp3c8_19720_int_F and Pp3c8_19720_int_R (Table 1).

553

554 ***Physcomitrium patens* transformation**

555 Before the transformation, large quantities of plasmid DNA were acquired by midiprep using

556 a QIAGEN plasmid midi kit. Approximately 20 µg of the plasmid was linearized by restriction

557 digest overnight, treated with CIAP and precipitated with sodium acetate. Before starting the

558 transformation, all solutions required were made fresh and filter sterilized using [filter]. The

559 transformation was carried out in a biological safety cabinet. 2 g of polyethylene glycol

560 (PEG) 6000 in a flat-bottomed vial, that had been sterilised in the autoclave, was melted in

561 the microwave for approximately 1 min. 5 ml mannitol/Ca(NO$_3$)$_2$ solution (0.8% mannitol, 0.1

562 M Ca(NO$_3$)$_2$, 10 mM Tris pH 8.0) was added to the molten PEG 6000, shaken and allowed to

563 cool for 2-3 h.

564   For each *P. patens* line that was to be transformed, 1-2 plates of tissue were harvested. 0.1

565   g Driselase enzyme was dissolved in 10 ml 8% mannitol for each digest. The tube containing

566   the enzyme solution was wrapped in foil and rocked for approximately 10 min at room

567   temperature. The tube was spun for 3 min in a centrifuge, and the supernatant was filter

568   sterilised. *P. patens* tissue was placed in the tube with the Driselase solution, wrapped in foil

569   and rocked very gently until the tissue appears well digested (at least 40 min). The digested

570   tissue was put through a 70 μm cell strainer to separate protoplasts from remaining debris.

571   The protoplasts were spun at no more than 120 xg, the supernatant was removed and the

572   protoplasts were resuspended in 6 ml mannitol. This wash was repeated twice more. The

573   cell density of the protoplasts was determined using a haemocytometer. The protoplasts

574   were spun down again and resuspended in MMM (0.5 M mannitol, 0.15 M $MgCl_2$, 0.1% MES

575   pH5.6), to achieve a cell density of 1.5×10^6 cells mL^{-1}. The next steps were carried out

576   without delay as the protoplasts cannot tolerate the MMM solution for very long. At least 10

577   μg of the linearised construct was pipetted into the bottom of a round-bottomed Falcon tube.

578   To this was added 300 μl of protoplast suspension. 300 μl of the PEG solution was added in

579   drops and the tube was swirled gently after the addition of each drop. The tube was heat-

580   shocked at 45 °C in a water bath for 5 min and then incubated at room temperature for a

581   further 5 min. Next, 300 μl 8% mannitol was added to the tube, 5 times at 3 min intervals and

582   the mixture was gently swirled after each addition. Then, 1 ml 8% mannitol was added to the

583   tube 5 times at 3 min intervals, gently tilting the tube after each addition to mix it. The tube

584   was centrifuged at 120 xg, supernatant removed, and the cells were resuspended in 3 ml 8%

585   mannitol. This suspension was gently pipetted onto three PRMB plates overlaid with

586   cellophanes (1 ml for each plate). These plates were sealed with micropore tape and

587   wrapped in foil for 24 h. Subsequently, they were unwrapped and placed in normal growing

588   conditions for 5-7 days. Then the cellophanes, with the regenerating protoplasts, were

589   transferred to selective BCDAT plates containing the appropriate selective agent. After

590   another week on selective plates, the cellophanes are transferred to BCDAT plates with no

591   selective agent to allow recovery. After another 1-2 weeks, the surviving *P. patens* colonies

592   were individually transferred to selective BCD plates, to select for stably transformed

593   colonies. Following another 1 – 2 weeks on selection, colonies that survived both rounds of

594   selection were transferred to non-selective BCD plates and allowed to grow and generate

595   tissue for further study.

596

597   **Generation of *snog1a* complementation lines**

598   RNA was extracted form two-week-old wild-type tissue using an RNeasy kit (Qiagen) and

599   treated with Turbo DNase (Ambion), according to the manufacturer's specifications. cDNA

600 was then synthesised using Superscript™ III Reverse Transcriptase (ThermoFisher

601 Scientific). Subsequently the Pp3c8_19720 transcript was PCR-amplified (excluding the stop

602 codon) using Pp3c8_19720.FSaII and Pp3c8_19720.R_NOSTOP_HindIII and ligated into

603 pZAG1 to create Act1p::Pp3c8_19720-GFP. This construct was linearised with SacII for

604 subsequent transformation into the *snog1a* mutant. Stable transformants were selected

605 using 100 µgml^{-1} Zeocin (Invitrogen; R25001). Genotyping was performed using the primers

606 putativeSNOG1A_genomic2_F and putativeSNOG1A_genomic2_R (Table 1, Fig. S6).

607

Generation of a *snog1a* deletion mutant

609 A deletion construct was designed. The construct consisted of the PpFLOE2L-1 5' flanking

610 sequence, with a small section of the PpFLOE2L-1 CDS and a PpFLOE2L-1 3' flanking

611 sequences. The 5' and 3' sequences were either side of a G418 resistance cassette. The

612 construct was synthesized by TWIST Bioscience. The product was verified by Sanger

613 sequencing. Prior to transformation into protoplasts isolated from the *nog1dis* mutant, the

614 plasmid was linearized with PvuI. Stable transformants were selected using 40 µgml^{-1} G418.

615 5' integration of the deletion construct was confirmed using the primers

616 snog1a_del_genotyping_F and snog1a_del_genotyping_R (Table 1, Fig. S7).

617

Phylogenetics

619 For each of the species included in the analysis, proteome sequences (primary transcript

620 only) were obtained. The data used were: *Physcomitrium patens* v3.3 (Lang et al., 2018),

621 *Marchantia polymorpha* v3.1 (Bowman et al., 2017), *Selaginella moellendorffii* v1.0 (Banks

622 et al., 2011), *Oryza sativa* v7.0 (Ouyang et al., 2007), *Zea mays* PH207 v1.1 (Hirsch et al.,

623 2016), *Sorghum bicolor* v3.1.1 (McCormick et al., 2018), *Brachypodium distachyon* v3.1

624 (Vogel et al., 2010), *Arabidopsis thaliana* Araport11 (Cheng et al., 2017), *Solanum*

625 *lycopersicum* ITAG2.4 (Sato et al., 2012), *Medicago truncatula* Mt4.0v1 (Tang et al., 2014),

626 *Populus trichocarpa* v3.1 (Tuskan et al., 2006), *Micromonas pusilla* CCMP1545 v3.0

627 (Worden et al., 2009) and *Ostreococcus lucimarinus* v2.0 (Palenik et al., 2007), *Chara*

628 *braunii* S276v1.0 (Nishiyama et al., 2018), *Chlamydomonas reinhardtii* v5.5 (Merchant et al.,

629 2007), *Amborella trichopoda* v1.0 (Albert *et* al., 2013), *Botryococcus braunii v2.1* (Browne *et*

630 *al*., 2017), *Anthoceros agrestis* Oxford (Li *et al*., 2020), *Azolla filiculoides* v1.1 (Li, *et al*.,

631 2018), *Brassica rapa* FPsc v1.3 (DOE-JGI, http://phytozome.jgi.doe.gov/), *Ceratodon*

632 *purpureus* R40 v1.1 (Carey *et al*., 2021), *Ceratopteris richardii* v2.1 (Marchant *et* al., 2022),

633 Chlorokybus atmophyticus CCAC 0220 v1.1 (Wang *et al*., 2020), *Klebsormidium nitens*

634 NIES-2285 v1.1 (Hori *et al*., 2014), *Mesostigma viride* NIES-296 (Liang *et al*., 2020),

635 *Spirogloea muscicola* CCAC 0214 (Cheng *et al.,* 2019), *Mesotaenium endlicherianum SAG*

636 *12.97* (Cheng *et al.,* 2019), *Coccomyxa subellipsoidea* C-169 v2.0 (Blanc *et al.,* 2012),

637 *Dunaliella salina* v1.0 (Polle, *et al.*, 2017), *Volvox carteri* v2.1 (Prochnik *eta l.*, 2010),

638 *Porphyra umbilicalis* v1.5 (Brawley *et al.*, 2017), *Sphagnum fallax* v1.1 (Healey *et al.*, 2023),

639 *Diphasiastrum complanatum* v3.1 (DOE-JGI, http://phytozome-next.jgi.doe.gov/), *Gingko*

640 *biloba* v2021 (Liu *et al.*, 2021), *Glycine max* Wm82 ISU-01 v2.1 (DOE-JGI,

641 http://phytozome.jgi.doe.gov), *Gossypium raimondii* v2.1 (Paterson *et al.*, 2012), *Musa*

642 *acuminata* v1 (D'Hont *et al.*, 2012), *Panicum hallii* v3.2 (Lovell *et al.*, 2018), *Salvinia*

643 *cucullata* v1.2 (Li *et al.*, 2018), *Spirodela polyrhiza* v2 (Wang *et al.*, 2014), *Thuja plicata* v3.1

644 (Shalev *et al.*, 2022), *Vitis vinifera* v2.1 (Jaillon *et al.*, 2007), *Saccharomyces cerevisiae* R64-

645 1-1 (Liachko et al., 2013), *Drosophila melanogaster* BDGP6.32 (Adams et al., 2000) and

646 *Homo sapiens* GRCh38 (Lander et al., 2001).

647 With this set of proteomes, OrthoFinder was used to identify orthogroups (Emms & Kelly,

648 2019). The orthogroup containing *Pp3c8_19720* was selected and the protein sequences

649 were aligned using MAFFT (L-IN-SI method) and a gene tree constructed using IQ-TREE,

650 with automatic model selection (ModelFinder) and ultrafast bootstrapping (UFBoot) with

651 1000 replicates (Hoang et al., 2018; Kalyaanamoorthy et al., 2017; Katoh & Standley, 2013;

652 Nguyen et al., 2015). Tree was rooted and edited in Interactive Tree of Life (iTOL) (Letunic &

653 Bork, 2016).

654

666

667 **AUTHOR CONTRIBUTIONS**

668   Z.W. conducted the experiments, with assistance from G.C., E.D. and L.A.M.; L.A.M.

669   conceived and designed the study; S.K. carried out the bioinformatics; Z.W. constructed the

670   phylogenetic tree; and L.A.M. and Z.W. wrote and edited the manuscript.

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1082 **FIGURE LEGENDS**

1083 **Figure 1. The nog1dis mutant fails to make the transition to 3D growth.** Representative
1084 images of 6-week-old Villersexel wild type (WT), *Ppnog1-R* and *nog1dis* plants showing the
1085 presence (WT) and absence (*Ppnog1-R* and *nog1dis*) of gametophores. Scale bars, 1 cm.

1086 **Figure 2. The *snog1a* mutant exhibits a partial restoration of 3D growth.** A)
1087 Representative images of 6-week-old Villersexel wild type (WT), *nog1dis* and *snog1a* plants
1088 showing the presence (WT and snog1a) and absence (nog1dis) of gametophores. B) Mean
1089 number of gametophores per culture (n=5) ± SEM (t test ***p < 0.05). C) Representative
1090 images of a gametophore from wild type (top) and stunted gametophore from the *snog1a*
1091 mutant (bottom). D) Mean height of gametophores from wild type (n=100) and *snog1a*
1092 (n=80) ± SEM (t test ***p < 0.05). Scale bars, 1 cm (A and C).

1093 **Figure 3. The *snog1a* mutant is cytokinin responsive.** Representative images of wild type
1094 (WT), *nog1dis* and *snog1a* plants cultured in the presence or absence of the cytokinin
1095 analogue 6-benzylaminopurine (BAP).

Figure 4. The *snog1a* mutant can establish and maintain a tetrahedral apical cell. Propidium-iodide-stained buds of wild type at the 2-cell (A), 3-cell (B) and 4-cell (C) and late stage (D); the nog1dis mutant at the 2-cell (E,F), 4-cell (G) and late stage (H); and the snog1a mutant at the 2-cell (I,J), 4-cell (K) and late stage (L). Red arrows denote the most recent division in each developing bud, and blue asterisks highlight misoriented division planes in *nog1dis* or *snog1a* mutants.

Figure 5. Identification of the causative mutation in the *snog1a* mutant. A) Phenotypic analysis of spore progeny derived from a cross between *snog1a* and the Reute::mCherry wild-type strain. B) Gene candidates identified following interrogation of the genomic locus on chromosome 8. C) Gene structure diagram of *Pp3c8_19720* highlighting the presence of two termination codons in the third exon (exons – blocks, introns – horizontal lines). D) The wild-type Pp3c8_19720 protein (top) and the truncated Pp3c8_19720 protein in *snog1a* (bottom).

Figure 6. Confirmation of the causative mutation in the *snog1a* mutant. (A,B) Representative images of 6-week-old snog1a (A) and snog1a complemented with pAct::PpFLOE2L-1 (B). (C-E) Representative images of 6-week-old nog1dis (C) and nog1dis/floe2l-1_4 (D) and nog1dis/floe2l-1_6 (E) double disruptants. Scale bars, 1 cm.

Figure 7. Speculative model for 3D growth regulation in *P. patens*. Top panel shows possible mechanism for cell-type specific LLPS of PpFLOE2L-1. Bottom panel shows possible relationship between PpFLOE2L-1 and other known regulators of 3D growth. PpNOG1 is proposed to act upstream of, and negatively regulate PpFLOE2-1, which in turn negatively regulates the expression of the *PpAPB* genes. Activation of the cytokinin signaling triggers the expression of cuticle-related genes, which act upstream of CLAVATA signaling components, which trigger an auxin-mediated repression of ectopic bud formation.

Supplementary Figure 1. Generation of the *nog1dis* line. A) Schematic of the construct designed to knockout the endogenous *PpNOG1* gene, and schematic of the modified *PpNOG1* locus in *nog1dis* following recombination. B) RT-PCR reveals the presence of the PpNOG1 transcript in wild type but not in the *nog1dis* mutant (tubulin – control).

Supplementary Figure 2. Gametophores formed in the *snog1a* mutant are stunted relative to wild type. Representative images of 2-month-old gametophores from wild type and *snog1a* plants. Scale bars, 0.5 cm.

1127 **Supplementary Figure 3. Bulk segregant analysis and the identification of the**
1128 **causative mutation in the *snog1a* mutant.** A) An outcrossing event between snog1a and
1129 the Reute::mCherry line yields a diploid sporophyte that undergoes meiosis to produce
1130 phenotypically segregating progeny (phenotypic outcomes highlighted). (B,C) Expected
1131 snog1a mutant, SNOG1A WT, nog1 mutant and NOG1 WT allele frequencies in the mutant
1132 (B) and wild-type (C) pools respectively. D) Allele frequency plot for segregants on
1133 chromosome 8 of the *P. patens* genome assembly.

1134 **Supplementary Figure 4. Phylogenetic analysis of FLOE-related homologues in the**
1135 **green lineage.** Bootstrap values have been indicated on each branch. Both FLOE1L and
1136 FLOE2L clades have also been indicated.

1137 **Supplementary Figure 5. Alignment of AtFLOE1, AtFLOE2, AtFLOE3 and PpFLOE2L-1.**
1138 Conserved domains have been highlighted as indicated.

1139 **Supplementary Figure 6. Generation of the *snog1a* complementation line.** A)
1140 Schematic of the construct used to complement the *snog1a* mutant phenotype, and the
1141 resulting targeted locus. B) Genotyping of the complementation line using
1142 putativeSNOG1A_genomic2_F and putativeSNOG1A_genomic2_R primers denoted by
1143 purple and green arrows in (A) respectively. The construct is only detected in the
1144 complemented line and not in wild type (tubulin – control).

1145 **Supplementary Figure 7.** (A) Schematic of the construct used to disrupt the *PpFLOE2L-1*
1146 locus in the *nog1dis* mutant, and the resulting targeted locus. B) Genotyping of the
1147 complementation line using snog1a_del_genotyping_F and snog1a_del_genotyping_R
1148 primers denoted by blue and red arrows in (A) respectively.

1149

1150 **Table 1. List of Primers**

Generation of *Vxnog1*	
NOG1.5FKpnI	AAAGGTACCCCATCCATGCACACAACCAA
NOG1.5RXhoI	AAACTCGAGCCTCCGCTCCAAACTCCCAC
NOG1.3FNotI	AAGCGGCCGCTAATCTGTGTATGAGTTCAG
NOG1.3RnotI	AAGCGGCCGCGAGTTATCTAGTTTTGTGGA

| Primers for RT-PCR	
PptubF	TGTGCTGTTGGACAATGAG
PptubR	ACATCAGATCGAACTTGTG
32970008(exon)_GSP.F	GTTGTAGGTTTGGAGTGGCG
32970008(exon)_GSP.R	GCAAGTTGAAAAGCCCACCT
snog1a mutation verification	
Pp3c8_19720_int_F	CAAGGCCTACCGTCTCATCC
Pp3c8_19720_int_R	GTGGAGGAGGGACCTCTTGA
Generation of *snog1a* complementation lines	
Pp3c8_19720.FSalI	aaagtcgacATGGATCATGTGGGATCC
Pp3c8_19720.R_NOSTOP_HindIII	aaaaagcttCCGGCCATACCAGC
Verification of *snog1a* complementation lines	
putativeSNOG1A_genomic2_F	CAGCTGACAACTTTGGTGCA
putativeSNOG1A_genomic2_R	CATCTGCTGCTGAGGAAGTG
Verification of *PpFLOE2L-1* disruption lines	
snog1a_del_genotyping_F	GTCCACCAAGACCACGAAAC
snog1a_del_genotyping_R	CATCAGAGCAGCCGATTGTC

1151

1152

FIGURE 1

FIGURE 2

FIGURE 3

WT - BAP

WT + 1μM BAP

nog1dis - BAP

nog1dis + 1μM BAP

snog1a - BAP

snog1a + 1μM BAP
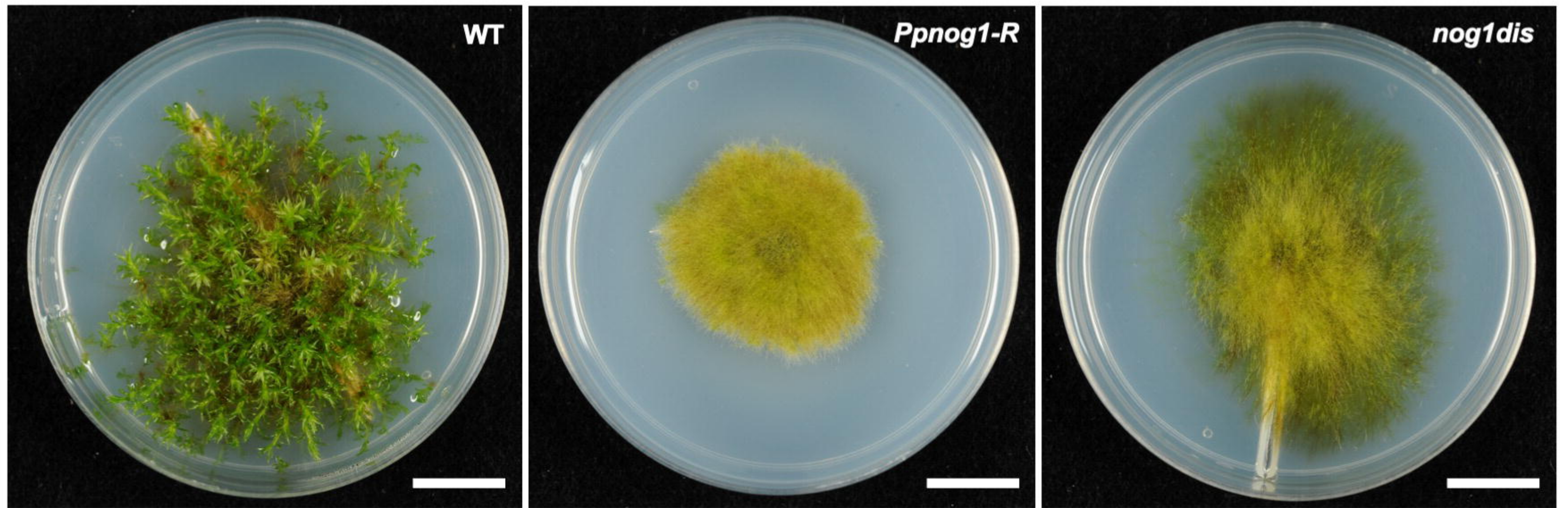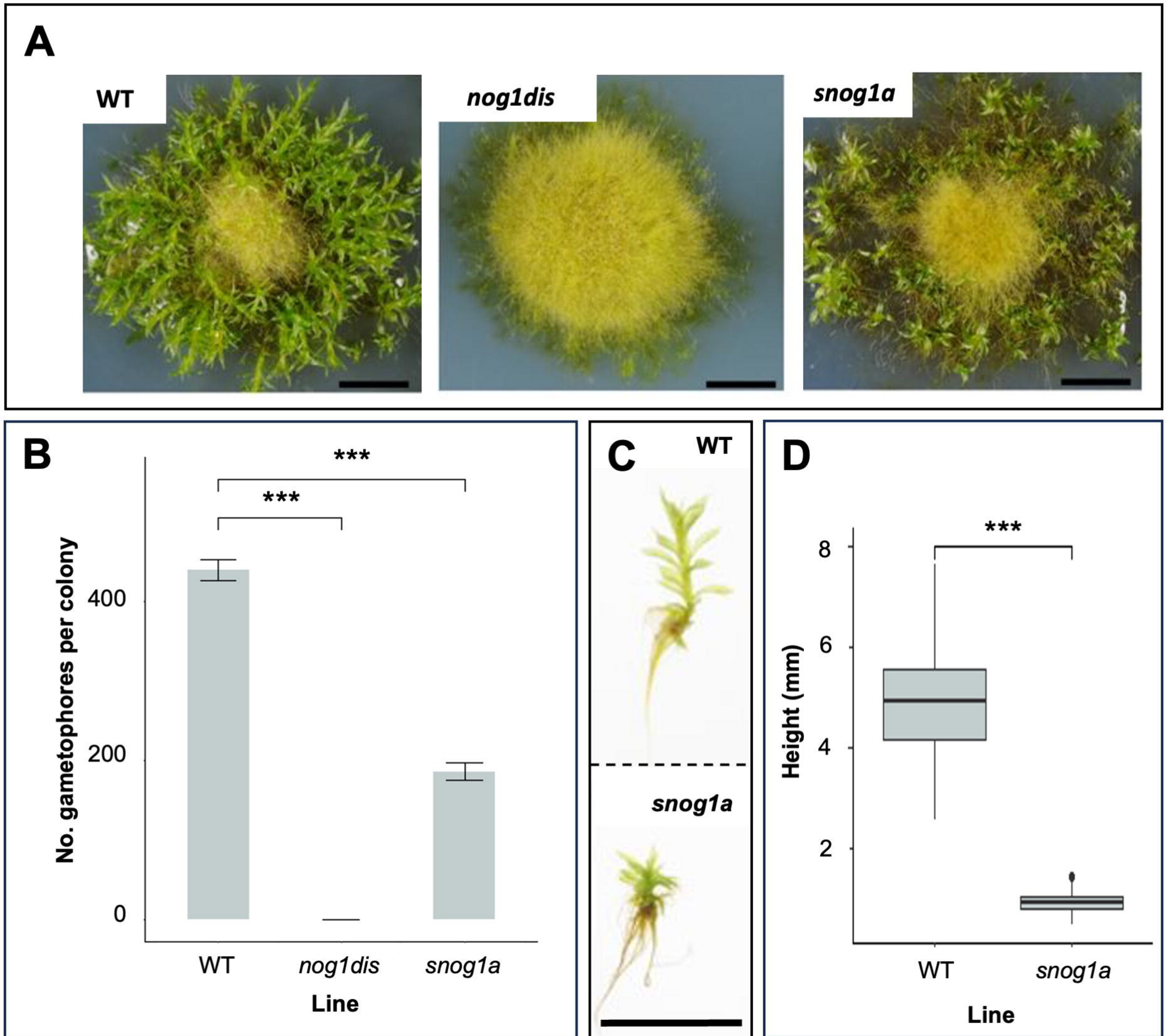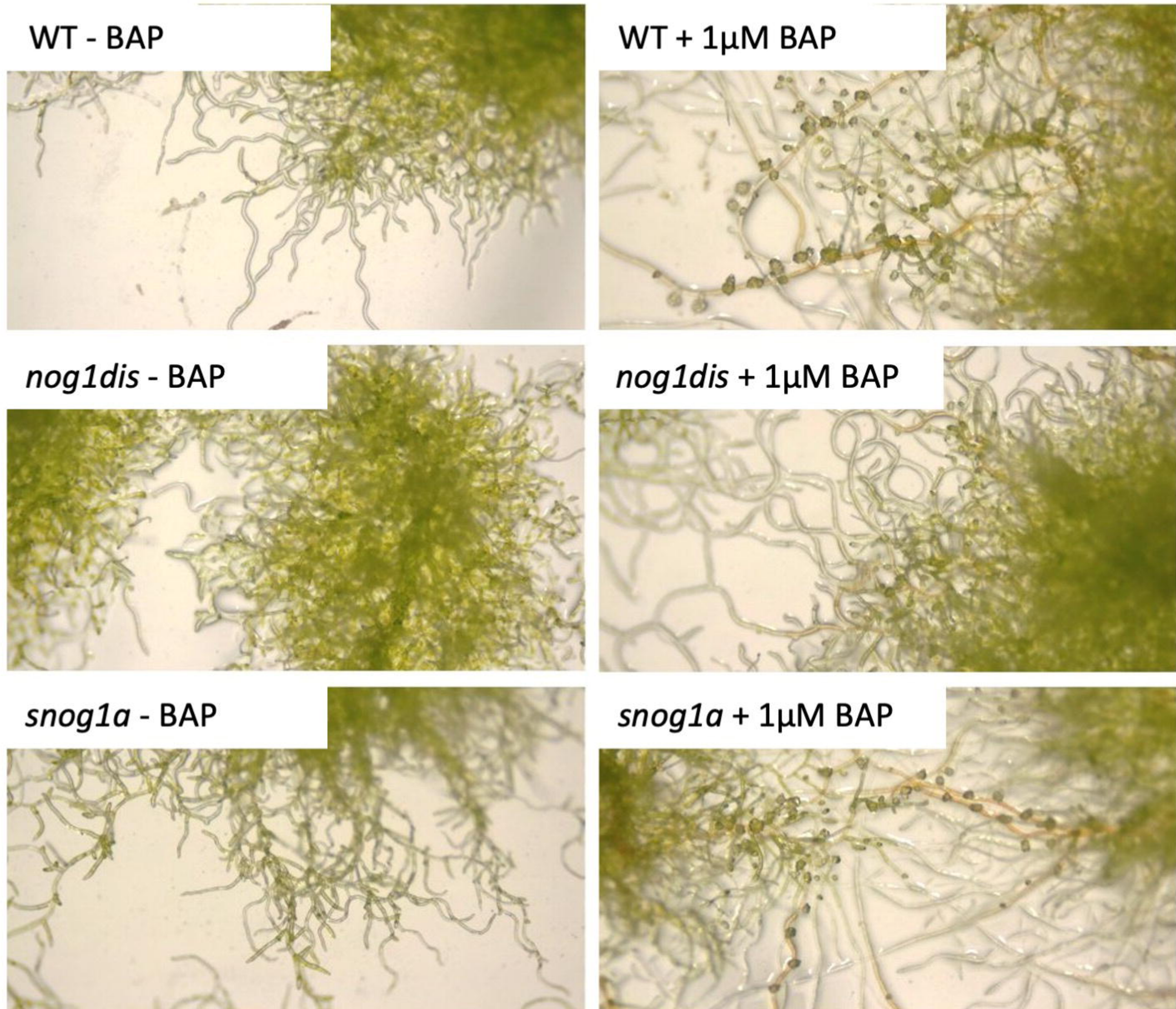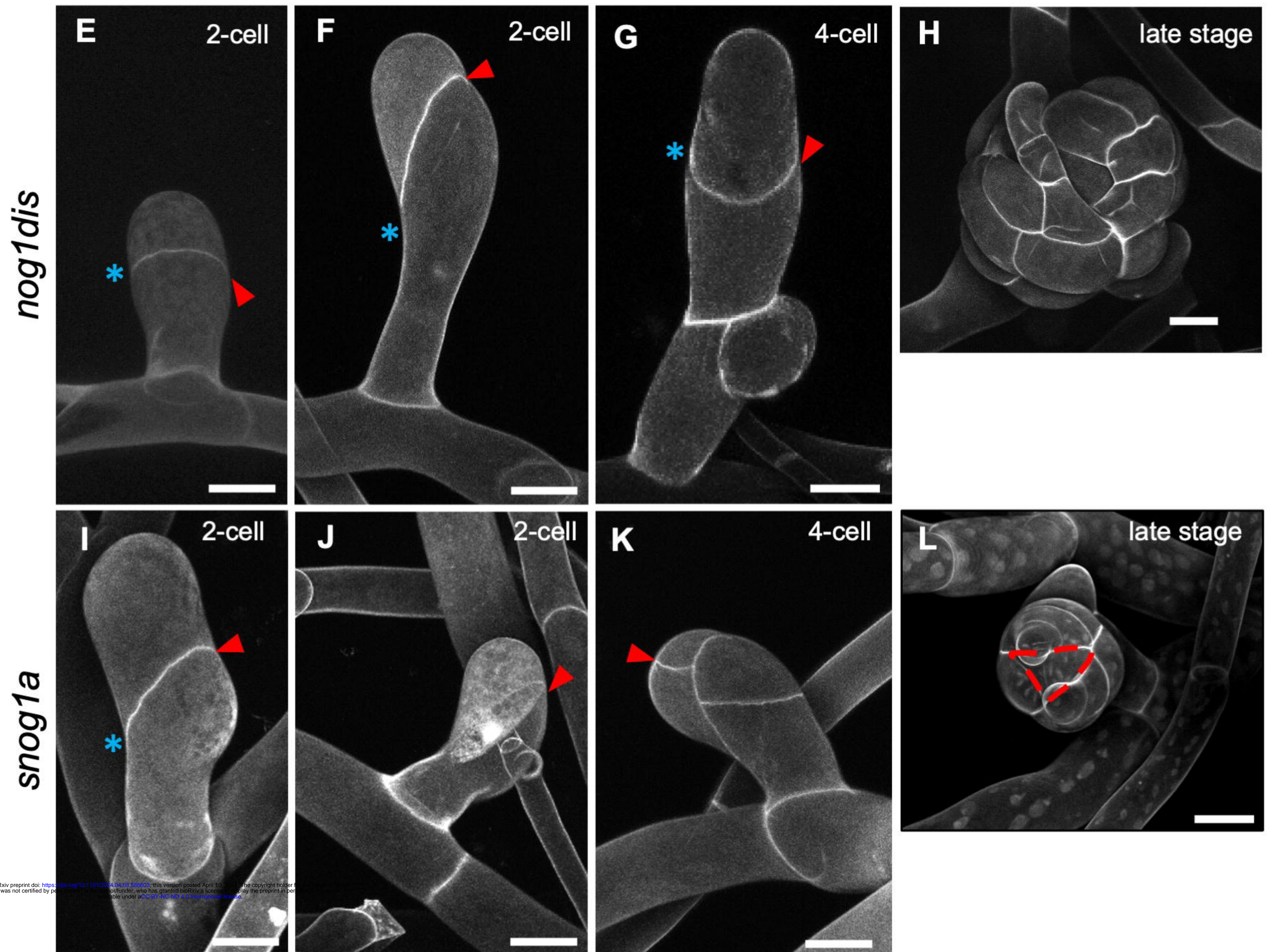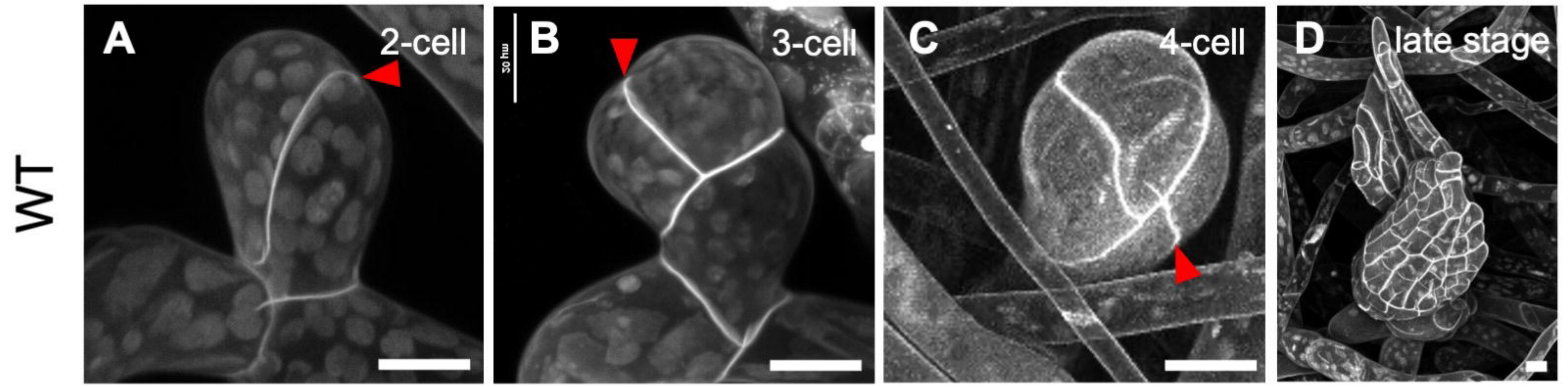
FIGURE 4

FIGURE 5

A

Observed			Expected				
3D growth	no 3D growth	Total	3D growth	no 3D growth	Total	Chi-square	Percentage
574	80	654	490.5	163.5	654	56.9	12.2324

B

Chromosome	Position	Reference	Variant	Gene	Location	Amino Acid Change
Chr08	13077134	C	T	Pp3c8_19720	exon 3	CAG (Gln) to UAG (stop)
Chr08	13077251	C	T	Pp3c8_19720	exon 3	CAA (Gln) to UAA (stop)

C

D

FIGURE 6

FIGURE 7