



Evolution of nuclear auxin signaling: Lessons from genetic studies with basal land plants

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1 **Title**

2 Evolution of nuclear auxin signaling; lessons from genetic studies with basal
3 land plants

4

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36

37 **Highlight**

38 Genetic studies using model bryophytes provided insights into the origin of
39 the nuclear auxin pathway, its critical roles in plant development, and the
40 increased complexity of the pathway during evolution.

41

42 **Abstract**

43 Auxin plays critical roles in growth and development through the regulation
44 of cell differentiation, cell expansion and pattern formation. The auxin signal
45 is mainly conveyed through a so-called nuclear auxin pathway involving the
46 receptor TIR1/AFB, the transcriptional co-repressor AUX/IAA, and the
47 transcription factor ARF with direct DNA-binding ability. Recent progress in
48 sequence information and molecular genetics in basal plants have provided
49 many insights into the evolutionary origin of the nuclear auxin pathway and
50 its pleiotropic roles in land plant development. In this review, we summarize
51 the latest knowledge of the nuclear auxin pathway gained from studies using
52 basal plants including charophycean green algae and two major model
53 bryophytes *Marchantia polymorpha* and *Physcomitrella patens*. In addition,

54 we discuss the functional implication of the increase in genetic complexity of
55 the nuclear auxin pathway during land plant evolution.

56

57 **Keywords**

58 TIR1/AFB, AUX/IAA, ARF, TPL, evolution, bryophytes, charophytes

59

60 **Abbreviations**

61 AD, ancillary domain; APB, AINTEGUMENTA, PLETHORA, BABY BOOM;
62 ARF, AUXIN RESPONSE FACTOR; AUX/IAA, AUXIN/INDOLE-3-ACETIC
63 ACID; BiFC, bimolecular fluorescence complementation; Cas9, CRISPR
64 associated endonuclease 9; CRISPR, clustered regularly interspaced short
65 palindromic repeats; DBD, DNA binding domain; DD, dimerization domain;
66 LRL, LOTUS JAPONICUS ROOTHAIRLESS1-LIKE; MR, middle region;
67 PB1, Phox and Bem1; RSL, ROOT HAIR DEFECTIVE SIX-LIKE; TAS3,
68 TRANS-ACTING SIRNA 3; TIR1/AFB, TRANSPORT INHIBITOR
69 RESPONSE 1/AUXIN SIGNALING F-BOX; TPL, TOPLESS; Y2H, yeast two-
70 hybrid

71

72 **Introduction**

73 The emergence of land plants approximately 450 million year ago was one of
74 the most important events in the history of life on Earth (Fig. 1, phylogenetic
75 tree). Land plants evolved from aquatic green algae consisting of two groups:
76 chlorophytes and charophytes, the latter of which forms a sister group of land
77 plants (Bowman, 2013; Wickett *et al.*, 2014). During the transition from
78 charophycean algae, the ancestor of land plants acquired many important
79 characteristics including three-dimensional growth from apical meristems,
80 and a multicellular diploid body (sporophyte or embryo). Thus, land plants
81 are also called embryophytes. After land invasion by embryophytes, there was
82 an explosion in land plant diversification, establishing major extant land
83 plant lineages including the bryophytes, lycophytes, ferns, and seed plants by
84 the end of the Devonian (360 million years ago) (Bowman, 2013).

85 One of key factors for morphogenesis and development in land plants
86 is auxin. The auxin signal in land plants is mainly conveyed through a
87 transcriptional pathway, the key components of which are TRANSPORT
88 INHIBITOR RESPONSE1/AUXIN SIGNALING F-BOX (TIR1/AFB) F-box
89 proteins, AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) transcriptional co-
90 regulators, and AUXIN RESPONSE FACTOR (ARF) transcription factors
91 (Fig. 1). Auxin stabilizes the interaction between TIR1/AFB and domain II of
92 AUX/IAA and promotes ubiquitin-dependent degradation of AUX/IAA
93 proteins in the 26S proteasome (Dharmasiri *et al.*, 2005a; Gray *et al.*, 2001;
94 Kepinski and Leyser, 2005). Therefore, mutations in domain II of AUX/IAA
95 proteins cause dominant auxin-resistant phenotypes (Gray *et al.*, 2001;

96 Ramos *et al.*, 2001). When the concentration of auxin is relatively low,
97 AUX/IAAs binds to ARFs through their shared C-terminal Phox and Bem1
98 (PB1) domain (Kim *et al.*, 1997). Through the N-terminal domain I, AUX/IAAs
99 recruit the co-repressor TOPLESS (TPL) and repress the transcription of
100 auxin-responsive genes (Szemenyei *et al.*, 2008). ARF proteins contain a DNA
101 binding domain (DBD) in their N-termini, which directly binds to a DNA
102 sequence called Auxin Responsive Element (AuxRE), and positively or
103 negatively regulate gene expression (Ulmasov *et al.*, 1997, 1999b). The DBD
104 of ARF proteins is further divided into the DNA-contacting B3 domain, its
105 flanking dimerization domains (DDs) that mediate an ARF-ARF interaction,
106 and the ancillary domain (AD) (Boer *et al.*, 2014). ARF proteins of land plants
107 are classified into three phylogenetically distinct clades, A, B and C (Finet *et*
108 *al.*, 2013). Class-A ARFs generally contain a glutamine-rich stretch in their
109 middle region (Finet *et al.*, 2013). Transactivation assays on model genes
110 demonstrated that class-A ARFs and class-B ARFs may function as
111 transcriptional activators and repressors, respectively (Ulmasov *et al.*, 1999a).
112 Class-C ARFs have been thought as transcriptional repressors because they
113 lack a glutamine-rich sequence in the middle region, although this assertion
114 has not yet been supported by experimental evidence. When did green plants
115 establish the nuclear auxin pathway and how did the pathway acquire
116 pleiotropic roles during evolution? The effect of auxin in growth and
117 development is widely observed among green plants including algal species
118 and bryophytes (Cooke *et al.*, 2002). However, past studies mostly focused on
119 angiosperms, and our knowledge of the origin and evolution of the auxin

120 signaling pathway has remained very limited. It is known that angiosperms
121 have high genetic redundancy in auxin signaling components (e.g. 23 ARFs,
122 29 AUX/IAAs and 6 TIR1/AFBs in *Arabidopsis*), allowing the formation of
123 complex interaction networks to regulate pleiotropic auxin responses. Modern
124 sequencing technologies make it possible to obtain genome and transcriptome
125 information in non-model organisms. In the past decade, genomic information
126 has become available in the moss *Physcomitrella patens* (Rensing *et al.*, 2008),
127 the lycophyte *Selaginella moellendorffii* (Banks *et al.*, 2011), the charophyte
128 *Klebsormidium flaccidum* (Hori *et al.*, 2014) and the liverwort *Marchantia*
129 *polymorpha* (<https://phytozome.jgi.doe.gov/>). In particular, experimental
130 studies with molecular genetic tools in the two bryophyte species *P. patens*
131 and *M. polymorpha* provided insights into the basics of nuclear auxin
132 signaling in land plants and its crucial roles in plant development. In this
133 review, we summarize the latest knowledge of nuclear auxin signaling in
134 basal plants and discuss the origin, evolution and fundamental
135 developmental roles in land plants.

136

137 **Origin of the nuclear auxin pathway**

138

139 *Establishment of the nuclear auxin pathway predated the emergence of land*
140 *plants*

141 Genome projects of *P. patens* and *S. moellendoffii* revealed that both species
142 encode all the components for the nuclear auxin pathway (Floyd and Bowman,
143 2007; Rensing *et al.*, 2008). Recently, genome data of another bryophyte model
144 *M. polymorpha* became available and revealed the existence of all canonical
145 components with minimal redundancy: only three ARFs, one AUX/IAA, and
146 one TIR1/AFB, all of which carry all conserved domains. Notably, each of the
147 three ARF proteins is phylogenetically classified into each of the three major
148 clades of ARF subfamilies in land plants (Flores-Sandoval *et al.*, 2015b; Kato
149 *et al.*, 2015).

150 In addition to bioinformatic analysis, biological functions of the
151 homologues of nuclear auxin signaling components have been investigated in
152 bryophytes. Prigge and colleagues found that 7 out of 17 auxin-resistant
153 mutants of *P. patens* that had been previously identified (Ashton *et al.*, 1979)
154 had a mutation in the degron motif (domain II) of either of the three AUX/IAA
155 genes that this plant possesses, and that the mutants showed reduced
156 expression of auxin responsive genes (Prigge *et al.*, 2010). They also confirmed
157 the auxin-dependent interaction between domain II of AUX/IAA and
158 TIR1/AFB proteins in *P. patens* using yeast two-hybrid (Y2H) and pull-down
159 assays (Prigge *et al.*, 2010). In *M. polymorpha*, transgenic plants expressing
160 AUX/IAA containing a mutation in domain II showed auxin-insensitive

161 phenotype and reduced expression of soybean-derived auxin response marker
162 GH3pro:GUS (Kato *et al.*, 2015). In addition, artificial microRNA (amiRNA)-
163 based knock-down of AUX/IAA showed auxin hyper-sensitivity (Flores-
164 Sandoval *et al.*, 2015b). Recently, a triple knock-out mutant lacking all
165 AUX/IAAs of *P. patens* was generated, and its transcriptome revealed that no
166 genes were differentially expressed upon auxin treatment in this mutant
167 (Lavy *et al.*, 2016). These findings indicate that bryophytes share the same
168 auxin perception mechanism with flowering plants, and it plays a major role
169 in transcriptional auxin responses (Fig .1).

170 The protein interaction for co-repressor complex formation is also
171 conserved in bryophytes. Y2H assay showed that TPL protein can interact
172 with all three AUX/IAAs in *P. patens* through their domain I (Causier *et al.*,
173 2012b). Transgenic plants of *M. polymorpha* expressing a chimeric fusion
174 protein of TPL and the PB1 domain from the AUX/IAA showed an auxin-
175 resistant phenotype (Flores-Sandoval *et al.*, 2015b). Y2H and bimolecular
176 fluorescence complementation (BiFC) assays demonstrated that the AUX/IAA
177 of *M. polymorpha* can interact with all the three ARFs through their PB1
178 domain (Kato *et al.*, 2015). These lines of evidence indicate that AUX/IAA
179 protein recruits the TPL co-repressor to ARFs and their target gene loci in
180 bryophytes as in angiosperms (Fig. 1).

181 Functional diversifications among the three classes of ARFs appear to
182 be conserved in bryophytes. Transactivation assays using BY-2 tobacco
183 cultured cells demonstrated that the single class-A and class-B ARFs in *M.*
184 *polymorpha* function as transcriptional activator and repressor, respectively,

185 as seen in angiosperms (Kato *et al.*, 2015; Ulmasov *et al.*, 1999a). Consistently,
186 mutants of the class-A ARF in *M. polymorpha*, which were generated by
187 various methods (homologous recombination, amiRNA, and CRISPR/Cas9),
188 showed an auxin-resistant phenotype and reduced expression of endogenous
189 auxin responsive genes (Eklund *et al.*, 2015; Flores-Sandoval *et al.*, 2015b;
190 Kato *et al.*, 2017; Sugano *et al.*, 2014). In *P. patens*, overexpression of class-A
191 ARF activates the expression of auxin-responsive genes. Overexpression of
192 class-B ARF suppressed the constitutive auxin response in complete knock-
193 out mutants of AUX/IAA. This suppression was presumably caused by
194 competition for target genes between class-A and class-B ARFs (Lavy *et al.*,
195 2016). Causier and colleagues presented that some of the class-B ARFs in *P.*
196 *patens* and *Arabidopsis* can interact with TPL, suggesting repression of auxin
197 responses by class-B ARFs partially through the recruitment of TPL co-
198 repressor without the aid of AUX/IAAs (Fig .1) (Causier *et al.*, 2012a; Causier
199 *et al.*, 2012b). This could provide a way to attenuate the strength of auxin
200 signaling independently of auxin levels.

201 In addition to protein characteristics, regulation of gene expression by
202 microRNA has also been investigated. In *Arabidopsis*, miR390 targets
203 *TRANS-ACTING SIRNA 3 (TAS3)* to trigger the biogenesis of trans-acting
204 siRNA (tasiRNA) which targets several class-B ARFs (ARF2, ARF3/ETTIN
205 and ARF4) (Allen *et al.*, 2005; Williams *et al.*, 2005). In *P. patens*, miR390
206 generates TAS3-derived tasiRNA which targets class-B ARFs (Axtell *et al.*,
207 2007), which was shown to confer robustness and sensitivity to auxin
208 response (Plavskin *et al.*, 2016). In *M. polymorpha*, miR390 and its target

209 TAS3 are also conserved and might target the class-B ARF (Tsuzuki *et al.*,
210 2016; Xia *et al.*, 2017). All of class-C ARFs in Arabidopsis are regulated by
211 miR160 (Mallory *et al.*, 2005; Wang *et al.*, 2005). miR160 and its target site
212 in class-C ARF are also conserved in both *P. patens* and *M. polymorpha* (Axtel
213 *et al.*, 2007; Lin *et al.*, 2016; Tsuzuki *et al.*, 2015), in the latter of which,
214 miR160 is used as backbone of artificial miRNA knock-down tool (Flores-
215 Sandoval *et al.*, 2015a). The lines of evidence suggest the importance of
216 miRNA-based regulation of class-B and C ARFs in the nuclear auxin pathway
217 throughout land plants.

218 These findings indicate that the ancestor of all land plants had already
219 established a basic nuclear auxin pathway consisting of TIR1/AFB-AUX/IAA
220 co-receptor and three classes of ARF transcription factors and its regulation
221 system mediated by microRNAs (Fig. 1). Moreover, recent studies
222 demonstrated that similar to angiosperms, the auxin biosynthesis pathway
223 via indole-3-pyruvic acid and PIN-mediated directional auxin transport is
224 critical for development of *M. polymorpha* and *P. patens*, respectively
225 (Bennett *et al.*, 2014; Eklund *et al.*, 2015; Viaene *et al.*, 2014). Taken together,
226 all aspects of the basic auxin system (biosynthesis, transport and signaling)
227 had been already established in the common ancestor of land plants.

228

229 *When and how was the nuclear auxin pathway established?*

230 Because, based on a few genomes, it has been proposed that chlorophytes do
231 not have any of the core components for the nuclear auxin pathway seen in
232 land plants (Lau *et al.*, 2009), the pathway has been supposed to be

233 established during evolution from charophycean green algae to embryophytes.
234 Presence of endogenous auxin in charophytes has been reported (Sztein *et al.*,
235 2000; Žižková *et al.*, 2016), even though it is controversial whether
236 charophycean algae have the indole-pyruvic acid-mediated auxin
237 biosynthesis pathway that accounts for the majority of auxin production in
238 land plants (Turnaev *et al.*, 2015; Yue *et al.*, 2014). Currently, genome
239 information of one species (*Klebsormidium flaccidum*) and transcriptome
240 data of several species are available within the charophytes (Hori *et al.*, 2014;
241 Ju *et al.*, 2015; Timme *et al.*, 2012), but the existence of nuclear auxin
242 pathway in charophytes is still unclear. Hori and colleagues reported that the
243 genome of *K. flaccidum* encodes none of the obvious homologues of nuclear
244 auxin pathway components (Hori *et al.*, 2014), while Wang and colleagues
245 reanalyzed the existing transcriptome data of charophytes and concluded that
246 the auxin system arose in charophytes (Wang *et al.*, 2015). However, the
247 “AUX/IAA” proteins from the charophyceae *Nitella mirabilis* and the
248 zygnematophyceae *Penium margaritaceum* lack domain I and II that are
249 critical for transcriptional repression and auxin perception. The longest ORF
250 of the “AUX/IAA” transcript from *K. flaccidum* (kfl00094_0070), in fact,
251 encodes a protein containing a PB1 domain and two DBDs, AP2 and B3
252 domains with neither DD nor AD. Furthermore, the deduced four domains
253 appear to contain multiple insertion sequences of various lengths and their
254 expected roles thus need to be experimentally verified. In addition,
255 charophyte F-box proteins are placed as sister to both TIR1/ABF and
256 CORONATINE INSENSITIVE 1 jasmonic acid receptor (Wang *et al.*, 2015).

257 What we can conclude at this moment would be that ancestral genes for an
258 ARF-like transcription factor and an auxin co-receptor were established in
259 charophytes, but it is unclear whether they are involved in auxin signaling
260 (Fig. 1). Most importantly, the current number of species from which
261 information is available is too small to reconstruct the evolutionary trajectory
262 that established the nuclear auxin pathway. It is necessary to collect precise
263 genome information from much broader species, especially in the
264 Zygnematophyceae, which forms the closest sister group of land plants
265 (Timme *et al.*, 2012; Turmel *et al.*, 2006; Wickett *et al.*, 2014; Wodniok *et al.*,
266 2011).

267 **The nuclear auxin pathway is critical for morphogenesis of land plants**

268

269 Auxin biology started from the study of phototropic responses in young
270 grass seedlings by Charles and Francis Darwin (Darwin, 1880). Tropic
271 responses are caused by differential cell elongation in opposite sides of plant
272 tissue. In *Arabidopsis*, many mutants of the nuclear auxin pathway, which
273 show defects in cell expansion and tropic responses, have been identified
274 (Harper *et al.*, 2000; Leyser *et al.*, 1996; Liscum and Reed, 2002; Möller *et al.*,
275 2010; Pérez-Pérez *et al.*, 2010; Tatematsu *et al.*, 2004). Following the
276 identification of auxin in the early 20th century, there was an extensive
277 literature reporting the effect of exogenous application of auxin on growth and
278 development of *M. polymorpha* including tropic responses (reviewed in
279 Bowman, 2015). Kohlenbach (1957) demonstrated that auxin causes positive
280 gravitropic growth of gemmaling, and Rousseau (1953) reported auxin-
281 induced elongation and abnormal tropic response in sexual organ. Flores-
282 Sandoval *et al.* (2015) and Kato *et al.* (2015) confirmed the auxin-induced
283 gravitropic growth in *M. polymorpha*, and also showed auxin-induced
284 elongation of cup-shaped structures (gemma cups) that are formed on the
285 dorsal side of the thallus, both of which are probably caused by elongation of
286 dorsal epidermal cells (Flores-Sandoval *et al.*, 2015b; Kato *et al.*, 2015).
287 Stabilized AUX/IAA protein-expressing plants showed suppression of this
288 auxin-dependent cell elongation and defects in tropic responses of sexual
289 organs as well (Kato *et al.*, 2015). In *P. patens*, perturbation of polar auxin
290 transport causes defects in size and shape of leaf cells, and in photo- and

291 gravi-tropic responses of gametophores (Bennett *et al.*, 2014; Viaene *et al.*,
292 2014). These findings suggest that regulation of cell elongation is a common
293 output of the nuclear auxin pathway in land plants.

294 Plant cells are immobile due to rigid cell walls, thus precise control of
295 developmental axes and cell division patterns is critical to achieve a three-
296 dimensional body plan of land plants. In *Arabidopsis*, it has been shown that
297 the nuclear auxin pathway is critical for apical-basal axis formation and
298 various formative cell divisions during embryogenesis (De Rybel *et al.*, 2013;
299 Friml *et al.*, 2003; Hamann *et al.*, 1999; Hardtke and Berleth, 1998; Möller *et al.*,
300 2017; Schlereth *et al.*, 2010; Weijers *et al.*, 2006; Yoshida *et al.*, 2014). *M.*
301 *polymorpha* thallus produces small discoid organs called gemmae for asexual
302 reproduction. The development of gemmae shows some similarity to
303 *Arabidopsis* embryogenesis. In both cases, a single initial cell first undergoes
304 a transverse asymmetric division creating the apical-basal axis. The apical
305 cell immediately or eventually undergoes longitudinal divisions and forms the
306 most part of the gemma or embryo. The basal cell undergoes limited numbers
307 of cell divisions, forming a supporting tissue called stalk or suspensor (Barnes
308 and Land, 1908; ten Hove *et al.*, 2015; Fig. 2). In *M. polymorpha*, the mutant
309 of the single class-A ARF exhibits defects in the transition of cell division
310 planes from transverse to longitudinal during early gemma development and
311 ectopic cell divisions of stalk cell (Kato *et al.*, 2017). In addition, the mutants
312 also had defects in meristem formation and positioning during gemma
313 development probably because of a defect in the formation of developmental
314 axis (Kato *et al.*, 2017; Fig. 2). In *Arabidopsis* embryogenesis, a class-A ARF

315 (ARF5) plays critical role to control the cell division plane in various processes
316 including root meristem formation (Hardtke and Berleth, 1998; Möller *et al.*,
317 2017; Schlereth *et al.*, 2010; Weijers *et al.*, 2006), and mutants of ARFs
318 expressed in the suspensor shows ectopic cell divisions there (Rademacher *et*
319 *al.*, 2012; Rademacher *et al.*, 2011; Fig. 2). These similarities of pattern
320 formation between *M. polymorpha* gemma development and Arabidopsis
321 embryogenesis give rise to the idea that some common mechanism exist,
322 nevertheless further investigation is necessary.

323 *M. polymorpha* thallus grows with periodical bifurcations.
324 Manipulations of auxin signaling by auxin biosynthesis inhibitor treatment
325 or overexpression of the class-A ARF caused decreased or increased
326 bifurcation rate, respectively (Flores-Sandoval *et al.*, 2015b). In *P. patens*,
327 auxin regulates the pattern of lateral branching of gametophore shoots via
328 bidirectional transport of auxin (Coudert *et al.*, 2015). Auxin transport
329 inhibitor treatment or knock-out of auxin transporter caused bifurcated
330 sporophytes which are rarely found under normal conditions (Bennett *et al.*,
331 2014; Fujita *et al.*, 2008). In any case, tissue growth occurs via divisions of a
332 single apical stem cell (Harrison *et al.*, 2009; Kofuji and Hasebe, 2014;
333 Shimamura, 2016). Even though the detailed mechanisms how the new apical
334 stem cell is formed during branching events remain to be elucidated, these
335 lines of evidence suggest that the nuclear auxin pathway plays pivotal roles
336 in the formation of new apical stem cells and branching in bryophytes.

337 In *M. polymorpha*, strong inhibition of auxin biosynthesis or signaling
338 by knocking out or down auxin biosynthesis genes or by introducing the stable

339 AUX/IAA protein caused defects in cell differentiation and produced callus-
340 like cell masses without the organs found in normal thallus (Eklund *et al.*,
341 2015; Kato *et al.*, 2015), suggesting that nuclear auxin pathway is critical for
342 cell differentiation processes. In *P. patens*, inhibition of auxin biosynthesis
343 caused defects in maturation of both male and female sexual organs probably
344 through the cell differentiation toward programmed cell death (Landberg *et*
345 *al.*, 2013). Protonemata of *P. patens* comprise two type of cells, chloroplast-
346 rich chloronema cells and long caulonema cells which differentiated from
347 chloronema cells. Exogenous auxin treatment promotes the cell fate
348 transition from chloronema to caulonema through the AUX/IAA-dependent
349 pathway (Ashton *et al.*, 1979; Prigge *et al.*, 2010). Caulonema cells sometimes
350 produce side branches, forming new protonemata or gametophores. Aoyama
351 and colleagues revealed that four AP2-type transcription factors, which are
352 orthologous to AINTEGUMENTA, PLETHORA and BABY BOOM (APB) in
353 Arabidopsis, are required for the formation of gametophore apical stem cells
354 in caulonema cells, and the expression of *APB* genes of *P. patens* are activated
355 by auxin (Aoyama *et al.*, 2012). Interestingly, most of the *APB* genes in
356 Arabidopsis were reported to function downstream of auxin pathways, and
357 regulate many aspects of plant development including stem cell niche
358 specification and meristem maintenance (reviewed in (Horstman *et al.*, 2014)).

359 More than half a century ago it has been reported that exogenous auxin
360 promoted rhizoid formation in both *M. polymorpha* and *P. patens* (Ashton *et*
361 *al.*, 1979; Rousseau, 1950, 1951a, b). Rhizoids are root hair-like structure seen
362 in a wide range of plants including green algae, bryophytes and pteridophytes.

363 Rhizoid formation caused by exogenous auxin is inhibited in undegradable
364 mutants of AUX/IAA in both *P. patens* and *M. polymorpha* (Kato *et al.*, 2015;
365 Prigge *et al.*, 2010). In *P. patens*, several auxin-inducible transcription factors
366 are identified as critical regulators of rhizoid formation (Jang and Dolan,
367 2011; Jang *et al.*, 2011; Sakakibara *et al.*, 2003; Tam *et al.*, 2015). Among
368 them, two different subgroups of basic helix-loop-helix transcription factors,
369 ROOT HAIR DEFECTIVE SIX-LIKE (RSL) and LOTUS JAPONICUS
370 Roothairless1-LIKE (LRL) are involved in root hair development in
371 angiosperms (Ding *et al.*, 2009; Karas *et al.*, 2009; Masucci and Schiefelbein,
372 1994; Menand *et al.*, 2007). Notably, class II *RSL* genes are also regulated by
373 auxin in *Arabidopsis* (Pires *et al.*, 2013; Yi *et al.*, 2010). *RSL* and *LRL* genes
374 in *M. polymorpha* are also involved in rhizoid formation, but if these genes
375 are also regulated by auxin is not clear (Breuninger *et al.*, 2016; Proust *et al.*,
376 2016).

377 The lines of evidence discussed above indicate that the nuclear auxin
378 pathway regulates various critical processes to achieve three-dimensional
379 body plans in land plants including cell expansion, tropic responses, cell
380 division pattern, axis formation, and cell differentiation. In some cases,
381 bryophytes and angiosperms may share the downstream system to regulate
382 similar developmental processes, exemplified possibly by *APB* and *RSL* genes.
383 In addition to morphogenesis, there are some reports showing similar
384 processes are regulated by auxin in bryophytes and angiosperms. For
385 example, in *M. polymorpha*, auxin, via the class-A ARF, functions as positive
386 regulator of gemma dormancy, which is reminiscent of the seed dormancy in

387 angiosperms (Eklund *et al.*, 2015)(Kato *et al.*, 2017). In *P. patens*, auxin
388 promotes resistance to oomycete infection as seen in angiosperms as well
389 (Mittag *et al.*, 2015). These findings support the idea that land plants share
390 some gene regulatory networks under nuclear auxin pathways, despite that
391 they have experienced more than 450 million years of evolution since
392 separation. Investigation using basal model plants would help us to
393 understand not only specific roles of the nuclear auxin pathway in basal
394 plants but also core regulatory systems under the control of auxin shared in
395 land plants.
396

397 **Impact of increasing complexity of auxin signaling pathway through plant**
398 **evolution**

399

400 *Increase of gene numbers through land plant evolution*

401 Among the land plant species of which genome sequences are available, *M.*
402 *polymorpha* has the simplest nuclear auxin pathway consisting of one
403 TIR1/AFB, one AUX/IAA, and three ARFs (Flores-Sandoval *et al.*, 2015b;
404 Kato *et al.*, 2015). In contrast, other land plants in which the genomes were
405 sequenced suggested that the number of components was increased during
406 the 450 million years of evolution. For example, there exist four TIR1/AFBs,
407 three AUX/IAAs, and 14 ARFs in *P. patens*, two TIR1/AFBs, four AUX/IAAs
408 and seven ARFs in *S. moellendorffii*, and six TIR1/AFBs, 29 AUX/IAAs and
409 23 ARFs in Arabidopsis. *P. patens* has a relatively high number of components
410 among basal land plants probably because of the whole genome duplication
411 event which occurred 30-60 million years ago (Rensing *et al.*, 2007).

412 Because there are only few streptophyte genomes available outside the
413 angiosperms, it is difficult to trace precisely how the components of the
414 nuclear auxin pathway increased throughout land plant evolution. Past
415 studies on TIR1/AFBs and ARFs presented that at least there are two major
416 gene increase events before and after the emergence of gymnosperms. The
417 common ancestor of gymnosperms is supposed to have three groups of
418 TIR1/AFB and six ARFs (three class-A, two class-B and one class-C), and they
419 were increased into six TIR1/AFB and 11 ARFs (five class-A, five class-B, one
420 class-C) in the ancestor of angiosperms (Finet *et al.*, 2013; Parry *et al.*, 2009).

421 These gene increase events are chronologically consistent with the predicted
422 whole genome duplication events (Finet *et al.*, 2013; Jiao *et al.*, 2011).

423

424 *Impact of increase in copy number*

425 What are the biological impacts of gene duplication in auxin signaling
426 components? Gene duplication events provide extra copies which can buffer
427 against gene loss by spontaneous mutations. Indeed, single loss-of-function
428 mutation in TIR1/AFB do not cause dramatic developmental defects in
429 Arabidopsis (Dharmasiri *et al.*, 2005b). None of loss-of-function mutants of
430 AUX/IAA with developmental phenotype have been reported except for IAA3,
431 IAA7 and IAA28 (Nagpal *et al.*, 2000; Overvoorde *et al.*, 2005; Tian and Reed,
432 1999; Wang *et al.*, 2015). Similarly, among 23 ARFs, only five members show
433 developmental defects as a single mutant (Okushima *et al.*, 2005). These
434 findings suggest that many of the auxin signaling components in Arabidopsis
435 have (at least partially) redundant paralogues.

436 Gene duplication also confers the capacity for functional diversification.
437 A way of diversification is to change the gene expression pattern. In
438 Arabidopsis embryos, ARF genes show different expression patterns and
439 some co-expressed ARFs function in the same biological processes
440 (Rademacher *et al.*, 2011). There are extensive reports showing importance of
441 small RNA-mediated spatio-temporal regulation for expression of nuclear
442 auxin pathway components. Among the six miRNAs conserved throughout
443 land plants, miR160 and miR390 are involved in repression of class-C and
444 class-B ARFs as described above (Lin *et al.*, 2016; Plavskin *et al.*, 2016;

445 Tsuzuki *et al.*, 2015; Xia *et al.*, 2017). In *Arabidopsis*, miR393 targets TIR1,
446 AFB1, AFB2 and AFB3 and reduces auxin responses (Navarro *et al.*, 2006;
447 Parry *et al.*, 2009). miR167 targets the two closely related class-A ARFs,
448 ARF6 and ARF8, regulating flower organ development (Wu *et al.*, 2006). The
449 origin of both miR393 and miR167 can be traced back at least to the common
450 ancestor of seed plants (Axtell and Bartel, 2005; Xia *et al.*, 2015). Some
451 species-specific miRNAs are also involved in the nuclear auxin pathway. In
452 *Arabidopsis*, auxin-induced miR847 cleaves IAA28 (Wang and Guo, 2015).
453 miR1219 targets two out of four class-B ARF in *P. patens* (Axtell *et al.*, 2007).
454 In the gymnosperm *Ginkgo biloba*, gbi-miRN41 and gbi-miRN45 are
455 predicted to target an AUX/IAA and an ARF, respectively (Zhang *et al.*, 2015).

456 Some promoter swap analyses in *Arabidopsis* suggested that there are
457 differences in protein characters even between closely related members of the
458 nuclear auxin pathway (Muto *et al.*, 2007; Parry *et al.*, 2009; Weijers *et al.*,
459 2005). Several lines of evidence indicate that six TIR1/AFB members in
460 *Arabidopsis* have differences in preference of interactions with AUX/IAAs or
461 auxin species and in efficiency to form ubiquitin ligase complex (Calderón
462 Villalobos *et al.*, 2012; Parry *et al.*, 2009; Prigge *et al.*, 2016; Yu *et al.*, 2015).
463 These differences presumably cause the difference in auxin-dependent
464 degradation rates of AUX/IAAs. Indeed, degradation assays of AUX/IAA
465 proteins in yeast showed that the degradation rates vary among AUX/IAA
466 proteins and depend on which TIR1/AFB and auxin species are present
467 (Havens *et al.*, 2012; Shimizu-Mitao and Kakimoto, 2014). Recently Guseman
468 and colleagues tested the impact of differences in AUX/IAA protein stability

469 *in vivo* and proposed that degradation rate of AUX/IAA determines the pace
470 of lateral root development (Guseman *et al.*, 2015).

471 Protein interactions between AUX/IAAs and ARFs through the PB1
472 domain could affect the sensitivity of ARFs to auxin signal. In Arabidopsis,
473 Y2H and BiFC assays showed that class-A ARFs could interact with almost
474 all AUX/IAAs, while most of the class-B, except for ARF4 and ARF9, and
475 class-C ARFs interacted with none or limited members of AUX/IAAs (Piya *et al.*
476 *et al.*, 2014; Vernoux *et al.*, 2011). These results suggest that class-B and class-
477 C ARFs are weakly regulated by AUX/IAAs and by the auxin signal. During
478 land plant evolution, truncation of PB1 domain from ARF protein has
479 happened many times in various species. *S. moellendorffii* and *P. patens* have
480 one truncated ARF without PB1, and these two truncated ARFs are
481 phylogenetically independent (Paponov *et al.*, 2009). Three out of 23 ARF
482 members in Arabidopsis do not have a PB1 domain (Guilfoyle and Hagen,
483 2001). Remarkably, more than 50% of the ARFs in *Medicago truncatula* lack
484 a PB1 domain (Chandler, 2016). ARF proteins without PB1 are presumably
485 independent from AUX/IAA and thus regulate downstream genes in an auxin-
486 independent manner. Indeed, Arabidopsis ARF5 which artificially lacks PB1
487 domain confers a gain-of-function phenotype and increases expression of
488 auxin-responsive genes (Krogan *et al.*, 2012). In addition, phosphorylation of
489 two class-A ARFs (ARF7 and ARF19) inhibits their interactions with
490 AUX/IAA proteins, resulting in auxin-independent activation of downstream
491 genes (Cho *et al.*, 2014).

492 Finally, target specificity of ARFs is supposed to be critical to generate

493 pleiotropic output of auxin signaling, but our current knowledge is
494 fragmented. Protein binding microarray unraveled that ARF5 (class-A) and
495 ARF1 (class-B) in *Arabidopsis* have little difference of specificity in intrinsic
496 binding preference (Boer *et al.*, 2014). On the other hand, recent cistrome
497 analysis by DNA affinity purification sequencing (DAP-seq) revealed that
498 *Arabidopsis* ARF5 and ARF2 (class-B), share only 9% of their binding sites on
499 the genome (O'Malley *et al.*, 2016). Crystal structural analysis revealed that
500 homo-dimerized ARF DBDs bind to palindromic AuxREs with space and that
501 ARF5 and ARF1 homodimers show different capacity in length of this space
502 (Boer *et al.*, 2014). However, synthetic auxin response circuits using yeast
503 suggested that the space between two AuxREs is not sufficient to confer
504 specificity to DNA binding of class-AARFs (Pierre-Jerome *et al.*, 2016). There
505 is an idea that interactions with other transcription factors modify target
506 specificity of ARFs, and in fact, many types of transcription factors are
507 reported to interact with various ARF proteins (Kelley *et al.*, 2012; Oh *et al.*,
508 2014; Pfluger and Zambryski, 2004; Shin *et al.*, 2007; Simonini *et al.*, 2016;
509 Varaud *et al.*, 2011). Notably, BRASSINAZOLE-RESISTANT 1 and
510 PHYTOCHROME INTERACTING FACTOR 4 largely share the downstream
511 genes with auxin-dependent pathway, and these proteins interact with ARF6
512 and ARF8, but not with ARF1 and ARF7 (Oh *et al.*, 2014). However, how
513 interactors modulate ARF functions remains to be elucidated. Furthermore,
514 our knowledge of target specificity of ARFs is mostly based on the study in
515 *Arabidopsis*. A recent study showed that *P. patens* ARFa8 (class-A) and
516 ARFb4 (class-B) can bind to the same DNA sequences in artificial and

517 endogenous auxin-responsive promoters *in vitro* (Lavy *et al.*, 2016).
518 However, further analysis is required to understand whether ARFs in basal
519 plants have specificity of target genes. Future studies using basal plants with
520 simpler systems would contribute to understand the molecular mechanism
521 for target specificity of ARF proteins.

522 Through the functional specification of nuclear auxin signaling
523 components as described above, pleiotropic auxin responses might be
524 regulated in cell-, tissue-, and stage-specific manner. However, an increase in
525 gene copies is not the only way to generate pleiotropic auxin responses. *M.*
526 *polymorpha* still has various kinds of cellular responses regulated by auxin
527 in spite of its simple nuclear auxin pathway. While relative auxin
528 concentration could in theory provide some specificity, it seems more likely
529 that the nuclear auxin pathway acts as a facilitator of the processes and
530 outcome specificity would be provided by interaction with other transcription
531 factors (Flores-Sandoval *et al.*, 2015b; Kato *et al.*, 2015). Therefore, further
532 analyses using various plant species are required to understand what is
533 commonly conserved during plant evolution and what is specifically
534 established in certain lineages.

535

536 **Future prospects**

537

538 Progress in the past decade has provided us with many insights into the
539 evolutionary origin of the nuclear auxin pathway, its crucial developmental
540 roles in land plants, and complexity surrounding the pathway. However, there
541 are still some large gaps in our understanding of the evolution, such as the
542 precise origin of this pathway, gene duplication events during the evolution
543 from bryophyte to seed plants, and biological functions in basal vascular
544 plants. Several studies using lycophytes and ferns showed that auxin is
545 involved in the important developmental processes including meristem
546 maintenance, root branching, phyllotaxy, and vascular development in basal
547 vascular plants as well (De *et al.*, 2016; de Vries *et al.*, 2016; Sanders and
548 Langdale, 2013), but the underlying molecular basis has remained unclear.
549 Currently, multiple projects are ongoing to enrich the genomic and
550 transcriptomic information of basal plants including charophytes and ferns
551 (reviewed in (Rensing, 2017)). Moreover, transformation techniques have
552 been established in the charophyte *Penium margaritaceum*, and the ferns
553 *Pteris vittata* and *Ceratopteris richardii* (Bui *et al.*, 2015; Muthukumar *et al.*,
554 2013; Plackett *et al.*, 2015; Sørensen *et al.*, 2014). Such progress will bridge
555 the gaps in our evolutionary understanding of the nuclear auxin pathway.

556 As we discussed in this review, class-C ARF was established before
557 land invasion by plants and has been conserved throughout land plant
558 evolution, suggesting its critical role in auxin signaling. However, our
559 knowledge of class-C ARF functions is very limited except for the regulation

560 by miR160. Classification as transcriptional repressor is based on only amino
561 acid composition in the MR. Some reports even support the opposite idea that
562 class-C ARFs in *Arabidopsis* function as transcriptional activators (Wang *et al.*
563 *al.*, 2005; Yang *et al.*, 2013). To understand how class-C ARFs are involved in
564 auxin responses, both genetic and biochemical analyses are necessary.
565 Especially, the question whether class-C ARFs share their targets with class-
566 A ARFs, as seen in class-B ARFs, and evaluation from an evolutionary point
567 of view would be critical.

568 Despite the lack of TIR1/AFB-, AUX/IAA- and ARF-mediated nuclear
569 auxin pathway, a wide range of algal species, including chlorophytes,
570 rhodophyta, ochrophyta, haptophyte, and cyanobacteria, produce auxin and
571 respond to exogenously supplied auxin (Labeeuw *et al.*, 2016; Zhang and van
572 Duijn, 2014; Žižková *et al.*, 2016). Bacteria and fungi also produce auxin and
573 use it for communication with land plants and algae (Amin *et al.*, 2015;
574 Bagwell *et al.*, 2014; Fu *et al.*, 2015). These facts suggest that algal species
575 have some different auxin response systems from those in land plants. To
576 investigate whether the auxin responses in algal species occur through a
577 transcriptional pathway and, if so, what its molecular mechanisms are would
578 be interesting challenges in the field of auxin research.

579

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581

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588

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1 **Figure legends**

2

3 **Fig. 1 Phylogeny of the green lineage and establishment of the nuclear auxin**
4 **pathway in embryophytes**

5 Box 1. ARF-like transcription factors, PB1-containing proteins, and proteins
6 consisting of F-box and LRR domains are present in charophyte green algae.
7 But its involvement in auxin signaling is unclear.

8 Box 2. Current model of the nuclear auxin pathway conserved in land plants.

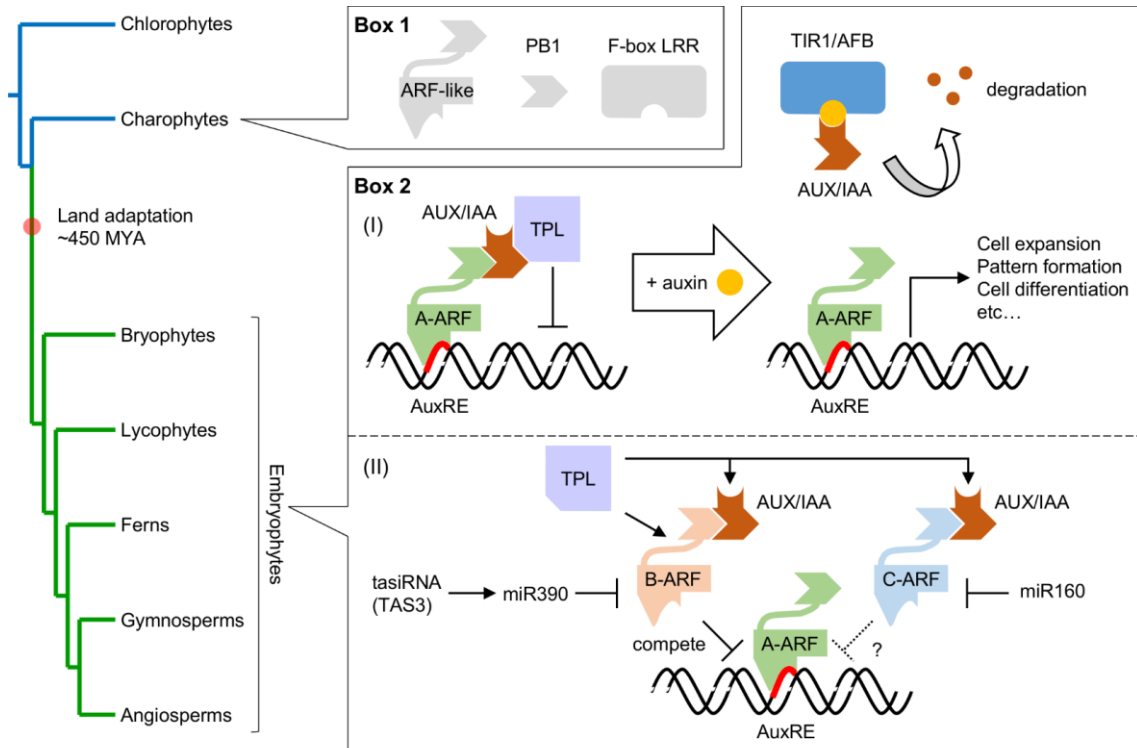
9 (I) Class-A ARFs directly bind to the AuxRE (red line). AUX/IAAs and TPLs
10 inhibit transcriptional activation by class-A ARFs in low auxin conditions,
11 and the inhibition is released by AUX/IAA degradation through its binding
12 with auxin and TIR1/AFB. (II) Class-B ARFs compete for target genes with
13 class-A ARFs. DNA binding mode of class-C ARFs is unclear. TPL protein can
14 interact with some of class-B ARFs. miR390 triggers production of tasiRNAs
15 targeting class-B ARFs. AUX/IAA proteins also interact with class-B and C
16 ARFs but its effect is unclear. Class-C ARF expression is regulated by miR160.
17

18 **Fig. 2 Similarities between gemma development in *M. polymorpha* and**
19 **embryogenesis in Arabidopsis**

20 Patterns of initial cell divisions during gemma and embryo development and
21 their final shapes are shown on the left. Cells are colored according to cell
22 lineage, as indicated in the box. ARFs that activate or repress the indicated
23 developmental processes are shown on the right.

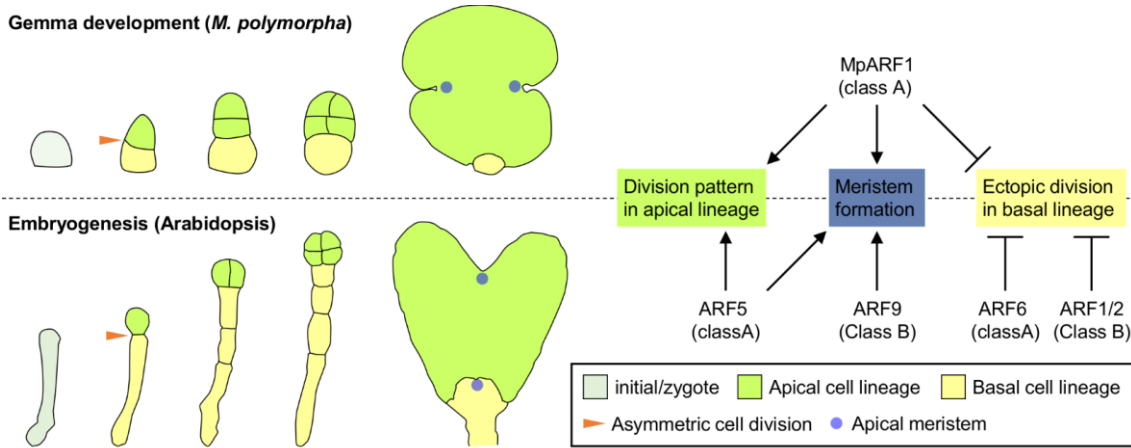
24

25 **Fig. 1**



26

27 **Fig. 2**



28