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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

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30 **Title**

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34 **Running title**

35 Evolution of nuclear auxin signaling

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,

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

Keywords

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

Abbreviations

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

Introduction

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

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

5

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

Origin of the nuclear auxin pathway

Establishment of the nuclear auxin pathway predated the emergence of land plants

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

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

phenotype and reduced expression of soybean-derived auxin response marker

GH3pro:GUS (Kato *et al.*, 2015). In addition, artificial microRNA (amiRNA)-

based knock-down of AUX/IAA showed auxin hyper-sensitivity (Flores-

Sandoval *et al.*, 2015b). Recently, a triple knock-out mutant lacking all

AUX/IAAs of *P. patens* was generated, and its transcriptome revealed that no

genes were differentially expressed upon auxin treatment in this mutant

(Lavy *et al.*, 2016). These findings indicate that bryophytes share the same

auxin perception mechanism with flowering plants, and it plays a major role

in transcriptional auxin responses (Fig .1).

The protein interaction for co-repressor complex formation is also

conserved in bryophytes. Y2H assay showed that TPL protein can interact

with all three AUX/IAAs in *P. patens* through their domain I (Causier *et al.*,

2012b). Transgenic plants of *M. polymorpha* expressing a chimeric fusion

protein of TPL and the PB1 domain from the AUX/IAA showed an auxin-

resistant phenotype (Flores-Sandoval *et al.*, 2015b). Y2H and bimolecular

fluorescence complementation (BiFC) assays demonstrated that the AUX/IAA

of *M. polymorpha* can interact with all the three ARFs through their PB1

domain (Kato *et al.*, 2015). These lines of evidence indicate that AUX/IAA

protein recruits the TPL co-repressor to ARFs and their target gene loci in

bryophytes as in angiosperms (Fig. 1).

Functional diversifications among the three classes of ARFs appear to

be conserved in bryophytes. Transactivation assays using BY-2 tobacco

cultured cells demonstrated that the single class-A and class-B ARFs in *M.*

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).

The nuclear auxin pathway is critical for morphogenesis of land plants

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

300   *al.*, 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

Impact of increasing complexity of auxin signaling pathway through plant evolution

Increase of gene numbers through land plant evolution

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

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

476　*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-A ARFs (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

Future prospects

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

As we discussed in this review, class-C ARF was established before land invasion by plants and has been conserved throughout land plant evolution, suggesting its critical role in auxin signaling. However, our 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*

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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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.

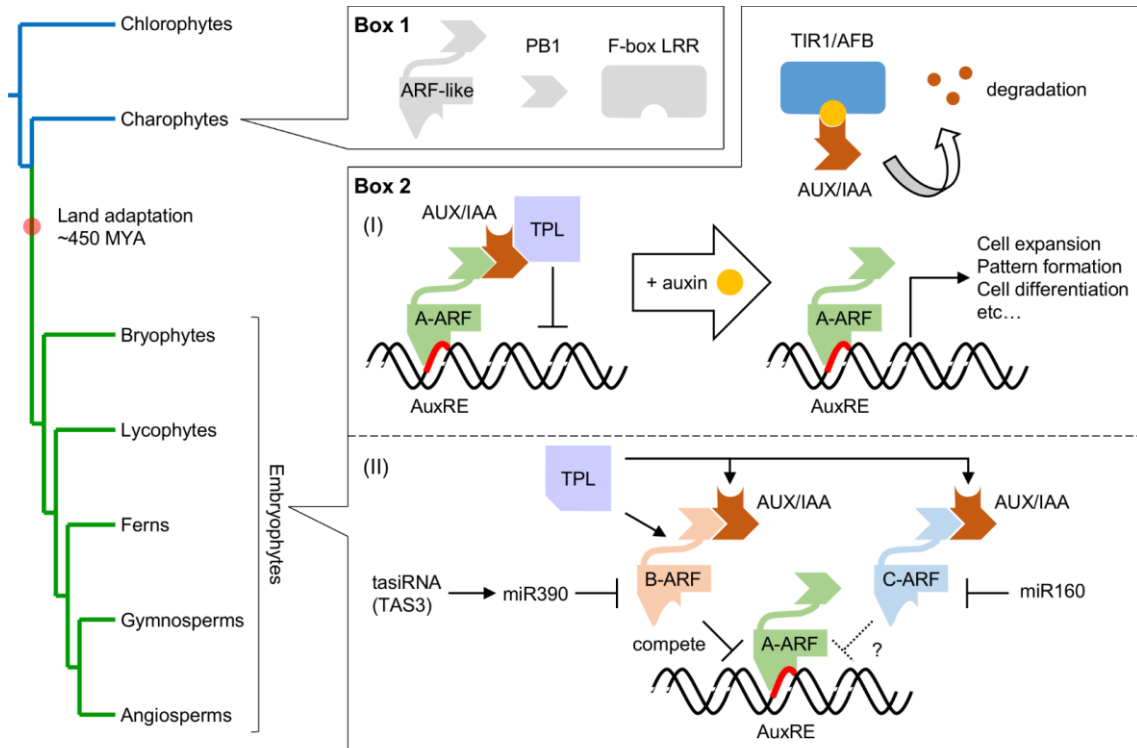8 Box 2. Current model of the nuclear auxin pathway conserved in land plants.

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
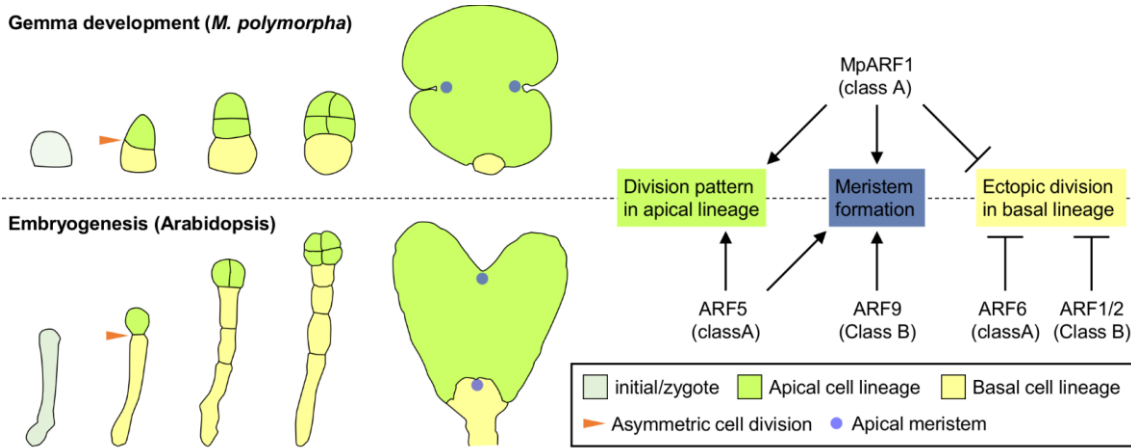
23 developmental processes are shown on the right.

24

25   **Fig. 1**

26

27   **Fig. 2**

28