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Hormonal diterpenoids derived from *ent*-kaurenoic acid are involved in the blue-light avoidance response of *Physcomitrella patens*

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Abbreviations: GA, gibberellin; KO, *ent*-kaurene oxidase; KAO, *ent*-kaurenoic acid oxidase; CPS, *ent*-copalyl diphosphate synthase; KS, *ent*-kaurene synthase; P450, cytochrome P450 monooxygenase; *nptII*, neomycin phosphotransferase II.

Gibberellins (GAs) are diterpenoid hormones that regulate growth and development in flowering plants. The moss *Physcomitrella patens* has part of the GA biosynthetic pathway from geranylgeranyl diphosphate to *ent*-kaurenoic acid *via ent*-kaurene, but it does not produce GA. Disruption of the *ent*-kaurene synthase gene in *P. patens* suppressed caulonemal differentiation. Application of *ent*-kaurene or *ent*-kaurenoic acid restored differentiation, suggesting that derivative(s) of *ent*-kaurenoic acid, but not GAs, are endogenous regulator(s) of caulonemal cell differentiation. The protonemal growth of *P. patens* shows an avoidance response under unilateral blue light. Physiological studies using gene mutants involved in *ent*-kaurene biosynthesis confirmed that diterpenoid(s) regulate the blue-light response. Here, we discuss the implications of these findings, and provide data for the *ent*-kaurene oxidase gene-disrupted mutant.

The diterpenoid plant hormone gibberellin (GA) regulates growth and development of vascular seed plants. In higher plants, this phytohormone has been extensively studied at the chemical, physiological, and biochemical levels. In contrast, little is known about endogenous GAs and their physiological roles in nonvascular plants. Here, we studied the roles of GA-like compounds in the moss *Physcomitrella patens*. The genome database of *P. patens* was released in 2008.[1] Homology searches of the database revealed that putative GA biosynthetic genes were present in the *P. patens* genome. Based on these results, we characterized a bifunctional *ent*-kaurene synthase (CPS/KS) and CYP701B1 *ent*-kaurene oxidase (KO), which catalyze reactions from geranylgeranyl diphosphate to *ent*-kaurene *via ent*-copalyl diphosphate and from *ent*-kaurene to *ent*-kaurenoic acid, respectively (**Fig. 1A**).[2,3] Previous studies on a *Ppcps/ks*-disrupted (*ent*-kaurene-deficient) mutant of *P. patens* showed that its caulonemal differentiation was suppressed under red light. The caulonemal differentiation was restored by application of either *ent*-kaurene or *ent*-kaurenoic acid, but not GA_4.[4] The germination and growth of positively photoblastic seeds of vascular plants such as *Arabidopsis* and lettuce are regulated by the red-light photoreceptor, phytochrome, and GAs.[5,6] The growth and development of photoblastic seed plants are positively regulated by GA biosynthesis and signaling under red light. These results suggested that caulonemal differentiation may be regulated by both red light and *ent*-kaurene-derived diterpenoids, but not by GAs in *P. patens* (**Fig. 1B**).

Recently, we observed unique responses of *P. patens* irradiated by blue light (**Fig. 1B**).[7] The wild-type protonema grew in the opposite direction to the light source under unilateral blue light, whereas the *Ppcps/ks* disruption mutant did not show this response. This avoidance response of the *Ppcps/ks* disruption mutant under blue light was rescued by application of *ent*-kaurenoic acid. In seed plants, blue light reduces the endogenous GA level and decreases shoot length.[8] In *P. patens*, diterpenoid regulator(s) derived from *ent*-kaurenoic acid may regulate its unique phototropism under blue light. To investigate the regulation of the diterpenoid biosynthetic genes, we analyzed the expression patterns of *PpCPS/KS* and *PpKO* in *P. patens* under red and blue light. *PpCPS/KS* gene expression was induced by blue light while that of *PpKO* was not regulated by either blue or red light. PpKO is a member of the cytochrome P450 monooxygenase (P450) superfamily, and the recombinant PpKO protein catalyzes the oxidation of *ent*-kaurene to *ent*-kaurenoic acid *in vitro*.[3] The responsiveness of *PpKO* gene expression to light stimuli was significantly different from that of *PpCPS/KS*. Since the level of *PpKO* expression was lower than that of *PpCPS/KS* and it did not respond to either blue or red light, we focused on the biosynthetic role of PpKO during protonemal growth and generated

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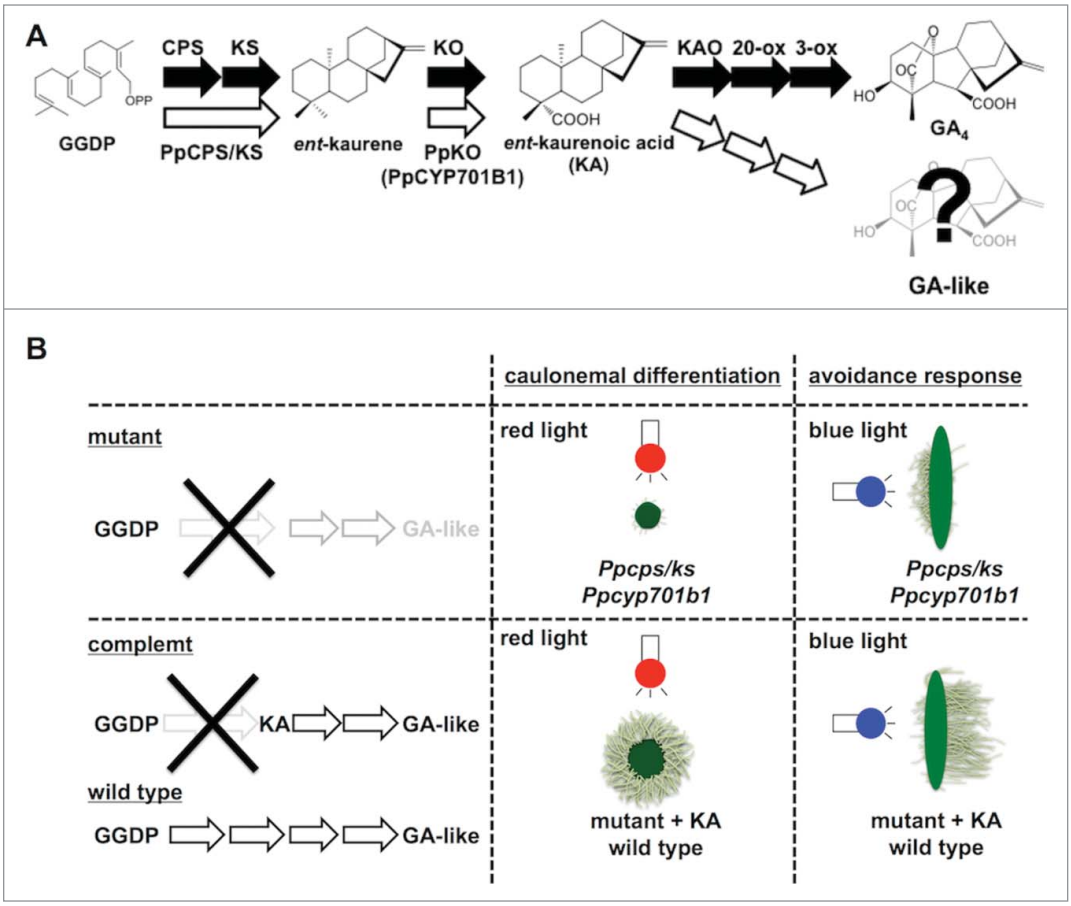

Figure 1. The biosynthetic pathway of diterpenoid regulator(s) and physiological responses under light sources in *P. patens*. (**A**), In *P. patens*, novel diterpenoid (GA-like) regulator(s) synthesized from *ent*-kaurenoic acid. PpCPS/KS and PpKO (CYP701B1) are involved in *ent*-kaurene and *ent*-kaurenoic acid biosynthesis, respectively. Black arrow shows the GA biosynthetic pathway in flowering plant. White arrow shows the GA-like biosynthetic pathway in *Physcomitrella patens*. (**B**), GA-like regulator(s) from *ent*-kaurenoic acid regulates the differentiation to caulonemal cells under red light and the avoidance response of protonemal cells to blue light. Mutants show the suppression of caulonemal differentiation under red light and that of avoidance response under unilateral blue light. These phenotypes are restored by application of *ent*-kaurenoic acid.

knockout mutant lines for *PpKO* by gene targeting according to the procedure reported previously.[9] There is a single copy of the KO gene, *PpKO* (*CYP701B1*), in the *P. patens* genome database.[3] To generate the Δ*cyp701b1* (*Ppko*-disruption) mutant, the exons of CYP701B1 were amplified and then inserted into the pTN182 vector containing the G418-resistance cassette (*nptII*, encoding neomycin phosphotransferase II) (**Fig. 2A**).[10-12] The transformants were selected on G418-containing medium. We obtained 2 mutant lines of *CYP701B1* (*Ppcyp701b1*). The insertion of the resistance cassette into the correct region in each line was confirmed by PCR (**Fig. 2B and C**). These two *Ppcyp701b1* mutants did not show any obvious differences in colony size and gametophore growth, compared with wild type. Under red-light irradiation, the caulonemal differentiation of *Ppcyp701b1* was suppressed as the same response as *Ppcps/ks* disruption mutant (**Fig. 3**). Protonemal colonies of *Ppcyp701b1* were inoculated into BCD-ATG medium containing 1 μM *ent*-kaurene or *ent*-kaurenoic acid under red-light. The suppression of caulonemal differentiation was recovered by the application of only *ent*-kaurenoic acid but not by the *ent*-kaurene (**Fig. 3**). Moreover, phenotypic analysis of *Ppcyp701b1* mutants revealed that neither *Ppcyp701b1* nor *Ppcps/ks* showed the avoidance response of protonemal growth to unilateral blue-light irradiation (**Fig. 4B and C**).[7] Protonemal colonies of *Ppcyp701b1* were inoculated into BCD-ATG medium containing 1 μM *ent*-kaurene or *ent*-kaurenoic acid and were incubated under blue-light conditions for 7 d As shown in **Fig. 4D**, protonemal growth was unchanged after *ent*-kaurene application. In contrast, application of *ent*-kaurenoic acid rescued the blue-light avoidance phenotype, and the protonemal growth occurred on the opposite side to the light source, just like in wild type (**Fig. 4A and E**). These results indicated that *ent*-kaurenoic acid is synthesized by PpKO (CYP701B1) in *P. patens,* and that PpKO is involved in the synthesis of the diterpene-derived regulator(s) involved in the blue-light avoidance response.

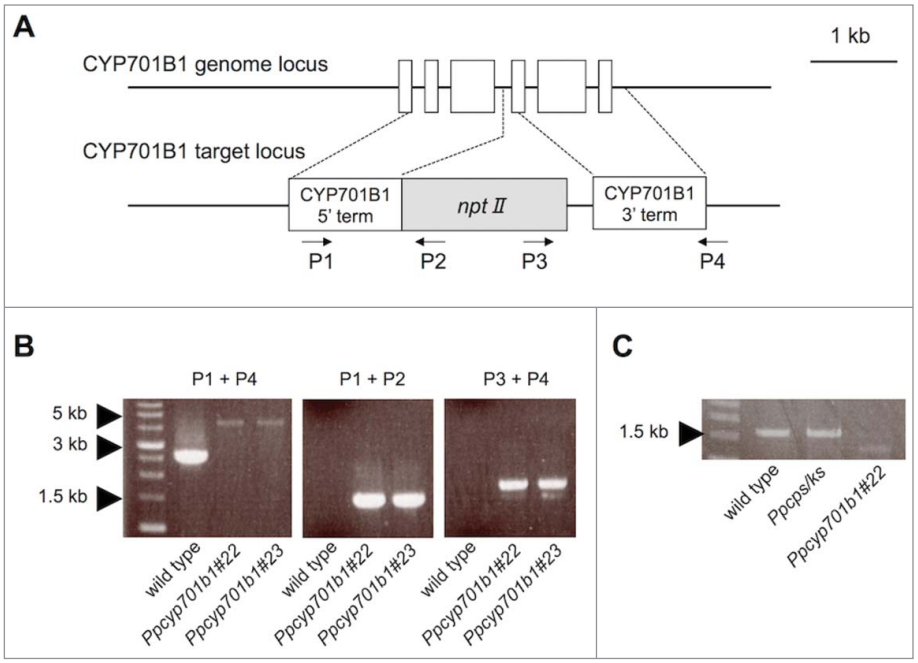

Figure 2. Generation of *CYP701B1* mutant. (**A**), Structure of genomic locus of *CYP701B1* and disruption constructs. White boxes represent exons; the line between the boxes represents an intron. Arrows show the primers used for PCR in (**B**). (**B**), Insertion of *nptII* in the correct region of *CYP701B1*. (**C**), The open reading frame of *CYP701B1* was amplified from cDNA by PCR.

Our research group has shown that diterpenoid compounds synthesized from *ent*-kaurenoic acid are involved in protonemal differentiation and the avoidance response to blue light (**Fig 1**). There is a significant difference between vascular and nonvascular plants, because no CYP88A P450 homolog was found in *P. patens*. CYP88A catalyzes the oxidation reaction of *ent*-kaurenoic acid to form GA_{12}. We hypothesize that the moss *P. patens* may convert *ent*-kaurenoic acid to a novel diterpenoid, but not GA_{12}, by a moss-specific member of the P450 family. Our current aim is to identify *ent*-kaurenoic acid oxidase and the metabolites converted from *ent*-kaurenoic acid in *P. patens*.

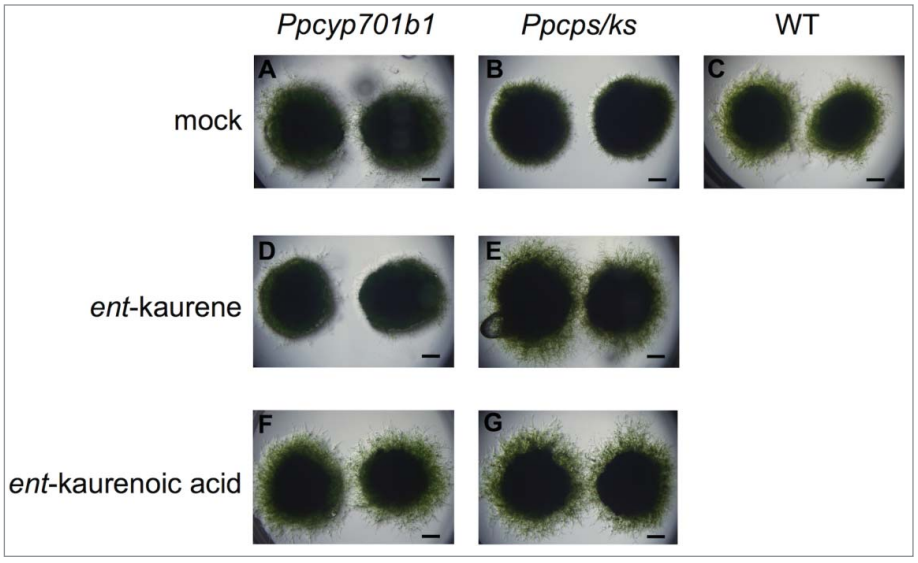

Figure 3. Photomorphogenesis of protonema in the wild type and mutant of *P. patens* under red-light irradiation. (**A**), *Ppcyp701b1* mutant. (**B**), *Ppcps/ks* mutant. (**C**), Wild type. (**D**), *Ppcyp701b1* mutant with application of 1 μM *ent*-kaurene. (**E**), *Ppcps/ks* mutant with application of 1 μM *ent*-kaurene. (**F**), *Ppcyp701b1* mutant with application of 1 μM *ent*-kaurenoic acid. (**G**), *Ppcps/ks* mutant with application of 1 μM *ent*-kaurenoic acid. Bars = 1 mm.

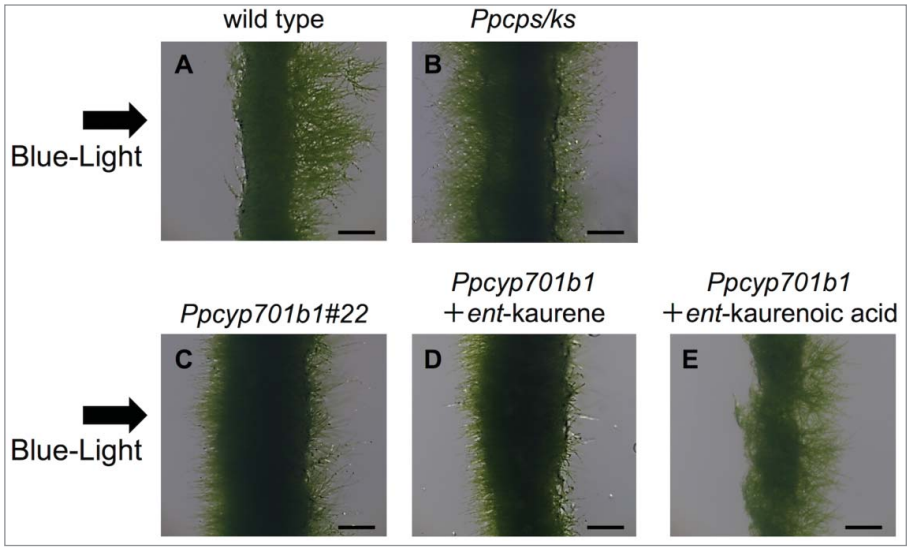

Figure 4. Photomorphogenesis of protonema in the wild type and mutant of *P. patens* under unilateral blue-light irradiation. (**A**), Wild type. (**B**), *Ppcps/ks* mutant. (**C**), *Ppcyp701b1* mutant. (**D**), *Ppcyp701b1* mutant with application of 1 μM *ent*-kaurene. (**E**), *Ppcyp701b1* mutant with application of 1 μM *ent*-kaurenoic acid. Bars = 5 mm.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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