The phytohormone auxin regulates many aspects of growth and development in land plants, but the origin and evolution of auxin signaling and response mechanisms remain largely unknown. Indeed, it remains to be investigated whether auxin-related pathways diverged before the emergence of land plants. To address this knowledge deficit, we analyzed auxin responses in the charophyte alga *Klebsormidium nitens* NIES-2285, whose ancestor diverged from a green algal ancestor during the evolution of land plants. This strain is the same as *Klebsormidium flaccidum* NIES-2285, for which the draft genome was sequenced in 2014, and was taxonomically reclassified as *K. nitens*. This genome sequence revealed genes involved in auxin responses. Furthermore, the auxin indole-3-acetic acid (IAA) was detected in cultures of *K. nitens*, but *K. nitens* lacks the central regulators of the canonical auxin-signaling pathway found in land plants. Exogenous IAA inhibited cell division and cell elongation in *K. nitens*. Inhibitors of auxin biosynthesis and of polar auxin transport also inhibited cell division and elongation. Moreover, exogenous IAA rapidly induced expression of a LATERNAL ORGAN BOUNDARIES-DOMAIN transcription factor. These results suggest that *K. nitens* has acquired the part of the auxin system that regulates transcription and cell growth without the requirement for the central players that govern auxin signaling in land plants.

The physiological and morphological effects of auxins have been well studied in land plants. Auxins play crucial roles in regulating many aspects of plant growth and development. For example, auxins are required for root hair initiation and elongation (Takahashi, 2013), formation of all primordia (Gallavotti, 2013), and developmental decisions to initiate various plant tissues such as cotyledons, roots, flowers, and leaves (Vanneste and Friml, 2009). Moreover, auxins regulate cell division and cell expansion during plant growth and development (Perrot-Rechenmann, 2010). Changes in auxin accumulation patterns mediate regulation of tropic growth in response to light and gravity (Adamowski and Friml, 2015). These auxin-dependent plant developmental processes are regulated by the combination of auxin metabolism, transport, and perception/signaling (Sauer et al., 2013).

Recent progress in genome analysis and molecular genetics of model bryophytes has uncovered auxin functions in bryophytes. The genome analysis of the moss *Physcomitrella patens* revealed the presence of principal gene families involved in auxin homeostasis and signaling (Rensing et al., 2008). In *P. patens*, auxin induces rhizoid development (Sakakibara et al., 2003). The auxin-signaling pathway is mediated by TRANSPORT INHIBITOR RESPONSE 1/AUXIN SIGNALING F-BOX (TIR1/AFB), and Auxin/INDOLE-3-ACETIC ACID (Aux/IAA) contributes to the chloronema-to-caulonema transition and normal rhizoid development (Prigge et al., 2010). Moreover, analysis of mutants for the auxin-efflux carrier PIN-FORMED (PIN) revealed that PINs in *P. patens* contribute to the regulation of auxin distribution in the gametophore (Bennett et al., 2014) and to fertility and development of sporophytes (Fujita et al., 2008; Bennett et al., 2014). PIN-mediated auxin transport regulates the chloronema-to-caulonema transition and gametophore leaf development (Viana et al., 2014). In the liverwort *Marchantia polymorpha*, no homolog of AUXIN BINDING PROTEIN (ABP1) was found in the genome.
have been reported in some charophyte algae (Cooke et al., 2015). Consequently, auxin-related genes show very few differences between land-plant lineages (Finet and Jaillais, 2012), suggesting that the last common ancestor of land plants had already acquired the core auxin machinery of land plants. However, the origin of the auxin system of land plants remains unclear.

Auxin has been detected in many algae (Niemann and Dörfling, 1980; Cooke et al., 2002; Tarakhovskaya et al., 2007; Stirk et al., 2013). Analyses of various algal genomes indicates that some algae have a subset of auxin-biosynthesis genes (De Smet et al., 2011; Le Bail et al., 2010; Finet and Jaillais, 2012), but these genomes do not harbor genes for the components of the central auxin-signaling pathway mediated by TIR1-Aux/IAA-ARF in land plants (Rensing et al., 2008; Riaño-Pachón et al., 2008; Lau et al., 2009). Furthermore, the effects of auxin have been reported in some algae. In the red alga Grateloupia dichotoma, for example, auxin induces the elongation of cut segments and inhibition of the formation of lateral branches (Yokoya and Handro, 1996). In the green alga Chlorella pyrenoidosa, auxin is involved in cell division (Vance, 1987) and promotes enlargement of Chlorella vulgaris cells (Yin, 1937). In the brown algae, the establishment of polarity in developing zygotes is impaired by auxin and auxin transport inhibitors in Fucus distichus (Basu et al., 2002; Sun et al., 2004) and by an auxin transport inhibitor in Fucus vesiculosus (Polevoï et al., 2003). In addition, in the brown alga Ectocarpus siliculosus, candidate genes involved in auxin metabolism and signaling have been identified, and auxin has been shown to modify the branching pattern via relaying cell-cell positional information in the sporophyte phase (Le Bail et al., 2010). Red and green algae, which are primary symbiotic algae, diverged long ago from these ancestors; moreover, brown algae are secondary symbiotic algae for which the genetic systems differ greatly from those of primary symbiotic algae. Nevertheless, it is curious that auxin may contribute to growth regulation of some algae. Although more detailed information at the molecular level may be needed to fully understand auxin signaling in algae, the relative lack of genetic information for algal auxin responses makes it difficult to understand not only the responses to auxins in algal cells but also the origin and evolution of auxin signaling in photosynthetic organisms.

The common ancestor of the land plants is believed to be closely related to charophyte algae (Lewis and McCourt, 2004; Leliaert et al., 2012). In fact, auxin has been detected in some charophyte algae (Cooke et al., 2002; Hori et al., 2014), and several auxin responses have been reported. In Microcystis thomasiiana, cell division is induced in the presence of auxin (Wood and Berliner, 1979). Depolymerization of microtubules with auxin was observed in Chara globularis (Jin et al., 2008).

Furthermore, PIN-family genes have been identified in the charophyte algae Spirogyra pratensis (De Smet et al., 2011), Klebsormidium flaccidum UTEX321 (Viaene et al., 2013; see below) and Klebsormidium nitens NIES-2285 (Hori et al., 2014; see below) by transcriptome and genome analyses, but their functions in auxin transport remain unclear. The existence of polar auxin transport is supported in several Chara species (Dibb-Fuller and Morris, 1992: Klambt et al., 1992; Boot et al., 2012; Zhang and van Duijn, 2014; Zabka et al., 2016). Therefore, unraveling the auxin system in charophyta—the polyphyletic group that diverged during the evolution of land plants from a green algae ancestor—will be helpful for clarifying the origin and evolution of auxin function and signaling.

We previously reported the draft genome sequence of K. nitens NIES-2285, which consists of simple, nonbranching, filamentous cells. K. nitens NIES-2285 is the strain formerly identified as K. flaccidum, the draft genome for which was reported by Hori et al. (2014). This strain was taxonomically reclassified as K. nitens. Moreover, it was also originally the same as K. flaccidum UTEX321. We previously identified gene homologs for several auxin-biosynthesis and auxin signaling-related factors, TRYPTOPHAN AMINOTRANSFERASE OF ARABIDOPSIS (TAA), YUCCA (YUC) flavin monooxygenase-like proteins, PIN, AUXIN RESISTANT 1/LIKE AUX1, and ABP1, in K. nitens; furthermore, the auxin IAA has been detected in K. nitens (Hori et al., 2014). On the other hand, the draft genome sequence suggested that K. nitens does not have the TIR1-Aux/IAA-ARF-mediated auxin-signaling pathway. Therefore, we investigated the effects of exogenous auxin and auxin inhibitors in K. nitens.

RESULTS

Exogenous Auxin and Auxin Transport Inhibitor Inhibit Growth of K. nitens

To investigate the effect of auxin on growth, K. nitens was cultured in the presence of IAA (0.1–100 μM) in liquid medium with aeration. An increase in exogenous IAA inhibited K. nitens growth (Fig. 1). The intracellular IAA concentration in the absence or presence of 100 μM exogenous IAA was quantified with liquid chromatography-tandem mass spectrometry (Supplemental Fig. S1). The concentration of endogenous IAA was normally several dozen pmol g⁻¹ dry weight. After 1 h of treatment with 100 μM IAA, the intracellular IAA level increased to >1000-fold (nmol order). This IAA level then decreased to approximately one-tenth after 24 h but still remained high at the 72 h time point. The IAA concentration-dependent inhibition of growth was probably affected by the stability of IAA in the medium. This result suggested that treatment of K. nitens with exogenous IAA (100 μM) may be necessary for long-term observation of an IAA effect. Therefore, we treated the cells with 100 μM IAA in subsequent experiments.
With respect to *K. nitens* growth, we also examined the effects of another natural auxin, indole-3-buthylic acid (IBA), as well as the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthalenacetic acid (NAA), the polar auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA), and N-1-naphthylphthalamic acid (NPA). At 100 μM, each of yucasin, PPBo, and BBo inhibited *K. nitens* growth (Fig. 2; Supplemental Fig. S2). BBo inhibited growth even at 10 μM and its inhibitory effect increased with its concentration (Supplemental Fig. S3). These results suggested that inhibition of YUCCA function inhibits cell growth.

**Cell Division of *K. nitens* in the Presence of IAA**

We established a method to observe cell division of *K. nitens* by transiently staining the cell wall with the fluorescent stain calcofluor white. After removal of the stain, *K. nitens* was cultured on solid medium. We

Figure 1. Growth of *K. nitens* in the presence of IAA. *K. nitens* was cultured in the presence of 0.1 to 100 μM IAA. A, Cell cultures were photographed at 0, 1, 3, and 7 d. B, Growth curves for *K. nitens* for each concentration of IAA. Error bars represent so of values for three replicates.

Figure 2. Growth of *K. nitens* in the presence of several auxins and auxin transport inhibitor. *K. nitens* was cultured in the presence of 100 μM IAA, IBA, 2,4-D, NAA, or TIBA. A, Cell cultures were photographed at 0, 1, 3, and 7 d. B, Growth curves for *K. nitens* for each compound. Error bars represent so of values for three replicates.
confirmed that the staining did not affect K. nitens growth (Supplemental Fig. S4). Although the cell wall on day 0 showed strong fluorescence, the fluorescence of de novo synthesized cell wall after staining was very weak. Thus, cell division over time (1–3 d) could be monitored by fluorescence microscopy (Fig. 4; Supplemental Fig. S5) and the effect of 100 μM IAA evaluated (Fig. 5A). By day 3, the untreated control cells had typically divided two times, and the sites of cell division were approximately equally distributed in the filaments of the alga. This suggested that K. nitens does not have specific zones of active cell division, i.e. such as occurs in the meristem of land plants (Fig. 5A; Supplemental Fig. S5A). In the presence of 100 μM IAA, however, almost all the cells did not divide, although the cells were elongated (Fig. 5, A and B; Supplemental Fig. S6). These results indicated that exogenous IAA inhibits cell division of K. nitens.

Cell Elongation of K. nitens in the Presence of IAA

Although IAA clearly inhibited cell division of K. nitens, under the fluorescence microscope, the cells cultured for 5 d in the presence of 100 μM IAA appeared to be longer than control cells (Fig. 6A). The distribution of cell length did not differ significantly between days 0 and 5 in the absence of exogenous IAA. However, the cell length at 5 d in the presence of 100 μM IAA was ~2 times greater than that at day 0 (Fig. 6B; Supplemental Fig. S7). There are two possibilities for the excessive elongation of the cells in our experiment: (1) IAA promoted cell elongation in K. nitens, or (2) the cells were elongated owing to the inhibition of cell division. Thus, we measured the length of the same undivided cell on day 0 and then again on day 1 post-IAA treatment, and this “elongation ratio” served as a measure of whether 100 μM IAA promoted cell elongation. Comparison of the elongation ratios revealed that exogenous IAA actually suppressed cell elongation (Fig. 6C; Supplemental Fig. S8). Accordingly, cell length in the presence of IAA gradually increased in cells in which cell division was inhibited.

Figure 3. Growth of K. nitens in the presence of yucasin, PPBo, and BBo. K. nitens was cultured in the presence of 100 μM yucasin, PPBo, or BBo. A, Cell cultures were photographed at 0, 1, 3, and 7 d. B, Growth curves for K. nitens for each compound. Error bars represent SD of values for three replicates.

Figure 4. Fluorescence micrographs of K. nitens transiently stained with calcofluor white. A, K. nitens was transiently stained with calcofluor white. B to D, The stained K. nitens cells were cultured for 1, 2, or 3 d on solid medium. Red and blue fluorescence indicate chloroplasts (chlorophyll autofluorescence) and cell wall (calcofluor white), respectively. White arrows indicate the cell wall for both sides of the same cell. Scale bars = 5 μm.
TIBA Slighty Inhibits Cell Division and Suppresses Cell Elongation

Similarly to the effect of IAA, TIBA also suppressed *K. nitens* growth (Fig. 2). It has been demonstrated that TIBA inhibits auxin efflux through inhibition of subcellular vesicle trafficking processes, including translocation of PIN by stabilizing actin (Michniewicz et al., 2007; Dhonukshe et al., 2008). It has also been reported that TIBA inhibits the elongation of Arabidopsis root cells (Rahman et al., 2007) and of *Nicotiana tabacum* cells (Vissenberg et al., 2001). Among basal land plants, e.g. the moss *Funaria hygrometrica*, TIBA inhibits IAA efflux from protonema and rhizoids (Rose et al., 1983; Rose and Bopp, 1983) and increases IAA accumulation in protoplasts (Geier et al., 1990). Therefore, we also measured cell division and cell elongation in the presence of 100 μM TIBA. The majority of *K. nitens* cells divided, as expected, two times within 3 d (Fig. 7A). Cell division was delayed, however, in the presence of 100 μM TIBA. Furthermore, the elongation ratio was slightly reduced in the presence of TIBA (Fig. 7B; Supplemental Fig. S9), whereas NPA had no effect on cell division or elongation (Supplemental Fig. S10). These effects of TIBA, although milder, were similar to those of IAA. It has been inferred that the mechanism of action differs between TIBA and NPA (Michniewicz et al., 2007; Dhonukshe et al., 2008). As such, the differential effects of these compounds on *K. nitens* growth may reflect differences in their intracellular targets. It is expected that polar auxin transport is involved in regulating normal cell division and elongation of *K. nitens*. Hence, an optimal level and specific distribution of auxin may be required for appropriate growth of *K. nitens*. However, the possibility cannot be excluded that TIBA affects cellular processes other than auxin transport that cause inhibition of cell division and elongation (Petrásek et al., 2003).

BBo Suppresses Cell Elongation

BBo inhibited the growth of *K. nitens* similarly to IAA and TIBA (Fig. 3; Supplemental Fig. S3). Therefore, we also investigated the effect of BBo on cell division and elongation. Cell division was not strongly inhibited at 30 μM BBo (Fig. 8A). The distribution of cell length after 5 d in the presence of BBo was rarely different from the control (Fig. 8B; Supplemental Fig. S11). As with IAA treatment, however, the elongation ratio was clearly suppressed (Fig. 8C; Supplemental Fig. S12). Therefore, it was obvious that inhibition of YUCCA by 30 μM BBo particularly caused suppression of cell elongation. These results suggested that auxin biosynthesis via YUCCA may contribute to cell elongation in *K. nitens*.
Transcriptome Analysis Reveals Auxin-Responsive Genes

The physiological effects of auxin on cell division and elongation suggested the existence of auxin signaling in *K. nitens*. Therefore, we assessed auxin-mediated regulation of gene expression by analyzing the *K. nitens* transcriptome. *K. nitens* was cultured for 10 h, 3 d, and 7 d in the presence or absence of 100 μM IAA. Subsequent analysis revealed 576 differentially expressed genes (DEGs) among all combinations of these six conditions. For comparison, between the presence or absence of IAA, 84, 170, and 106 DEGs were identified at 10 h, 3 d, and 7 d, respectively (Fig. 9A). The 576 DEGs were clustered into 16 groups by quality threshold clustering (Supplemental Table S1), which facilitated the identification of consistently up- or down-regulated genes following exogenous IAA application (Fig. 9B). Subsequently, we investigated the initial expression changes of some genes in the upregulated Cluster11 and downregulated Cluster8 to search for early-response genes; the real-time PCR analysis identified three up-regulated genes and one down-regulated gene (Fig. 10A; Supplemental Fig. S13). Notably, one of three upregulated genes was a LATERAL ORGAN BOUNDARIES-DOMAIN (LBD) transcription factor, which responded to IAA as early as 1 h of treatment (Fig. 10A). In Arabidopsis, some LBD transcription factors are upregulated via auxin signaling during the formation of lateral organs (Okushima et al., 2007; Lee et al., 2009; Berckmans et al., 2011; Goh et al., 2012; Lee et al., 2013; Lee and Kim, 2013). Therefore, we investigated the expression of the *K. nitens* LBD gene (*KnLBD1*) in more detail by real-time PCR. *KnLBD1* expression was upregulated in an IAA concentration-dependent manner (Fig. 10B). Moreover, *KnLBD1* responded to IAA in the presence of the translation inhibitor cycloheximide (Fig. 10C), suggesting direct transcriptional regulation by auxin. Although *K. nitens* does not have clear homologs of the TIR1-Aux/IAA-ARF auxin-signaling pathway components of land plants (Hori et al., 2014), *KnLBD1* was induced early by exogenous IAA via some constitutively expressed signal transduction factor(s). Induction of *KnLBD1* did not seem to be an indirect effect of the inhibition of cell division and elongation, as *KnLBD1* did not respond to TIBA (Fig. 10D).
DISCUSSION AND CONCLUSION

Our results show that exogenous IAA inhibits cell division and cell elongation and regulates gene expression in *K. nitens*, suggesting that endogenous auxin likely also regulates cell division/elongation. Concentration-dependent growth inhibition by IAA has been reported in the auxin response of bryophytes. In the moss *P. patens*, for example, growth of the gametophore is inhibited in the presence of auxin, and increasing the auxin concentration elicits more severe phenotypes (Ashton et al., 1979; Jang and Dolan, 2011; Bennett et al., 2014). In the liverwort *M. polymorpha*, exogenous auxin promoted abnormal growth phenotypes such as inhibition of thallus growth and rhizoid formation on the dorsal surface and production of many rhizoids (Tarén, 1958; Kaul et al., 1962; Maravolo and Voth, 1966; Otto and Halbgsuth, 1976; Maravolo, 1980; Ishizaki et al., 2012). Hence, the inhibition of cell growth in the presence of exogenous IAA is conserved between *K. nitens* and bryophytes. However, cell differentiation has not been observed in *Klebsormidium* except for zoosporogenesis (Marchant et al., 1973; Lokhorst, 1991; Rindi et al., 2008; Skaloud and Rindi, 2013). Indeed, all cells in *K. nitens* filaments appeared to divide equally (Fig. 5; Supplemental Fig. S5), and there was no local area in the filamentous cells of active cell division. These observations suggest that auxin regulates cell division in *K. nitens* but does not play a role in cellular differentiation. Unlike land plants including bryophytes, the auxin signaling system composed of TIR1-Aux/IAA-ARF has not been found in *K. nitens*. These components must have emerged after the divergence of the ancestor of *K. nitens* and probably allowed for more complex auxin signaling in land plants with multiple cell types and differentiated cells.

The transcriptome analysis of *K. nitens* revealed that IAA treatment rapidly induced *KnLBD1* expression (Fig. 10A). Although *K. nitens* has five *KnLBD* genes (group I type) (Supplemental Fig. S14), only *KnLBD1* responded to IAA. The accumulation of *KnLBD1*...
mRNA was dependent on IAA concentration but independent of translation (Fig. 10, B and C). In Arabidopsis, some LBD genes respond to auxin via Aux/IAA-ARF and regulate lateral root development (Okushima et al., 2007; Lee et al., 2009, 2013; Berckmans et al., 2011; Goh et al., 2012; Lee and Kim, 2013). It is estimated that the LBD genes were acquired from the common ancestor of K. nitens and land plants. Our phylogenetic tree suggests that LBD genes diverged substantially after land colonization of plants.

Figure 9. Transcriptome analysis of K. nitens grown in the presence of IAA. A, MA plots of mRNAs counted in each of the paired control and IAA treatment groups at 10 h, 3 d, and 7 d of cell culture. Red dots represent each DEG at 10 h (left, 84 DEGs), 3 d (middle, 170 DEGs), and 7 d (right, 106 DEGs). B, Heatmaps of the consistently downregulated genes (Cluster8) and upregulated genes (Cluster11) in the presence of IAA. Clusters were abstracted from log2 fold changes (relative to 10 h control) of 576 DEGs by quality threshold clustering.

Figure 10. Changes in expression of KnLBD1 mRNA. Changes in the expression of KnLBD1 mRNA were analyzed by real-time PCR of samples from cells incubated for (A) 1, 3, and 6 h in the absence or presence of 100 μM IAA, (B) 6 h in the presence of 0.01, 0.1, 1, 10, or 100 μM IAA, (C) 6 h in the absence or presence of 100 μM IAA and 10 μg/mL cycloheximide (CHX), and (D) 6 h in the absence or presence of 100 μM IAA, 100 μM TIBA, or 100 μM NPA. All error bars represent SD of values for three replicates. B and D, Different lowercase letters (a, b) denote a statistically significant difference between treatments. B, P < 0.05; D, P < 0.01 (Tukey’s test). A and C, *P < 0.1; **P < 0.01 (Student’s t test).
Auxin Regulates Growth in *K. nitens*

(Supplemental Fig. S14). However, the early stage of divergence was unclear because of low support values. In Arabidopsis, *LBD16* and *LBD29* are directly regulated by the transcription factors *ARF7* and *ARF19* (Okushima et al., 2007). In contrast, *K. nitens* has no *ARF* genes, and therefore other transcription factors probably regulate *KnLBD1*. The identification of this transcription factor(s) and its target sequence will help clarify the evolutionary relationship between auxin response of *K. nitens* and Arabidopsis.

Because *K. nitens* lacks the Aux/IAA-ARF pathway, it remains unclear which signaling mechanism mediates auxin’s effects on *K. nitens* growth and *KnLBD1* transcription. The *K. nitens* genome contains a candidate homolog of the ABP1 auxin receptor. In Arabidopsis, the physiological importance of ABP1 is unclear because *abp1* null mutants show no obvious phenotype (Gao et al., 2015), and previously reported embryonic-lethal *abp1* alleles as well as conditional knockdown lines have only off-target effects (Michalko et al., 2015, 2016). In addition, the liverwort *M. polymorpha* lacks an ABP1 homolog (Kato et al., 2015).

However, mutational analysis of the amino acid residues involved in the formation of the auxin-binding pocket of ABP1 supports a role for ABP1 as an auxin receptor during development (Grones et al., 2015). These auxin-binding residues are conserved in the ABP1-like protein encoded in the *K. nitens* genome (Supplemental Figure S15), indicating that the ABP1 homolog of *K. nitens* may also be able to bind auxin. Thus, elucidation of the auxin-response mechanism of *K. nitens*, which lacks TIR1-Aux/IAA-ARF, may help clarify the function of ABP1.

In addition to mediating auxin signaling, polar auxin transport is involved in the normal growth of land plants. Changes in auxin distribution by auxin transporters contribute to tropism and developmental decisions (Tanaka et al., 2006; Vanneste and Friml, 2009). In *P. patens*, regulation of auxin distribution by PIN auxin efflux carriers plays important roles in the development of protonemata, gametophores, and sporophytes (Fujita et al., 2008; Bennett et al., 2014; Viaene et al., 2014). The *K. nitens* genome also has a putative PIN homolog (Hori et al., 2014). We investigated the effect of the polar auxin transport inhibitors, TIBA and NPA, on *K. nitens* growth. TIBA inhibited cell division and suppressed cell elongation (Fig. 7; Supplemental Fig. S9), whereas NPA had no such effects (Supplemental Fig. S10). Although it is expected that auxin transport mediated by the PIN homolog is involved in controlling endogenous auxin levels and thus regulating cell elongation and division in *K. nitens*, further validation is required.

Furthermore, *K. nitens* has a homolog of YUCCA, which is a component of the main auxin biosynthesis pathway in land plants (Mashiguchi et al., 2011; Hori et al., 2014). We thus tested the effect of BBo, a potent inhibitor of YUCCA, on cell division and elongation in *K. nitens*. BBo suppressed cell elongation more so than cell division (Fig. 8), suggesting that suppression of cell elongation was caused by down-regulation of auxin biosynthesis through inhibition of YUCCA function by BBo. It is possible that the basal level of auxin before BBo treatment was sufficient for cell division. If true, then activation of YUCCA-mediated auxin biosynthesis may be required for normal cell elongation in *K. nitens*, although a higher concentration of IAA (such as that for exogenous IAA in our experiments) may inhibit both cell division and elongation. It is well established that, with respect to plant growth, the optimal concentration of auxin differs depending on organ type and among land plant species. Inhibition of cell elongation of *K. nitens*—whether with IAA biosynthesis inhibitors or with exogenous IAA treatment—suggests that normal cell elongation requires an optimal concentration of auxin.

Our study suggests that, during evolution, *K. nitens* acquired a part of the auxin system that regulates cell division and elongation. Although the auxin system of *K. nitens* may be relatively simple, it probably provided the basis for the evolution of the more complex auxin system that exists in modern plants. It is possible that the acquisition of the primitive auxin-response pathway occurred before the divergence of Klebsormidiophyceae and was one of the important steps that enabled evolution from a simple body plan to a more complex morphology to adapt to harsh terrestrial environments. In future studies, a more comprehensive examination of auxin systems of charophyte algae, including *K. nitens*, will help elucidate the origin and evolution of the plant auxin system.

**MATERIALS AND METHODS**

**Culture Conditions and Chemicals**

The strain *Klebsormidium nitens* NIES-2285 was found to be the same as *Klebsormidium flacccatum* NIES-2285, for which the draft genome was reported by Hori et al. (2014). This strain was taxonomically reclassified as *K. nitens*. *K. nitens* NIES-2285 was subcultured in 50 mL liquid 0.1% Glc + BCDAT medium (Nishiyama et al., 2009) with aeration at 23°C under 10 to 20 μmol photons m⁻² s⁻¹ of light for 2 to 4 weeks. The cells were harvested by centrifugation for 10 min at 130 g (LC-1000 7000402 swinging-bucket rotor, TOMY, Tokyo, Japan). The loose cell pellet was transferred to a 10-mL centrifuge tube, resuspended in fresh medium, and collected by centrifugation for 3 min at 130g. The pellet was similarly washed again. Finally, the pellet was resuspended in an 8-fold packed cell volume of liquid C medium (ichimura, 1971), and 1 mL of cell suspension was inoculated into 50 mL of 0.1% Glc + BCDAT medium. IAA, IBA, 2,4-Cl, NAA, TIBA, and NPA were first dissolved with dimethyl sulfoxide to make 100 μmol/L stock solutions. Yucasin, PPBo, and BBo were first dissolved with dimethyl sulfoxide to make 100 μmol/L stock solutions.

**IAA Quantification**

IAA was extracted and purified as described (Hori et al., 2014) with some modifications. Samples of *K. nitens* cells (20–80 mg, dry weight) were used for extraction, which was carried out overnight. Extracts were subjected to purification via a single passage through an Oasis WAX cartridge column (60 mg, 3 mL, Waters). Liquid chromatography-tandem mass spectrometry analysis was performed as described by Kanno et al. (2016).

**Observation of Cell Division and Measurement of Cell Elongation by Calcofluor White Staining**

*K. nitens* was cultured in liquid C medium with aeration at 23°C under 10 to 20 μmol photons m⁻² s⁻¹ of light for 4 d. A 1-mL aliquot of culture was plated on solid C medium. *K. nitens* cultured for 7 d on solid medium was transferred to liquid C medium and stained for 1 h with calcofluor white (final...
concentration: 10 μg/mL. The cells were pelleted in a mini-centrifuge for ∼10 to 20 s (Force Mini Centrifuge III). The pellet was resuspended with C medium, left to stand for 10 min and then collected by centrifugation. This washing procedure was repeated twice. The cell suspension was plated on solid C medium in the absence or presence of 100 μM IAA, TIBA, NPA, or BBo. The cultures were observed by fluorescence microscopy (ECLIPSE 80i equipped with a V-2A filter, Nikon). To calculate the cell elongation ratio, the length of individual K. nitens cells was measured at day 0 (control) and day 1 posttreatment using ImageJ (Abramoff et al., 2004). For analysis of cell-length distribution, unstained K. nitens was cultured and stained with calcofluor white just before fluorescence imaging.

RNA Extraction
Total RNA was extracted from K. nitens cells grown in liquid 0.1% Glc + BCDAT medium in the absence or presence of 100 μM IAA. Cells were harvested by vacuum filtration through a nitrocellulose transfer membrane (Protran, 0.45 μm pore size, Whatman). The pelleted sample was frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. At least three volumes of RNA extraction buffer (0.8% sodium dodecyl sulfate, 25 mM Tris-HCl, pH 7.6, 25 mM MgCl₂, 25 mM KCl); acid phenol (1:1) was added, and the samples were further ground. The aqueous phase was extracted three times by adding an equal volume of acid phenol:chloroform (1:1). The RNA was precipitated by adding an equal volume of isopropanol, and the precipitate was rinsed with 70% ethanol. After the ethanol was removed, the RNA was treated with DNase I at 37°C for 30 min and extracted twice with acid phenol:chloroform (1:1). The RNA was precipitated by adding 2.5 volumes of 100% ethanol. The RNA was dissolved in RNase-free water and used for real-time PCR. For RNA sequencing, the RNA was precipitated by adding one-third volume of 10 mM lithium chloride, incubating for 1 h at 20°C, and centrifuging at 10,000 g for 10 min. The RNA pellet was washed with a buffer containing 2 mM LiCl and 50 mM EDTA. The dissolved RNA was further purified using the RNeasy Mini or Midi kit (Cat. No. 74104/75142, Qiagen).

RNA Sequencing and Data Analysis
The cDNA library for RNA sequencing was prepared following the protocol of the Illumina TruSeq RNA Sample Preparation kit v2. Sequencing of 76-bp single reads was performed on the Illumina GAIIx platform. The sequencing data were deposited in the DNA Data Bank of Japan Sequence Read Archive (PRJDB4958. GenBank accession numbers for ABP1 proteins are as follows: Arabidopsis (NP_192207), Populus trichocarpa (XP_006386838), Oryza sativa (XP_015620272), O. sativa (ABA99317), Selaginella moellendorffii (XP_002979034), P. patens (XP_001782753), K. nitens (GAQ82792), Chlamydomonas reinhardtii (XP_001692979), Chlorella variabilis (XP_005853097), and C. variabilis (XP_005844798). KGBD1 (k100147_0060) is located in scaffold00147 (GenBank accession number DF270996) within KFL_001470060 locus. KGBD1 (k100147_0060) is located in scaffold00147 (GenBank accession number DF270996) within KFL_001470060 locus. KGBD1 (k100147_0060) is located in scaffold00147 (GenBank accession number DF270996) within KFL_001470060 locus. KGBD1 (k100147_0060) is located in scaffold00147 (GenBank accession number DF270996) within KFL_001470060 locus. KGBD1 (k100147_0060) is located in scaffold00147 (GenBank accession number DF270996) within KFL_001470060 locus.

Supplemental Data
The following supplemental materials are available.

Supplemental Figure S1. Quantification of the IAA concentrations of K. nitens in the absence or presence of exogenous 100 μM IAA.

Supplemental Figure S2. Growth of K. nitens in the presence of NPA.

Supplemental Figure S3. Growth of K. nitens in the presence of BBo.

Supplemental Figure S4. Growth of K. nitens in the presence of calcofluor white.

Supplemental Figure S5. Measurement of the frequency of cell division in K. nitens by calcofluor white staining.

Supplemental Figure S6. Measurement of the frequency of cell division in K. nitens by calcofluor white staining in the presence of IAA.

Supplemental Figure S7. Distribution of cell length at 5 d in the absence or presence of 100 μM IAA.

Supplemental Figure S8. Cell elongation ratio as calculated in the absence or presence of 100 μM IAA.

Supplemental Figure S9. Cell elongation ratio as calculated in the absence or presence of 100 μM TIBA.

Supplemental Figure S10. Cell division and cell elongation of K. nitens grown in the presence of NPA.

Supplemental Figure S11. Distribution of cell length at 5 d in the absence or presence of 30 μM BBo.

Supplemental Figure S12. Cell elongation ratio as calculated in the absence or presence of 30 μM BBo.

Supplemental Figure S13. The initial expression changes of certain mRNAs in the downregulated Cluster8 and upregulated Cluster11.

Supplemental Figure S14. Phylogenetic tree analysis of LBD genes.

Supplemental Figure S15. Multiple sequence alignment of ABP1 homologs.

Supplemental References. A list of supplemental references.

Supplemental Table S1. The 576 differentially expressed genes between all combinations of six conditions in the presence or absence of IAA.

Supplemental Table S2. Datasets for phylogenetic analysis.

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