Expression of the AtGH3a Gene, an Arabidopsis Homologue of the Soybean GH3 Gene, is Regulated by Phytochrome B

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Light is one of the most crucial environmental cues for plants. Phytochrome, one of the major photoreceptors of plants, regulates expression of many genes. We screened for Arabidopsis promoter trap lines that exhibited light-dependent reporter gene expression. Molecular analysis of one such line revealed that a reporter gene was inserted near an Arabidopsis homologue of the soybean GH3 gene, AtGH3a. We performed RNA gel blot analysis to further characterize the response of the AtGH3a gene to light. In response to the end-of-day far-red light treatment, the expression increased substantially. Analysis of the phyB-deficient mutant indicated that this light response is under the control of phytochrome B. The expression was also induced by exogenous auxin. Furthermore, the light response was substantially reduced in the auxin-related aux2 mutant. Taken together, it is suggested that phytochrome B regulates the expression of genes by altering the levels of auxin.

Key words: Auxin — End-of-day far-red light — Gene expression — Phytochrome B — Promoter trap lines — Shade avoidance.

Abbreviations: 2,4-D; 2,4-dichlorophenoxyacetic acid; FR, far-red light; GUS, β-glucuronidase; 4-MU, 4-methylumbelliferone; phyA, phytochrome A; phyB, phytochrome B; R, red light; T-DNA, transfer DNA; X-Gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronide.

Introduction

Light is one of the most crucial environmental cues for plants. Plants utilize light not only as an energy source but also as a critical signal to control physiological as well as developmental processes. Higher plants have evolved complex photoreceptive systems consisting of distinct families of photoreceptors to perceive the light signals (Kendrick and Kronenberg 1994). The phytochrome family is one of the best-characterized families of plant photoreceptors. Phytochrome is reversibly photoconverted by red light (R) and far-red light (FR) between two distinct forms, Pfr and Pr (Quail et al. 1995). The Arabidopsis phytochrome family includes five members, phytochrome A to E, among which phytochrome A (phyA) and phytochrome B (phyB) are the best characterized. Both phyA and phyB accumulate in the nucleus (Yamaguchi et al. 1999, Hisada et al. 2000). A transcription factor PIF3, which binds with a consensus DNA sequence found upstream of the light-regulated genes (Martínez-Garcia et al. 2000), physically interacts with phytochrome (Ni et al. 1998).

Phytochrome is thought to cause photomorphogenesis through the regulation of gene expression (Kendrick and Kronenberg 1994). Expression of many genes are under the control of phytochrome (Terzaghi and Cashmore 1995). The CAB gene, which is up-regulated by light, is a good example. Promoter elements needed for regulation by light and/or circadian oscillation have been identified in this gene (Anderson and Kay 1995). Furthermore, transcription factors involved in the phytochrome regulation of the CAB gene expression have been identified (Wang et al. 1997). By contrast, information on the genes down-regulated by phytochrome is limited. Only two genes other than PHYA have been studied extensively. Expression of the ATHB2 gene is up-regulated by a brief FR irradiation, suggesting that the phyB Pfr represses the expression of this gene (Carabelli et al. 1996). In pea, one of the GTPase family genes, prad2, is repressed by phytochrome (Nagano et al. 1995). A promoter element conferring dark-induction and phytochrome-directed repression in the prad2 gene has been identified recently (Inaba et al. 2000).

Phytohormones such as auxin are believed to be involved in various photomorphogenic processes (Kendrick and Kronenberg 1994). Auxin has been shown to be involved in phototropism (Briggs and Huala 1999, Harper et al. 2000). Physiological studies suggest the importance of light-directed production of an auxin gradient in stems in this response. Complex models on the light-regulated changes in axial and/or lateral carri- ers of auxin has been proposed to explain this phenomenon (reviewed in Pickard 1985). Inhibition of stem elongation by R was also suspected to be the result of light-directed changes in the levels of auxin. R has been shown to decrease the level of auxin in etiolated maize mesocotyls, pea internodes and Phaseolus seedlings (Fletcher and Zalik 1964).

Intensive work has been done to identify genes whose expression is regulated by auxin. Several genes have been found to be activated by application of exogenous auxin. These genes are classified into several classes (Sitbon and Perrot-Rechenmann 1997). The IAA/Aux family was initially isolated in pea (Sitbon and Perrot-Rechenmann 1997). Recent analysis of auxin-resistant mutants has indicated that the ectopic activa-
tion of the IAA/Aux genes disturbs the auxin-responsiveness in plants (Nagpal et al. 2000). Two other auxin-responsive gene families, SAUR and GH3, have been analyzed for their expression patterns and stability (Hagen and Guilfoyle 1985, Newman et al. 1993). However, their biological roles in auxin responses remain less clear.

Recent genetic studies provide evidence for the involvement of auxin-related genes in the phytochrome responses. The shy2 mutation, initially characterized as a suppressor mutation of the long-hypocotyl phenotype of a phytochrome-deficient mutant, is caused by a dominant mutation of one of the IAA/Aux genes (Tian and Reed 1999). The mutant cop1 is a constitutive photomorphogenic mutant of Arabidopsis. A suppressor mutant of the temperature-sensitive cop1-1, the fin219 mutant, is FR-insensitive (Hsieh et al. 2000). This mutation is caused by a loss-of-function mutation in one of the auxin-responsive genes, GH3. Together with another study on an activation-tagged line (Nakazawa et al. 2001), the GH3 family has been proposed to be involved in the phytochrome signal transduction.

These observations prompted us to examine whether expression of the auxin-responsive genes is controlled by phytochrome. Though IAA/Aux family genes in Arabidopsis have been examined for their expression under different light conditions, the results varied with the gene (Abel et al. 1995). Detailed analyses have not been done to identify the photoreceptors controlling these genes. Although two GH3-like genes are proposed to be involved in the phytochrome signal transduction (see above) (Hsieh et al. 2000, Nakazawa et al. 2001), it remains unclear whether the expression of these genes is controlled by phytochrome.

In the present study, we screened for promoter trap lines that exhibit phytochrome-dependent repression of reporter gene expression. Molecular analysis of one such line suggested that the reporter gene was inserted near an Arabidopsis homologue of the auxin-responsive GH3 gene, AtGH3a. Responses of this gene to light and auxin in the wild type as well as an auxin-related mutant of Arabidopsis were examined. The results suggested that phyB regulates the expression of AtGH3a by changing the levels of auxin.

**Results**

*Analysis of a promoter trap line that exhibits phytochrome-dependent GUS expression*

We screened for promoter trap lines that show reporter gene expression specifically in darkness. Seeds of promoter trap lines donated from INRA (Versailles, France) to NASC (Nottingham Arabidopsis Seed Stock Center, Nottingham, U.K.) were sown and grown for 7 d either under continuous white light or in darkness. The seedlings were then subjected to β-glucuronidase (GUS) histochemical staining. We re-isolated the line N35 that showed intense GUS staining in hypocotyls only in darkness from the pool N5335, which consists of 20

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**Fig. 1** Regulation of GUS expression in the N35 line. Seedlings were grown under different light conditions and subjected to the GUS histochemical assay (A–D). (A) a seedling grown under continuous white light for 6 d; (B) a seedling grown in darkness for 6 d; (C) a seedling grown for 5 d under continuous white light was treated with a 10 min FR pulse and then placed in darkness for 18 h; (D) same as in (C) except that the FR treatment was omitted. (E) Time course of the induction of GUS gene activity. Seedlings grown under continuous white light for 5 d were treated with (open circle) or without (closed triangle) a FR pulse and transferred to darkness. Seedlings were harvested and assayed at indicated time points during the dark incubation. Negative controls (open square) were placed under continuous white light. Average of three independent experiments are shown. Bars indicate SDs.
Phytochrome B regulates \textit{AtGH3a} expression in independent lines (Fig. 1A, B).

To test whether phytochrome regulates the GUS expression in the N35 line, we examined the effects of the end-of-day FR treatment. The light-grown seedlings were treated with or without a FR pulse and then transferred into darkness. After 18 h in darkness, seedlings were harvested and RNA was extracted. Negative controls were placed under continuous white light (cW). Each lane contains 5 μg of total RNA.

We determined the insertion point of the reporter gene in the N35 line to identify the tagged gene. Genome fragments flanking the inserted transfer DNA (T-DNA) were cloned from the N35 genomic DNA by the TAIL-PCR (thermal asymmetric interlaced-PCR) and sequenced. All three independent clones matched the same sequence in the Arabidopsis genome database (Sequences from BAC M4I22, The Kazusa Arabidopsis Data Opening Site, KAOS) (Fig. 2A). The reporter gene was inserted between two predicted genes M4I22.70 and M4I22.80 on chromosome 4. The M4I22.70 gene exhibits strong similarity to the soybean auxin-induced \textit{GH3} gene (Hsieh et al. 2000). The M4I22.80 gene has similarity with the \textit{LEDI-3} gene of \textit{Lithospermum erythrorhizon}, which shows homology to a tryptophan repressor binding protein WrbA of \textit{E. coli} (Yazaki et al. 1997). Both genes had several expressed sequence tags, indicating that they are expressed. According to their similarity to known genes, we hereafter refer to the M4I22.70 and M4I22.80 genes as \textit{AtGH3a} and \textit{AtLEDI3a} genes, respectively.

Expression of the \textit{AtGH3a} and \textit{AtLEDI3a} genes in the wild-type plants

We examined whether expression of the \textit{AtGH3a} and \textit{AtLEDI3a} genes respond to the FR treatment in the wild-type background. RNA was extracted from seedlings treated with or without the end-of-day FR and probed with the specific DNA fragments amplified by PCR. As a negative control, RNA was extracted from seedlings placed under continuous white light. As shown in Fig. 2B, almost no \textit{AtGH3a} signal was detected under continuous white light. Incubation for 6 h in darkness without the FR treatment barely affected the expression. By
of-day FR treatment (Fig. 3). This observation suggests that the expression of the \textit{AtGH3a} gene is suppressed by the \textit{PrF} form of stable phytochrome. In Arabidopsis, phyB is a major molecular species of the stable phytochromes (Furuya 1993). So, we examined whether phyB is involved in this response with the aid of the phyB mutant. We extracted RNA from 6-day-old seedlings of the phyB mutant and performed the RNA gel blot analysis for the \textit{AtGH3a} expression (Fig. 4A). In the phyB mutant, clear expression of the \textit{AtGH3a} gene was observed regardless of the light conditions (lanes 4–6). Hence, we concluded that the major photoreceptor for this response is phyB.

As described above, exogenous auxin induced expression of the \textit{AtGH3a} gene in the light (Fig. 3C). Hence, auxin is suspected to be involved in the light regulation of the \textit{AtGH3a} gene expression. To test this possibility, we examined the light response in the \textit{axr2} mutant, in which the induction by auxin of the auxin-responsive genes such as \textit{SAUR} and \textit{IAA} is substantially reduced (Timpte et al. 1992). We treated the seedlings of the \textit{axr2} mutant with the end-of-day FR. RNA was extracted from the seedlings and subjected to the RNA gel blot analysis (Fig. 4B). As expected, the FR induction of \textit{AtGH3a} expression was substantially reduced in the \textit{axr2} mutant.

The T-DNA insertion can alter the expression patterns of several neighboring genes (Amedeo et al. 2000). In some cases, unusual fusion transcripts are detected (Wei et al. 1997). So, we examined the expression of the \textit{AtGH3a} gene in the homozygous seedlings of the N35 promoter trap line (Fig. 5). As shown in the figure, the response to the end-of-day FR treatment was abolished in the N35 lines (lanes 1–4), indicating that \textit{cis}-elements required for the normal \textit{AtGH3a} expression resides around the T-DNA insertion point. We also tested the response of the \textit{AtGH3a} gene to exogenous auxin in the N35 line (Fig. 5). The response was substantially reduced in the N35 line, although a weak response was observed.

\textit{Morphological responses of the N35 line to light}

The morphology of the homozygous N35 seedlings was indistinguishable from that of the wild-type seedlings under
Phytochrome B regulates *AtGH3a* expression

Fig. 6 Photomorphogenic responses of the N35 seedlings. (A) Hypocotyl responses to the end-of-day FR treatment in N35 (left) and wild-type (ecotype Wassilewskija) (right) seedlings. Seedlings were placed under dark/light cycles for 3 d with (hatched column) or without (closed column) the end-of-day FR treatment and the hypocotyl lengths were determined. As negative controls, seedlings were placed under continuous white light for 2 d were placed under light-dark cycles with or without the end-of-day FR treatments for 3 d. Hypocotyl lengths and cotyledon areas were determined for those seedlings. The responses of the N35 seedlings to the end-of-day FR treatment in N35 (left) and wild-type (right) seedlings. Light treatments and symbols are as described for panel A.

Discussion

The N35 promoter trap line

We re-isolated a promoter trap line that expressed the GUS reporter gene in hypocotyls in response to the end-of-day FR treatment (Fig. 1). The results of molecular analysis of this line, N35, suggested that the T-DNA was inserted between two putative genes, *AtGH3a* and *AtLED13a*, on chromosome 4 (Fig. 2A). The RNA gel blot analysis indicated that the *AtGH3a* gene but not the *AtLED13a* gene responds to the light treatment (Fig. 2B, C). Since the GUS gene in the T-DNA lacks the promoter (Bouchez et al. 1993), it remains unclear how the GUS gene was expressed in the N35 line. We speculate that the sequence around the insertion point acted as a promoter by chance and an enhancer element situated downstream of the *AtGH3a* gene affected the expression of the GUS gene. A similar mechanism has been proposed for other promoter trap lines (Fobert et al. 1994). The fact that the response of the *AtGH3a* gene to light and auxin was diminished by the T-DNA insertion in the N35 line (Fig. 5) and the fact that the GUS reporter gene expression was induced by auxin in the N35 line (Tanaka, unpublished results) are consistent with this interpretation.

As shown in Fig. 1 and 2, both the GUS expression in the N35 line and the *AtGH3a* expression in the wild type were induced by the end-of-day FR treatment. In this regard, the GUS reporter gene reproduced the expression pattern of the endogenous *AtGH3a* gene. The spatial patterns of expression, however, were somewhat different between these two genes. We detected the *AtGH3a* expression in hypocotyls as well as in cotyledons in the wild-type seedlings (Fig. 3), whereas the GUS expression in the N35 line was confined to the hypocotyls (Fig. 1B, C). This kind of discrepancy has been reported in other promoter trap lines. For example, a T-DNA insertion line that showed the reporter expression specifically in root caps was identified, but the tagged gene, *RCP1*, was expressed in leaves in addition to the roots (Tsugeki and Fedoroff 1999). We speculate that the putative enhancer situated near the insertion point was active only in hypocotyls.

Expression of *AtGH3a* is regulated by phyB

The expression of *AtGH3a* was induced by the end-of-day FR treatment (Fig. 2, 3). This treatment is known to convert the \( P_{FR} \) phytochrome generated during the day to the inactive \( P_{R} \) form. Without such treatments, the light-stable phytochrome remains in the active \( P_{aq} \) form for a longer time in the following dark period. The end-of-day FR treatment has been widely used to mimic the effects of the shaded light (Smith and Whitelam 1997). In response to this treatment, hypocotyls, internodes and petioles are elongated and the areas of leaves are reduced (Devlin et al. 1998). The treatment affects the flowering time in some cases (Smith 1995). Hence, the *AtGH3a* gene might be involved in some of these physiological processes.

In Arabidopsis and other plant species, the responses to the end-of-day FR are mainly mediated by phyB (Reed et al. 1993). We observed that the *AtGH3a* expression in the phyB mutant was high regardless of the light conditions (Fig. 4A). So, we concluded that phyB is a major photoreceptor mediating the response of the *AtGH3a* gene to the end-of-day FR treatment as is the case with the other responses. The *ATHB2* gene is one of few genes that have been shown to be up-regulated by the end-of-day FR treatment (Carabelli et al. 1996). Like the
AtGH3a, the ATHB2 expression shows marked increase after the end-of-day FR treatment in mature Arabidopsis plants.

Involvement of auxin in the light-regulation of AtGH3a expression

The GH3 gene was initially identified in soybean as a gene that exhibits an acute response to exogenous auxin. In soybean, expression of the GH3 gene is induced by the $10^{-7}$ M exogenous auxin. We found that the AtGH3a gene responded to the same concentration of auxin (Fig. 3C). This observation suggests that phyB might regulate the AtGH3a expression by changing the endogenous level of auxin. To test this, we examined the response in an auxin-related mutant, axr2, to the end-of-day FR treatment for the induction of the AtGH3a expression (Fig. 4B). The axr2 mutant, which is resistant to a high concentration of exogenous auxin, is known to be less sensitive to auxin (Nagpal et al. 2000). The induction of auxin-induced genes such as SAUR and IAA is abolished in this mutant (Timppe et al. 1992). As expected, light induction of the AtGH3a gene expression was substantially reduced in the axr2 mutant. Hence, the involvement of auxin in the phyB regulation of the AtGH3a expression was suggested.

The above results suggest that the level of auxin increases in response to the end-of-day FR treatment. This speculation is consistent with the data obtained in other plant species. In corn and oat seedlings, continuous irradiation with R reduces the level of auxin in the coleoptiles (Briggs 1963). The level of auxin in etiolated pea seedlings has been reported to be reduced in response to a R pulse (Behringer and Davies 1992). Furthermore, there is evidence that light affects various aspects of auxin metabolism such as synthesis, degradation, inactivation and transport. Auxin synthesis is reduced by a R pulse in etiolated maize mesocotyls (Iino 1982). R induces auxin oxidation and transport. Auxin synthesis is reduced by a R pulse in etiolated Phaseolus seedlings (Fletcher and Zalik 1964). In rice etiolated coleoptiles, auxin transport is suggested to be regulated in the R/FR reversible manner (Sherwin and Furuya 1973). These results suggest that the P$_{FR}$ photoreceptor reduces the level of active auxin. Hence, it is not surprising that the level of auxin is altered in response to the end-of-day FR treatment in light-grown seedlings of Arabidopsis.

Although the physiological evidence suggests that phytochrome regulates the level of auxin, the molecular mechanism remains unclear. So far, only a few genes are known to be involved in auxin synthesis (Bartel 1997, Zhao et al. 2001) and auxin transport (Galweiler et al. 1998, Utsuno et al. 1998). Whether these genes are under the control of phytochrome has not been examined. Besides the gene involved directly in the auxin metabolism, a transcription factor, ATHB2, might be involved in the process (Steindler et al. 1999). As mentioned above, the expression of the ATHB2 gene is up-regulated by the end-of-day FR treatment. Interestingly, transgenic plants over-expressing this gene exhibit phenotypes that can be explained by increased lateral transport of auxin. Hence, phytochrome may regulate the level of auxin by changing the expression of the ATHB2 gene.

Light responses of other auxin-related genes

The FIN219 gene on chromosome 2 has been identified to be responsible for the suppressor mutation in cop1 (Hsieh et al. 2000). The expression of this gene is induced by auxin. However, the effects of light on its expression have not been examined. The DFL1 gene on chromosome 5 shows the highest similarity to the AtGH3a gene. The over-expression of this gene causes the short hypocotyl phenotype only in the light (Nakazawa et al. 2001). Interestingly, the genomic sequence around the DFL1 gene shows high synteny with that around the AtGH3a gene (Arabidopsis Genome Initiative 2000, Vision et al. 2000). Nevertheless, the DFL1 gene is not induced by the end-of-day FR treatment regardless of the fact that it responds to auxin (Nakazawa et al. 2001). Hence, not all the members of the GH3 family are down-regulated by phytochrome signal. This discrepancy might be due to the differences in the sensitivities of these genes to auxin. Alternatively, differences in the tissue specificity might determine the light responsiveness. For instance, the DFL1 gene might be expressed only in roots, where the levels of auxin might not be altered by phytochrome.

A question arises: Are other classes auxin-responsive genes regulated by light? In fact, some IAA/Aux genes in Arabidopsis show higher expression in the dark than under light (Abel et al. 1995). Induction of the SAUR-AC1 gene expression in hypocotyls grown in darkness has been reported in Arabidopsis (Gil and Green 1997). Hence, some of these genes might be under the control of phytochrome.

The whole genome sequence of Arabidopsis has been determined recently (Arabidopsis Genome Initiative 2000). Using the DNA array technique, the expression of a large number of genes can be examined simultaneously (Wisman and Ohlrogge 2000). The expression of about 8,000 Arabidopsis genes has been examined in the wild type and the phyA mutant using this technique (Tepperman et al. 2001). About 10% of the genes tested were found to be under the control of phyA. Among them were found two GH3-like genes, four SAUR-like genes and three IAA/AUX genes. These data indicate the involvement of auxin in the signal transduction of phytochrome. However, not all the auxin-responsive genes appear to respond to light in the same way. For example, one of the GH3-like genes is up-regulated but another one is down-regulated by phyA (Tepperman et al. 2001). A more comprehensive analysis focused on auxin-related genes under different light conditions is awaited.

Spatial pattern of AtGH3a expression

Under continuous white light, low but significant expression of the AtGH3a gene was detected in cotyledons, hypocotyls and roots (Fig. 3). After the end-of-day FR, expression of the AtGH3a gene was substantially increased in cotyledons and hypocotyls but not in roots. This pattern of expression is somewhat different from that reported for the soybean GH3 gene. In situ hybridization assay revealed expression of the soybean GH3 only in root vascular tissues in the light (Gee et al. 1991).
Plant materials and growth condition

Arabidopsis thaliana (L.) Heynh promoter trap lines established by INRA (Versailles, France) were used. As controls, the wild-type A. thaliana (ecotype Wassilewskija, Landsberg erecta and Columbia) were used. Mutant Arabidopsis lines used in the present study were phyB-1 (formerly Bo64) (Reed et al. 1993) and axr2-1 (Nagpal et al. 2000). We re-isolated the N35 line from a batch of the promoter trap lines (stock number N5353) provided by NASC (Nottingham Arabidopsis Seed Stock Center, Nottingham, U.K.)

Plant growth conditions were as previously described (Nakamura et al. 2000). Seeds were sown on 0.8% Phytagor plates composed of MS inorganic (Murashige and Skoog 1962) and B5 vitamins (Gamborg et al. 1968) supplemented with 2% sucrose. Mature plants were transferred to OASIS growing medium (Smithers-OASIS, Nihon-Soda, Tokyo) placed on vermiculite soil for seed setting. Arabidopsis (BETATECH bvba, Gent, Belgium) was applied for collecting seeds. For physiological experiments, we used a sucrose-free medium.

**End-of-day FR treatment**

The white light source was as described previously (Yamaguchi et al. 1999). The FR was from fluorescent tubes (FL20S FR-74, TOSHIBA, Tokyo) filtered through a 3-mm plastic plate (Delglass A-900, Asahi Chemical Industry, Tokyo). The intensities of white and FR were 13.0 and 2.55 W m\(^{-2}\), respectively. For induction of gene expression, 5-day-old seedlings grown under white light were treated with 10 min FR and transferred into darkness. For \(\beta\)-glucuronidase (GUS) histochemical staining, seedlings were kept in darkness for 18 h and then stained. For RNA gel blot analysis, RNA was extracted 6 h after the transfer to darkness. For morphological observation, seedlings were grown under white fluorescent light for 2 d in 25°C, then kept under 13 h dark/11 h light cycles for 3 d on black paper (hypocotyl measurements) or white paper (cotyledon measurements). The end-of-day FR treatment was given as described above on each day.

**Auxin treatment**

Synthetic auxin 2,4-D was dissolved in ethanol at 220 µg ml\(^{-1}\) and stored at \(-20°C\). The auxin solution was added to the low melting point agarose (SEA PLAQUE, Takara, Tokyo) solution at a 1 : 1,000 ratio immediately before use. The agarose solution kept at 40°C was then poured onto seedlings grown on agar plates. The seedlings were placed under white light for 6 h and then subjected to the RNA extraction. We confirmed that the maximum concentration of ethanol exhibited no significant effect on the \(A IGH3a\) gene expression.

**GUS activity detection**

Histochemical and quantitative GUS enzyme activity detection (Gallagher 1992, Jefferson 1987) was performed as follows. For the histochemical assay, GUS substrate 5-bromo-4-chloro-3-indolyl \(\beta\)-glucuronide (X-Gluc) (Roche Scientific, Edmonton, Canada) was dissolved in dimethylformamide at 50 mg ml\(^{-1}\). The solvent was added to the GUS histochemical staining buffer (100 mM Na\(_2\)HPO\(_4\), pH 7.0, 10 mM Na\(_2\)EDTA, 0.1% Triton X-100) so as to make the final concentration of X-Gluc 0.5 mg ml\(^{-1}\). Seedlings were soaked in the buffer and evacuated twice in order to fully permeate GUS substrate. After incubating appropriate time at 37°C in an air incubator, the buffer was replaced with the fixative solution (ethanol : water : acetic acid = 8 : 1 : 1) (Aida et al. 1997). The seedlings were then observed under a stereoscopic microscope.

Quantitative assay was performed as described by Gallagher (1992). GUS substrate, 4-methylumbelliferyl-glucuronide, was dissolved in GUS extraction buffer (50 mM Na\(_2\)HPO\(_4\), pH 7.0, 10 mM mercaptoethanol, 1 mM Na\(_2\)EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) at the concentration of 1 mM. The amount of the product, 4-methylumbelliferone (4-MU), was determined with a spectrophotometric photometer (Perkin-Elmer). The excitation and emission wavelengths were 365 and 455 nm, respectively. The amounts of total protein were determined using the Protein Assay Kit (Bio-Rad). Since extracts from the wild-type seedlings constantly exhibited a slight signal (about 10.0 nmol 4-MU min\(^{-1}\) (mg protein\(^{-1}\)), this value was used as the background.
Tail-PCR and Northern blot analysis

Plant genomic DNA preparation and oligomer synthesis were as described previously (Nakamura et al. 2000). We followed the Tail-PCR (thermal asymmetric interlaced-PCR) protocols reported (Liu et al. 1995). Specific primers for the transfer DNA (T-DNA) right borderer were 5'-TGATGGTAAACTGGCTTGCA-3' (primary reaction), 5'-CAATTCGCGCTTCTTGAAC-3' (secondary reaction), and 5'-GATCCAGATGAATGCCACAG-3' (tertiary reaction). DNA fragments obtained from the reactions were cloned into a vector using the TA-cloning system (Promega) and subjected to DNA sequence determination. The position of the T-DNA insertion was determined by BLAST homology search (Altschul et al. 1990). Total RNA isolation from seedlings and RNA gel blot analysis were described (Mochizuki et al. 2001).

Plant total RNA was isolated using SEPAZOL (Nacalai Tesque, Kyoto) and electrophoresed in denaturing agarose gel. The sequence of the binary vector pGKB5 was blotted onto Nytran Supercharge nylon membrane (Schleicher & Schuell, Dassel, Germany). The AtLED13a gene-specific DNA probe was obtained by PCR using oligonucleotide primers set in the first and second introns: 5'-TTCTTGAGGTTGGACAAGATTGGTG-3' and 5'-CAAAAGCCTGCACAAATACAC. The AtGH3a gene-specific probe was obtained with primers 5'-CCCTGCAAGAGTATTGGGCA-3' and 5'-GAGCTAGCGTTTGTTCTTGACAGG-3'. This region correspond to the second exons of the AtGH3a gene, which shows 86% similarity to the most similar Arabidopsis gene, DFL1 (Nakazawa et al. 2001). Thus, we hybridized the blots with the DFL1 probe amplified in a similar manner. Since no signal was detected with this probe, we concluded that our AtGH3a probe indeed detected the AtGH3a gene expression in the present work. The specific probe for ribosomal DNA used as a control was as previously described (Mochizuki et al. 2001). We labeled the probes using the BcaBEST labeling kit (Takara, Tokyo) with 32P. Hybridization analysis were performed using 32P-labeled probes in the hybridization buffer (0.5 M sodium phosphate buffer pH 7.2, 1% bovine serum albumin, 1 mM EDTA, 7% SDS). Membrane was exposed to X-ray films in the cassette for the appropriate time (5–48 h).

Determination of morphological parameters

Hypocotyl lengths and cotyledon areas were measured in 20–30 seedlings. Enlarged images of seedlings were captured with a video camera (HAMAMATSU Photonics, Hamamatsu) and the hypocotyl lengths were determined on the printed images. For cotyledon area measurements, a tracing software (Flexitrace, TREE STAR INC., CA, U.S.A.) was used.

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