

Gibberellin-regulated gene in the basal region of rice leaf sheath encodes basic helix–loop–helix transcription factor

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Abstract Genes regulated by gibberellin (GA) during leaf sheath elongation in rice seedlings were identified using the transcriptome approach. mRNA from the basal regions of leaf sheaths treated with GA₃ was analyzed by high-coverage gene expression profiling. 33,004 peaks were detected, and 30 transcripts showed significant changes in the presence of GA₃. Among these, basic helix–loop–helix transcription factor (AK073385) was significantly upregulated. Quantitative PCR analysis confirmed that expression of AK073385 was controlled by GA₃ in a time- and dose-dependent manner. Basic helix–loop–helix transcription factor (AK073385) is therefore involved in the regulation of gene expression by GA₃.

Keywords Gibberellin · High-coverage gene expression profiling · Rice · Basal region of leaf sheath · Basic helix–loop–helix transcription factor

Abbreviation

GA Gibberellin

Introduction

Gibberellins (GAs) are plant hormones that play an important role in regulating many physiological processes involved in the growth and development of plants, including seed germination, shoot and stem elongation, and flower

development (Hooley 1994). GA biosynthesis (Sakamoto et al. 2004) and signaling pathway (Ueguchi-Tanaka et al. 2005) in rice have been characterized by using mutants. A topographical study showed that the expression of the genes for GA biosynthesis and signaling pathway in rice is apparently restricted to the basal and the peripheral regions of the shoot apex meristem, where the cell fate is determined (Kaneko et al. 2003). The downstream section of GA-response pathways is integrated with other signaling pathways to regulate plant growth and development (Tanaka et al. 2005). These reports indicate that GAs, through complex signaling pathways, affect several physiological processes in the growth and development of rice.

The technique of differential display represents a powerful approach for studying several genes downstream of GA signal transduction. cDNA microarrays from rice seedlings (Yang et al. 2004), callus (Yazaki et al. 2003), and aleurone layer (Bethke et al. 2006) treated with GA were used to identify genes with altered expression levels. By using this technique, xyloglucan endotransglucosylase/hydrolase 8 (Jan et al. 2004), pyruvate dehydrogenase kinase1 (Jan et al. 2006a), and a novel GA-enhanced gene1 (Jan et al. 2006b) have been characterized as GA-induced genes. Transgenic rice plants with repression of these genes showed a reduction in height (Jan et al. 2004, 2006a, b). These reports highlight the importance of a comprehensive analysis, such as provided by the cDNA microarray approach; this has also been demonstrated in an analysis of the mechanism of rice internode elongation by GA.

While the microarray technique can be used to identify GA-regulated genes in rice (Yang et al. 2004; Yazaki et al. 2003; Bethke et al. 2006), the hybridization technique is subject to variability as a result of differences in probe hybridization (Audic and Claverie 1997) and cross-reactivity (Richmond et al. 1999). Moreover,

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Table 1 GA₃ up-regulated genes detected by high-coverage gene expression profiling analysis

No.	Gene name	AC	Ratio	F
1	Basic helix–loop–helix transcription factor	AK073385	8.71	T
2	Myosin heavy chain	AK101972	5.2	C
3	<i>O. sativa</i> genomic sequence	AK066135	3.35	G
4	Allinase	AK064938	3.35	M
5	Superoxide dismutase [Cu–Zn]	AK061662	3.3	D

After determining the sequences, genes were assigned using the classifications described by Bevan et al. (1998). Ratio means “expression volume of gene by plants treated with GA₃ to expression volume of gene by control plants”

F function of genes, *T* transcription, *C* cell growth/division, *M* metabolism, *D* disease/defense, *U* unclear classification, *G* genomic sequence

this method requires sequence information for functional analysis.

In an attempt to clarify gene profiles, the available techniques for analyzing gene expression are limited. In this situation, the application of high-coverage gene expression profiling by using massively parallel signature sequencing was reported for *Arabidopsis* (Meyers et al. 2004) and rice (Nobuta et al. 2007). Among the substantial improvements made to high-coverage gene expression profiling analysis is a reduction in the rate of false positives by means of the amplified-fragment-length polymorphism technique (Vos et al. 1995). Applications of this technique have been reported for the mouse (Fukumura et al. 2003) and for rice (Suzuki et al. 2005).

High-coverage gene expression profiling analysis permitted the detection and analysis of approximately 70% of all genes expressed in rice (Suzuki et al. 2005). These reports show that high-coverage gene expression profiling analysis is a well-developed method and that it might be applied in high-coverage and quantitative gene expression analysis. In this study, we used the high-coverage gene expression profiling technique as a quantitative transcriptomic tool to identify changes in the expression of GA₃-regulated genes during rice leaf-sheath elongation. Furthermore, we analyzed the GA₃ dependence of the basic helix–loop–helix transcription factor (AK073385), which is expressed to a significant extent in the basal region of the rice leaf sheath in the presence of GA₃.

Materials and methods

Plant material and chemicals

Rice (*Oryza sativa* L.) cv. Nipponbare was grown in a granulated nutrient soil (Kureha Chemical, Tokyo, Japan)

Table 2 GA₃ down-regulated genes detected by high-coverage gene expression profiling analysis

No.	Gene name	AC	Ratio	F
1	Senescence-associated protein	AK063042	0.06	U
2	Hypothetical protein	AK101059	0.07	U
3	Blue copper protein precursor	AK101397	0.13	D
4	Short-chain dehydrogenase/reductase	AK065290	0.13	U
5	Cellulose synthase	AK065259	0.13	M
6	Caffeoyl-CoA 3-O-methyltransferase	AK071482	0.13	S
7	<i>O. sativa</i> genomic sequence	AK120228	0.14	G
8	Resurrection1 (<i>Arabidopsis thaliana</i>)	AK120051	0.14	M
9	Ribosomal RNA	AK105002	0.18	Ps
10	Basic helix–loop–helix transcription factor	AB040744	0.18	T
11	Xylulose phosphate synthase 2	AK100909	0.18	S
12	Ribophorin family protein	AK101333	0.18	U
13	Ribosomal RNA	AK062277	0.19	Ps
14	Disease resistance protein	AK119460	0.19	D
15	Naringenin–chalcone synthase	AK067907	0.20	U
16	No hit	–	0.20	N
17	Allene oxide synthase	AK068620	0.21	D
18	T29M8.5 protein (<i>Arabidopsis thaliana</i>)	AK108738	0.21	M
19	<i>O. sativa</i> genomic sequence	AK073589	0.21	G
20	26S proteasome regulatory subunit	AB070252	0.22	Pd
21	<i>O. sativa</i> genomic sequence	AK105002	0.26	G
22	No hit	–	0.26	N
23	No hit	–	0.28	N
24	Ketol-acid reductoisomerase	AK072075	0.30	U
25	Myb	AK111571	0.30	T

After determining the sequences, genes were assigned using the classifications described by Bevan et al. (1998). Ratio means “expression volume of gene by plants treated with GA₃ to expression volume of gene by control plants”

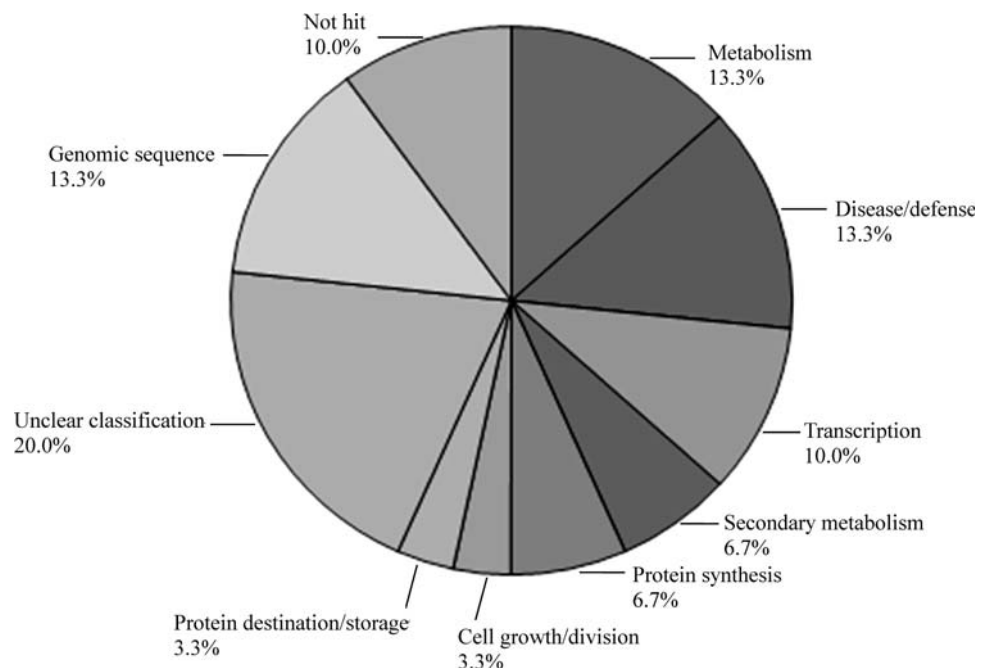
F function of genes, *T* transcription, *M* metabolism, *S* secondary metabolism, *Ps* protein synthesis, *Pd* protein destination/storage, *D* disease/defence, *U* unclear classification, *G* genomic sequence, *N* no hit in available database

under white fluorescent light (600 μmol/m²/s, 12 h light per day) at 28°C and 70% relative humidity in a growth chamber. Stock solutions of gibberellic acid (GA₃; Wako, Osaka, Japan), 2,4-dichlorophenoxyacetic acid (2,4-D, Wako), brassinolide (BL, Fuji Chemical, Toyama, Japan), and abscisic acid (ABA, Wako) were prepared in N,N-dimethylformamide (DMF; Wako); the control treatment contained the same amount of DMF. NaCl (Wako) and mannitol (Wako) solutions were prepared with water.

RNA isolation and high-coverage gene expression profiling analysis

Rice seedlings 10 days old were treated with 5 μM GA₃ for 24 h and mRNA was isolated from the basal regions of the

Fig. 1 Assignment of the identified genes to functional categories. Genes were assigned by using the classifications described by Bevan et al. (1998) (Tables 1, 2) and the total number and the percentage of genes in each category were determined. The distribution of genes in each functional category is shown as the ratio of the number of GA₃-regulated genes to the total number of genes identified in the basal region of the rice leaf sheath. “Unclear classification” corresponds to genes of unknown function. “Genome sequence” corresponds to genes that are not named genes, although they are part of the *O. sativa* genome sequences



leaf sheaths. mRNA samples isolated from three independent experiments were mixed and analyzed twice by using the same procedure of high-coverage gene expression profiling. Differences in fluorescent signals of more than twofold between the control and GA₃-treated samples were selected as being significant. Samples were rapidly frozen in liquid nitrogen and ground to a powder by using a mortar and pestle. Total RNA was extracted by using the RNeasy plant mini kit (Qiagen, Chatsworth, CA, USA) according to the manufacturer's protocol, and digested with 1.5 U/ml DNase I at 37°C for 10 min. mRNA was purified from the total RNA by using Oligotex-dT30 (Takara Bio, Otsu, Japan).

The high-coverage gene expression profiling analysis reaction was performed according to a previous report (Fukumura et al. 2003). Briefly, total RNA was converted to single strand cDNA by reverse transcriptase with 5' biotinylated oligo-d(T) primer and the second strand was synthesized. The double strand cDNA was cut with MspI and ligated with MspI adapter using T4 DNA ligase. The ligand products bearing biotin at the 3' terminus were collected by streptavidin-coated magnetic beads and washed twice with 1.0 ml of washing buffer containing 5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1.0 M NaCl. The cDNA fragments on the magnetic beads were digested with *MesI* and the supernatants were collected. Ligation was performed with *MesI* adapter using T4 DNA ligase in the presence of *MesI* in reaction mixture. The resulting solution was used as a template for the selective PCR and then the PCR products were analyzed by electrophoresis of ABI PRISM 3100 (Applied Biosystems, Foster City, CA, USA). Electropherograms of PCR products were analyzed with the MS-3000 Analyzer (Maze, Inc., Tokyo, Japan). The

peaks of interest were fractionated with standard slab gel (20 cm × 40 cm × 4 mm). The gel slices corresponding to the peak of interest were excised and used for sequencing with dye terminator method or after the cloning with a TA cloning kit (pGEM-T Easy, Promega, Madison, WI, USA).

Selected peaks were annotated by comparison with the database of the Rice Annotation Project Database (The Rice Annotation Project 2007: <http://rapdb.lab.nig.ac.jp/>) and full-length cDNA from rice (Kikuchi et al. 2003: <http://cdna01.dna.affrc.go.jp/cDNA/>) by using BLAST programs. The identified genes were categorized by using the criteria described by Bevan et al. (1998) for the analysis of the 1.9-Mb sequence of chromosome 4 from *Arabidopsis*.

Quantitative real-time PCR analysis

The same mRNA that was used for high-coverage gene expression profiling analysis was used for quantitative real-time PCR analysis. The mRNA (1.5 µg) was used with a Superscript™ first-strand synthesis system (Invitrogen, Carlsbad, CA, USA); the reaction was performed according to the manufacturer's protocol with an oligo(dT)18 primer. The first-strand cDNA was used as the template for the quantitative real-time PCR. The real-time PCR (SYBR Green PCR Master Mix; Applied Biosystems, Framingham, MA, USA) was performed and analyzed by using an ABI Prism 7700 (Applied Biosystems). The PCR conditions were 95°C for 10 min and 50 cycles of 95°C for 15 s, 60°C for 30 s, and 78°C for 40 s. The data were normalized in relation to the level of expression of the actin gene.

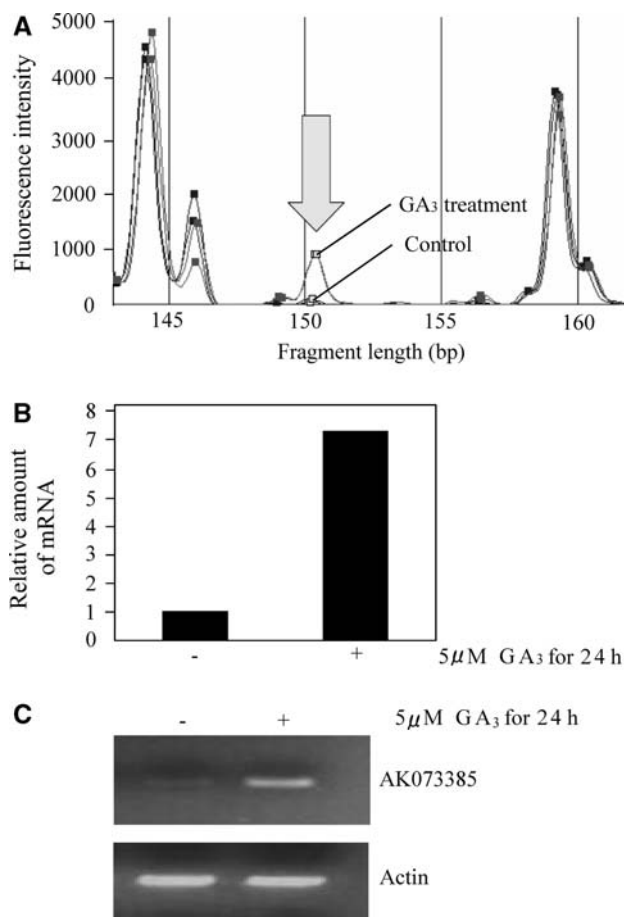


Fig. 2 Expression analysis of a GA₃-regulated gene (AK073385) identified by high-coverage profiling analysis. Total RNA was extracted from the basal region of leaf sheath from 10-day-old rice seedlings treated with 5 μM GA₃ for 24 h, followed by isolation of mRNAs. **a** mRNAs were subjected to high-coverage gene expression profiling analysis. The result from the control and GA₃ treatment overlap and two independent experiments are shown. The arrow indicates the peak changed by GA₃ that corresponds to AK073385. **b** mRNA was subjected to quantitative real-time PCR by using AK073385 primers. The y-axis indicates the relative amount of mRNA. Quantitative real-time PCR data were normalized relative to the expression level of actin gene. **c** mRNA was subjected to semiquantitative reverse transcription PCR. After reverse transcription, cDNAs were used as templates for PCR using AK073385 primers. Actin gene was used as the internal control

RNA isolation and semiquantitative reverse transcription PCR analysis

Total RNA was isolated according to the procedure of Chomczynski and Sacchi (1987) and digested with 1.5 U/ml DNase I at 37°C for 10 min, and then the mRNA was purified by using a QuickPrep Micro mRNA purification kit (GE Healthcare, Piscataway, NJ, USA). cDNA was generated by using a ReverTra-Plus-RT-PCR kit (Toyobo, Tokyo, Japan), and used as a template for PCR. The primers for the AK073385 gene were 5'-GGATTTGG

GAACAGGACATT-3' and 5'-GCTTTGCTTTGTTCCT-GACG-3'. The PCR conditions were 94°C for 4 min and 30 cycles of 94°C for 30 s, 60°C for 30 s, and 74°C for 30 s. The actin gene was used as an internal control.

Results and discussion

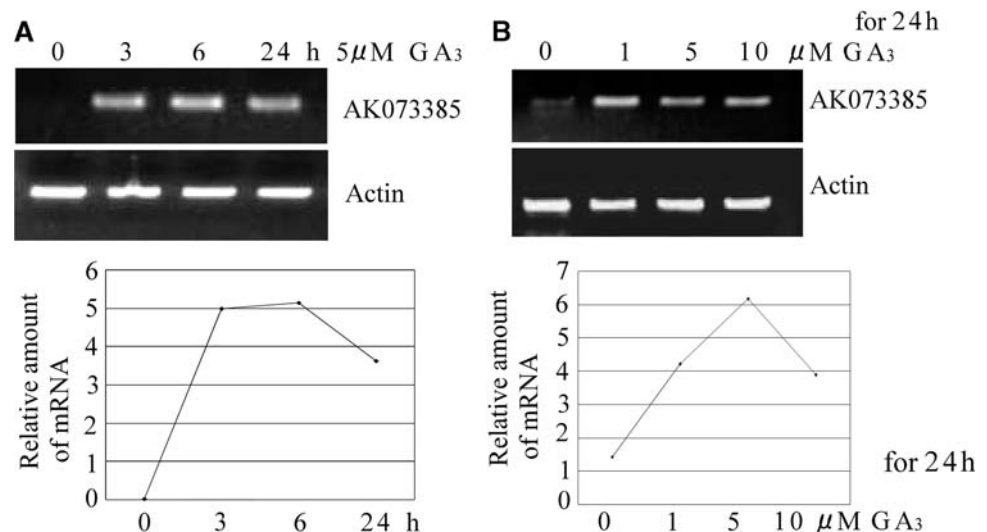
High-coverage gene expression profiling analysis selects 30 unique genes as GA₃ regulated genes

The downstream GA response pathways in rice are integrated with a number of signaling pathways to regulate plant growth and development (Tanaka et al. 2005; Yang et al. 2004). Genetic analysis of GA-response mutants of rice and cloning of the respective genes revealed that DELLA proteins function as negative regulators of GA signaling pathway, and *gid1* has been identified as a soluble GA-receptor in rice (Ueguchi-Tanaka et al. 2005). However, more GA-related genes are needed for characterization of GA signal transduction in rice, because GA affects the growth and development through complex signaling pathways in several physiological processes. Rice leaf sheath is an important part where considerable critical metabolic and regulatory activities take place, which eventually control rice height and robustness. Rice leaf sheath elongates rapidly with the treatment of GA (Shen et al. 2003). To understand the mechanism by which GA regulates rice leaf sheath growth, it is necessary to identify more genes involved in the process.

In this study, to identify the changes in expression of GA₃-regulated genes in rice leaf sheath elongation, the high-coverage gene expression profiling technique (Fukumura et al. 2003; Suzuki et al. 2005) was used as a quantitative transcriptomic tool. With this technique, 420 peaks were found to be changed by GA₃ treatment: 115 peaks were up-regulated and 305 were down-regulated (data not shown). By using the microarray technique, Yang et al. (2004) found that 96 clones were changed more than twofold between control and GA₃-treated samples. Among these 96 clones, 40 were up-regulated and 56 were down-regulated (Yang et al. 2004). These results confirm that high-coverage gene expression profiling is more sensitive than microarray analysis. Fluorescent signal differences of more than threefold between control and GA₃-treated samples were selected as being significant and subjected to further analysis. Thirty genes were significantly affected by GA₃ treatment; five peaks were up-regulated and 25 peaks were down-regulated. This tendency, that the number of up-regulated genes was lower than the number of down-regulated genes, was also reported by Yang et al. (2004).

The peaks were sequenced and searched in the Rice Annotation Project database (The Rice Annotation Project

Fig. 3 Analysis of the time course and GA₃-dose dependence of the expression of AK073385. Total RNAs were extracted from the basal regions of leaf sheaths from 10-day-old rice seedlings treated with 5 μ M GA₃ for 3, 6, or 24 h (a), or with 1, 5, or 10 μ M GA₃ for 24 h (b). mRNA was isolated and subjected to semiquantitative reverse transcription PCR using AK073385 primers. Actin gene was used as the internal control to normalize the amount of cDNA template. The values shown are the mean of relative amounts from three independent experiments



2007) and the full-length cDNA database (Kikuchi et al. 2003). Among the five genes up-regulated by GA₃ (Table 1) were basic helix–loop–helix transcription factor (AK073385), myosin heavy chain (AK101972), allinase (AK064938), and superoxide dismutase [Cu–Zn] (AK061662); the remaining gene (AK066135) was not an identified gene, although it is in the *O. sativa* genomic sequence. Among the 25 GA₃ down-regulated genes (Table 2), 19 encoded products. These were basic helix–loop–helix transcription factor (AB040744), blue copper protein precursor (AK101397), cellulose synthase (AK065259), caffeoyl-CoA 3-O-methyltransferase (AK071482), resurrection 1 (AK120051), two ribosomal RNAs (AK105502 and AK062277), xylulose phosphate synthase 2 (AK100909), disease resistance protein (AK119460), allene oxide synthase (AK068620), T29M8.5 protein (AK108738), 26S proteasome regulatory subunit (AB070252), senescence-associated protein (AK063042), hypothetical protein (AK101059), short-chain dehydrogenase/reductase (AK065290), ribophorin family protein (AK101333), naringenin–chalcone synthase (AK067907), ketol-acid reductoisomerase (AK072075), and Myb (AK111571). Three genes (AK120228, AK073589, and AK105002) were unidentified genes, although there were in the *O. sativa* genomic sequence, and remaining three genes were not in the databases that we searched.

To understand the function of the GA₃-regulated genes, the identified genes were categorized by using the criteria described by Bevan et al. (1998) for the analysis of the 1.9-Mb sequence of chromosome 4 from *Arabidopsis* (Tables 1, 2; Fig. 1). The genes were categorized as metabolism (13.3%), disease/defense (13.3%), transcription (10.0%), secondary metabolism (6.7%), protein synthesis (6.7%), cell growth/division (3.3%), and protein destination/storage (3.3%). For 20% of the sequences

homologous genes have been characterized, but their functions were not identified. The sequences of 13.3% of genes showed hits in terms of their genome sequence, but there was no information on the annotated genes. The sequences of the remaining 10.0% of genes could not be found in the available database. These results indicate that there are many unidentified genes among the GA₃-regulated genes. The use of high-coverage gene expression profiling for identifying GA₃-regulated genes provides an efficient high-throughput approach for assessing the possible functions of large numbers of genes.

Basic helix–loop–helix transcription factor (AK073385) is involved in the regulation of gene expression by GA₃

To evaluate the results obtained by high-coverage gene expression profiling analysis, GA₃ up-regulated gene AK073385, which is basic helix–loop–helix transcription factor (Table 1), was selected for further analysis. mRNA samples were isolated from basal regions of leaf sheaths of 10-day-old rice seedlings with or without treatment by 5 μ M of GA₃ for 24 h. An 8.71-fold difference was observed by high-coverage gene expression profiling analysis in the fluorescent signal for peak number 150, which corresponding to AK073385 (Fig. 2a). Furthermore, the results of quantitative real-time PCR and semiquantitative reverse transcription PCR using AK073385 confirmed that levels of transcription of AK073385 were indeed increased by GA₃ (Fig. 2b, c).

The time dependence of the response to GA₃ was also examined for AK073385. Basal regions of leaf sheaths from 10-day-old rice seedlings were treated with 5 μ M GA₃ for 3, 6, or 24 h. AK073385 was up-regulated by

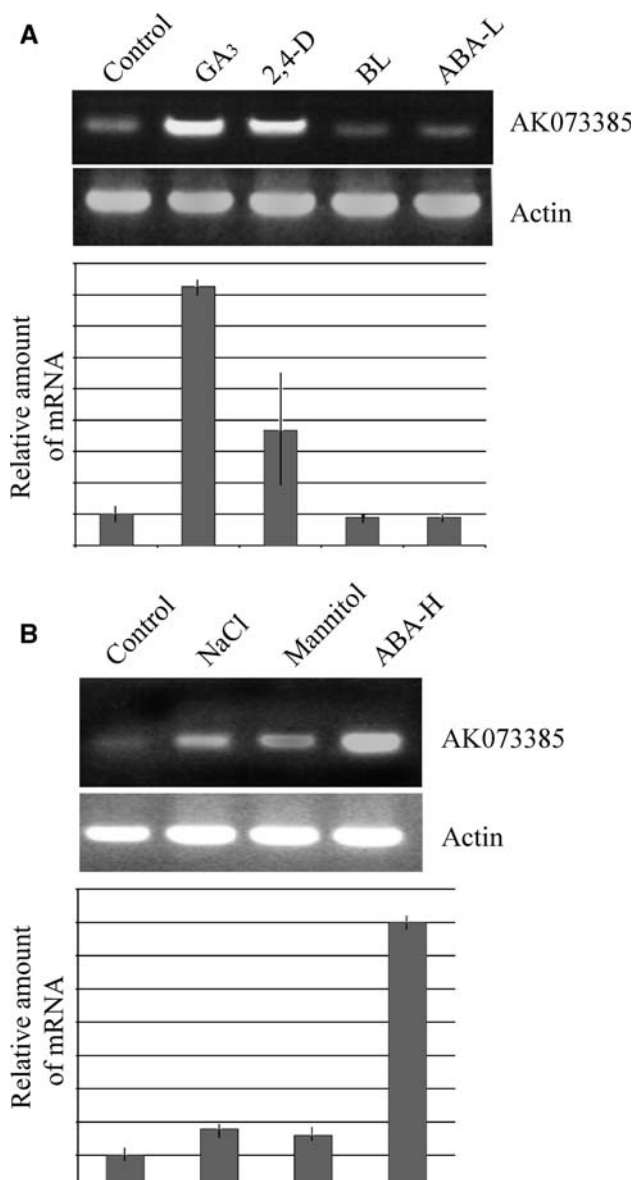


Fig. 4 Hormone- and stress-regulated expression analysis of AK073385. Rice seedlings 10 days old were treated with 1 μ M BL, or 5 μ M GA₃, 2,4-D, and ABA (a), and/or 200 mM NaCl, 400 mM mannitol, and 1 mM ABA (b) for 24 h. Total RNAs were extracted from the basal regions of leaf sheaths. mRNA was isolated and subjected to semiquantitative reverse transcription PCR using AK073385 primers. Actin gene was used as internal control to normalize the amount of cDNA template. The values shown are the mean $\pm$ SE of relative amounts from three independent experiments

5 μ M GA₃ for 3 h (Fig. 3a). Furthermore, the dose dependence of the response to GA₃ was examined for AK073385. Basal regions of leaf sheaths from 10-day-old rice seedlings were treated with 1, 5, or 10 μ M GA₃ for 24 h. AK073385 was up-regulated by 1 μ M of GA₃ for 24 h (Fig. 3b). These results indicated that AK073385 was up-regulated in the early stages of leaf sheath elongation by a low concentration of GA₃.

To identify the role of AK073385, the effects of other plant hormone and stresses were analyzed. Rice seedlings 10 days old were treated with 1 μ M BL or 5 μ M GA₃, 2,4-D and ABA, and/or 200 mM NaCl, 400 mM mannitol and 1 mM ABA for 24 h. Total RNAs were extracted from the basal region of the leaf sheaths. mRNA was isolated and subjected to semiquantitative reverse transcription PCR. AK073385 was significantly up-regulated by GA₃ compared with other plant hormones (Fig. 4a). Furthermore, AK073385 was also up-regulated by high concentrations of ABA (Fig. 4b).

A phytochrome-interacting basic helix–loop–helix protein PIL5 regulates GAs responsiveness by binding directly to the GAI and RGA promoters in *Arabidopsis* seeds (Oh et al. 2006, 2007). Basic helix–loop–helix protein NAM regulates cell elongation and seed germination in *Arabidopsis* (Kim et al. 2005). Cold- and light- controlled seed germination is regulated by the basic helix–loop–helix protein SPATULA in *Arabidopsis* (Penfield et al. 2005). In rice, the basic helix–loop–helix family proteins have been characterized as a group of functionally diverse transcription factors, and 167 corresponding genes have been identified in rice genome (Li et al. 2006). In this study, AK073385 and AB040744 were identified as genes of the basic helix–loop–helix family regulated by GA₃ in the basal region of the leaf sheath; such GA-regulation has not been reported previously.

Basic helix–loop–helix transcription factor (AB040744) was down-regulated by GA₃ in this study, and it has been reported as jasmonic acid-responsive gene RERJ1 (Kiribuchi et al. 2004), and also as wounding- and drought stress-responsive gene (Kiribuchi et al. 2005). Analysis of the phenotypes of rice plants expressing sense- and anti-sense-RERJ1 mRNA indicated that RERJ1 was involved in the inhibition of shoot growth caused by exogenously applied jasmonic acid (Kiribuchi et al. 2004). This report suggested that RERJ1 might be related to the growth of the rice plant, but the function of this protein in most plants is unknown.

On the other hand, basic helix–loop–helix family protein (AK073385) has been reported as a novel iron-regulated basic helix–loop–helix family gene IRO2 (Ogo et al. 2006, 2007). Overexpression of transcription factor IDEF1, which specifically binds to iron deficiency-responsive element 1, leads to the enhanced expression of the IRO2 (Kobayashi et al. 2007). IRO2 was involved in the regulation of gene expression under iron-deficiency conditions; this results in reduced crop yields (Ogo et al. 2006). When rice plants were grown in an iron-deficient hydrophobic culture medium, RNAi transgenic plants stopped growing, whereas over-expressing transgenic plants kept growing and their height exceeded that of control plants (Ogo et al. 2007). Iron is an essential element for plant growth, and

plants grown in calcareous soil often exhibit severe chlorosis as a result of iron deficiency.

These reports and our results indicate that basic helix–loop–helix transcription factor (AK073385) is controlled by iron-deficient conditions and GA₃ treatment. Lange et al. (1994) reported that GA₂₀-oxidase, a multifunctional enzyme involved in GA biosynthesis, contains regions that are highly conserved in a group of non-heme iron-containing dioxygenases. The capacity of the protein to oxidize GA₁₂ was absolutely dependent on the addition 2-oxoglutaric acid, and was reduced by 67% in the absence of iron and by 87% in the absence of ascorbic acid (Lange et al. 1994). On the other hand, superoxide dismutases exist as a Fe- and a CuZn-containing enzymes and are transcriptionally controlled by stress. The expression of these enzymes is up-regulated by GA₃ (Kurepa et al. 1997). These results suggest that basic helix–loop–helix transcription factor (AK073385) is involved in the regulation of gene expression by GA₃, and this expression might be related to iron concentration.

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