Interaction of N-Acetylglutamate Kinase with a PII-Like Protein in Rice

Kenjiro Sugiyama 1, Toshihiko Hayakawa 1,3, Toru Kudo 1, Takashi Ito 1 and Tomoyuki Yamaya 1,2

1 Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai, 981-8555 Japan
2 Plant Science Center, RIKEN, 1-7-22 Suehiro, Tsurumi-ku, Yokohama, 230-0045 Japan

PII protein in bacteria is a sensor for 2-oxoglutarate and a transmitter for glutamine signaling. We identified an OsGlnB gene that encoded a bacterial PII-like protein in rice. Yeast two-hybrid analysis showed that an OsGlnB gene product interacted with N-acetylglutamate kinase 1 (OsNAGK1) and PII-like protein (OsGlnB) itself in rice. In cyanobacteria, NAGK is a key enzyme in arginine biosynthesis. Transient expression of OsGlnB cDNA or OsNAGK1 cDNA fused with sGFP in rice leaf blades strongly suggested that the PII-like protein as well as OsNAGK1 protein is located in chloroplasts. Both OsGlnB and OsNAGK1 genes were expressed in roots, leaf blades, leaf sheaths and spikelets of rice, and these two genes were coordinately expressed in leaf blades during the life span. Thus, PII-like protein in rice plants is potentially able to interact with OsNAGK1 protein in vivo. This finding will provide a clue to the precise physiological function of PII-like protein in rice.

Keywords: N-acetylglutamate kinase — PII-like protein — Rice — Yeast two-hybrid analysis.

Introduction

PII protein in bacteria is important in the transduction of signals of nitrogen and carbon status in cells (Arcondégy et al. 2001). In Escherichia coli, GlnB encodes PII protein and homotrimeric proteins of PII act as a signal transmitter for nitrogen and as a signal sensor for carbon. The trimeric PII complex interacts with 2-oxoglutarate (2-OG), as the carbon-status indicator molecule, and ATP. Monomeric PII protein possesses one 2-OG binding site and hence sensing of cellular conditions for nitrogen limitation or carbon excess is conducted by the 2-OG number per trimeric PII complex (Ninfa and Atkinson 2000). PII is also reversibly uridylylated on a tyrosine residue (Tyr-51) (Arcondégy et al. 2001) by a glutamine-sensor protein, PII-uridylyltransferase/uridylyl-removing enzyme (UTase/UR = GlnD) under a low concentration of glutamine (Jiang et al. 1998, Maheswaran and Forchhammer 2003). These signals are transmitted via a two-component system NtrB/NtrC, and finally down-regulates the activity of glutamine synthetase (GS) by modulating its adenylylation state under nitrogen-excess or carbon-limited conditions (Arcondégy et al. 2001). A PII homolog, GlnK, was identified recently. The deuridylylated GlnK protein interacted with the high-affinity ammonium transporter and inactivated the transport activity under a high concentration of glutamine (Javelle et al. 2004). PII protein in cyanobacteria also interacts with 2-OG and ATP (Forchhammer and Hedler 1997). PII in cyanobacteria appears to control the activities of nitrate-nitrile (Lee et al. 1998) and bicarbonate (Hisbergues et al. 1999) transporters. In contrast to the enteric bacteria, PII in cyanobacteria is phosphorylated on a serine residue (Ser-49) rather than uridylylation at Tyr-51 (Forchhammer and Tandeau de Marsac 1995, Lee et al. 1999, Arcondégy et al. 2001). A gene for type 2C protein phosphatase was identified as a PII phosphatase from Synechocystis sp. strain PCC 6803 (Ruppert et al. 2002). By a yeast two-hybrid screening of Synechococcus sp. strain PCC 7942 libraries, Burillo et al. (2004) and Heinrich et al. (2004) recently found that N-acetylglutamate kinase (NAGK, an ArgB product) could be a PII receptor protein. In the same report, Burillo et al. (2004) showed an interaction between a PII-like protein and NAGK in Arabidopsis thaliana using the yeast two-hybrid system. On the cyclic pathway of arginine biosynthesis in cyanobacteria, NAGK represents the main controlling step, which is feedback regulated by arginine (Caldivic and Tuchman 2003). Under nitrogen-excess conditions, non-phosphorylated PII interacted with NAGK and activated this enzyme (Heinrich et al. 2004).

In higher plants, glutamine could also be a signaling molecule up-regulating genes for ammonium transporters, OsAMT1;1 and OsAMT1;2 (Sonoda et al. 2003), and the NADH-glutamate synthase (NADH-GOGAT) gene (Hirose et al. 1997, Hirose and Yamaya 1999) in rice and down-regulating the gene for an ammonium transporter, AtAMT1;1, in A. thaliana (Rawat

Abbreviations: ATase, adenylyltransferase; 3AT, 3-amino-1,2,4-triazole; CTAB, cetyltrimethyl-ammonium bromide; Dig, digoxigenin; 5-FOA, 5-fluoroorotic acid; GFP, green fluorescent protein; GOGAT, glutamate synthase; GS, glutamine synthetase; NAGK, N-acetylglutamate kinase; 2-OG, 2-oxoglutarate; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR; UTase, uridylyltransferase.

The nucleotide sequences reported in this paper have been submitted to DDBJ, EMBL, GenBank under accession numbers of AB119279 for OsGlnB cDNA and AB183868 for OsNAGK2 cDNA by us, and AK104439 for OsNAGK1 cDNA by the Rice Full-Length cDNA Consortium, National Institute of Agrobiological Sciences Rice Full-Length cDNA Project Team, Foundation of Advancement of International Science Genome Sequencing and Analysis Group and RIKEN (Kikuchi et al. 2003).
et al. 1999) and a nitrate reductase gene in tobacco leaves (Vincentz et al. 1993). From analysis in silico, however, there is no gene that is homologous to GlnD in E. coli and there is no uridylylation site on PII genes in plants (Hsieh et al. 1998, Moorhead and Smith 2003) as well as cyanobacteria (Forchhammer and Tandeau de Marsac 1995). Thus, occurrence of a glutamine sensor and transmitter molecules could be expected in plants, but the signal transduction system is probably different from the bacterial UTase-PII system.

A gene GLB1, which is homologous to the bacterial GlnB, was first identified from A. thaliana (Hsieh et al. 1998). The transcript of this gene was accumulated under carbon-excess conditions, such as in the light or with a supply of sucrose (Hsieh et al. 1998). A PII-like protein in A. thaliana has been detected in isolated chloroplasts (Hsieh et al. 1998). Binding of ATP or 2-OG to the PII-like protein has been indicated using isothermal titration calorimetry (Smith et al. 2003), but the function of the PII-like protein is still unknown largely because the phenotype of transgenic A. thaliana overexpressing GLB1 was not much different from that of the wild-type plant (Hsieh et al. 1998). Although the interaction between PII-like protein and NAGK from A. thaliana was shown by Burillo et al. (2004), nothing is known about plant NAGK, such as gene expression and intracellular localization. If plant PII-like protein interacted with NAGK in vivo, genes for these proteins would be expressed in a coordinated manner and the gene products should be located in the same compartment.

In this study, we identified in rice a nuclear gene, OsGlnB, homologous to A. thaliana GLB1. A yeast two-hybrid screening of a rice cDNA library successfully identified two proteins, OsNAGK1 and the PII-like protein itself, which interact with the PII-like protein in rice. Both rice PII-like protein and OsNAGK1 migrated to chloroplasts in rice leaf blades following the transient expression of these genes and the gene expression profile of OsNAGK1 was similar to that of OsGlnB in rice leaf blades during the life span.

Results and Discussion

Structures of the OsGlnB gene and its protein in rice

We isolated an 868 bp cDNA for a PII-like protein, a product of the OsGlnB (accession number AB119279) gene on chromosome 5 (BAC accession number AC118288) from rice, using 3′-rapid amplification of cDNA ends (RACE) and 5′-RACE against rice leaf poly(A)+ RNA. Sequencing the OsGlnB cDNA revealed a 639 bp open reading frame (ORF) coding for 212 amino acids with a predicted molecular mass of 22.7 kDa. Genomic Southern blot analysis suggested that OsGlnB is a single copy gene in the rice genome (Fig. 1A). This was supported by the result of a search in the rice genome sequence database (http://RiceBLAST.dna.affrc.go.jp/). The result of sequence alignment of the OsGlnB gene with the OsGlnB cDNA showed that the 2.9 kb structural gene consisted of seven exons separated by six introns (Fig. 1B), whereas the dicotyledonous A. thaliana GlnB (GLB1) gene (1.8 kb) was composed of eight exons and seven introns, which was deduced from sequence alignments of the putative A. thaliana GlnB gene on chromosome 4 (BAC accession number AC007138) with the corresponding cDNA (accession number AF095455; Hsieh et al. 1998) (Fig. 1B).

The alignment of the deduced amino acid sequences of PII proteins from E. coli, Synechococcus sp. PCC 7942 and Porphyra purpurea, and PII-like proteins from rice, A. thaliana, Medicago sativa, Lycopersicon esculentum and Pinus pinaster is shown in Fig. 1C. The predicted peptide sequences for rice PII-like protein and other PII proteins were highly conserved, except for 83 and 17 amino acid residues at the N- and C-termini, respectively. Three functionally essential regions determined in E. coli are highly conserved in plant PII-like proteins. These are the T-loop, which is essential for interaction with NtrB, adenyllyltransferase (ATase) and UTase in E. coli PII (Jaggi et al. 1996, Jiang et al. 1997, Martinez-Argudo and Contreras 2002), and B-loop and C-loop, which form a cleft between the T-loop for interaction with ATP (Carr et al. 1996, Xu et al. 2001). Ser-49 in Synechococcus sp. PII, which is the phosphorylation site (Forchhammer and Tandeau de Marsac 1995), is highly conserved in PII-like proteins from rice and other higher plants and PII protein from red alga, such as Ser-131 for rice, Ser-122 for A. thaliana, Ser-118 for M. sativa, Ser-119 for L. esculentum, Ser-152 for P. pinaster and Ser-49 for P. purpurea. Tyr-51 in E. coli PII, which is covalently modified by uridylylation (Arcondéguy et al. 2001), is replaced by Phe in PII-like proteins from plants, which is located at the C-terminal third position from the conserved Ser. This result suggests that the PII-like protein in plants is also modified via phosphorylation/dephosphorylation, like PII protein in cyanobacteria.

Identification of proteins interacting with rice PII-like protein

We searched for proteins that interact with rice PII-like protein with a yeast two-hybrid system using a cDNA library constructed with the mRNA from unexpanded leaf blades of rice. The OsGlnB expression vector encoding the GAL4 DNA binding domain of yeast GAL4 transcription factor (GAL4BD) fused to a partial cDNA for the conserved region (257–649 bp) of rice PII-like protein, which was used as bait, and plasmids containing a rice cDNA expression library fused to the GAL4 activation domain (GAL4AD) were co-transformed into yeast. From the population of approximately 2.5×106 yeast transformants, we isolated 11 colonies that showed a specific interaction with GAL4BD:OsGlnB (Fig. 2A). Ten transformants (A3 in Fig. 2A) of 11 contained an identical cDNA for the NAGK homolog (for cDNA, accession number AK104439; for the gene, BAC accession number AL663000). The other transformant (B2 in Fig. 2A) contained a cDNA for PII-like protein, itself, in a prey vector. While we were preparing this communication, Burillo et al. (2004) and Heinrich et al. (2004) reported the interaction of NAGK, found to be a receptor protein, with...
PII protein in cyanobacteria. PII in *E. coli* is a homotrimeric protein (Arcondéguy et al. 2001). Recombinant PII in *A. thaliana* has also been shown to be a homotrimer (Smith et al. 2003). Our yeast two-hybrid analysis showed that the PII-like protein interacted with the PII-like protein itself (Fig. 2A). This is in good agreement with the previous studies. We also found
an NAGK interacting with the PII-like protein in rice. NAGK is the enzyme catalyzing conversion from N-acetylglutamate to N-acetylglutamate-5-phosphate, which is a precursor of ornithine (Verma and Zhang 1999). Ornithine can be metabolized to arginine and putrescine in plants.

The database searching on rice genomic sequences using the predicted amino acid sequence encoded in the rice NAGK cDNA isolated here (named OsNAGK1) showed the presence of another gene encoding an NAGK homolog (named OsNAGK2, BAC accession number AP005289) on rice chro-
mosome 2. The entire cDNA encoding OsNAGK2 was amplified by reverse transcription (RT)-PCR from shoots of rice seedlings and cloned (accession number AB183868). The rice NAGK homolog proteins (OsNAGK1 and OsNAGK2) contain a putative plastid-targeting polypeptide [predicted by the PSORT (http://psort.ims.u-tokyo.ac.jp/) and ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) programs], the conserved sequence observed in the nitrogen regulatory protein PII family (PII-signature, BLOCKS Database accession number IPB002187A, http://www.blocks.fncrc.org/), and amino acid kinase domain (Protein Family Database (Pfam) accession number PF00696, http://www.sanger.ac.uk/Software/Pfam), as in NAGKs in other plants, an alga and prokaryotic bacteria (Fig. 2B). In rice, none of the introns was inserted in the structural gene of OsNAGK1, whereas the gene of OsNAGK2 consisted of two exons separated by an intron, which was inserted just before the region encoding the amino acid kinase domain (data not shown).

Immunological identification and intracellular localization of PII-like protein and OsNAGK1 protein in rice

We found a plastid-targeting peptide at the N-terminus of rice PII-like protein using the PSORT and ChloroP programs, as identified in the PII-like protein in A. thaliana (Moorhead and Smith 2003) (Fig. 1C). The putative N-terminal plastid-targeting peptide was also detected in PII-like proteins from M. sativa (accession number AY027892), L. esculentum (accession number AY442185) and P. pinaster (accession number AJ489604). For immunological detection of PII-like protein in rice, an antibody raised against the purified recombinant OsGlnB protein, expressed in E. coli, was prepared (Fig. 3). For expression in E. coli, the native OsGlnB was slightly modified as follows: the N-terminal region from 1 to 80, where the predicted plastid-targeting peptide was included, was deleted, Ser-81 was replaced with Met-81, and the modified gene was fused with the poly-histidine tag at the N-terminus. The expressed polypeptide was purified to near homogeneity using metal-chelate affinity chromatography with Ni-NTA resin (data not shown).

**Immunodetection of PII-like protein as the OsGlnB product in extract from leaf blades of rice plants with anti-recombinant OsGlnB antiserum.** (A) Expression of recombinant 6×His:OsGlnB protein in E. coli BL21-AI. Total proteins from uninduced E. coli cells (lane 1), total proteins from α-arabinose-induced cells (lane 2) and 1 µg of recombinant 6×His:OsGlnB protein purified by metal-chelate affinity chromatography using Ni-NTA resin (lane 3) were subjected to SDS-PAGE at a 14.0% (w/v) gel concentration and analyzed by Coomassie brilliant blue R-250 staining. (B) Two nanograms of recombinant 6×His:OsGlnB protein purified from E. coli (lane 1) and 25 µg of crude protein from rice leaf blades (lane 2) were subjected to SDS-PAGE [14.0% (w/v) gel] followed by immunoblotting with antirecombinant OsGlnB antiserum. The numbers at the left refer to the positions of molecular weight markers in kDa. The white and black arrowheads indicate polypeptides of recombinant 6×His:OsGlnB (rOsGlnB) and PII-like protein (an OsGlnB product), respectively.

To have direct evidence for its localization in plastids/chloroplasts, the entire OsGlnB ORF, but without its stop codon, was fused in-frame to the sGFP gene (OsGlnB:sGFP), and transient expression of this fusion protein driven by the cauliflower mosaic virus 35S (CaMV35S) promoter was monitored in rice leaves, using a confocal laser-scanning fluorescence microscope. The signal of OsGlnB:sGFP co-localized with the autofluorescence of chloroplasts (Fig. 4A, B, C), suggesting detection at least 2 ng of recombinant OsGlnB protein (Fig. 3B, lane 1). In the crude extract from rice leaf blades, a single protein band at 15.2 kDa was detected (Fig. 3B, lane 2). This peptide size was in good agreement with that from computergenerated mature rice PII-like protein (15.6 kDa).

Fig. 2 (A) The interaction between OsGlnB and the A3 and B2 protein in the yeast two-hybrid system. The protein fused to the GAL4 binding domain (GAL4BD) or GAL4 activation domain (GAL4AD) is indicated at the left column. In the right panel, the yeasts containing both GAL4BD and GAL4AD fusion proteins were plated on selective media, SC-Leu-Trp-His+25 mM 3AT (-His + 3AT), a YPAD plate containing a nylon membrane (+-gal), SC-Leu-Trp-Ura (+Ura), and SC-Leu-Trp + 0.2% 5-fluoroorotic acid (+0.2% 5FOA). Two cDNA clones, A3 and B2, encode NAGK homolog (OsNAGK1) and PII-like protein (OsGlnB) itself, respectively. dDP and dE2F are proteins known to interact with each other and used as a positive control. Empty vector, represented as none in the left two columns, is used as a negative control. (B) Alignment of the deduced amino acid sequences of NAGKs from plants, an alga, bacteria and an archaebacterium. The alignment was performed by CLUSTAL W (http://www.clustalw.genome.ad.jp/). Identical and similar residues are shaded in black and gray, respectively, by BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). The accession numbers for various NAGKs used were: rice NAGK1, AK104439; rice NAGK2, AB183868; T. aestivum, BT009246; A. thaliana, AT5G09300; P. purpurea, U38804 (AAC108214); Synechococcus sp. PCC 7942, AP005373 (BAC08960); E. coli, M21446 (AA324378); Methanosarcina mazei, AE013352 (AA03916). The putative plastid-target sequence, which was predicted by the PSORT (http://psort.ims.u-tokyo.ac.jp/) and ChloroP (http://www.cbs.dtu.dk/services/ChloroP/) programs, and the amino acid (AA)-kinase domain (Pfam accession number PF00696, http://www.sanger.ac.uk/Software/Pfam) are boxed. The conserved signature sequence of nitrogen regulatory protein PII (IPB002187A) from the Blocks database (http://www.blocks.fncrc.org/) is also indicated by overlining.
gesting that rice PII-like protein is located in plastids/chloroplasts.

To confirm the plastid localization of OsNAGK1, the entire OsNAGK1 ORF, but without its stop codon, was fused in-frame to the sGFP gene (OsNAGK1:sGFP) and the transient expression analysis of this fusion protein driven by the CaMV35S promoter was also performed in rice leaves. The OsNAGK1:sGFP signal (Fig. 4E) was clearly co-localized with the autofluorescence of chloroplasts (Fig. 4D, F). As in the rice PII-like protein, OsNAGK1 protein is possibly located in plastids/chloroplasts. Phylogenetic analysis of the deduced amino acid sequences by the neighbor-joining method in the CLUSTAL W program (Thompson et al. 1994) showed that NAGKs in three plant species, namely, rice, *Triticum aestivum* and *A. thaliana*, were grouped with algal and cyanobacterial NAGKs (data not shown). A similar phylogenetic relationship was also observed among PII-like proteins from plants and PII proteins from algae and cyanobacteria (data not shown; Hsieh et al. 1998).

**Expression of OsGlnB and OsNAGK genes in rice**

RT-PCR analysis showed that transcripts for *OsGlnB*, *OsNAGK1* and *OsNAGK2* were expressed in roots, fully expanded leaf sheaths, unexpanded leaf blades, fully expanded leaf blades, senescent leaf blades and spikelets in rice plants (Fig. 5A). The accumulation pattern of the *OsGlnB* transcript was similar to that of *OsNAGK1*. Although *OsNAGK2* was expressed constitutively, the accumulation level of its transcript was much lower than that of *OsNAGK1* transcript. The yeast two-hybrid screening with PII-like protein as bait showed

---

**Fig. 4** Transient expression of OsGlnB:sGFP (A, B, C) or OsNAGK1:sGFP (D, E, F) fusion protein in leaf blades of rice. The images were monitored by a confocal laser-scanning microscope. The light-microscopic observations were performed with Nomarski differential interference contrast (DIC) optics. (A) and (D) The images of red autofluorescence of chlorophyll detected at an excitation wavelength of 560 nm and an emission wavelength of >560 nm. (B) and (E) The images of green fluorescence of sGFP, which was detected at an excitation wavelength of 488 nm and an emission wavelength of 505–520 nm, and DIC observation. (C) and (F) The merged images of sGFP and chlorophyll. The cell shapes are surrounded by white lines. The scale bars are 10 µm.

**Fig. 5** Expression of *OsGlnB*, *OsNAGK1* and *OsNAGK2* genes in various organs of rice. (A) RT-PCR analyses of transcripts of *OsGlnB*, *OsNAGK1*, *OsNAGK2*, and GAPDH as a control. (B) Immunoblot analysis of PII-like protein, the *OsGlnB* product, with anti-recombinant *OsGlnB* antiserum. Total RNA and crude soluble protein were extracted from 26-day-old roots (lane 1), fully expanded leaf sheaths at the 10th nodal position (lane 2), unexpanded leaf blades inside sheaths at the 10th nodal position (lane 3), fully expanded leaf blades at the 10th nodal position (lane 4), senescent leaf blades at the 6th nodal position (lane 5) and spikelets at 5 d after flowering (lane 6) in rice plants grown until the 10-leaf stage or until the early grain-filling stage on the main stems in hydroponics. Equal amounts (25 µg) of crude soluble protein from various organs of rice were loaded in lanes 1–6 of (B).
PII-like protein in rice

no association with OsNAGK2, suggesting that the dominant protein interacting with the PII-like protein is the OsNAGK1 protein in rice.

Both transcript and protein for OsGlnB and the transcript for OsNAGK1 were abundant in senescent leaf blades rather than in unexpanded leaf blades (Fig. 5). Age-dependent expression of OsGlnB and OsNAGK1 was followed in leaf blades of rice (Fig. 6). The expression of GS genes in these leaf blades was also examined as controls. The accumulation of transcript and protein for GS2 decreased during senescence (Fig. 6A, B). On the other hand, expression of OsNAGK2 decreased during this period (Fig. 6A). In Synechococcus sp. PCC 7942 cells supplied with exogenous NH$_4^+$ as sole nitrogen source, non-phosphorylated PII protein interacts with NAGK and the NAGK is activated (Burillo et al. 2004, Heinrich et al. 2004). This PII phosphorylation state-dependent activation of NAGK could be important for the control of arginine biosynthesis, linked to the global carbon/nitrogen status of the cyanobacterial cells. In plants, on the other hand, nothing is clearly understood about the precise physiological importance of PII-like protein and that of NAGK. Current findings of OsNAGK1 as an interactive protein with PII-like protein in rice will be a clue to the function of PII-like protein in rice.

Conclusion

This is the first paper to report that OsNAGK1, as a protein interactive with a PII-like protein, was screened from rice cDNA libraries using the yeast two-hybrid approach. Both PII-like protein and OsNAGK1 protein were detected in chloroplasts and their expression profiles were similar in leaf blades during the life span. Unlike prokaryotic NAGK, however, there is little knowledge available on the characteristics of NAGK in plants. Further studies, such as analysis of transgenic rice knocked down on the gene expression of OsGlnB and OsNAGKs using the RNA interference method, will help to understand the precise function of NAGK, as well as the biochemical/physiological meaning of its interaction with PII-like protein.

Materials and Methods

Plant materials

Rice (Oryza sativa L. cv. Sasanishiki) plants were grown in hydroponic culture in a greenhouse either until the three- or four-leaf stages (Yamaya et al. 1995), until the 10-leaf stage (Yamaya et al. 1992), until 42 d after full expansion of the 12th leaf blade on the main stem (Kamachi et al. 1991) or until the early grain-filling stage (Hayakawa et al. 1993). For preparations of protein extracts and/or total RNA, the following plant organs were harvested: 26-day-old roots and shoots, fully expanded leaf sheaths and blades at the 10th nodal position, unexpanded leaf blades at the 11th nodal position before emerging from the 10th sheaths, senescent leaf blades at the 6th nodal position and spikelets at 5 d after flowering. The 12th leaf blades on the main stem were also harvested every 7 d from –7 to 42 d. The day when the 12th leaf blade on the main stem was fully expanded was defined as day 0. The samples were weighed, frozen in liquid nitrogen and stored at –80°C until they were extracted.

Preparation of total RNA and poly(A)$^+$ RNA

Total RNA was extracted from various organs of rice plants as described above using the cetyltrimethyl-ammonium bromide (CTAB) method with Sepasol-RNA I (Nacalai Tesque, Kyoto, Japan) as described previously (Suenaga et al. 2003). The poly(A)$^+$ RNA was
prepared from the total RNA using Oligo(dT)-latex resin (Oligotex™-dT30; Takara, Tokyo, Japan).

Cloning and sequencing of cDNAs for OsGlnB, OsNAGK1 and OsNAGK2

A predicted coding sequence for OsGlnB (BAC accession number AC118288, location bp 42,650–45,583, chromosome 5) was picked out from the database for the rice genomic sequence using the deduced amino acid sequence for A. thaliana GlnB cDNA (GLB1, accession number AF005455; Hsieh et al. 1998). A full-length cDNA for OsGlnB was isolated from rice leaf poly(A) RNA using the 5′- and 3′-RACE methods (Marathon cDNA Amplification Kits; Clontech, Palo Alto, CA, U.S.A.) as described previously (Suenaga et al. 2003). The following primers were used: as a forward primer, 5′-ACCAGCGGGAGTCGGAGTTCTACAAG-3′; as a reverse primer, 5′-CATGCTCTCTCTATGAGCTCCGCAATT-3′; and as an adaptor primer, 5′-ACTCTAGATAGGCTCGACGCGC-3′. The amplified cDNA fragments obtained from 5′- and 3′-RACE were cloned into pGEM-T Easy vectors (Promega, Madison, WI, U.S.A.) by the TA cloning method according to the manufacturer’s protocol. Sequencing of these cDNA clones was carried out by the dideoxy chain-termination method using an automated DNA sequencer (Genetic Analyzer 310; Applied Biosystems Japan, Tokyo, Japan).

The partial cDNA clone encoding OsNAGK1 was isolated by the yeast two-hybrid screening as described below and the entire OsNAGK1 cDNA clone (accession number AK104439) was provided from the Rice Genome Project of the National Institute of Agrobiological Sciences (NIAS) and the Rice Genome Resource Center (RGRC) (Tsukuba, Ibaraki, Japan). The predicted coding sequence for OsNAGK2 (BAC accession number AP005289, location bp 24,627–25,921, chromosome 2) was found by database searching in the rice genome sequence using the deduced amino acid sequence for OsNAGK1 cDNA. The cDNA encoding the entire ORF for OsNAGK2 was amplified from shoots of rice seedlings by RT-PCR. The first-strand cDNA was prepared from the total RNA by using a SuperScript First-Strand Synthesis System for RT-PCR kit (Invitrogen, Carlsbad, CA, U.S.A.), as described previously (Suenaga et al. 2003). The 1,130 bp cDNA fragment was obtained from rice shoot cDNA as a template by PCR using KOD-plus DNA polymerase (Toyobo). The following primers were used: 5′-TGAGATCGGCATTGCTGAGGACAGC-3′, as a forward primer, and 5′-TGAGATCGGCATTGCTGAGGACAGC-3′, as a reverse primer. The amplified PCR product was cloned inside the pGEM-T Easy vectors (Promega, Madison, WI, U.S.A.), as described previously (Du et al. 1996) and a plasmid pair, 5′-GACACAAATAGTTGTGGACTCAC-3′. This blunt-ended cDNA was cloned at the HindIII site of pUC118 vector (Takara) and sequenced.

Genomic Southern hybridization

Genomic DNA was prepared from 26-day-old shoots of rice seedlings by the CTAB method and used for DNA gel blot analysis, as described previously (Goto et al. 1998). The DNA (500 ng) was digested with EcoRⅤ, HindⅢ or XbaⅠ and the digested DNA was electrophoresed through a 0.8% agarose gel, denatured, transferred to a nylon membrane and probed with the digoxigenin (DIG)-labeled cDNA fragment (399 bp), whose sequence corresponds to that from +253 to +652 of OsGlnB cDNA.

Yeast two-hybrid screening

Poly(A)+ RNA was prepared from non-green 12th leaf blades inside of the 11th leaf sheaths of rice plants. Double-stranded cDNA was synthesized using a SuperScript™ Plasmid System for cDNA Synthesis and Plasmid Cloning kit (Invitrogen) and cloned inside attB1 and attB2 of the yeast expression vector, pEXP-AD502, containing the GAL4 activation domain (GAL4AD) outside the attB1 sequence according to the instruction protocol (Invitrogen). These plasmids con-taining cDNA were introduced into E. coli cells (ElectroMAX™ DH10B™ cells; Invitrogen) by electroporation. The resulting cDNA expression library, fused downstream of the activation domain of the GAL4 transcription factor, contained 1.1 × 107 independent recombinants with an average insert length of 1.7 kb. These cDNA expression plasmids (GAL4AD,cDNAs) were purified from E. coli and were used as prey constructs in the yeast two-hybrid screen.

The partial cDNA (257–649 bp) of OsGlnB, which encodes the conserved peptide region with bacterial PII, was amplified from rice leaf cDNA as a template by PCR using KOD-plus DNA polymerase (Toyobo). The following primers were used: 5′-caccATGGAGTCTCTTACAGGTTGAGGACAGC-3′, as a forward primer, and 5′-TGAGATCGGCATTGCTGAGGACAGC-3′, as a reverse primer. The additional first ATG codon in the forward primer is indicated in bold letters and four additional CACC bases at the 5′-terminus used for directional cloning using topoisoerase I are indicated in lower case. The PCR product was cloned inside the attL1 and attL2 sequences of pENTR/D-TOPO vector (pENTR Directional TOPO™ Cloning Kit; Invitrogen) via the reaction with topoisoerase I, according to the manufacturer’s protocol. The resulting Gateway (GW) entry plasmid was used in an attL × attR-recombinational reaction (LR reaction) with the destination vector pDEST32 (Invitrogen) containing the GAL4 DNA binding domain (GAL4BD) outside the attR1 and attR2 sequences. The C-terminal fusion of OsGlnB to GAL4BD (GAL4BD:OsGlnB), as bait, was generated using the destination vector, according to the manufacturer’s protocol (version B: www.invitrogen.com).

The cDNA library plasmids (GAL4AD,cDNAs) as prey constructs were co-transformed into the Saccharomyces cerevisiae strain MaV203, containing three reporter genes (HI3, URA3 and lacZ), with the expression construct as bait, GAL4BD:OsGlnB, by the lithium acetate method. Approximately 2.5 × 106 transformants were screened on selective synthetic complete (SC) media lacking leucine, tryptophan and histidine and supplemented with 25 mM 3-amino-1,2,4-triazole (SC-Leu-Trp-His+3AT). The His+ colonies grown on selective media were chosen and patched on master plates (SC-Leu-Trp). After incubation of the master plates for 30 h at 30°C, the replica was transferred onto the selective plates, i.e. SC-Leu-Trp-Ura, SC-Leu-Trp+0.2% 5-fluoroorotic acid (5-FOA) and SC-Leu-Trp+His+3AT, and nylon membranes overlaid on YPAD media to analyze the activity of the reporter genes, i.e. HI3, URA3 and lacZ. The activity of lacZ was determined by assessment of white/blue colonies using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The DP transcription factor (dDP) and E2F1 protein (dE2F) from Drosophila melanogaster are known to interact with each other (Du et al. 1996) and a plasmid pair, pPC97-CYH2-dDP (expresses GAL4BD:dDP) and pPC86-dE2F (expresses GAL4AD:dE2F), was used as a positive control. Both pPC97 and pPC86 vectors were used as a negative control. The pEXP-AD502 plasmids containing cDNA encoding a candidate protein that interacted with OsGlnB were isolated from yeast colonies. These plasmids were co-transformed again into MaV203 with the constructs of either GAL4BD:OsGlnB or GAL4BD alone (pDBLeu), as control bait, and their phenotypes were assessed after being grown on each selective plate. Finally, 11 cDNA clones were isolated and cDNA inserts of these clones were sequenced.

Preparation of anti-recombinant OsGlnB antibody

The partial cDNA (257–652 bp) encoding the peptide (Glu-82 to C-terminus Ser-212) of OsGlnB was amplified by PCR using the primers, 5′-CACCAGGGAGTCGGAGTTCTACAAGGAGC-3′, as a forward primer, and 5′-TCATGAGATCCGACTCTTACAGGAGGACAGC-3′, as a reverse primer, followed by cloning into the pENTR/D-TOPO vector as described above. From this entry plasmid, the cDNA fragment was transferred into the bacteriophage T7 promoter (Prot7) reg-
ulated expression vector, pDEST17 (Invitrogen), which contained a poly-histidine (6 × His) tag outside the attR1 and attR2 sequences, by a LR-recombinational reaction. The resulting construct (ProT7::6′ × His:OsGlnB) was transformed into E. coli BL21-AI (Invitrogen) containing the T7 RNA polymerase gene downstream of the araBAD promoter, whose expression was controlled by α-arabinose. The BL21-AI harboring the expression vector construct was pre-cultured at 37°C until the OD600 reached 0.4 in Luria-Bertani (LB) broth containing carbenicillin (50 µg mL⁻¹) and then treated with 0.2% α-arabinose to express T7 RNA polymerase and recombinant 6 × His:OsGlnB, followed by culture for 3 h at 37°C. The inclusion bodies containing overexpressed 6 × His:OsGlnB protein were recovered from E. coli cells by sonication and centrifugation and further purified by ultracentrifugation through a sucrose step gradient (Taniguchi and Sugiyama 1996). The 6 × His:OsGlnB protein was solubilized from inclusion bodies in 10 mM Tris-HCl (pH 8.0) containing 100 mM Na₂HPO₄ and 8 M urea by gently mixing for 30 min and purified to near homogeneity by metal-chelate affinity chromatography using Ni-NTA resin (Qiagen, Hilden, Germany) in accordance with the manufacturer’s instructions. To confirm the internal amino acid sequence of expressed 6 × His:OsGlnB protein, the purified protein was digested with Achromobacter Protease I (Takara) for 12 h at 37°C according to the manufacturer’s instructions and the resulting proteolytic peptides were separated by SDS-PAGE (Laemmli 1970), blotted on a polyvinylidene fluoride (PVDF) membrane and sequenced using a protein sequencer (ABI 473A; Applied Biosystems Japan).

A rabbit-polyclonal antibody raised against the purified 6 × His: OsGlnB protein, as an antigen, was prepared essentially as described previously (Ishiyama et al. 2004).

Preparation of protein extracts and immunoblotting analysis

The frozen samples were first ground to a fine powder in a mortar with a pestle in the presence of washed quartz sand with liquid nitrogen. The powder was homogenized in 50 mM KH₂PO₄–KOH buffer (pH 7.5) containing 5 mM diithiothreitol, 1 mM EDTA, 200 µM leupeptin and 500 µM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) [2 ml (g FW)⁻¹]. The homogenate was centrifuged at 18,500 × g for 20 min at 4°C and the supernatant was used as a soluble protein fraction for immunoblotting analysis. The protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Immunoblotting after SDS-PAGE was performed as described previously (Yamaya et al. 1992). The dilutions of primary antibodies used in immunoblotting analyses were at 1 : 1,000 for the anti-recombinant OsGlnB antiserum and at 1 : 500 for anti-recombinant rice GS1 antiserum (Ishiyama et al. 2004), respectively. After primary immunostaining, the reacted peptides were detected with a goat anti-rabbit IgG alkaline phosphatase conjugate (Promega) using nitroblue tetrazolium (NBT; Promega) and 5-bromo-4-chloro-3-indolyl-1-phosphate (BCIP; Promega) as substrates.

Expression of transcripts for OsGlnB, OsNAGK1 and OsNAGK2

The first-strand cDNA was prepared from total RNA extracted from various organs of rice plants as described above. Gene-specific primer pairs of OsGlnB, OsNAGK1 and OsNAGK2 used for RT-PCR were: 5′-ACCCAGCGGAGTCCGAGGCTGACCGAAG-3′ as a forward primer and 5′-CTCATGAGATCGGCAATGCTGAGGAC-3′ as a reverse primer for OsGlnB; 5′-GGTGATGCTCCGCCATTCTTTC-3′ as a forward primer and 5′-CTGACAGTGGACCAGGAC-3′ as reverse primer for OsNAGK1; and 5′-TGAATTTCTGAAAGTCGATGGACGAG-3′ as a forward primer and 5′-CTGTTACATGCTTCGAACGGG-3′ as a reverse primer for OsNAGK2, respectively. Gene-specific primer pairs of GS1 (accession number X14245), GS2 (accession number X14246) and the gene encoding cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH, accession number AK103777) from rice, as controls, were: 5′-GCTCTTTTGACGCCACATCCTC-3′ as a forward primer and 5′-GGACGATGATCCACAGGGATGGGAC-3′ as a reverse primer for GS1; 5′-GAAGAATGGCAGTAATGGCTTCGGGC-3′ as a forward primer and 5′-GAAGGAGGAGGCAGGCAGTCAAGA-3′ as a reverse primer for GS2; and 5′-GGCGTCGAATTGTCTTCAAC-3′ as a forward primer and 5′-CAGCATGACAAAGCATTACC-3′ as a reverse primer for GAPDH, respectively. PCR reaction using the first-strand cDNA as template and a gene-specific primer pair with Ex Taq DNA polymerase (Takara) was performed as follows: 2 min at 94°C followed by 25 cycles for OsGlnB, GS1, GS2 and GAPDH, and 28 cycles for OsNAGK1 and OsNAGK2 of 30 s at 94°C, 30 s at 60°C and 1 min at 72°C, and 1 cycle of 7 min at 72°C to be in a semi-quantitative range. It should be noted that several different amplification cycles of PCR (22–30 cycles) were adopted for each first-strand cDNA sample in order to amplify double-stranded cDNA in a semi-quantitative range and the PCR cycles described above gave the most quantitative results for each transcript. RT-PCR products were analyzed by 2.0% agarose gel electrophoresis. To confirm gene-specific amplification, the amplified products were cloned and fully sequenced.

Transient expression of the sGFP fused protein in rice leaf cells

The DNA fragment, which contained the entire ORF for OsGlnB and added attB sequences at both 5′- and 3′-termini, was amplified by two sequential steps of PCR, according to the manufacturer’s protocol (version B; Invitrogen). The primer pair used in the first PCR for OsGlnB-specific amplification was: 5′-AAAAACGACGTCCTCAGCT-CTGCCTGCGTCCTG-3′ as a forward primer and 5′-AGAAAAGCTG- GTATGATCCGCAATTCTTTCGAG-3′ (the TGA stop codon was removed) as a reverse primer. The 12 bp of 3′-terminal sequences of attB1 (forward primer) and attB2 (reverse primer) are indicated in bold letters. The primer pair for the second PCR for installation of the complete attB sequences was: 5′-GGGACACGTTGTGTACAAAAAGCGAGC-3′ as the attB1 adaptor primer (forward) and 5′-GGGACACCTTTTGACGAGATGA-3′ as the attB2 adaptor primer (reverse), respectively. The resulting PCR product with attB sequences at both 5′- and 3′-sides was cloned inside the attP1 and attP2 sequences of pDONR221 vector (Invitrogen) by attBx attP-recombinational reaction (BP reaction). The DNA fragment containing the complete ORF for OsNAGK1 was amplified by PCR using the primers, 5′-CAGCATGACAAAGCATTACC-3′ as a forward primer and 5′-GCCAATGATCATAGTGCCATGCCGTC-3′ as a reverse primer, followed by cloning into the pENTR/D-TOPO vector as described above.

Two resulting entry plasmids were used in LR reactions with the destination binary vector pGW85 [Pro35S::attR1::CmR (Chloramphenicol resistance gene)::c:cd::attR2::sGFP(S65T)], which was kindly provided by Dr. T. Nakagawa (Department of Molecular and Functional Genomics, Center for Integrated Research in Science, Shimane University, Matsue, Japan). The C-terminal fusions of sGFP(S65T) to OsGlnB (OsGlnB:sGFP) or to OsNAGK1 (OsNAGK1:sGFP) were generated using the binary vector, respectively, according to the manufacturer’s protocol (version B; Invitrogen). Pro35S::OsGlnB::sGFP or Pro35S::OsNAGK1::sGFP, as a fusion construct, was introduced into cells of rice leaf blades by a particle bombardment method (Biologic PDS-1000/He particle Delivery System; Bio-Rad, Hercules, CA, U.S.A.) as described previously (Goto et al. 1998). Green fluorescence for sGFP was detected at an excitation wavelength of 488 nm and an emission wavelength of 505–520 nm using a confocal laser-scanning microscope system (FV1000; Olympus, Tokyo, Japan). Red autofluorescence from chlorophyll was also detected at an excitation wavelength of 543 nm and an emission wavelength of
>560 nm. Light-microscopic observations were also performed with Nomarski differential interference contrast optics.

Acknowledgments

We thank Dr. T. Nakagawa of Shimane University for providing the binary vector, pGBW5, and the Rice Genome Project of the National Institute of Agrobiological Sciences (NIAS) and the Rice Genome Resource Center (RGRC), Japan, for providing a rice full-length cDNA clone (accession number AK104439). This work was supported in part by a program of CREST of JST (Japan Science Technology), in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 14360035) and in part by The Project for Rice Genome Research (IP6001) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

References


(Received July 16, 2004; Accepted September 15, 2004)