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Cloning of cDNA for UDP-glucose pyrophosphorylase and the expression of mRNA in rice endosperm

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Abstract Rice endosperm UDP-glucose pyrophosphorylase (UGPase) cDNA clones were isolated by screening a λ ZAP II library prepared from poly (A^+) RNA of *japonica* rice (cv Sasanishiki) endosperm with a probe of potato UGPase cDNA. One cDNA clone, possessing about 1,700 nucleotides, contained the complete open reading frame of rice UGPase. At the nucleotide-sequence level, the UGPase cDNA of rice endosperm had high homology with the UGPase cDNA of barley endosperm (84%) and potato tuber (71%). The calculated molecular weight (50 kDa) agrees with the value determined by SDS-PAGE (51 kDa). At the amino-acid sequence level, rice UGPase has high homology with the UGPase of barley (92%) and potato (85%). The enzyme contained conserved sequence elements which are thought to be involved in substrate binding and catalytic activity. A Southern-blot analysis indicated that the gene existed as a single copy. Expression of the enzyme in rice endosperm examined by Northern-blot analysis was high at 10–15 days after heading.

Keywords UDP-Glucose pyrophosphorylase (UGPase) · Endosperm · cDNA cloning · Rice (*Oryza sativa* L.)

Introduction

UGP-glucose is a key metabolite for carbohydrate biosynthesis in non-photosynthetic tissues of plants, such as developing endosperm, root and tuber tissue, to which sucrose is translocated and stored as starch (Keeling et al. 1988; Kleczkowski 1994). The conversion of sucrose to starch is generally considered to begin with a reversal of the sucrose synthase reaction from sucrose and UDP to fructose and UDP-glucose. In the cytoplasm, UDP-glucose is degraded to glucose-1-phosphate and UTP by the reaction of UDP-glucose pyrophosphorylase, UGPase (EC 2.7.7.9). Hexose-phosphates or their derivatives, triose-phosphates, are transferred to the starch-synthesizing plastid, the amyloplast, by the activity of a translocator and are utilized for the synthesis of starch via ADP-glucose. (Echeverria et al. 1988; Keeling et al. 1988; Lin et al. 1988; Tyson and Ress 1988; Nakamura et al. 1989; Tetlow et al. 1994). UDP-glucose is also considered to be utilized as a substrate in the synthesis of starch in the amyloplast. Although, clear evidence of this has not been reported, there are several reports which show the involvement of UDP-glucose and UGPase in starch synthesis. Echeverria et al. (1988) reported that starch synthesis occurred when lysed amyloplasts of corn kernels were incubated in vitro with UTP. In developing rice endosperm, 90% of UGPase was found to be located in the cytosol and the rest was distributed in the amyloplasts and Golgi membranes (Kimura et al. 1992). In addition to being used in sucrose pathways, UDP-glucose is employed in the biosynthesis of cell-wall components including beta-glucans and cellulose (Kleczkowski 1994; Delmer and Amor 1995). In a previous experiment, we purified UGPase from rice endosperm, analyzed it by two-dimensional gel-electrophoresis (2D-PAGE), and determined its molecular weight and N-terminal amino-acid sequence (Gusti et al. 1999). The enzyme consisted of a single polypeptide of approximately 51 kDa. The cDNA of a plant UGPase was first isolated from a cDNA library of immature potato tuber and sequenced by Katsube et al. (1990). The potato enzyme had blocked N-terminal amino acids but no transit peptide. The structure of the active site of the enzyme was investigated using affinity labeling regents and site-directed mutagenesis in which Lys residues were converted to Gln residues (Katsube et al. 1991).

Until now, the complete cDNA of rice UGPase, the derived primary structure of the enzyme, and the expression of its mRNA have not been reported. Despite many studies it is still unclear whether UGP-glucose is directly

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involved in starch synthesis as a substrate in the developing endosperm. In this study, we successfully cloned the complete cDNA of rice UGPase from rice immature endosperm. Thus, the aims of this study were to elucidate the complete coding region of the mRNA, the putative primary structure of the rice UGPase, and the gene expression in rice endosperm during grain development. In addition, based on a comparison of the deduced and actual N-terminal amino-acid sequences, we discuss the possibility that rice endosperm UGPase is transported from the cytosol to amyloplasts.

Materials and methods

Plant materials

Rice plants (*Oryza sativa* L. cv Sasanishiki) were grown in a paddy field in the Yamagata University farm. Endosperm samples were collected at 10 days after flowering for preparing a cDNA library and at 5-day intervals from 5 to 35 days after flowering for a Northern-blot analysis. Samples were stored at –80 °C until use.

cDNA library construction and screening

All solutions were prepared using water treated with 0.1% diethyl pyrocarbonate (DEPC) to inactive ribonuclease. Total RNA was isolated from endosperm 10 days after flowering by the SDS-phenol method (Palmiter 1974). Poly (A^+) RNA was separated with a poly A tract system according to the specifications of the supplier (BIO-RAD, Tokyo, Japan). A cDNA library was prepared by inserting fragments in a λ ZAP-II vector (Stratagene, La Jolla, Calif., USA) between *Eco*RI and *Xho*I sites. The library was plated onto a 140 × 100 mm rectangular plate and the plaques were lifted with a nylon membrane (Hybond N, Amersham Buckinghamshire, England). A potato cDNA clone, pTUG (Katsube et al. 1991), which was labeled with digoxigenin-dUTP, was used as a probe. Hybridization and detection of positive plaques were carried out using a DIG-High prime nonradioactive DNA labeling and detection kit plus AMPPD [3-(2-spiroadamantane)-4-methoxy-4-(3-phosphoryloxy)phenyl-1,2-dioxethane, disodium salt] according to the specifications of the supplier (Boehringer Biochemical, Mannheim, Germany). The chemiluminescent signal was recorded with Kodak XAR-5 film. After the third screening, λ ZAP-cDNA clones were subcloned to the pBluescript SK vector through in vivo excision according to the instructions of the supplier (Stratagene, La Jolla, Calif., USA).

Nucleotide sequence analysis

One cDNA clone had a full length of 1.7 kb. This clone was digested with exonuclease III as described by Henikoff (1984). Two deletion fragments (1.25 and 0.85 kb) were obtained after the digestion. Nucleotide sequencing was carried out by the dideoxy chain-termination method (Sanger et al. 1977) using IRD-41-labeled M13 forward and M13 reverse primers and DNA polymerase (Thermo Sequenase, Amersham). Sequencing was done with a LI-COR 4000L sequencer (LI-COR, U.S.A) and sequence data were analyzed with Sequencher (Hitachi Software, Japan), DNASIS (Hitachi Software, Japan) and the CLUSTAL W algorithm (Thompson et al. 1994).

Northern and Southern analyses

All solutions and equipment were treated with DEPC. Total RNA was extracted from rice endosperm 5 to 35 days after flowering. Total RNA (20 μg for each sample) was separated by 1.2% (w/v) agarose-gel electrophoresis and transferred onto a positively charged nylon membrane (Roche Molecular Biochemicals). An antisense RNA probe was labeled by an in vitro transcription reaction with T7 RNA polymerase and dNTP containing digoxigenin-11-UTP. Hybridization with a digoxigenin-labeled RNA probe and detection were performed using a DIG Northern starter kit (Roche Molecular Biochemicals). Signal intensities of the transcripts were measured through scanning of the spots using a computer program, Diversity Database V. 1.1 (Toyobo, Japan). Genomic Southern hybridization was also performed using a digoxigenin-labeled DNA probe according to the instructions of the supplier (Roche Molecular Biochemicals). After hybridization, filters were washed at 68 °C in washing solution (0.5 × SSC and 0.1% SDS) for 15 min × 2. Detection of a genomic Southern blot was conducted using a DIG labeling and detection kit according to the instructions of the supplier (Roche Molecular Biochemicals).

Results and discussion

Cloning of UGPase cDNA

The rice endosperm cDNA library used in this study was previously constructed in λ ZAP phage. The library was plated on *Escherichia coli*, XL1-Blue MRF' cells. Approximately 50,000 phages from the rice endosperm cDNA library were plated and transferred onto nylon filters. Among the phages plated, six clones were isolated by screening using potato UGPase cDNA (pUTG: Katsube et al. 1991). These phage clones were subcloned into pBluescript SK(-) vector by in vivo excision. The insert sizes were 0.88 kb (three clones), 1.3 kb (two clones) and 1.7 kb (one clone) in six clones. The structure of the longest clone (1.7 kb) was determined by DNA sequence analysis. The 1.7-kb cDNA clone was named pRUG.

Nucleotide sequence analysis

The 1.7-kb cDNA clone which contained a complete open reading flame (ORF) of the UGPase was first sequenced using the M13 reverse primer. Thus, the 1.7-kb cDNA clone and its deletion clones (1.25 and 0.85 kb) were sequenced on both strands by the dideoxyribonucleotide chain-termination reaction method. A restriction map of the 1.7-kb insert of the cDNA clone is shown in Fig. 1. A single ORF of 1,407 nucleotides starting with the initiation codon ATG at position 68 and terminating

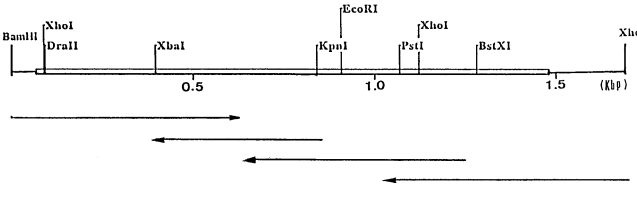

Fig. 1 Restriction map and sequence strategy of a rice UGPase cDNA fragment. Restriction sites are shown on the map. The *open bar* represents the coding sequences for the rice UGPase. *Arrows* show the direction and the extent of sequence determination

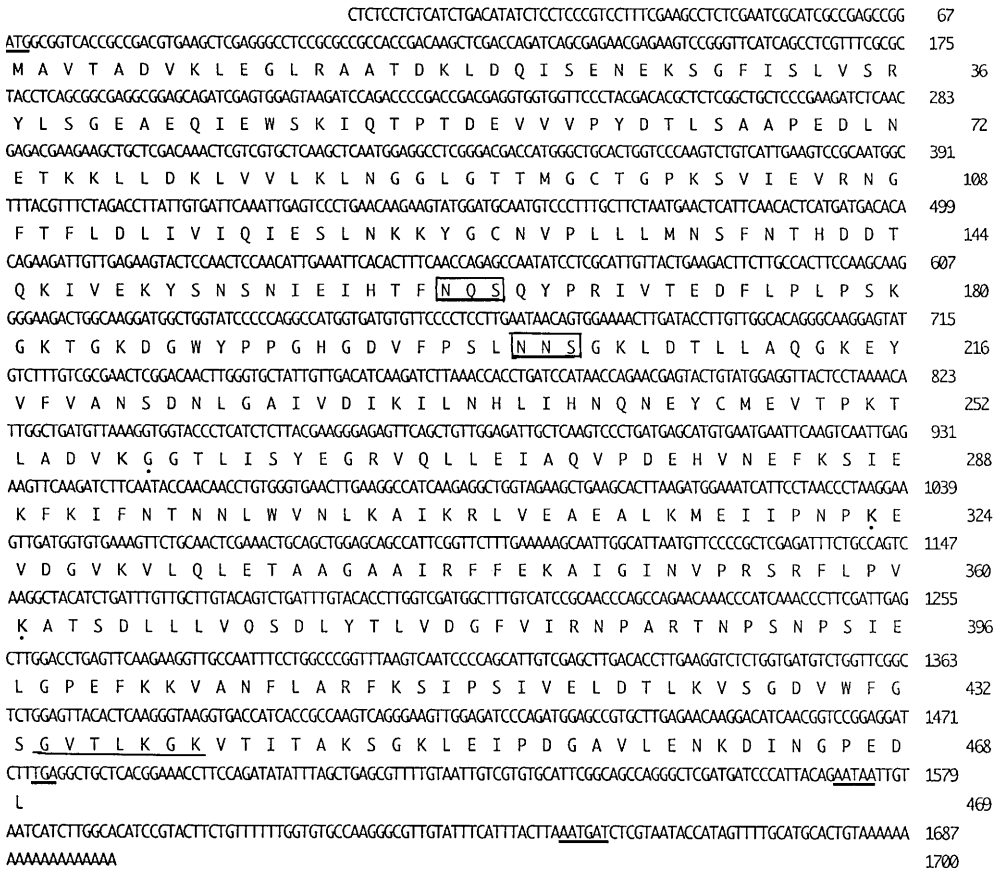

Fig. 2 cDNA and deduced amino-acid sequence of UGPase from rice endosperm. *Numbers* on the right are nucleotide base pair or amino-acid residues. The translation initiation, termination and polyadenylation signals are *underlined*. Motifs for putative N-glycosylation are *boxed*. The consensus sequence for putative nucleotide binding is *underlined*. Lys residues which are homologous to those of potato UGPase and which are assumed to be essential for substrate binding and catalytic activity are marked with a *dot*

in the stop codon TGA at position 1,475 was found (Fig. 2). A polyadenylation signal, AATAA, was found at position 1,571 and was 130 bp upstream from the polyadenylation addition site. Other than the above polyadenylation signal, the DNA sequence, AATGAT, which is a variant form of the polyadenylation signal, was also observed 35 bp upstream from the poly (A) site. In this experiment, we did not determine the transcription initiation site. However, we assume that the clone is a nearly full-length cDNA for the rice UGPase. At the cDNA level, rice UGPase has a high homology with the UGPase of barley (84%; Eimert et al. 1996) and potato (71%; Katsube et al. 1990).

Deduced amino-acid sequence of the rice UGPase

In the ORF of the rice UGPase (positions 68 to 1,474), cDNA is translatable to a polypeptide composed of 469 amino-acid residues (Figs. 2 and 3). The predicted molecular weight is 51.6 kDa. The deduced amino-acid sequence of rice endosperm UGPase was 92% homologous to that of barley and 85% homologous to that of potato. These high similarities suggest that these enzymes have a common evolutionary origin. In the N-terminal sequence of the derived protein, amino-acid residues 24–30 (E-N-E-K-S-G-F) are identical to the N-terminal amino-acid sequence of the polypeptide blotted onto the PVDF membrane after two-dimensional gel electrophoresis (Gusti et al. 1999). This suggests that the protein was processed after translation and that a proteolytic cleavage occurred between residues 23 and 24 (Fig. 2). The N-terminal 23 amino-acid residues were assumed to be a transit peptide. The molecular weight of the enzyme was calculated to be 50 kDa, which agrees with the value determined by SDS-PAGE (51 kDa) (Gusti et al. 1999).

Concerning the subcellular location of UGPase in rice cultured cells, immunocytochemical studies using antibodies against rice UGPase suggested that 90% of the enzyme is localized in the cytosol, while the remaining 10% is localized in the amyloplasts and Golgi membranes (Kimura et al. 1992). Another rice enzyme, ADP-glucose pyrophosphorylase (AGPase), is thought to be transported to chloroplasts or amyloplasts, and the putative transit peptide is estimated to have 28 amino-acid residues (Anderson et al. 1989). However, in the developing endosperm of maize, 95% of the total AGPase activity was found to be extra-plastidial (Denyer et al. 1996). These reports suggest that AGPase and UGPase in cereal endosperm each exist in two forms, cytosolic and plastidial forms. They also suggest that a part of the UGPase in rice developing endosperm is transported to amyloplasts, although there is evidence that most of the rice UGPase is present in the cytosol (Nakamura et al. 1989; Kimura et al. 1992).

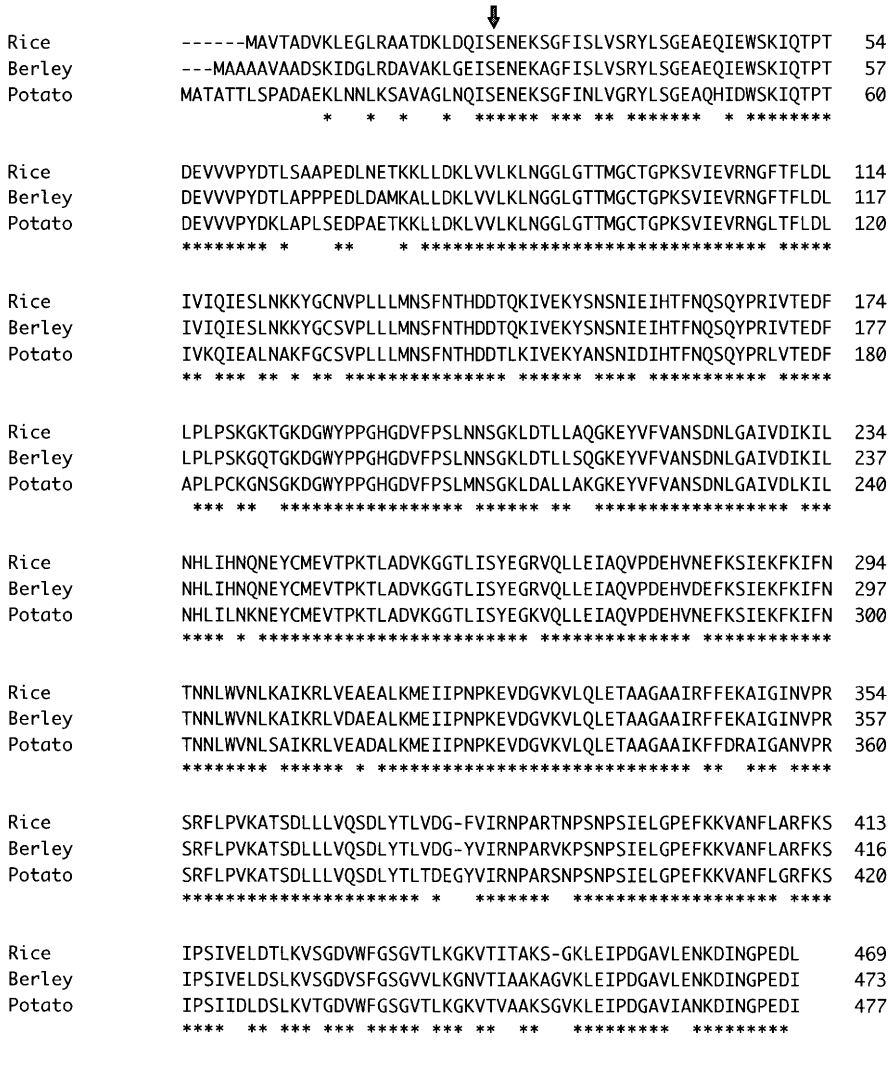

Fig. 3 Alignment of deduced amino-acid sequences of UGPase from rice, barley (Eimert et al. 1996) and potato (Katsube et al. 1990). *Gaps* (-) were introduced to achieve maximum homology. Identical amino acids among the three enzymes are indicated by *asterisks*. The *arrow* designates the putative proteolytic cleavage site identified by N-terminal amino-acid sequence analysis (Gusti et al. 1999)

Catalytic, nucleotide-binding and glycosylation sites

Several nucleotide-binding enzymes have a consensus sequence of G-X-X-X-X-G-K, where X represents an unspecified amino-acid residue (Walker et al. 1982; Fry et al. 1986). We found this consensus sequence at positions 434–440 in rice UGPase. Lys-440 with a preceding glycine-rich sequence is assumed to participate in nucleotide binding. On the other hand, from intensive studies of potato UGPase with affinity labeling regents and site-directed mutagenesis, Katsube et al. (1991) reported that Lys-367 is essential for the catalytic activity of potato UGPase, and Lys-263 and Lys-329 may participate in binding of the substrate. Rice UGPase has an identical positioning of Lys residues, Lys-257, Lys-323 and Lys-361, in rice and have the same relative positions as Lys-263, Lys-329 and Lys-367 in potato. These results suggest that Lys-361 is responsible for the catalytic activity and that Lys-257, Lys-323 and Lys-440 are responsible for substrate binding including nucleotide and sugar-nucleotide binding. Furthermore, plant UGPases are reported to have a membrane-bound activity (Kleczkowski 1994; Becker et al. 1995). The sequence involved in the glycosylation is N-X-S/T, where X stands for any amino acid other than Pro (Staehelin and Moore 1995). Rice UGPase has two such motifs, one at 162–164 (N-Q-S) and the other at 201–203 (N-N-S). In these sequences, the first N is a potential glycosylation site.

Genomic Southern-blot analysis of the rice UGPase gene

To investigate the copy number of UGPase in the rice genome, total DNA (2 μg) was digested with *Bam*HI or *Hin*dIII and hybridized with pRUG (Fig. 4). The probe hybridized to one 9.5-kb DNA fragment produced by *Bam*HI and one 3.0-kb DNA fragment produced by *Hin*dIII. Thus, the rice UGPase is a single-copy gene in the rice genome, although a weak band was observed at *Hin*dIII-2, in which the total DNA was digested by *Hin*dIII, suggesting the existence of a pseudogene.

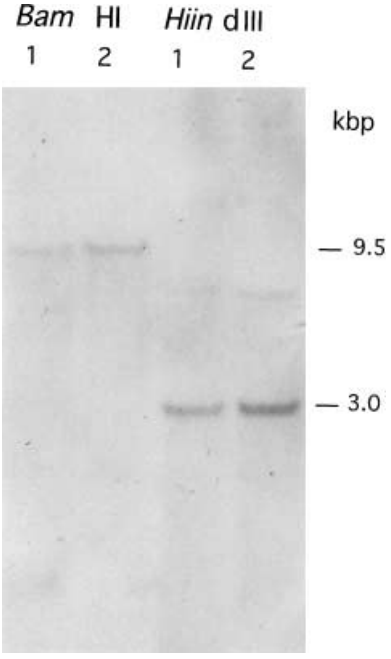

Fig. 4 Genomic Southern-hybridization analysis of rice total DNA (*1*: *japonica* cv Sasanishiki; *2*: *indica* cv Te-tep) digested with *Bam*HI and *Hin*dIII. Hybridization was performed with a probe which is specific for the coding region of rice UGPase (pRUG). The *numbers* on the right indicate the size of the fragments

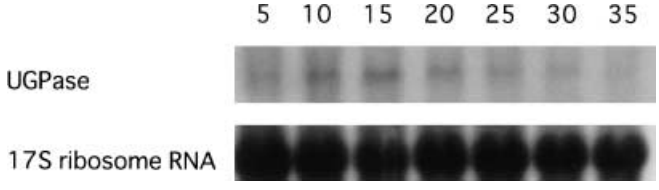

Fig. 5 Northern-blot analysis of transcripts of UGPase in rice endosperm. Total RNA was isolated from rice endosperm tissue at 5, 10, 15, 20, 25, 30 and 35 days after flowering. The upper column (*A*) shows the transcription levels of UGPase. The lower column (*B*) shows equal loadings of total RNA checked by the density of 17S ribosome RNA

Northern-blot analysis of rice UGPase mRNA expression

The expression of rice UGPase mRNA was examined by a Northern-blot analysis using a digoxigenin-labeled RNA probe of pRUG. The total RNA isolated from the endosperm of rice cv Sasanishiki, was used for the experiment. The relative abundance of rice UGPase mRNA was detected at 5-day intervals through seed development (Fig. 5) and was estimated from the intensity of the blots. The size of the transcript that hybridized with pRUG was 1.7 kb. The level of expression was high at 10–15 days after flowering, and then slightly decreased up to 35 days after flowering. This expression pattern was almost the same as that reported for AGPase in barley endosperm (Doan et al. 1999). The mRNAs of UGPase and AGPase might work in concert during seed development.

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