PERGAMON

Phytochemistry 62 (2003) 47–52

PHYTOCHEMISTRY

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Isolation and characterization of two fructokinase cDNA clones from rice☆

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Received 3 April 2002; received in revised form 29 August 2002

Abstract

Two cDNA clones, *OsFKI* and *OsFKII*, encoding fructokinase (EC 2.7.1.4) were isolated from immature seeds of rice (*Oryza sativa* L.) by PCR. *OsFKI* cDNA encoded a deduced protein of 323 amino acids that was 59–71% identical to previously characterized plant fructokinases. In contrast, *OsFKII* cDNA encoded a deduced protein of 336 amino acids that shared only 64% amino acid identity with OsFKI. The deduced proteins both possessed an ATP-binding motif and putative substrate recognition site sequences that were previously identified in bacterial fructokinases. Genomic DNA blot analysis also revealed that each fructokinase gene exists as a single copy in the rice genome. The identity of OsFKI and OsFKII as fructokinases was confirmed by the expression of enzyme activity in *E. coli*. Although both OsFKI and OsFKII utilized fructose as substrate, only OsFKII activity was strongly inhibited at a high fructose concentration. The mRNA corresponding to *OsFKII* accumulated at high levels in developing rice grains, whereas there were only low levels of *OsFKI* transcripts in immature seeds. These results indicate that fructokinase in rice endosperm is encoded by two divergent genes, which play different roles in rice grains for starch storage based on their sensitivity to substrate inhibition and level of transcripts in endosperm.

Keywords: Fructokinase; Rice (*Oryza sativa* L.); Gramineae

1. Introduction

Sucrose is the major carbon source for starch biosynthesis in reserve tissues such as potato tubers, tomato fruit and cereal grains. In these tissues, sucrose is hydrolyzed to UDP-glucose and fructose by sucrose synthase (SuSy), whose activity is correlated with starch synthesis (Wang et al., 1994; Asano et al., 2002). In SuSy-mediated metabolic pathways, the activities of SuSy and fructokinase are subject to feedback inhibition by free fructose, and effective fructose phosphorylation appears necessary for maintaining carbon flux toward starch formation and for respiration. Fructose can be phosphorylated by fructokinase (EC 2.7.1.4) or hexokinase (EC 2.7.1.1). Since fructokinase has a much higher affinity for fructose than hexokinase (Renz and Stitt, 1993), fructokinase may play a critical role in the sink metabolism of sucrose (Doehlert, 1989; Gardner et al., 1992; Martinez-Barajas and Randall, 1996; Schaffer and Petreikov, 1997; Kanayama et al., 1998; Guglielminetti et al., 2000). On the other hand, fructokinase may also be involved in a sugar-sensing pathway in plants, since a *mig* (mannose-insensitive germination) mutant of *Arabidopsis* lack the specific fructokinase activity of FrK2 (Pego and Smeekens, 2000).

In potato tubers, a cDNA encoding fructokinase was cloned (Smith et al., 1993) and its physiological role in starch synthesis investigated (Taylor et al., 1995). Two cDNA clones (*FrK1* and *FrK2*) encoding fructokinase were also isolated from tomato: *FrK1* is constitutively expressed throughout the entire tomato plant, while *FrK2* seems to be sink-specific and is present in source leaves only at low levels (Martinez-Barajus et al., 1997; Kanayama et al., 1997, 1998). In addition, tomato fructokinase gene expression is regulated by sugars in a

☆ The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank Nucleotide Sequence Database with the following accession number: *OsFKI*, AF429948; *OsFKII*, AF429947.

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0031-9422/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
PII: S0031-9422(02)00428-4

coordinated manner with other genes involved in sucrose-starch conversion (Kanayama et al., 1998).

Little is known about fructokinase in cereal grains. The only fructokinase analysis data available were obtained with maize, where three fructokinases differed with regard to their specificity for nucleoside triphosphates (Doehlert, 1990). As a first step toward investigating more fully the role of fructokinase in carbohydrate metabolism in rice grains, two cDNA fructokinase clones were isolated by PCR from rice endosperm and designated *OsFKI* and *OsFKII*. Their enzymatic properties and their transcript levels in endosperm were also examined.

2. Results and discussion

2.1. Isolation of OsFK cDNA clones

To obtain fructokinase cDNA clones from rice, four primers for RT-PCR were designed based on the nucleotide sequences of rice Expressed Sequence Tags (EST's) homologous to fructokinase genes from a few plant species. As a result, two bands of about 1100 and 750 bp were obtained with the primer pairs F1/F3 and F2/F4 (see Experimental), which were then purified, subcloned and sequenced. The resulting longer DNA fragments were 1135 bp (from primers of F1/F3) and 1164 bp (from primers of F2/F4), and defined as *OsFKI* and *OsFKII*, respectively. *OsFKI* cDNA contains an open reading frame (ORF) of 323 amino acids with a calculated molecular mass of 34,719 Da, whereas *OsFKII* cDNA contains an ORF of 336 amino acids with a calculated molecular mass of 35,515 Da. The same initiation codon (CAATGGC) in the two cDNA's matches the known consensus sequence of plant translational start sites (CXATGXC) (Lutche et al., 1987). In addition, the shorter sequences obtained from the PCR products had lost one or two fragments within the longer cDNA fragments (Fig. 1). Whether this is the result of post-transcriptional processing of fructokinase transcripts remains to be determined.

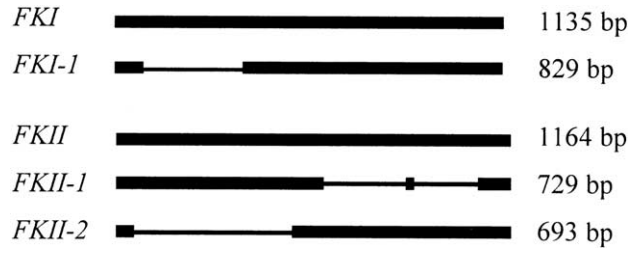

Fig. 1. DNA fragments obtained by RT-PCR. *FKI* was obtained using primers F1 (5′-TCT TTC ATC GCC TCT TGT GTT CG-3′) and F3 (5′-AAA AGG CAG GCA TCT TGG AGT ACC-3′), while *FKII* was obtained using primers F2 (5′-GTT TCG TTC TCG CTG TTG ATT TGT TC-3′) and F4 (5′-CCA AGC CCC TCC TCT TCC TTG TC-3′). The cDNA sequences are indicated as black boxes and missing nucleotides are shown as solid lines.

2.2. Amino acid sequence alignment

The deduced amino acid sequences from *OsFKI* and *OsFKII* cDNAs were aligned (Fig. 2) and the sequences of 12 plant fructokinases available were analyzed with the J. Hein method using DNASTAR software (Fig. 3). The 12 plant fructokinases were separated into three classes (Fig. 3). The OsFKI sequence, which was most similar to AtFK-AF387001 (71% identity) fell into Class I, while OsFKII was most similar to AtFK-AF096373 (75% identity) and was in Class II. OsFKI and OsFKII shared only 64% sequence identity. Two regions have been proposed to be conserved functional domains in fructokinase (Fennington and Hughes, 1996), and each domain is indicated in Fig. 2. The proposed ATP-binding motif (Domain A in Fig. 2), which contains the GD motif essential for activity, was conserved in the OsFKI and OsFKII sequences. Domain B, which was also found in the OsFKI and OsFKII sequences, is unique to fructokinases and has been thought to represent an Frc substrate recognition site. These sequence comparisons strongly suggest that the *OsFKI* and *OsFKII* cDNAs identified here encode fructokinases.

2.3. Southern blot analysis

DNA isolated from 14-day-old leaves of rice was used for genomic DNA blot analysis. Genomic DNA was digested with *Dra*I, *Eco*RI, *Eco*RV and *Xba*I, respectively. The digested DNA fragments were hybridized with *OSFKI* and *OSFKII* specific probes. A single band in each lane was observed with the *OsFKI* probe, suggesting the presence of a single copy in the rice genome. For *OsFKII*, a single fragment, except for the line of *Eco*RV, was generated, again suggesting a single copy of the gene (Fig. 4). Digestion with *Eco*RV yielded two bands. Lack of an *Eco*RV site within the *OsFKII* cDNA sequence indicated that an *Eco*RV cleavage site should be present within an intron of the *OsFKII* gene. The *OsFKI* cDNA probe hybridized to single restriction fragments that were distinct from those identified by *OsFKII*. These results indicate that two distinct fructokinase genes exist in rice.

2.4. Characterization of OsFKI and OsFKII products expressed in Escherichia coli

To determine whether *OsFKI* and *OsFKII* encode authentic fructokinase isoforms, these two genes were expressed in *E. coli* (BL21) and their enzymatic activity was tested at different concentrations of fructose and ATP. The total activities of these two fructokinases

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OsFKI      MA----------GRSELVVSFGEMLIDFVPTVAGVSLAEAPAFVKAPGGAPANVAIAVA 49
OsFKII     MAPLGDGAAAAAAAEPNLVVSFGEMLIDFVPDVAGVSLAESGGFVKAPGGAPANVACAIS 60
           **           ...:************** ********: .************ *::

OsFKI      RLGGGAAFVGKLGDDEFGRMLAAILRDNGVDDGGVVFDAGARTALAFVTLRADGEREFMF 109
OsFKII     KLGGSSAFVGKFGDDEFGHMLVDILKKNGVNAEGCLFDEHARTALAFVTLKSNGEREFMF 120
           :***.:*****:******:**. **:.***:  * :**  **********:::*******
                                                        B
OsFKI      YRNPSADMLLTHAELNVELIKRAAVFHYGSISLIAEPCRSAHLRAMEIAKEAGALLSYDP 169
OsFKII     YRNPSADMLLTEAELNLDLIRRAKIFHYGSISLITEPCRSAHVAAMRAAKSAGILCSYDP 180
           ***********.****::**:** :*********:*******: **. **.** * ****
                      B
OsFKI      NLREALWPSREEARTKILSIWDQADIVKVSEVELEFLTGIDSVEDDVVMKLWRPTMKLLL 229
OsFKII     NVRLPLWPSEDAARAGILSIWKEADFIKVSDDEVAFLTQGDANDEKNVLSLWFDGLKLLI 240
           *:* .****.: **: *****.:**::***: *: ***  *: ::. *:.**   :***:
                                                  A
OsFKI      VTLGDQGCKYYARDFRGAVPSYKVQQVDTTGAGDAFVGALLRRIVQDPSSLQDQKKLEEA 289
OsFKII     VTDGEKGCRYFTKDFKGSVPGFSVNTVDTTGAGDAFVGSLLVNVAKDDSIFHNEEKLREA 300
           ** *::**:*:::**:*:**.:.*: ************:** .:.:* * :::::**.**

OsFKI      IKFANACGAITATKKGAIPSLPTEVEVLKLMESA-- 323
OsFKII     LKFSNACGAICTTKKGAIPALPTVAVAQELISKAAN 336
           :**:****** :*******:*** . .  :*:..*
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Fig. 2. Comparison of the deduced amino acid sequences of the cDNA clones *OsFKI* and *OsFKII*. The sequences were aligned using the CLUSTAL program. ATP- (A) and sugar-binding domains (B), and identical (*) and conserved (.:) residues are indicated.

were increased 9.9- and 17.5-fold, respectively, at 2 mM fructose and 2 mM ATP, relative to baseline levels of *E. coli* fructokinase activity. On the other hand, the activity of glucokinase increased only slightly relative to baseline levels. These results confirmed that *OsFKI* and *OsFKII* encode fructokinase. Enzyme assays showed that the two activities were almost completely inhibited at 50 mM ATP. OsFKII activity, but not OsFKI activity, was inhibited by 50 mM fructose (Fig. 5a). In addition, the affinity of OsFKII for fructose was higher than that for OsFKI (Fig. 5b), with the respective K_m values for fructose of 3.3 mM (OsFKI) and 0.34 mM (OsFKII) calculated from Lineweaver–Burke plots.

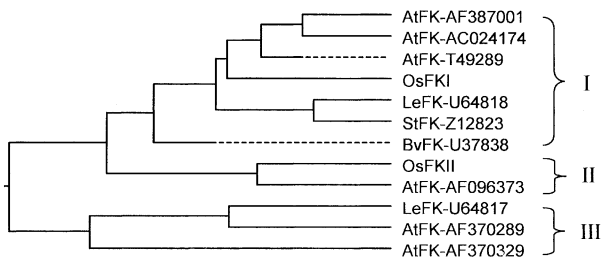

Fig. 3. Phylogenetic tree of the deduced amino acid sequences of twelve plant fructokinases (FK) using the J. Hein method in DNASTAR software. At, *Arabidopsis thaliana*; Bv, *Beta vulgaris*; Le, *Lycopersicon esculentum*; St, *Solanum tuberosum*. GenBank accession numbers are shown.

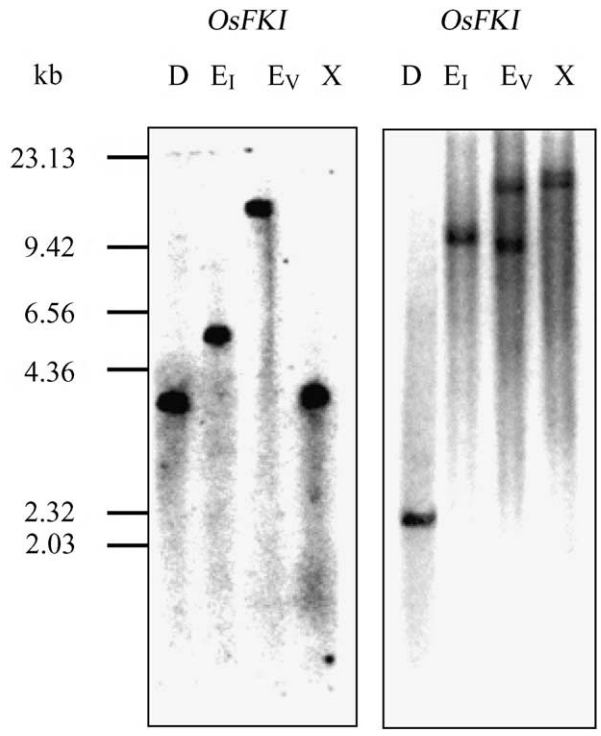

Fig. 4. Genomic DNA gel blot analysis. Genomic DNA (10 μg/lane) was digested with *Dra*I (D), *Eco*RI (E_I), *Eco*RV (E_V) and *Xba*I (X), and separated on 0.8% agarose gel, transferred to a Hybond N^+ nylon membrane and hybridized with gene-specific probes. The positions of *Hind* III-λDNA size markers are also indicated.

2.5. Analysis of fructokinase mRNA

The spatial expression patterns of *OsFKI* and *OsFKII* were investigated by Northern blot analysis and RT-PCR. The *OsFKI* transcripts were detected in root, endosperm and leaf tissues (Fig. 6A). In rice endosperm, there were much higher levels of the *OsFKII* transcript than the *OsFKI* transcript (Fig. 6B). The transcript of *OsFKII* was detected predominantly in root and endosperm and at only low levels in mature leaf (Fig. 6C). Developmentally, *OsFKII* was expressed as early as 3 days after flowering, but its expression level decreased at 8 days after flowering and then remained constant (Fig. 6D). The higher expression level in the endosperm and the property of substrate inhibition for OsFKII suggests that it may play an important role in sucrose metabolism and in starch accumulation in rice endosperm. In tomato (Kanayama et al., 1997, 1998), potato (Renz et al., 1993), sugar beet (Chaubron et al., 1995), and *Arabidopsis* (Gonzali et al., 2001), the findings regarding activity and gene expression seem to point to a sink-specific function for *FrK2*, whereas *FrK1* is thought to be primarily source-specific. The present results suggest that expression of both *OsFKs* in rice may be sink-specific.

3. Concluding remarks

The following three lines of evidence support the conclusion that *OsFKI* and *OsFKII* encode fructokinase in rice. The predicted gene products of *OsFKI* and *OsFKII* share 64% amino acid sequence identity and are 59–75% homologous to the fructokinases cloned from other plant species (Fig. 3). The two proteins contain conserved regions of specific domains characteristic of the ATP-binding motif and putative substrate recognition site sequences that have been identified in bacterial fructokinases (Fig. 2). We also examined the expression of fructokinase activity in *E. coli* (Fig. 5). OsFKII can be distinguished from OsFKI by its sensitivity to substrate inhibition and the higher level of transcripts in

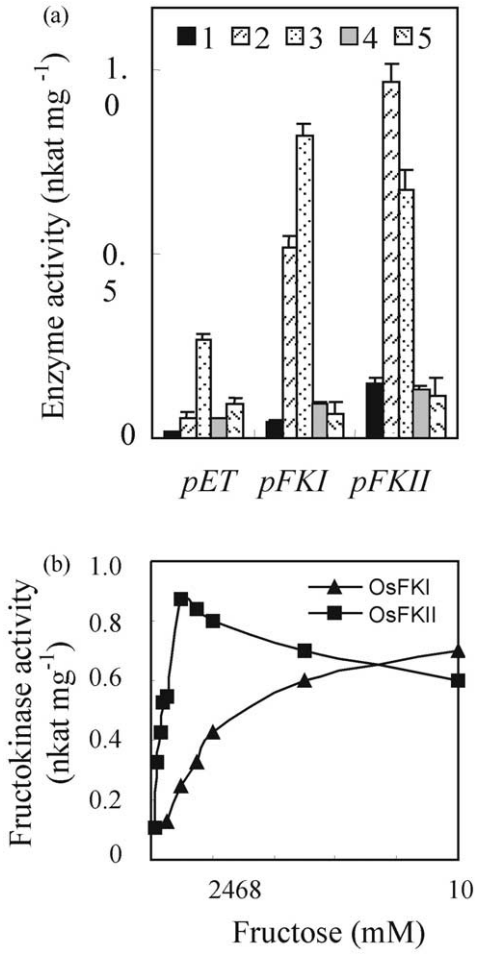

Fig. 5. (a) Characterization of the fructokinases expressed in *E. coli*. Gene expression was induced for 5 h in the exponential phase of *E. coli* cells transformed with the indicated plasmid. Total soluble extracts were assayed for fructokinase and glucokinase activity using different substrates. Quadruplicate analyses were performed and the mean and standard deviation are shown. The substrates used in the enzyme assays were: (1) 0.2 mM fructose and 2 mM ATP; (2) 2 mM fructose and 2 mM ATP; (3) 50 mM fructose and 2 mM ATP; (4) 4 mM glucose and 2 mM ATP; and (5) 2 mM fructose and 50 mM ATP. (b) Fructokinase activity of the expressed OsFKI and OsFKII in *E. coli* at various concentration of fructose. The baseline frucrokinase activities in *E. coli* transformed with pET-29b(+) were subtracted.

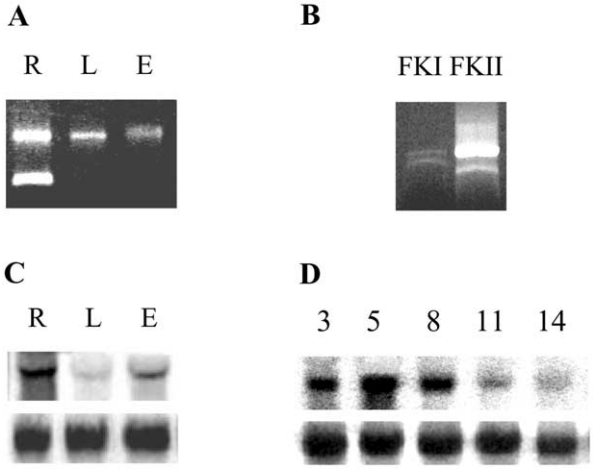

Fig. 6. (A) RT-PCR analysis of *OsFKI* transcript levels. R, root; L, leaf (5 days after flowering flag leaf); E, endosperm (5–10 days after flowering). cDNA was synthesized from total RNA extracted from the tissues indicated, and RT-PCR was performed as described in the text. The second band in lane R was about 300 bp long, and should be the product of nonspecific amplification. (B) RT-PCR analysis of the distinct abundance of *OsFKI* and *OsFKII* transcripts in rice endosperm. (C) Gel analysis of the tissue specificity of *OsFKII*. (D) Gel analysis of the expression of *OsFKII* in developing rice endosperms. Each lane is labeled with the number of days after flowering. Lower panel shows the normalization of total RNA levels in each sample by the hybridization of 18S rRNA on the same blot with a tomato 18S rDNA probe.

endosperm, which suggest that it may play a primary role in starch storage in rice grains.

4. Experimental

4.1. Plant material and growth conditions

Rice (*Oryza sativa* L., Zhe 733, indica) was grown under greenhouse conditions at 22/28 °C (night/day) and 80% relative humidity. Roots from 7-day old rice seedlings, leaves from 14-day old rice seedlings, flag leaves from 5 days after flowering and immature seeds from 3 to 14 days after flowering rice plants were harvested.

4.2. PCR amplification and sequencing

Complementary-DNA clones for rice endosperm fructokinases were isolated from polymerase-chain-reaction (PCR) products that were generated by primers designed from the nucleotide sequences of rice ESTs homologous to the fructokinases of *Arabidopsis*, potato, tomato, and sugar beet. Sense primers F1 (5′-TCT TTC ATC GCC TCT TGT GTT CG-3′) and F2 (5′-GTT TCG TTC TCG CTG TTG ATT TGT TC-3′), antisense primers F3 (5′-AAA AGG CAG GCA TCT TGG AGT ACC-3′) and F4 (5′-CCA AGC CCC TCC TCT TCC TTG TC-3′) were synthesized. PCR was carried out with the template of a cDNA library of immature rice seeds, and using 0.2 μM primers (primer pairs are F1/F3, F1/F4, F2/F3 and F2/F4, respectively), 100 μM dNTPs, and 1 unit Taq DNA polymerase. Amplifications were for 35 cycles, each consisting of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C. The resulting DNA fragments were gel-purified (QIAquick® Gel Extraction Kit, QIAGEN) and cloned into pUC-T plasmid. Sequence analysis was performed using MegaBACE™ 1000 (Amersham Pharmacia Biotech).

4.3. Genomic DNA blot analysis

Rice genomic DNA (10 μg) was digested with restriction enzymes, *Dra*I, *EcoR*I, *EcoR*V and *Xba*I, separated on 0.8% agarose gel, and blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia). The probe was prepared by random priming of the cDNA fragments of ORF, stringency used for hybridization and washing was the same as that described by the nylon membrane manufacturer's instructions. After washing, the blots were analyzed using Typhoon-8600 (Amersham Pharmacia Biotech).

4.4. Construction of expression vectors

The following primers were used for PCR modification of the putative full length ORF of *OsFKI* and *OsFKII*: FKI-E-sense (5′-gaa ggg ccc ata tgg cgg gga gga gcg ag-3′) and FKI-E-antisense (5′-gtg gtg ctc gag agc act ctc cat caa ctt caa gac c-3′), FKII-E-sense (5′-gaa ggg ccc ata tgg ctc ctc tcg gtg acg-3′) and FKII-E-antisense (5′-gtg gtg ctc gag gtt ggc tgc ctt gct gat gag-3′). The reconstructed sequences of *OsFKI* and *OsFKII* were subcloned into the *Nde*I/*Xho*I sites of pET-29b(+), sequenced, and defined as *pFKI* and *pFKII*, respectively.

4.5. Expression in E. coli

E. coli strain BL21 containing pET-29b(+), *pFKI* and *pFKII* were each grown in Luria-Bertani medium. Overnight cultures were inoculated into fresh medium at a 1:100 dilution and grown at 37 °C until the A_{600} was 0.6. IPTG was added to 0.5 mM and the cultures were grown for 5 h at 28 °C. Cells were collected by centrifugation, suspended in one-twentieth culture volume of sonication buffer (50 mM Tris–acetate, pH 7.5, 10 mM EDTA, and 5 mM DTT cocktail), and broken by sonication. Lysates were cleared by centrifugation at 10,000 *g* for 10 min, and the supernatants were used for subsequent analyses.

4.6. Fructokinase assays

Fructokinase activity was measured by an enzyme-linked assay. Assays contained, in a total volume of 100 μl, 30 mM HEPES-KOH (pH 7.5), 1 mM $MgCl_2$, 0.6 mM EDTA, 9 mM KCl, 1 mM $NADP^+$, 0.2 unit of $NADP^+$-dependent Glc 6-phosphate dehydrogenase, and 0.2 unit of phosphoglucoisomerase. The reaction was initiated with ATP at 2 mM, fructose at 0.1–50 mM, respectively, and fructose at 2 mM and ATP at 50 mM. Reactions were carried out at 37 °C and A_{340} was monitored continuously (Beckman-DU640). Similarly, the activity of glucokinase was measured with 4 mM glucose and 2 mM ATP in the absence of phosphoglucoisomerase. Fructokinase activity was expressed in katal units, which are equivalent to the conversion of 1 mol of fructose per second. K_m values for fructose were calculated from Lineweaver–Burke plots.

4.7. Northern blot and RT-PCR analysis

Total cellular RNA (20 μg) prepared from various rice tissues was separated on 1% formaldehyde-agarose gels, and transferred onto Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech). Total RNA was isolated using Trizol (GIBCOBRL) following the kit protocols. The probe was prepared by random priming of the ORF fragment of *OsFKII*. Hybridization was carried out at 42 °C for 16 h in 5×SSPE (0.6 M NaCl, 40 mM NaH_2PO_4, 5 mM EDTA, pH7.4), 50% formamide, 0.1% Ficoll 400, 0.1% PVP, 1% BSA, 0.5% SDS, 0.2 mg/ml of denatured salmon sperm DNA. After washing, the blots were analyzed using

Typhoon-8600. Subsequent hybridization of the blots was performed using an 18S rRNA probe to show the relative amount of RNA present in each lane.

The total RNA was used for reverse transcriptase polymerase chain reaction (RT-PCR) to quantify the RNA expression level of *OsFKs*. First-strand synthesis for RT-PCR was carried out using M-MuLV reverse transcriptase and oligo (dT) primers. Two μl of cDNA was used for the PCR reaction with the above primers (in 4.2). The program consisted of 35 cycles, each consisting of 30 s at 94 °C, 30 s at 60 °C, 1 min at 72 °C.

Acknowledgements

This work was financially supported by National Natural Science Foundation of China (39830250) and Natural Science Foundation of Zhejiang Province, China (ZA0106).

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