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Altered Sucrose Synthesis in Rice Plants with Reduced Activity of Fructose-6-Phosphate 2-Kinase/Fructose-2,6-Bisphosphatase

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Fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase (F2KP) is a bifunctional enzyme involved in regulating photosynthetic carbon metabolism by modulating the cellular level of fructose-2,6-bisphosphate (Fru-2,6-P_2) in plants. Rice appears to have two *F2KP* genes -- *OsF2KP1* and *OsF2KP2* -- on chromosomes 5 and 3, respectively. RT-PCR analysis showed that both genes are primarily expressed in the leaves, with transcripts somewhat less detectable in the flowers, roots, and seeds. To elucidate the functions of these OsF2KPs, we generated a *Tos17* insertion mutant of *OsF2KP1* and an RNAi mutant of *OsF2KP2*. In mutant plants, expression of the corresponding *OsF2KP*s was inhibited mainly in the leaves. At the end of day, sucrose contents had increased in the leaves of mutant plants while both fructose-6-phosphate 2-kinase activities and Fru-2,6-P_2 levels had declined. Our results suggest that the two OsF2KPs are involved in the regulation of sucrose synthesis by controlling the levels of Fru-2,6-P_2 during the daytime.

Keywords: fructose-2,6-bisphosphate, fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase, primary carbon metabolism, rice, sucrose

Fructose-2,6-bisphosphate (Fru-2,6-P_2) is a regulatory metabolite that controls primary carbon metabolism in all eukaryotes (Hue and Rider, 1987; Okar et al., 2001; Nielsen et al., 2004). The target metabolic step is an interconversion of fructose-6-phosphate and fructose-1,6-bisphosphate, which is a key component in controlling carbon flux (Hue and Rider, 1987; Stitt, 1990; Nielsen et al., 2004). In mammals and yeast cells, Fru-2,6-P_2 stimulates glycolysis and inhibits gluconeogenesis by acting as an activator of phosphofructokinase (PFK) and an inhibitor of cytosolic fructose-1,6-bisphosphatase (cFBPase) (Hue and Rider, 1987; Dihazi et al., 2001; Okar et al., 2001). In plants, Fru-2,6-P_2 is thought to be involved in the regulation of sucrose and starch synthesis by inhibiting cFBPase and activating pyrophosphate:fructose-6-phosphate 1-phosphotransferase (PFP), rather than by activating PFK, which is insensitive to Fru-2,6-P_2 (Stitt, 1990; Trevanion, 2002; Nielsen et al., 2004). In transgenic tobacco with increased levels of Fru-2,6-P_2, sucrose synthesis is diminished, whereas starch synthesis is enhanced (Scott et al., 1995). In contrast, the synthesis of sucrose is increased in transgenic tobacco and *Arabidopsis* when the level of Fru-2,6-P_2 declines (Scott et al., 2000; Draborg et al., 2001; Rung et al., 2004; Lee et al., 2006).

The formation and degradation of Fru-2,6-P_2 are catalyzed by a bifunctional enzyme -- fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatase (F2KP) (Nielsen et al., 2004). Plant F2KPs exist as homotetramers, with a subunit size of ~90 kDa (Larondelle et al., 1986; Villadsen and Nielsen, 2001; Markham and Kruger, 2002). Each subunit consists of a large, variable N-terminal region that is unique to plants, and a highly conserved catalytic C-terminal region of kinase and phosphatase (Nielsen et al., 2004). The cellular level of Fru-2,6-P_2 is controlled by the ratio of fructose-6-phosphate 2-kinase (F6P2K) and fructose-2,6-bisphosphatase (F26BPase) activities. Those ratios are allosterically regulated by several metabolites, such as fructose-6-phosphate, fructose-1,6-bisphosphate, pyruvate, and inorganic ortho- and pyrophosphates (Nielsen et al., 2004). In mammals, the enzyme (called PFK-2/FBPase-2) activities are also modulated by phosphorylation in response to certain hormones, e.g., insulin and glucagons (Bertrand et al., 1999; El-Maghrabi et al., 2001). For example, the insulin/growth factor-stimulated phosphorylation of heart PFK-2/FBPase-2 results in the binding of 14-3-3 proteins (Bertrand et al., 1999; Pozuelo Rubio et al., 2003). Recombinant *Arabidopsis* F2KP phosphorylated by protein kinases is bound to 14-3-3s (Kulma et al., 2004). The phosphorylation status of F2KP in *Arabidopsis* leaves depends upon their developmental stage, which is supported by the discovery of a highly enriched phosphorylated form of F2KP in young rosette leaves (Furumoto et al., 2001). However, the modulation of plant F2KP activity by phosphorylation has not yet been clarified.

Our homology search with *Arabidopsis F2KP* revealed that two *F2KP* homologues are present in rice. Therefore, to elucidate a role for those rice F2KPs in primary carbon metabolism, we examined the levels of metabolites, such as glucose, sucrose, and starch, in mutant rice plants with reduced F2KP activity.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

OsF2KP2-RNAi mutant plants were generated by transforming japonica rice (*Oryza sativa* cv. Dongjin). Rice seeds (cv. Nipponbare) carrying the insertion of a retrotransposon,

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Tos17, in *OsF2KP1* (NG0654) were obtained from the National Institute of Agrobiological Sciences (NIAS) (Tsukuba, Japan). A homozygous line of the *Tos17* insertion mutant of *OsF2KP1* (*osf2kp1*) was screened via genomic PCR, using *OsF2KP1*-specific primers (forward, 5′-TGTACGTGTCGCTCAAGATGGAGA-3′ and reverse, 5′-GACATGCTTCTCAGTGATGGCATC-3′) and the combination of a gene-specific forward primer and a *Tos17*-specific reverse primer (5′-CTGGACATGGGCCAACTATACAGT-3′). Seeds of wild-type and mutant rice plants were germinated and grown for 21 d in a growth chamber (23°C, 16 h light/8 h dark cycle). The seedlings were then transferred for further growth in the greenhouse (typically 30°C/20°C day/night, under a 14 h light/10 h dark cycle).

RNA Isolation and RT-PCR Analysis

Total RNA was prepared from various rice tissues by using Trizol reagent (Invitrogen, USA). First-strand synthesis was conducted with isolated RNAs, employing a first-strand cDNA synthesis kit (Roche, Germany) with an oligo-dT primer. The resulting first-strand cDNAs were used in RT-PCR reactions with gene-specific primers and control primers for *18S rRNA* and *actin*. To confirm the expression of rice *F2KP* genes in our mutant rice plants, we performed RT-PCR analysis under the following conditions: 5 min at 95°C; 27 cycles of 95°C for 1 min, 56°C for 1 min, 72°C for 1 min; then an additional incubation at 72°C for 7 min. These gene-specific and control primers included: *OsF2KP1*, 5′-TCAGAGACTGGAACCTGTTATC-3′ and 5′-GAATGCTTCTTTTTACAGTGATTC-3′; *OsF2KP2*, 5′- CACGGTGAAAGTTTAGATAATGT-3′ and 5′-GTCCATGAGTTTGTATCTTTTCTC-3′; *18S rRNA*, 5′-TCCATCTTGGCATCTCTCAG-3′ and 5′-GTACCCGCATCAGGCATCTG-3′; and *actin*, 5′-GGAACTGGTATGGTCAAGGC-3′ and 5′-AGTCTCATGGATACCCGCAG-3′.

Vector Construction for *OsF2KP2*-RNAi

A cDNA clone (E2263) containing full-length *OsF2KP2* cDNA in pBluescriptII SK+ was obtained from the NIAS DNA Bank. For the RNAi construct, a portion of *OsF2KP2* (1348 to 1731 bp in the cDNA) that showed low homology to *OsF2KP1* was amplified by using gene-specific primers selectively amplifying *OsF2KP2* from the cDNA clone E2263. These primers included: 5′-GCACTAGTGGCGCGCCAGTCCTGATTATGCGGAACAGACA-3′ and 5′-GCGAGCTCGCCTAGGAGTCCATATAGATGCAGT- TCTCTC-3′. The amplified fragment of *OsF2KP2* was inserted into a binary vector, JJ374, that contains a hygromycin resistance gene as a selective marker, in sense and antisense orientations, with separation by the rice Waxy-a intron under the control of the CaMV35S promoter.

Generation of *OsF2KP2*-RNAi Rice Plants

The RNAi construct of *OsF2KP2* was introduced into *Agrobacterium tumefaciens* strain LBA4404 by electroporation (Hoekema et al., 1983). To generate a loss-of-function mutant, the *Agrobacterium*-mediated transformation was performed according to the procedure previously reported (Lee et al., 1999).

Assay of F6P2K Activity and Determination of Fru-2,6-P_2 Content

F6P2K activity and Fru-2,6-P_2 content in rice leaves were determined by measuring the formation of Fru-2,6-P_2 according to the method described previously (van Schaftingen et al., 1982; Nielsen, 1992; Lee et al., 2006). The assay mixture (1 mL total volume) contained 50 µL crude extract, 50 mM Tris/acetate (pH 8.0), 5 mM MgOAc, 10 mM fructose-6-phosphate, 0.75 mM NADH, 4 U aldolase, 4 U triosephosphate isomerase, 4 U glucose-6-phosphate dehydrogenase, and 0.0087 U PFP from potato (van Schaftingen et al., 1982). One unit of F6P2K was defined as the capacity to generate 1 µmol of Fru-2,6-P_2 per min. Results were represented as the means ±SD of three individual plants.

Purification of Potato Tuber PFP

PFP was partially purified from potato tubers according to the method described previously (van Schaftingen et al., 1982), with minor modification. For PEG fractionation, 3 to 14% PEG 6000 fractionation was performed. The active fractions resulting from DEAE-Sepharose column chromatography were combined and concentrated to a small volume with an 80% saturation of ammonium sulfate. The PFP preparation was then stored in -20°C until use.

Extraction and Measurement of Metabolites

Leaf materials (~0.1 g) were collected at the end of night and the end of day, based on a 14 h light/10 h dark cycle. After the samples were ground with liquid nitrogen to a fine powder, their soluble sugars and starch were extracted according to the method described by Lu and Sharkey (2004). The contents of those two components were assayed according to the enzymatic method of Stitt et al. (1989), using a UV/Vis spectrophotometer (CARY300 Bio; Varian, USA). All results were represented as the means ±SD of three individual plants.

RESULTS AND DISCUSSION

Unlike a range of tissue-specific isoforms of F2KPs are present in mammals (Okar et al., 2001), it has been suggested that *Arabidopsis* F2KP is encoded by a single gene (Villadsen et al., 2000; Nielsen et al., 2004). However, blast searches of the amino acid sequence for *Arabidopsis* F2KP identified two *F2KP* homologues (designated as *OsF2KP1* and *OsF2KP2*) in the rice genome. *OsF2KP1* and *OsF2KP2* reside on rice chromosomes 5 and 3 and show 79 and 76% amino acid sequence similarities with the *Arabidopsis* enzyme, respectively (Fig. 1). As with other plant F2KPs (Nielsen et al., 2004), the rice F2KPs consist of a plant-specific N-terminal region and a highly conserved catalytic region (Fig. 1). The alignment of the deduced amino acid sequences for *Arabidopsis* and rice F2KPs revealed that the N-terminal regions are variable, with 53% to 62% similarity (39% to 50% identity), while the C-terminal regions are highly conserved, with 92% to 94% similarity (83% to 87% identity) (Fig. 1). The N-terminal region of plant F2KPs

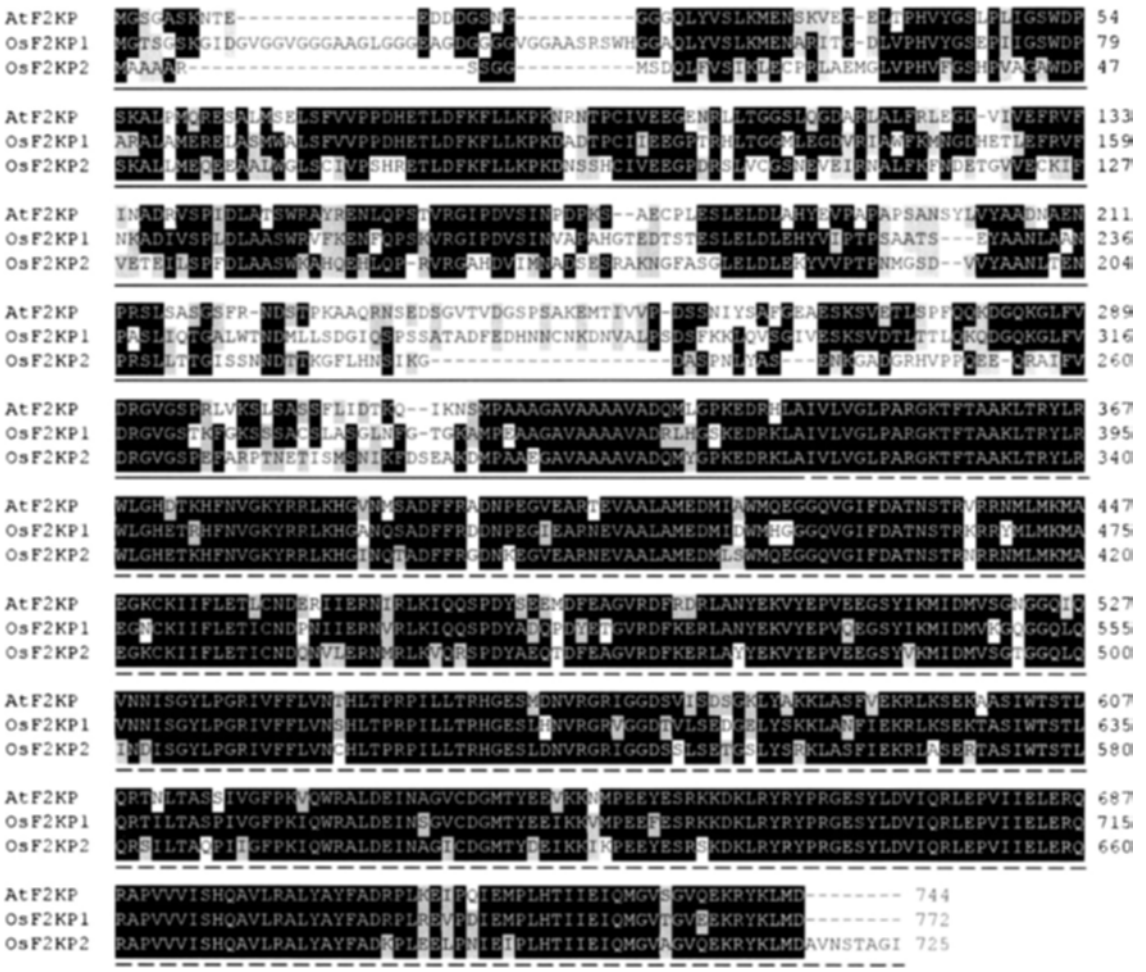

Figure 1. Alignment of deduced amino acid sequences for *Arabidopsis* F2KP (AtF2KP) and rice F2KPs (OsF2KP1 and OsF2KP2). N-terminal region and C-terminal catalytic region of F2KPs are underlined with solid and dashed lines, respectively. N-terminal region is variable, with 53% to 62% similarity (39% to 50% identity); C-terminal region is highly conserved, with 92% to 94% similarity (83% to 87% identity). Identical and similar amino acid residues are shown in black and gray, respectively.

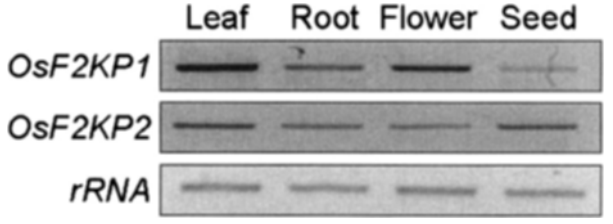

Figure 2. Expression of *OsF2KP*s in different tissues. RT-PCR analysis showed that both *OsF2KP1* and *OsF2KP2* were primarily expressed in leaves, but transcripts were also detected in roots, flowers, and seeds.

shares no homology with the N-terminal extension of mammalian PFK-2/FBPase-2s, and its function is unclear. However, Villadsen and Nielsen (2001) have demonstrated that this N-terminal region is important for subunit assembly, and also affects the kinetic properties of the enzyme.

To examine the expression of these two *OsF2KP*s in the leaf, root, flower, and seed, we performed RT-PCR analysis with primer sets to selectively amplify individual *OsF2KP*s. *OsF2KP1* transcripts were detected mainly in the leaves, with considerable expression also found in the flowers and roots, but relatively low transcript levels measured in the seeds (Fig. 2). For *OsF2KP2*, expression also was the highest in the leaves, with transcript amounts being lower, but fairly even among the roots, flowers, and seeds (Fig. 2). Northern blot analysis of potato *F2KP* produced similar expression patterns, i.e., greater levels of transcript in the leaves (primarily in source leaves) and flowers but little in the tubers (Draborg et al., 1999). Our results and the previous report (Draborg et al., 1999) are consistent with the fact that F2KP plays a role in coordinating primary carbon metabolism in plant leaves by controlling the cellular levels of Fru-2,6-P_2 (Scott et al., 1995; Draborg et al., 2001; Nielsen et al., 2004; Rung et al., 2004).

To elucidate the function of these two OsF2KPs in regulating primary carbon metabolism, we produced mutant rice plants with suppressed expression of either *OsF2KP1* or *OsF2KP2*. A plant (*osf2kp1*) with insertion of *Tos17* in the third exon of *OsF2KP1* was isolated from the *Tos17* mutant populations (http://pfg101.nias.affrc.go.jp/~miyao/pub/tos17/index.html.en) (Fig. 3A). The homozygous *osf2kp1* mutant rice plant was screened by genomic PCR, using sets of

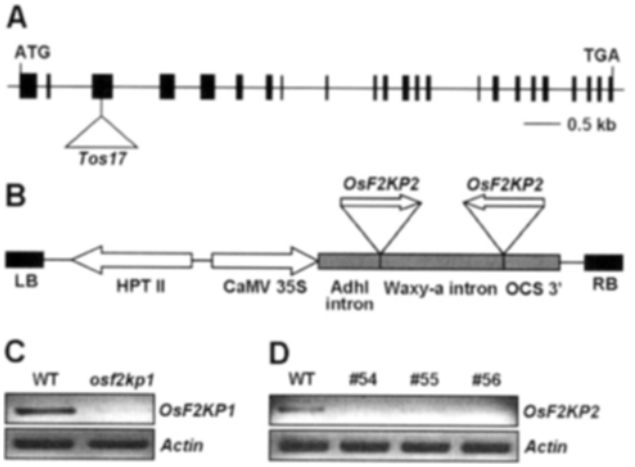

Figure 3. Generation of mutant rice plants with reduced F2KP activity, and expression of corresponding *OsF2KPs* in the mutants. (**A**) Insertion of retrotransposon, *Tos17*, in the third exon of *OsF2KP1*. (**B**) *OsF2KP2*-RNAi construct. The cDNA fragment of *OsF2KP2* (1348 to 1731 bp in cDNA) was inserted into binary vector in sense and antisense orientations, with separation by rice Waxy-a intron under control of CaMV35S promoter. (**C**) RT-PCR analysis showed that *OsF2KP1* expression was almost completely inhibited in leaves of *osf2kp1* mutant plants. (**D**) Three *OsF2KP2*-RNAi mutant lines (#54, #55, and #56) showed suppressed expression of *OsF2KP2* in leaves.

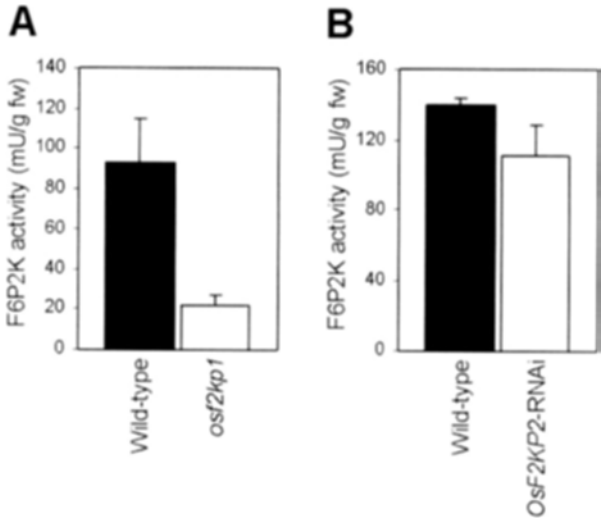

Figure 4. F6P2K activities were reduced to 23.3% and 79.6% of wild type in leaves of *osf2kp1* mutant (**A**) and *OsF2KP2*-RNAi mutant (**B**), respectively. *fw*, fresh weight.

OsF2KP1-specific primers and a combination of gene-specific and *Tos17*-specific primers. RT-PCR analysis showed that the *OsF2KP1* transcript was undetectable in the *osf2kp1* mutant leaves (Fig. 3C). By RNAi technique, we also generated loss-of-function mutants (*OsF2KP2*-RNAi) for *OsF2KP2* (Fig. 3B, D). Among the three *OsF2KP2*-RNAi mutant lines (#54, #55, and #56) that exhibited suppressing *OsF2KP2* expression in their leaves (Fig. 3D), we chose line #56 for further analysis. Although expression of the corresponding genes was almost entirely inhibited, 23.3 and 79.6% of F6P2K activity remained in the *osf2kp1* and the *OsF2KP2*-RNAi mutants, respectively (Fig. 4). This activity in the latter was most likely due to OsF2KP2 enzyme activity. Moreover,

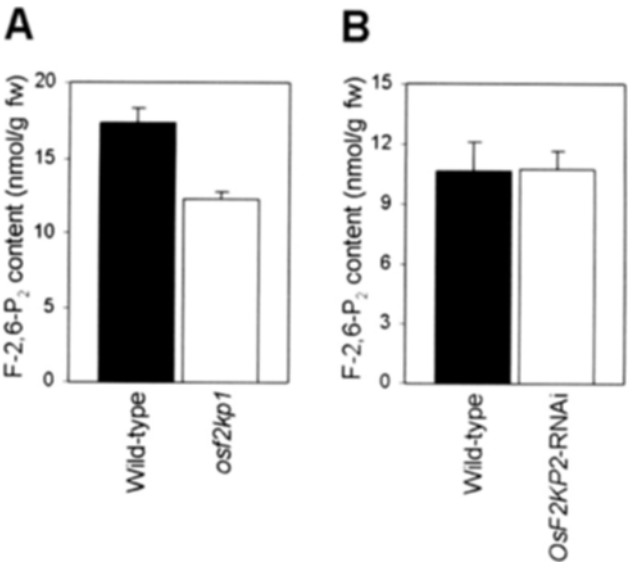

Figure 5. Fru-2,6-P_2 levels were decreased in *osf2kp1* mutant (**A**) but similar in *OsF2KP2*-RNAi mutant (**B**) plants when compared with wild type.

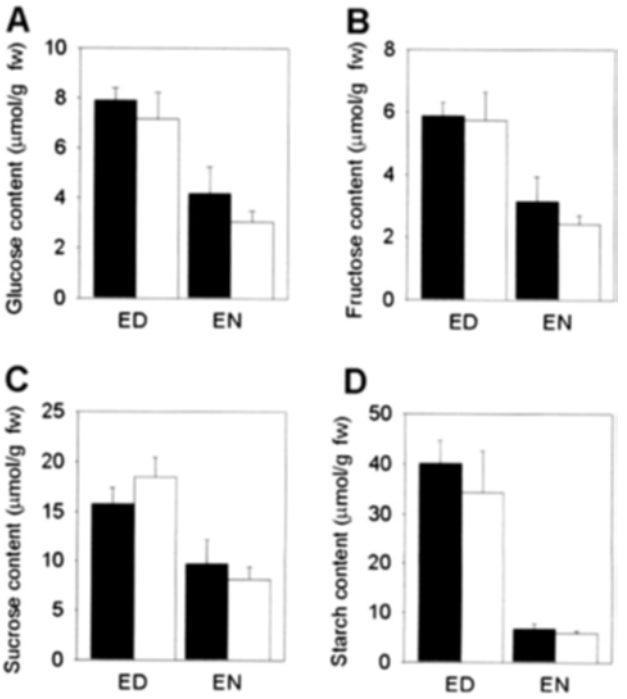

Figure 6. Analysis of metabolites in leaves of 6-week-old wild-type (black bar) and *osf2kp1* mutant (white bar) plants at end of 14-h day (ED) and end of night (EN): contents of glucose (**A**), fructose (**B**), sucrose (**C**), and starch (**D**).

compared with the wild-type, the level of Fru-2,6-P_2 decreased by ~30% in the *osf2kp1* plants, and to a similar degree in the *OsF2KP2*-RNAi plants (Fig. 5). These discrepancies in gene expression, residual F6P2K activities, and amounts of Fru-2,6-P_2 were presumably due to the redundancy of OsF2KPs. In transgenic *Arabidopsis* with reduced F2KP, enzyme activity is closely correlated with the level of Fru-2,6-P_2 (Draborg et al., 2001). In rice, both *OsF2KP1* and

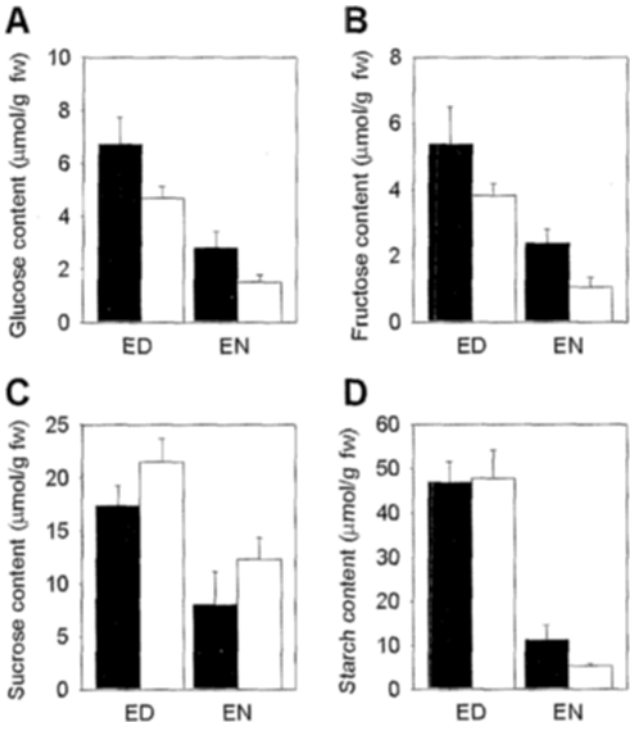

Figure 7. Analysis of metabolites in leaves of 6-week-old wild-type (black bar) and *OsF2KP2*-RNAi mutant (white bar) plants at the end of day (ED) and the end of night (EN): contents of glucose (**A**), fructose (**B**), sucrose (**C**), and starch (**D**).

OsF2KP2 were primarily expressed in the leaves (Fig. 2). These results indicate that, to some extent, the defect of one OsF2KP is compensated by another isozyme in mutant rice. Consequently, we observed no detectable changes in growth phenotypes of mutant plants with reduced activity of F2KP.

To examine the effects of this reduced F2KP activity on primary carbon metabolism, we determined the levels of metabolites in rice leaves. At the end of day (ED), sucrose contents in both the *osf2kp1* and the *OsF2KP2*-RNAi mutant plants were higher than in the wild type (Fig. 6C, 7C). This result is consistent with reports from transgenic *Arabidopsis* and tobacco leaves with reduced F2KP activity, in which sucrose contents increase during the light period compared with those of the wild-type plants (Draborg et al., 2001; Rung et al., 2004). At the end of night (EN), the sucrose content in *osf2kp1* mutant plants was slightly lower than in the wild type (Fig. 6C). However, sucrose levels were higher in the *OsF2KP2*-RNAi transgenics than in the wild-type plants (Fig. 7C). Based on these data, as well as previous reports that F2KP activity is reduced in transgenic *Arabidopsis* at the end of night without apparent effect (Draborg et al., 2001), we suggest that OsF2KPs may not contribute significantly to the regulation of carbon metabolism during the dark period. We also measured the contents of starch, glucose, and fructose in our transgenic rice plants (Fig. 6, 7). In the leaves of *osf2kp1* plants, metabolite amounts did not differ significantly from those found in the wild type (Fig. 6). Glucose and fructose levels were slightly decreased in *OsF2KP2*-RNAi transgenic plants, compared with the wild type (Fig. 7).

In summary, we have now determined that rice possesses two *F2KP* genes, unlike *Arabidopsis* has a single gene that encodes F2KP (Villadsen et al., 2000; Nielsen et al., 2004). A minor effect on Fru-2,6-P_2 levels and no changes on growth phenotypes in the mutant plants with reduced F2KP activity are likely due to the redundancy of OsF2KPs. Spatially, the expression patterns for *OsF2KPs* are found mainly in the leaves, and sucrose levels are increased in transgenic plants. These findings suggest that both OsF2KPs are involved in the regulation of primary carbon metabolism in rice leaves during the light period.

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