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Coordinated Regulation of the Genes Participating in Starch Biosynthesis by the Rice *Floury-2* Locus

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The recessive *floury-2* (*flo-2*) locus of rice (*Oryza sativa* L.), which is located on chromosome 4, causes a strong reduction in expression of the gene encoding an isoform of branching enzyme RBE1 in immature seeds 10 d after flowering. Mapping of the RBE1 gene demonstrated the localization on rice chromosome 6, suggesting that the wild-type *Floury-2* (*Flo-2*) gene regulates RBE1 gene expression in trans. However, reduced expression of the genes encoding some other starch-synthesizing enzymes, including another isoform of branching enzyme RBE3 and granule-bound starch synthase, was also found in the *flo-2* seeds. In spite of the low level of RBE1 gene expression in the immature seeds of the *flo-2* mutants, the RBE1 gene was equally expressed in the leaves of the wild type and *flo-2* mutants. Thus, these results imply that the *Flo-2* gene may co-regulate expression of some of the genes participating in starch synthesis possibly in a developing seed-specific manner.

Starch synthesis occurs in both photosynthetic and nonphotosynthetic tissues. In the former tissues such as leaves, starch is accumulated during the light period and then is hydrolyzed during the dark period. The nonphotosynthetic tissues, including seeds and tubers, also accumulate a large amount of starch as an important energy source in germination. The D-Glc polymer is believed to be synthesized through the enzymatic processes catalyzed by AGPP, starch synthase, and BE (Preiss, 1991).

To elucidate the molecular basis of starch synthesis in plants, starch-synthesizing enzymes have been identified, characterized, and cloned. In rice (*Oryza sativa* L.), the cDNA clones encoding AGPP, GBSS, SSS1, RBE1, and RBE3 have been isolated (Anderson et al., 1989; Mizuno et al., 1992, 1993; Okagaki, 1992; Baba et al., 1993). These five genes are all highly expressed in the developing seeds, although the genes coding for SSS1 and RBE1 are also expressed in the leaves (Baba et al., 1993; Kawasaki et al., 1993b). It has been reported that expression of the AGPP and BE genes is induced or elevated by treatment with Suc (Müller-Röber et al., 1990; Kossmann et al., 1991). This result suggests that nutrient flow may be a key step of regulatory stimulus for these genes (Lopes and Larkins, 1993). However, the regulatory mechanisms of the genes participating in starch synthesis are still unclear. Moreover, evidence for the existence of the transcriptional factor(s) controlling starch synthesis has not yet been provided.

Multiple forms of BE are present in endosperm tissues of various plants, including maize and rice (Preiss and Sivak, 1995). The role of each of the BE isoforms in vivo has been examined mostly by the use of endosperm mutants missing either of the BE isoforms. For instance, the lack of BE-II and RBE3 in the *amylose-extender* mutants of maize (Boyer and Preiss, 1978a) and rice (Mizuno et al., 1993), respectively, is implicated in the production of abnormally branched glucans in starch synthesis (Boyer and Preiss, 1978a; Baba and Arai, 1984; Yano et al., 1985). However, no mutant missing another isoform of BE (BE-I and RBE1 in maize and rice, respectively) (see Boyer and Preiss, 1978b; Mizuno et al., 1992) has been identified so far.

In this study, to examine the in vivo function of rice RBE1, we initially attempted to identify the rice RBE1-deficient mutants. Instead of the rice mutants completely lacking the RBE1 activity, we found that the recessive *flo-2* endosperm mutants exhibit an extremely reduced level of RBE1 in the immature seeds 10 DAF. This mutation also decreases the production of other starch-synthesizing enzymes, including RBE3 and GBSS. Moreover, RFLP analysis demonstrates that the RBE1 gene is located on rice chromosome 6, which is a different location than the *flo-2* locus (chromosome 4). Therefore, the wild-type *Flo-2* gene may modulate expression of the genes participating in starch synthesis in trans. A common regulatory system for the genes encoding starch-synthesizing enzymes in rice is discussed.

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Abbreviations: AGPP, ADP-Glc pyrophosphorylase; BE, branching enzyme; DBE, starch debranching enzyme; *flo-2*, *floury-2*; GBSS, starch granule-bound starch synthase; GluA-1, type I glutelin; RBE, rice branching enzyme; RFLP, restriction fragment length polymorphism; SPK, seed-specific protein kinase; SSS1, a form of soluble starch synthase.

MATERIALS AND METHODS

Materials

A rice cultivar, Kinmaze, and its mutant lines were grown in the agronomy farm of Kyushu University (Fukuoka, Japan) and the research farm of Mitsui Toatsu Chemicals, Inc. (Chiba, Japan). The cDNA clones encoding RBE1, RBE3, GBSS, SSS1, and SPK were isolated from a cDNA library prepared from immature rice seeds 10 to 15 DAF (Mizuno et al., 1992, 1993; Baba et al., 1993; Kawasaki et al., 1993a; Shimada et al., 1993). A cDNA clone for AGPP was isolated from the same library, using a PCR fragment as a probe. The PCR fragment was obtained by using 5′ and 3′ primers synthesized according to the known sequence (Anderson et al., 1989). A cDNA clone for GluA-1 (pREE61) was kindly provided by Dr. F. Takaiwa (Takaiwa et al., 1987).

RFLP Analysis

For RFLP analysis (Kishimoto et al., 1994), an F_2 population (144 plants) was generated by a cross between Kasalath (*indica* type rice, maternal parent) and FL134 (*japonica* type rice, paternal parent). Genomic DNAs prepared from leaves of the parental lines and F_2 plants were digested with each of several restriction enzymes, separated by electrophoresis on 0.8% agarose gels, and blotted onto nylon membranes (Hybond-N, Amersham). Southern blot hybridization was carried out using a 0.9-kb *Eco*RI/*Eco*RI fragment of the RBE1 cDNA (Mizuno et al., 1992). The recombination values between the RBE1 gene locus and the marker loci previously determined on a rice RFLP map (Saito et al., 1991) were estimated using a maximum likelihood method (Allard, 1956).

Western Blot Analysis

Rice seeds were ground in a solution containing 125 mM Tris-HCl, pH 6.8, 4% SDS, 4 M urea, and 20% glycerol in a mortar and pestle. The homogenate was kept overnight at room temperature and centrifuged at 9000*g* for 10 min. Proteins (20 μg) in the supernatant were separated by 10% (w/v) SDS-PAGE. The gels were stained with Coomassie brilliant blue R-250 (Sigma). For western blot analysis, proteins were separated by 7.5% (w/v) SDS-PAGE and transferred onto Immobilon-P polyvinylidene difluoride membranes (Millipore), using a fast semidry blotter (Biometra, Gottingen, Germany). After blocking with 1% BSA, the blots were probed by an antibody against rice RBE1, RBE3, GBSS, or DBE (Toguri, 1991; Mizuno et al., 1992, 1993). The immunoreactive proteins were detected using an ECL western blotting detection kit (Amersham).

Northern Blot Analysis

Total cellular RNA was prepared from immature seeds and leaves of rice plants as described previously (Kawasaki et al., 1993b). RNA was separated by electrophoresis on 1% agarose gels containing formaldehyde and then blotted onto nylon membranes (Hybond-N, Amersham). The blots were hybridized with cDNA probes that had been labeled with ^{32}P using a random primer DNA-labeling kit (Boehringer Mannheim). Hybridization was carried out at 60°C according to the manufacturer's protocol. The positive signals were analyzed using a Bio-imaging Analyzer BS2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Isolation of Nuclei and Nuclear Run-On Transcription

Nuclei were isolated from immature rice seeds 10 DAF based on the procedures of Takaiwa and Oono (1990) and Kodrzycki et al. (1989). The procedures for the nuclei isolation were carried out at 4°C. The immature seeds were ground to a fine powder in liquid nitrogen using a blender and homogenized in a nuclear extraction buffer (buffer A: 10 mM Mes-NaOH, pH 6.0, containing 10 mM NaCl, 5 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 2 mM DTT, 0.2 mM PMSF, 0.4 mM salicylhydroxamic acid, 10 μg/mL butylated hydroxytoluene, 0.6% Triton X-100, and 0.25 M Suc). The homogenate was filtered through two layers of Miracloth (Calbiochem) and centrifuged for 10 min at 2000*g*. The nuclear pellet was washed once in buffer A and resuspended in buffer B consisting of 5× buffer A (6 g) and Percoll (45 g). The suspension was centrifuged for 5 min at 4000*g*, and the white floating nuclei were collected and diluted with buffer A. The nuclei were pelleted by centrif-

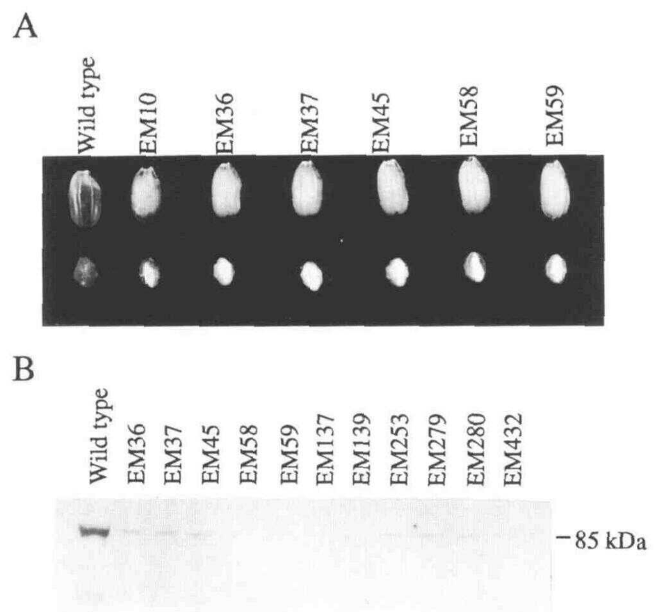

Figure 1. Endosperm mutants affecting RBE1 production. A, Mutant seeds forming floury endosperm. The endosperm mutants were prepared from a rice *japonica* cultivar, Kinmaze, by treatment of fertilized egg cells with *N*-methyl-*N*-nitroso urea. Among them, 11 mutants producing floury endosperm exhibited the reduced level of RBE1. Five of these 11 mutants are represented together with an *amylose-extender* mutant lacking RBE3 (EM10; see Mizuno et al., 1993). EM36 and EM37 were genetically characterized as *flo-2* (Satoh et al., 1984). B, A reduced level of RBE1 production in the mature mutant seeds. Proteins (20 μg) extracted from the wild type (Kinmaze) and 11 mutants were subjected to western blot analysis using an affinity-purified anti-RBE1 antibody.

ugation at 2000*g* for 10 min, resuspended in buffer C (25 mM Tris-HCl, pH 7.8, containing 0.25 M Suc, 10 mM $MgCl_2$, 2.5% Ficoll, 5.0% dextran, and 10 mM 2-mercaptoethanol), repelleted by centrifugation at 2000*g* for 10 min, and then suspended in buffer C containing 20% glycerol.

Procedures for nuclear run-on RNA synthesis were essentially similar to the method of Kodrzycki et al. (1989). Briefly, the reaction mixture contained 320 mM $(NH_4)_2SO_4$, 0.35 mM each ATP, CTP, and GTP, 4.3 μM UTP, and 500 μCi of [α-^{32}P]UTP. Freshly prepared nuclei in buffer C containing 20% glycerol were diluted 9-fold in the mixture. The DNA content was about 150 μg per reaction. The transcription reaction was carried out at 25°C for 20 min and followed by treatment with 40 μg/mL DNase I for 15 min. The mixture was adjusted to 5 mM Tris (pH 7.6), 0.5 mM EDTA, and 0.5% SDS and digested with 8 μg/mL proteinase K at 42°C for 1 h. The RNA transcripts were extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v). Unincorporated [^{32}P]UTP was removed by TCA precipitation and three precipitations with ethanol.

Plasmids (5 μg) containing cDNA inserts were linearized by restriction enzyme digestion and applied to nylon membranes (Hybond-N, Amersham). The membranes were hybridized to the isolated ^{32}P-labeled transcripts and washed according to the procedure of Giroux et al. (1994). Signals on the membranes were quantified using a Bio-imaging Analyzer BS2000. Values were standardized using the SPK signal as a control.

Determination of Soluble Sugar Contents

Immature rice seeds (0.1 g) were homogenized using a disposable grinder (Treff AG, Degersheim, Switzerland; catalog No. 34400) in water (1 mL) at 50°C and incubated at 80°C for 5 min to destroy activities of amylolytic enzymes. After the sample was centrifuged at 15,000 rpm for 5 min, the supernatant solution was used for measurements of Glc and Suc contents using a commercially available kit (Boehringer Mannheim) according to the manufacturer's protocol.

RESULTS

Identification of Endosperm Mutants Affecting RBE1 Production

Endosperm mutants were prepared from *japonica* rice, cv Kinmaze, by treatment of fertilized egg cells with *N*-meth-

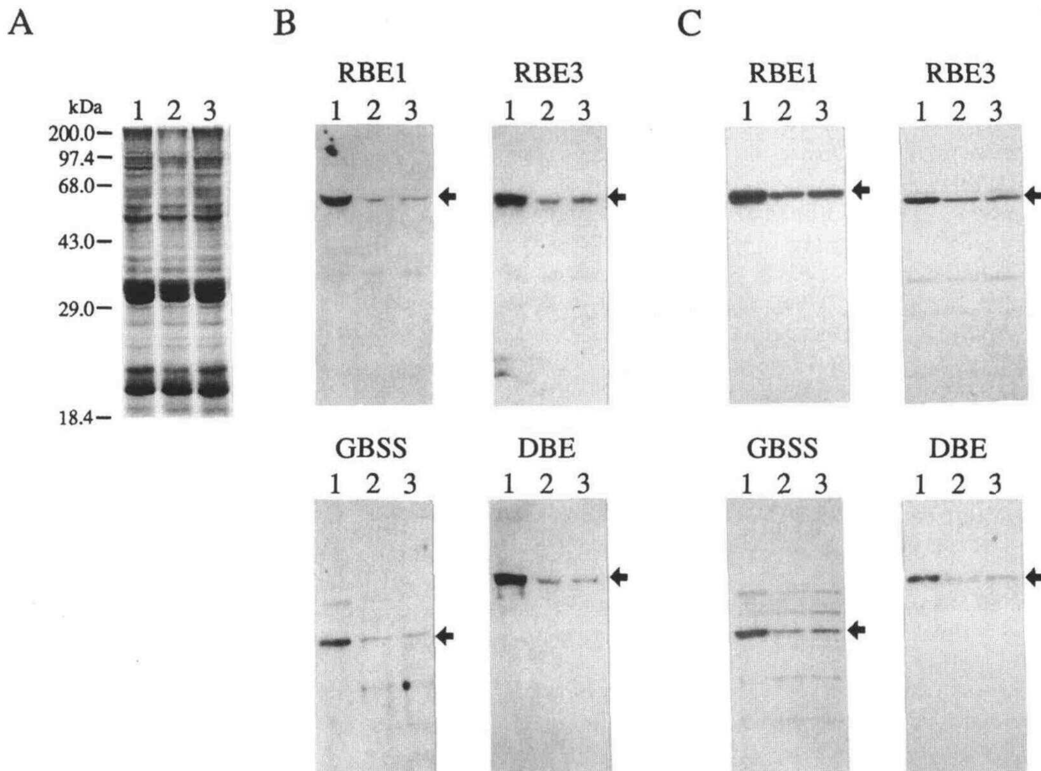

Figure 2. Reduced levels of starch-synthesizing enzymes in mature seeds and immature seeds 10 DAF of the rice *flo-2* mutants. A, SDS-PAGE analysis of total mature seed proteins. Proteins were extracted from the mature seeds of the wild type (1) and *flo-2* mutants EM36 (2) and EM37 (3), as described in "Materials and Methods." The extracted proteins (20 μg) were separated by 10% (w/v) SDS-PAGE and stained with Coomassie brilliant blue R-250. B, Western blot analysis of starch-synthesizing enzymes in the mature seeds. C, Western blot analysis of starch-synthesizing enzymes in the immature seeds 10 DAF. The extracted proteins (20 μg) from the mature seeds or immature seeds of the wild type (1) and *flo-2* mutants EM36 (2) and EM37 (3) were separated by 7.5% (w/v) SDS-PAGE and then subjected to western blot analysis. The blots were probed by affinity-purified anti-RBE1 or anti-RBE3 antibody or by antiserum raised against GBSS or DBE. The immunoreactive bands corresponding to the 85-kD RBE1, 87-kD RBE3, 60-kD GBSS, and 100-kD DBE are indicated by arrows.

yl-*N*-nitroso urea (Satoh and Omura, 1981) and established as independent mutant lines. To identify mutants affecting RBE1 production, screening of the mature seeds from 84 mutant lines, which formed floury endosperms, was carried out using an affinity-purified anti-RBE1 antibody as a probe. As described by Smyth (1988) and Mizuno et al. (1992), the mature rice seeds still contained sufficient amounts of BE. The RBE1 content was extremely low in 11 mutant lines (Fig. 1). Two of these 11 lines, EM36 and EM37, were genetically characterized as *flo-2* (Satoh et al., 1984). The mutations in the remaining 9 lines have not been tested for allelism with *flo-2*. The *flo-2* locus has been reported to be located on rice chromosome 4 (Kaushik and Khush, 1991). If the *flo-2* mutation has occurred in the RBE1 gene, the *flo-2* and RBE1 loci must be identical. Recently, Nakamura et al. (1994) reported that the RBE1 gene is located on rice chromosome 6. Our data regarding mapping of the RBE1 gene were consistent with theirs (data not shown). These results demonstrate that the *Flo-2* locus is not the structural gene coding for RBE1.

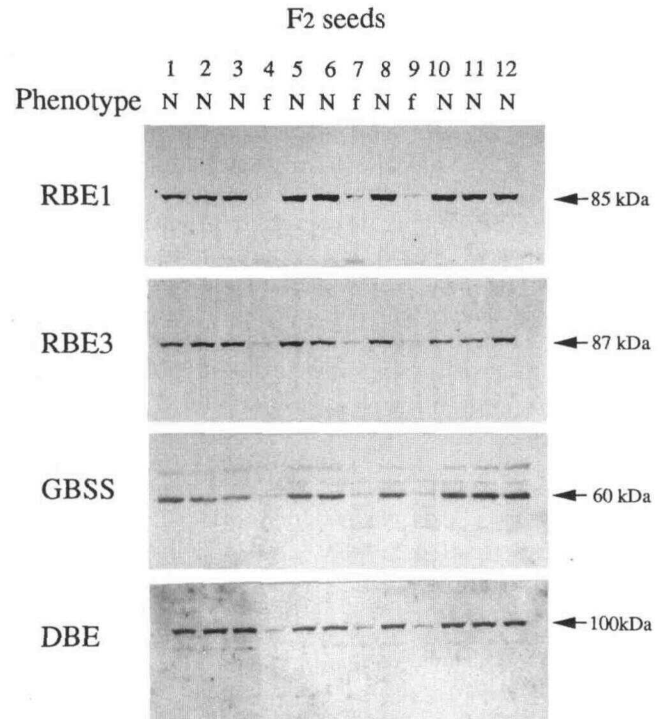

Figure 3. Co-segregation between phenotypic expression of *flo-2* and reduction of starch-synthesizing enzymes. The mature F_2 seeds were obtained by self-pollination of an F_1 plant (wild type × EM36 as the *flo-2* mutant line). Proteins (20 μg) extracted from the F_2 seeds were subjected to western blot analysis using an antibody against RBE1, RBE3, GBSS or DBE, as described in Figure 2. The phenotype of each endosperm (1–12) is indicated as N (normal endosperm) and f (floury endosperm).

Production of Starch-Synthesizing Enzymes in Mutant Seeds

To examine the effects of the *flo-2* mutation on accumulation of mature seed proteins, including starch-synthesizing enzymes, proteins were extracted from the wild-type and *flo-2* mutant seeds and analyzed by SDS-PAGE. No significant difference of the pattern of the seed proteins was found between the wild-type and *flo-2* seeds (Fig. 2A). When several enzymes involved in starch biosynthesis were examined by western blot analysis, the level of RBE1 was extremely low in the mature seeds of the *flo-2* mutants (almost 30% of that in the wild type, as determined by densitometric analysis; Fig. 2B). Unexpectedly, the levels of RBE3, GBSS, and DBE (Toguri, 1991) were also reduced in the *flo-2* mutants (approximately 40, 40, and 30% of those in the wild type, respectively). Moreover, western blot analysis was carried out to examine the levels of these starch-synthesizing enzymes in the immature seeds 10 DAF. The immature seeds of the *flo-2* mutants also contained the reduced levels of these enzymes, as in the case of the mature seeds (Fig. 2C).

To confirm the effects of the *flo-2* mutation on the production of starch-synthesizing enzymes, an F_1 plant was generated by crossing EM36 with the wild-type Kinmaze, and the F_2 individuals were examined. The F_2 population consisted of normal and *flo-2* seeds, segregating in a ratio of approximately 3:1 (184:66, normal seeds:*flo-2* seeds; $\chi^2 = 0.26$, $P > 0.5$). This supports the previous result that *flo-2* is a single recessive allele (Satoh et al., 1984). The F_2 seeds with the floury endosperms exhibited the reduced levels of RBE1, RBE3, GBSS, and DBE, whereas the levels of these enzymes were normal in the wild-type F_2 seeds (Fig. 3). These results indicate that phenotypic expression of *flo-2* co-segregates with the reduced levels of the starch-synthesizing enzymes. Thus, the reduced production of these enzymes is most likely due to the *flo-2* mutation.

Expression of the Genes Encoding Starch-Synthesizing Enzymes in the *flo-2* Mutants

Northern blot analysis was carried out using total RNAs prepared from immature seeds 10 DAF to verify whether the reduced production of starch-synthesizing enzymes in the *flo-2* mutants was due to a low level of gene expression. As a control experiment, we measured the mRNA level of GluA-1 (Takaiwa et al., 1987), which is one of the major rice seed storage proteins. The amount of GluA-1 mRNA was almost equal between the wild type and *flo-2* mutants (Fig. 4). As expected, the level of RBE1 mRNA was strongly reduced in the 10-DAF stage seeds of the *flo-2* mutants. The RBE1 mRNA level was estimated to be less than 20% of that in the wild type. The amounts of RBE3, GBSS, and AGPP mRNAs were also reduced in the *flo-2* mutants (approximately 50, 40, and 60% of those in the wild type, respectively). However, the mRNAs of SSS1 (Baba et al., 1993) and SPK (Kawasaki et al., 1993a) were at the same levels between the wild type and the *flo-2* mutants, as in the case of GluA-1.

Run-on transcription assays were carried out using nuclei prepared from the 10-DAF immature seeds of the wild type and a *flo-2* mutant, EM36 (Fig. 5). The radioactive RNA products were hybridized to the cDNA fragments encoding RBE1, RBE3, GBSS, SSS1, AGPP, SPK, and

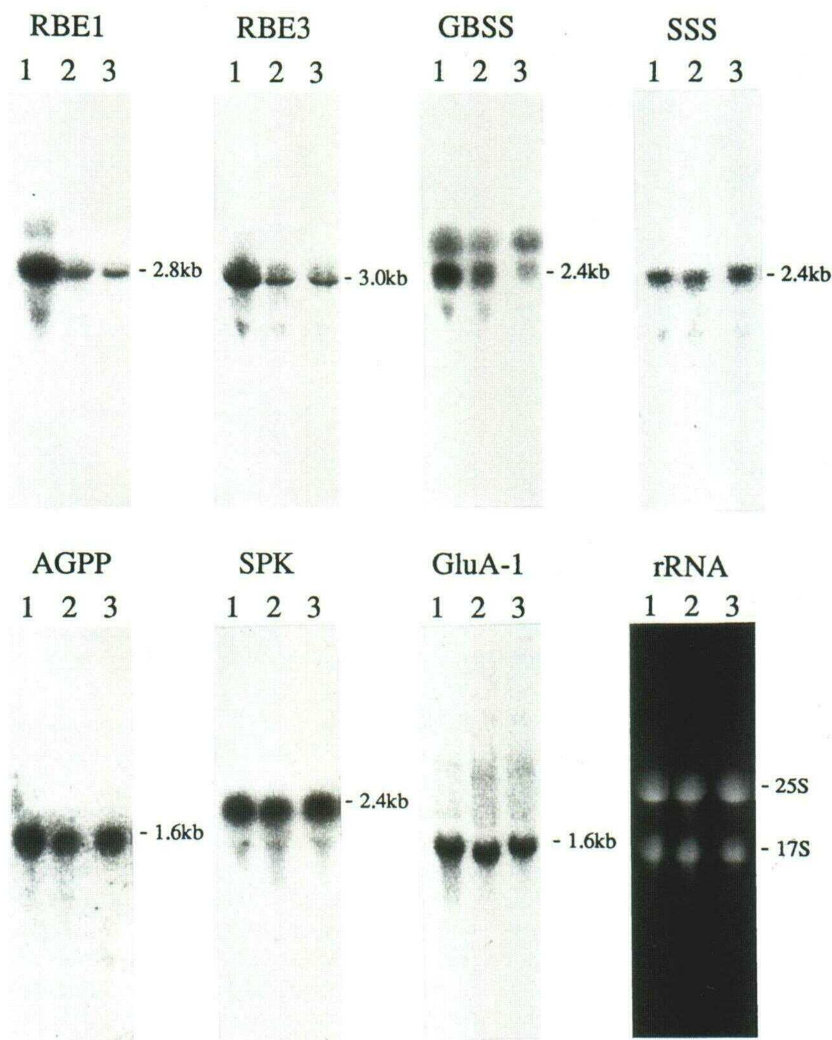

Figure 4. Expression levels of the genes encoding starch-synthesizing enzymes in the immature seeds of the *flo-2* mutants 10 DAF. Total cellular RNA (30 μg) from the immature seeds of the wild type (1) and *flo-2* mutants EM36 (2) and EM37 (3) were separated by agarose gel electrophoresis, stained with ethidium bromide, and transferred onto nylon membranes. The blots were then probed by the cDNA fragment encoding RBE1, RBE3, GBSS, SSS1, AGPP, SPK, or GluA-1. Rice 25S and 17S rRNA were used as internal standards.

GluA-1. The transcriptional activities of the genes encoding RBE1, RBE3, GBSS, and AGPP were reduced in the *flo-2* mutant, although the reduction ratios were slightly different from those found by northern blot analysis. The differences of the reduction ratios may be explained by the fact that the northern and the run-on transcription analyses have different denominators, since the northern and run-on transcription analyses were done on a per total RNA basis and a per nuclear DNA basis, respectively. Moreover, SSS1 gene expression was decreased in the *flo-2* mutant, which was not consistent with the data obtained by northern analysis. The reason for this discrepancy is unknown at the present time. At any rate, it is probable that reduction of the mRNA levels of starch-synthesizing enzymes in the *flo-2* mutants is due to the reduced transcription rather than the posttranscriptional controls.

The RBE1 gene is a single-copy gene in the rice genome and is expressed in the leaves and stems as well as in the developing seeds (Kawasaki et al., 1993b). However, the amount of the RBE1 mRNA in the developing seeds is much larger than those in the leaves and stems. In the present study, northern blot analysis demonstrated that the expression level of the RBE1 gene in the leaves was equal between the wild type and *flo-2* mutants (Fig. 6). Although we examined at a specific stage of seed development (10 DAF), these data appear to reflect the effects of the *flo-2* mutation that may be specific for the developing seeds.

Carbohydrate Contents in the *flo-2* Endosperms

Expression of the genes encoding starch-synthesizing enzymes has been reported to be enhanced by metabolizable carbohydrates such as Suc (Müller-Röber et al., 1990; Kossmann et al., 1991; Giroux et al., 1994). To examine whether the reduced levels of gene expression in the *flo-2* mutants are implicated in the levels of the metabolizable carbohydrates, the contents of Suc and Glc in the immature seeds 10 DAF were measured (Table I). These carbohydrate contents were almost equal in the the wild-type and *flo-2* mutant immature seeds. It is therefore unlikely that this *flo-2* mutation affects the levels of Suc and Glc.

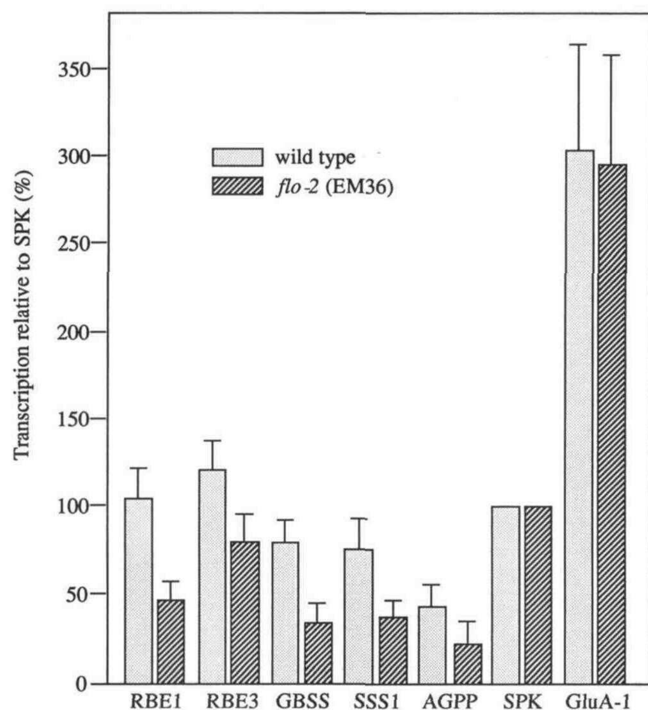

Figure 5. Transcription rates of the genes encoding starch-synthesizing enzymes in the immature seeds of the *flo-2* mutant 10 DAF. Run-on transcription assays were performed using the nuclei prepared from the immature rice seeds as described in "Materials and Methods." After digestion with restriction enzyme, plasmids containing cDNA inserts were applied to nylon membranes. The membranes were probed by the radioactive products from the run-on transcription reaction. The radioactivity was measured using a Bio-imaging Analyzer BS2000, and the values were standardized using the SPK signal as a control. Error bars indicate SDs ($n \geq 3$).

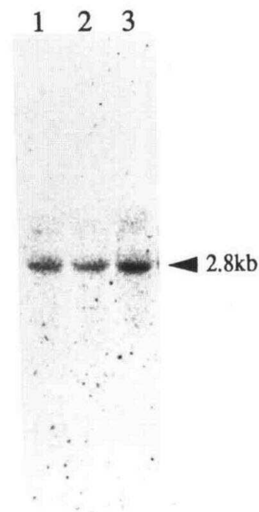

Figure 6. RBE1 gene expression in leaves. Total RNAs (30 μg) from leaves of the wild type (1) and *flo-2* mutants EM36 (2) and EM37 (3) were subjected to northern blot analysis using a cDNA fragment encoding RBE1 as a probe. Note that the signal for the RBE1 mRNA is found at the same level between the wild type and *flo-2* mutants.

DISCUSSION

In this study, we found that the rice *flo-2* mutation results in a drastic reduction of the RBE1 production in immature seeds 10 DAF (Figs. 1, 2, and 4). This mutation also causes reduced expression of the genes encoding some other starch-synthesizing enzymes (Figs. 2 and 4). In maize, the *flo-2* mutant has already been characterized as a mutant producing a floury endosperm. Although the rice *flo-2* mutants also produce the floury endosperm (Fig. 1), the rice mutant is distinguishable from the maize mutant because the maize mutation is mainly correlated with the formation of a storage protein, zein (Jones, 1978; Lopes et al., 1994; Coleman et al., 1995). Since the recessive *flo-2* mutants of rice were generated by using *N*-methyl-*N*-nitroso urea (Satoh and Omura, 1981), a point mutation(s) has likely occurred on the *Flo-2* locus, which may lead to no production or inactivation of a protein encoded by the *Flo-2* locus. However, it is also possible that the rice *flo-2* mutants are leaky mutants. At any rate, the *Flo-2* gene product appears to regulate expression of some genes participating in starch synthesis in trans, because the chromosomal localization of the rice *Flo-2* locus (Kaushik and Khush, 1991) is quite different from the rice genes encoding RBE1, RBE3 (E. Kobayashi, N. Kishimoto, and T. Baba, unpublished data), and GBSS (Saito et al., 1991).

Although the expression level of the RBE1 gene is equal in the leaves between the wild type and *flo-2* mutants (Fig. 6), this gene expression is drastically reduced in the immature seeds of the *flo-2* mutants 10 DAF. These data suggest that the Flo-2 protein may function to enhance RBE1 gene expression possibly in a seed-specific manner. Moreover, the genes for some other starch-synthesizing enzymes are also expressed at low levels in the *flo-2* mutant (Figs. 2 and 4). Thus, the Flo-2 protein may affect expression of different genes to different degrees. On the contrary, expression of the SPK and GluA-1 genes is not changed by the *flo-2* mutation (Fig. 4), indicating that the Flo-2 protein does not interact with these two genes as a gene regulator. We speculate that the Flo-2 gene may regulate coordinately some of the genes encoding starch-synthesizing enzymes in a developing seed-specific manner.

Metabolizable carbohydrates, including Suc, have been reported to induce coordinate expression of several starch synthetic genes (Müller-Röber et al., 1990; Kossmann et al., 1991; Giroux et al., 1994). Maize high-sugar mutants such as *shrunken-2* and *brittle-2* indicate coordinated elevation of gene expression of starch-synthesizing enzymes, including AGPP and GBSS, in the developing kernels (Giroux et al.,

Table I. *Carbohydrate contents in the immature rice seeds 10 DAF*

Values for Suc and Glc are given as percentages of fresh weight of the immature seeds 10 DAF. Values are the averages of two or more experiments.

Seeds	Suc	Glc
	%	%
Wild type	1.52 ± 0.18	0.53 ± 0.05
EM36	2.01 ± 0.15	0.66 ± 0.08
EM37	1.76 ± 0.10	0.50 ± 0.05

1994). Because the accumulation of Suc in the developing seeds and tubers leads to secondary effects such as the increment of osmotic stress, it is possible that the coordinated elevation arises through the secondary effects (Bhattacharyya et al., 1993; Giroux et al., 1994). In potato leaves, expression of the genes encoding AGPP and BE is also induced strongly by Suc (Müller-Röber et al., 1990; Kossmann et al., 1991). However, in the case of transgenic potato plants that suppress the production of AGPP and contain a large amount of Suc in the tubers, expression of the genes encoding another form of AGPP and two other starch-synthesizing enzymes (GBSS and BE) is not affected (Müller-Röber et al., 1992). Thus, it is still unclear at present whether Suc actually plays an important role in the regulation of the genes encoding starch-synthesizing enzymes in natural plant cells. Our results indicate that the Suc content in the immature seeds 10 DAF is almost equal between the wild type and *flo-2* mutants (Table I). Thus, reduced gene expression of some starch-synthesizing enzymes in the *flo-2* mutants appears not to be related to the Suc level.

In flavonoid biosynthesis, many mutants that impaired expression of the flavonoid biosynthetic genes have been identified in various plants, including maize and snapdragon (Martin et al., 1987; Dooner et al., 1991). Genetic analysis of these mutants has revealed that the coordinate regulation occurs in the flavonoid biosynthesis (Dooner, 1983; Martin et al., 1991). Characterization of the genes of the mutant loci demonstrated that these mutations result in the functional loss of transcriptional factors that regulate several biosynthetic genes (Paz-Ares et al., 1987; Ludwig et al., 1989; Grotewold et al., 1991; Goodrich et al., 1992). Therefore, the genes involved in the flavonoid biosynthesis probably possess a common regulational cascade for gene transcription (Grotewold et al., 1994; Sablowski et al., 1994). Starch granules are formed by the combined activities of starch-synthesizing enzymes in the amyloplast of seed endosperm during development. Since the simultaneous production of these enzymes is probably essential for starch synthesis, a co-regulational system for the genes encoding starch-synthesizing enzymes may be present; starch synthesis in plant storage tissues appears to be controlled by a cascade of genes whose expression is consecutively regulated. If so, an intriguing question is what gene in the cascade regulates the *Flo-2* gene. Moreover, *cis*-element(s) in the promoter region of the genes encoding starch-synthesizing enzymes, including RBE1, which may be regulated by the Flo-2 protein, remains to be identified.

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