Original Paper Plant Biotechnology, **21** (3), 183-189 (2004)

Molecular approaches for producing low-phytic-acid grains in rice

Xiaoge FENG and Kaoru T. YOSHIDA*

Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi Bunkyo-ku, Tokyo 113-8657, Japan

**Corresponding author* E-mail address: ayosida@mail.ecc.u-toko.ac.jp

Received 18 August 2003; accepted 12 April 2004

Abstract

Phytic acid (*myo*-inositol hexa*kis*phosphate) is the storage form of phosphorus in plant seeds. Reduction of phytic acid levels in seeds leads to both an increase in the availability of phosphorus and a reduction in the environmental load. *Myo*-inositol-1-phosphate ($Ins(3)P_1$) synthase (EC 5.5.1.4) catalyzes the first step in phytic acid biosynthesis. We attempted to reduce the phytic acid content of seeds through manipulation of the expression of the $Ins(3)P_1$ synthase gene. A rice $Ins(3)P_1$ synthase gene, *RINO1*, was transformed into rice plants in the antisense or sense orientation under the control of the CaMV 35S promoter or the *RINO1* promoter. Some T_1 seeds that transformed both with *RINO1* promoter::antisense *RINO1* gene and with *35S* promoter::sense *RINO1* gene increased the amount of inorganic phosphates, as compared to those of non-transgenic plants. This result suggested a molar-equivalent decrease in phytic acid. We also examined *RINO1* promoter activity using a β-glucuronidase (GUS) reporter gene. Finally, we discuss the strategy of molecular breeding to reduce phytic acid levels in seeds.

Key words: β-glucuronidase, *Myo*-inositol-1-phosphate synthase, Phytic acid, Promoter, Rice.

Abbreviations

GUS, β-glucuronidase; $Ins(3)P_1$, *Myo*-inositol-1-phosphate; *lpa, low-phytic acid*; NOS, nopaline synthase; Pc, inorganic P.

Introduction

Many human activities, including agricultural runoff and production of sewage, pollute lakes and marshes with phosphorus from fertilizers, human and domestic animal wastes, detergents and industrial sources (Berner and Berner, 1996). Influx of phosphorus into bodies of water leads to eutrophication. Plant seeds contain a major storage compound for phosphorus, phytic acid, which is the molecule inositol hexa*kis*phosphate. Up to 80% of total phosphorus in seeds is contained in phytic acid, and the remaining phosphorus consists of soluble inorganic phosphate and the cellular phosphorus contained in nucleic acids, proteins, lipids and sugars (Raboy, 1990). Phytic acid strongly binds various metal cations, such as K^+, Mg^{2+}, and Fe^{2+}, to form a salt mixture termed phytin (Ashton, 1976). Because monogastric animals digest phytin poorly, grains and legumes in feed are responsible for a large amount of phosphorus in waste matter (Ashton, 1976). Reducing the phytin content of seeds is a major breeding target, both to elevate the availability of nutrients and to reduce the environmental load.

Because phytic acid chelates positively charged cations, it causes mineral deficiency in animals, including human beings, when consumed in feeds and foods (Erdman, 1981). Iron deficiency is a serious global nutritional problem, especially in populations in which vegetable-based diets are dominant (Craig, 1994). To address the problem of iron deficiency in human populations, a transgenic rice variety that accumulates the soybean iron-storage protein ferritin in its endosperm tissues has been produced (Goto *et al.*, 1999). The bioavailable iron is dependent both on the intake and on its absorption. Even if increasing seed ferritin leads an increase in iron intake, the availability of iron is greatly reduced by the presence of phytic acid, a potential inhibitor of iron absorption (Gillooly *et al.*, 1983). To improve the availability of iron in foods, it would be desirable to reduce the phytin content of crop plants, including varieties such as the above transgenic rice.

Phytic acid is synthesized from 1L-*myo*-inositol

-1-phosphate (Ins(3)P_1) (**Fig. 1**). Ins(3)P_1 is formed from D-glucose-6-phosphate by the action of Ins(3)P_1 synthase (EC 5.5.1.4), a reaction that represents the first steps in inositol metabolism and phytic acid biosynthesis. Biochemical pathways from Ins(3)P_1 to Inositol (1,2,3,4,5,6) P_6 and enzymes catalyzing these steps are not fully understood. We isolated an Ins(3)P_1 synthase gene, *RINO1*, from rice, demonstrated that the accumulation of *RINO1* transcripts coincides well with the localization of phytin-containing particles, and also showed that this accumulation precedes the appearance of the phytin particles (Yoshida *et al.*, 1999). These findings suggest that Ins(3)P_1 synthase plays a key role in phytic acid biosynthesis in developing rice seeds. A reduction in the expression of the Ins(3)P_1 synthase gene in developing seeds might lead to a valuable reduction in the phytin content of seeds.

Recently, low-phytic acid (*lpa*) mutants have been isolated in maize, barley, rice and soybean (Raboy *et al.*, 2000, Rasmussen and Hatzack, 1998, Larson *et al.*, 1998, Larson *et al.*, 2000, Wilcox *et al.*, 2000, Hitz *et al.*, 2002). Most *lpa* mutants produce seeds that have normal levels of total P but reduced levels of phytic acid, accompanied by a near-molar increase in inorganic P (Pi) and/or lower levels of inositol phosphates. These findings imply that manipulation of the expression of phytic-acid-biosynthesis-related genes could lead to crop seeds with low levels of phytic acid and high levels of Pi, which could make highly nutritious food.

In this study, we attempted to reduce the phytic acid content of rice seeds through reduction of *RINO1* expression in transgenic rice.

Materials and Methods

Plant materials

Oryza sativa L. (cv. Yamahoushi) was used in this study.

Plasmid construction and transformation procedure

The *RINO1* cDNA, with a length of about 1,800 bp (Yoshida *et al.*, 1999), was ligated in the antisense orientation to a *RINO1* promoter fragment of about 2,160 bp (Yoshida *et al.*, 2002) and to transcription termination sequences derived from the *Agrobacterium tumefaciens* nopaline synthase (*NOS*) gene. The resultant gene cassette was named RINO1::anti. The *RINO1* cDNA was also ligated in the sense and antisense orientations to the CaMV 35S promoter and to the *NOS* terminator, and the resultant gene cassettes were named 35S::sense and 35S::anti. The 2,160-bp 5'-flanking region of the *RINO1* gene was fused to the β-glucuronidase (GUS) reporter gene followed by the *NOS* terminator to produce the gene cassette RINO1::GUS. To compare the CaMV 35S promoter activity with that of the *RINO1* promoter, we used pBI221, which contains the CaMV 35S promoter, the *GUS* gene, and the *NOS* terminator. The nucleotide sequences of the chimeric genes were determined using ABI PRISM 310 Genetic Analyzer. The above constructs were inserted into the binary vector pTF338 (Fukuda *et al.*, 1999) and transferred into *A. tumefaciens* strain C58 using the triparental mating method. A culture of *A. tumefaciens* that harbored this plasmid was used to infect scutellum-derived calli of rice, according to the method of Yokoi *et al.* (1998). Putative transgenic plants were selected on media containing 50 mg ml^{-1} hygromycin, transplanted into soil, and grown in a growth chamber.

Western blot analysis

Total soluble proteins from each immature seed at seven days after anthesis were extracted in 1 ml of extraction buffer [(0.5% (v/v) Triton X-100 in PBS, pH 7.4) : (1M Tris-HCl (pH6.8), 4% SDS, 20% glycerol, 0.01% BPB) = 1:1]. The extract was centrifuged for 10 min at 12,000*g* at 4℃ and the supernatant was used for Western blot analysis. Following SDS-PAGE (10% polyacrylamide gel), the separated proteins were transferred to PVDF membrane (Immun-Blot PVDF Membrane, Bio

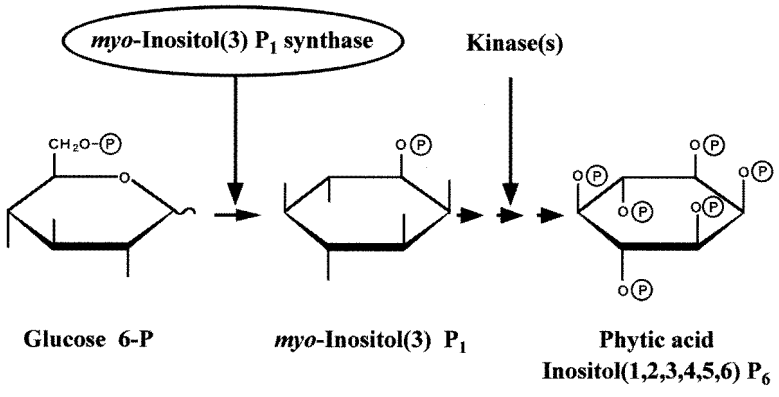

Fig. 1 Biosynthetic pathways to phytic acid.

Rad, Richmond, CA). RINO1 protein produced in *E. coli* was used to raise antibodies in a rabbit. The membranes were incubated with rabbit anti-RINO1 antiserum, and RINO1 protein was visualized with a goat anti-rabbit IgG alkaline phosphatase conjugate, according to the instructions provided by the supplier (ProtoBlot II AP System with Stabilized Substrate, Promega).

Analysis of seed phosphorus levels

All experiments were performed with self-fertilized T_1 seeds from T_0 plants. Dehusked seeds were dried for 24 h at 60℃. In the preliminary experiments, the experimental error in the measurement of phytic acid P (Raboy *et al.*, 1990) was rather large. In contrast, the value for Pi was stable among the control non-transgenic seeds. We then measured Pi, which represented the storage form of phosphorus in the transgenic seeds.

Each seed was weighed, crushed and extracted in 12.5% (w/v) trichloroacetic acid + 25 mM $MgCl_2$. Following centrifugation (12,000*g*, 10 min), the Pi in the supernatant was assayed colorimetrically as described by Chen *et al.* (1956). Seed total P was determined as described by Raboy *et al.* (2000). Each seed was weighed, crushed and total P was extracted by the wet-ashing method, and then a colorimetric assay was performed.

Promoter activities

Panicles at the spikelet differentiation stage, flowers just before heading, immature seeds at seven or fourteen days after anthesis, and seedlings at two or four days after germination were collected and fixed in 0.3% (w/v) formalehyde/10 mM MES/0.3 M mannitol. Histochemical analyses of GUS activity were performed according to Jefferson (1987). To observe GUS activity in leaves of seedlings at four days after germination, the leaves were embedded in 5% agar and 100-200 μm sections were cut. The sections were dehydrated through a graded ethanol series and cleared with clearing fluid (Herr, 1982). Flowers at the spikelet differentiation stage were also observed after the clearing treatment to determine the precise location of GUS expression.

Results

Production of transgenic rice plants

Twenty-one, eleven, and eight hygromycin-resistant independent lines were obtained from transformations with the RINO1::anti, 35S::anti, and 35S::sense constructions, respectively. None of the rice plants containing these constructs showed any difference in morphology, as compared to non-transgenic rice plants. Self-pollinated T_1 seeds were obtained from the transgenic T_0 plants.

RINO1 protein in transgenic seeds

The expression of the *RINO1* gene in transgenic plants was assessed by immunoblotting. As copies of the transgenes were expected to separate in the T_1 seeds, we analyzed seeds individually. Total protein homogenates of T_1 immature seeds from some *RINO1*::anti lines (RINO1-A), *35S*::anti lines (35S-A), *35S*::sense lines (35S-S) and non-transgenic seeds at seven days after anthesis were analyzed by Western blotting using RINO1 antiserum. The RINO1 polyclonal antibody detected a band of about 56 kDa (**Fig. 2**). RINO1 protein levels varied between different lines and among individual T_1 seeds. This result suggested that the repression of the *RINO1* gene was mediated by production of the antisense *RINO1* mRNA in RINO1-A-16 and 17, and that co-suppression occurred in line 35-S-6. In contrast, some seeds of the 35S-S-2 line had a higher level of RINO1 protein than did control seeds, indicating possible overexpression of the *RINO1* gene.

Phosphorus in transgenic plants

Seeds of non-transgenic rice had Pi concen-

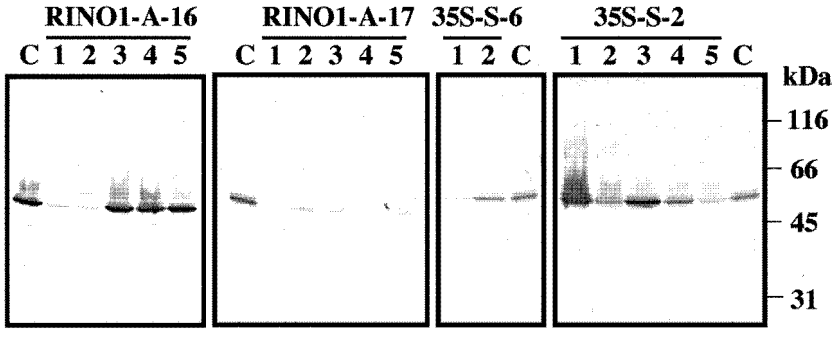

Fig. 2 Immunoblot analyses of RINO1 proteins in transgenic T_1 seeds of *RINO1*::antisense (RINO1-A) lines and of *35S*::sense (35S-S) lines. Each lane contains total proteins in 10 mg of each seed at seven days after anthesis.

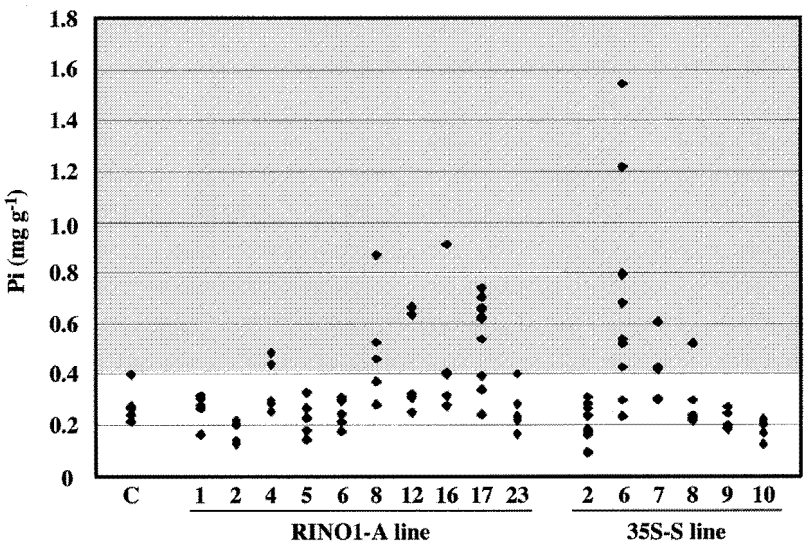

Fig. 3 Comparison of Pi contents in transgenic rice T_1 seeds. Each dot represents the Pi content of one seed. Shadow means the range above Pi content of non-transgenic seeds. C: non-transformant.

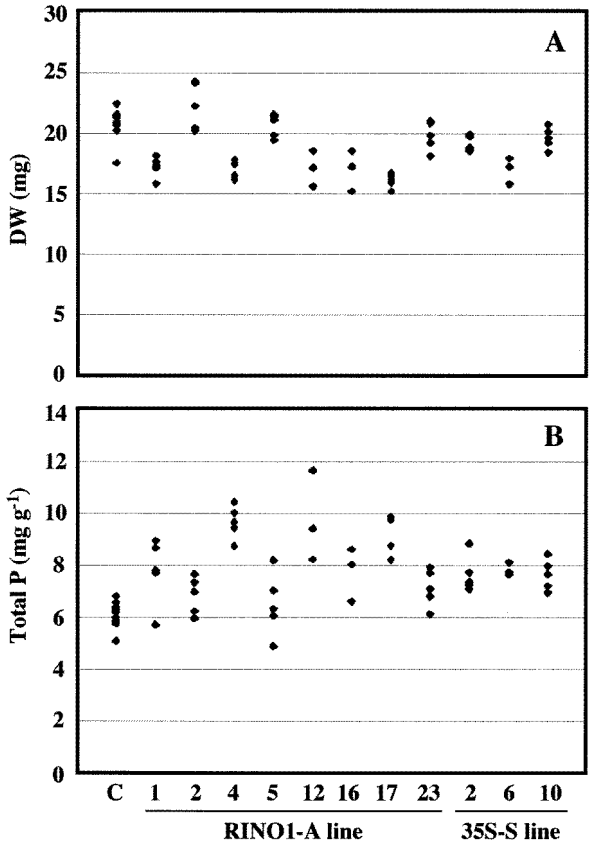

Fig. 4 Dry weights (A) and total P concentrations (B) in transgenic rice T_1 seeds. Each dot represents the value of one seed. C: non-transformant.

trations of 0.21 to 0.40 mg g^{-1} (**Fig. 3**). Seed total P ranged from 5.05 to 6.80 mg g^{-1} (**Fig. 4B**) with an average of 6.05 mg g^{-1}. Pi content ranged from 3.49% to 6.53% of total P, with an average of 4.56%. The values for seeds from non-transgenic plants were very stable.

To examine the storage form of phosphorus in transgenic seeds, the Pi levels in the T_1 seeds were determined. Some RINO1-A lines and 35S-S lines had elevated Pi concentrations (**Fig. 3**). Some seeds of the RINO1-A-8, 12, 16 and 17 lines, and 35S-S-7 and 8 lines had approximately 1.5- to 2-fold greater levels of Pi than did non-transgenic seeds. Among the transgenic lines, 35S-S-6 line produced seeds with Pi concentration above 1.20 mg g^{-1} (**Fig. 3**). The greatest Pi concentration in this line was 1.54 mg g^{-1} and was about four-fold greater than that in non-transgenic seeds. No T_1 seeds from any of the 35S::anti lines, in contrast, showed elevated Pi concentrations (data not shown). T_1 seeds of the 35-S-2 and 35S-S-10 lines, in contrast, had decreased levels of Pi. The lowest Pi concentrations among seeds of 35-S-2 and 35-S-10 were 0.09 mg g^{-1} and 0.12 mg g^{-1}, respectively.

The dry weight and total P concentration in T_1 seeds were examined (**Fig. 4**). Though the dry weights of the transgenic seeds were found to be rather lower than those of non-transgenic seeds, the large difference was not observed. The total P concentrations of transgenic seeds did not decrease and were rather higher than those of non-transgenic seeds (**Fig. 4B**). The differences in dry weight and total P between transgenic and non-transgenic seeds were probably experimental errors due to small number of seeds examined. The presence of the transgenes probably had no discernible effect both on seed dry weight and on seed total P. It seems likely that a molar-equivalent decrease in phytic acid in the Pi-elevated seeds occurred.

As shown in **Fig. 2**, the higher levels of Pi in the RINO1-A-12, 16, and 35S-S-6 were probably

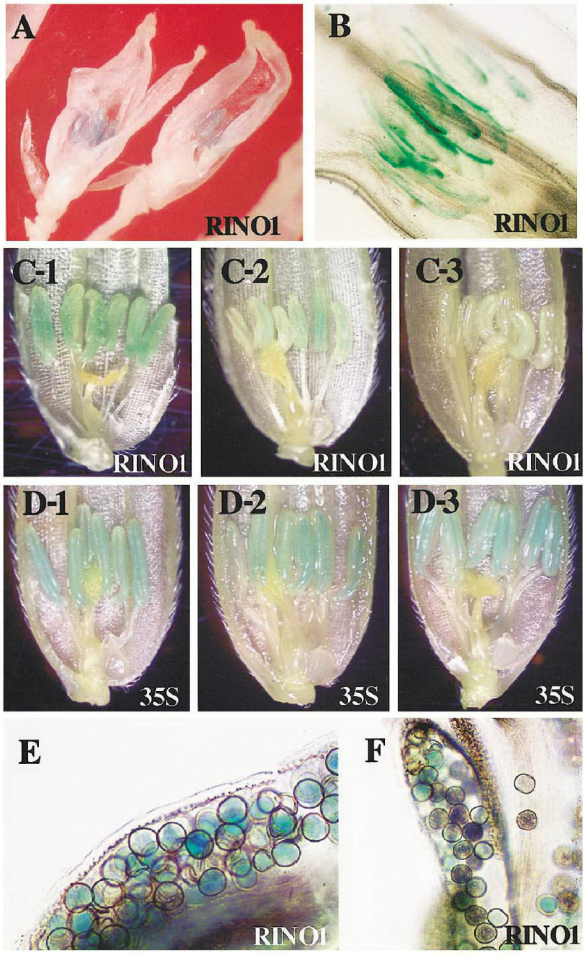

Fig. 5 GUS staining analysis of spikelets in *RINO1::GUS* and *35S::GUS* plants. A and B were spikelets at late spikelet differentiation stage. C and D were spikelets in a panicle at one to two days before heading. The spikelets were on the lower (C-1 and D-1), the middle (C-2 and D-2), and the upper (C-3 and D-3) rachis branches. E and F represent the GUS activities in pollen grains of spikelets at the lower and the upper rachis branches, respectively. Anthers of E and F were treated with clearing fluid. A, B, C, E, and F : *RINO1::GUS*, D : *35S::GUS*.

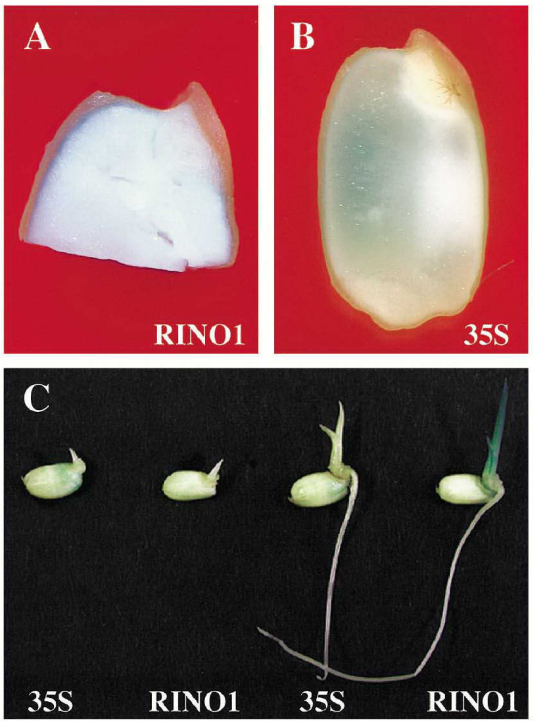

Fig. 6 GUS staining analysis of T_1 seeds and seedlings of *RINO1::GUS* and *35S::GUS* plants. Hand-cut sections of seeds of *RINO1::GUS* (A) at seven days after anthesis and of *35S::GUS* (B) at fourteen days after anthesis were stained with X-Gluc solution. T_1 seedlings at two (left side) and four (right side) days after imbibition were GUS stained (C).

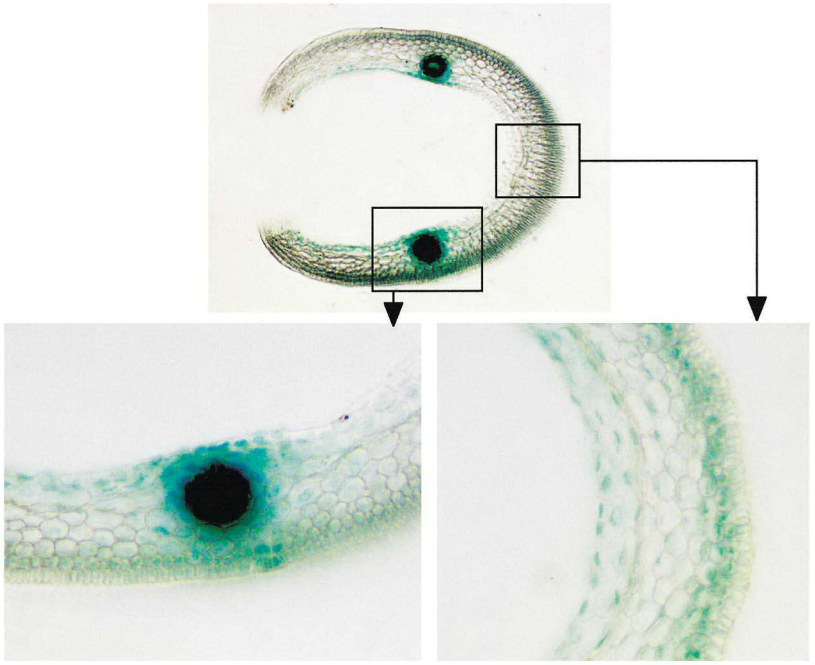

Fig. 7 Histochemical analysis of GUS expression in a coleoptile of T_1 plant of RINO1:: GUS plant. A GUS-stained cross section was observed after clearing treatment.

related to the lower level of RINO1 protein. Also, it seems likely that the lower level of Pi in the 35S-S-2 was related to the higher level of RINO1 protein.

GUS expression

To know the promoter activities of the transgenes used in this study, RINO1::GUS and pBI221 (35S::GUS) were introduced into rice plants. In transformations with the RINO1::GUS and 35S::GUS constructs, six and seven hygromycin-resistant fertile lines, respectively, were obtained. The GUS activity in young panicles, spikelets of T_0 plants, and T_1 seeds and seedlings was examined.

GUS expression was detected in anthers of RINO1::GUS transgenic plants after the late spikelet differentiation stage (**Fig. 5A**). A clearing treatment of these spikelets revealed GUS expression in both the inner cell layer of the endothecium, which is fated to become the tapetum, and in immature pollen grains (**Fig. 5B**).

Spikelets in a panicle at one to two days before heading were also analyzed for GUS activity. The spikelets on the upper, middle, and lower rachis branches were analyzed separately, as the development of spikelets and anthesis in rice proceed in this order (Hoshikawa, 1993). Both the RINO1 and the 35S promoter directed expression of the *GUS* gene only in anthers (**Fig. 5C, D**). The expression patterns were rather different between RINO1::GUS and 35S::GUS transgenic plants. In RINO1::GUS plants, the younger spikelets on the lower rachis branches had stronger GUS activity than the older spikelets on the upper rachis branches. In contrast, the relationship between the strength of GUS activity in anthers and the developmental stage of the spikelets was not clear in 35S::GUS plants. The 35S promoter directed expression of the GUS gene in both the anther wall and pollen grains. In contrast, a RINO1::GUS plant displayed GUS activity only in pollen grains (**Fig. 5E**). That is, the GUS activity in the inner cell layer of the endothecium of anthers of the RINO1::GUS plant was transient at the late spikelet organ differentiation stage. GUS activity in pollen grains from RINO1::GUS plants decreased with their development. Several days before anthesis, starch grains begin to accumulate in the pollen grains. Pollen grains containing large amounts of starch exhibited low GUS activity (**Fig. 5F**).

To determine the GUS activity in developing caryopses, transgenic rice grains at seven and fourteen days after anthesis were collected. Seeds of RINO1::GUS plants had low GUS activity, with signal barely detectable in the grains at fourteen days after anthesis. GUS activity was detected only in the aleurone layer at seven days after anthesis (**Fig. 6A**). GUS activity was not detected in the embryos at either seven or fourteen days after anthesis. Grains from 35S::GUS plants displayed higher GUS activity than did those of RINO1::GUS plants. GUS signals were detected in seeds of 35S::GUS plants in endosperms and embryos at fourteen days after anthesis (**Fig. 6B**).

Next, we investigated the GUS activity in T_1 seedlings at two and four days after imbibition. GUS activity was detected in the coleoptiles and first leaves of RINO1::GUS seedlings at four days after imbibition (**Fig. 6C**). Cross sections of cleared leaves revealed GUS activity in both epidermal cells and mesophyll cells. The highest signals were detected around the vascular bundles (**Fig. 7**). In contrast, the signal was only detected in germinated 35S::GUS T_1 seeds at two days after imbibition (**Fig. 6C**). GUS signals were barely detectable in leaves and seminal roots of 35S::GUS seedlings at four days after imbibition.

Discussion

In this study, manipulation of *RINO1* gene expression led to increases in inorganic phosphate levels in transgenic rice seeds, without reduction in seed total P levels. Approximately 20% of the total phosphorus was in the Pi form in the 35S-S-6 T_1 seeds, which had the highest Pi concentration of the seeds examined in this study. This suggested a molar-equivalent phytic acid decrease in seeds of this line. Western blot analysis showed that the levels of Pi in the transgenic seeds corresponded relatively well to the levels of RINO1 protein. These results point to the potential feasibility of molecular breeding to alter the storage form of phosphorus in seeds through the manipulation of *RINO1* gene expression. This idea is also supported by studies of *lpa* mutants. In maize, a gene homologous to Ins(3)P_1 synthase was mapped to a site near the *lpa1-1* mutation on the 1S chromosome (Larson and Raboy, 1999, Raboy *et al.,* 2000). Recently, a low phytic acid mutant in soybean was reported to contain a mutation in a seed-expressed Ins(3)P_1 synthase. A single amino acid change at residue 396 of this protein caused a decrease of about 90% in the specific activity of the seed-expressed Ins(3)P_1 synthase (Hitz *et al.,* 2002).

In some seeds of the 35S-S-2 and -10 lines, the Pi concentration was reduced, as compared to wild-type. In 35S-S-2 seeds, the reduction in Pi concentration was accompanied by an increase in the quantity of RINO1 protein. These results suggest that overexpression of the *RINO1* gene leads to a

decrease in Pi. In general, phytic acid is not synthesized in large quantities in vegetative organs. If phytic acid could be abundantly synthesized and accumulated in vegetative organs such as leaves or roots, the plants could be used as hyperaccumulators of phosphates from eutrophicated lakes. The total P and phytic acid P levels in the vegetative organs of these transgenic lines would need to be analyzed to confirm the creation of a hyperaccumulating line.

GUS expression analysis revealed that the *RINO1* promoter was active in the endothecium of immature anthers at the late spikelet organ differentiation stage, as well as in immature pollen, the aleurone layer, the coleoptile and the first leaf of a young seedling. Mature pollen grains of many plant species contain large amounts of phytic acid, which provides free phosphates, *myo*-inositol and cations to the pollen tube during its elongation (Jackson *et al.*, 1982). This was consistent with the activity of the RINO1 promoter in immature pollen grains observed in this study. The lack of GUS activity in immature embryos was inconsistent with the high level of *RINO1* gene expression that has been observed in Northern blot and *in situ* hybridization analyses (Yoshida *et al.*, 1999). The *RINO1* promoter used in this study was about 2,160 bp in length. Several motifs responsible for seed-specific expression are present in the sites between -2,160 and -3,000 bp (Yoshida *et al.*, 2002). There is a possibility that some *cis*-acting elements responsible for embryo-specific expression are present in the regions before -2,160 bp, which were not included in the constructions generated in this study. In young seedlings, the highest GUS activity was observed around the vascular bundles. Previously, we showed the induction of *RINO1* gene expression by sugar in sugar-starved rice suspension cells (Yoshida *et al.*, 2002). The high GUS expression around the vascular bundle can be partly explained by this induction by sugar.

The effects of the chimeric transgenes used in this study were rather low, as compared to the effects of the *lpa1* mutations. In seeds of the rice mutant *lpa1-1*, Pi represents 32% of the total P (Larson *et al.*, 2000). The *lpa1-1* mutations in maize and barley result in reductions in seed phytic acid P, ranging from 50% to 95% (Raboy *et al.*, 2001). The less-pronounced effect of the transgenes in this study was probably partly due to the promoters. As stated above, *RINO1* promoter used in this study was not sufficient to induce gene expression in developing embryos, one of the phytic acid synthetic tissues (Yoshida *et al.*, 1999). As seen in the GUS expression analysis, both of *RINO1* and *35S* promoters were active in several tissues other than developing seeds. It is plausible that the RINO1 protein has an important role in these tissues and that a complete repression of *RINO1* expression leads to death of the transgenic plant, preventing isolation of transformants that exhibit a severe phenotype. Another possible reason for the less-pronounced effect of the transgenes was the copy number of Ins(3)P_1 synthase genes in the rice genome. Two rice Ins(3)P_1 synthase genes, one of which is *RINO1*, are present in the National Institute of Agrobiological Sciences DNA Bank in Japan (http://rgp.dna.affrc.go.jp/). RT-PCR analysis indicates that *RINO1* gene expression is strong, and expression of the other gene is weak, in every analyzed tissue, including developing seeds (data not shown). This means that complete repression of *RINO1* could be strongly detrimental to plants, as Ins(3)P_1 synthase catalyzes the first step in *myo*-inositol biosynthesis and the resulting metabolites are essential for the survival of plant cells (Bonert *et al.*, 1995).

The GUS expression analysis suggested that an understanding of the promoter activity would be very important in future molecular breeding efforts. It is crucial to use a promoter that has activity only in developing seeds, and especially in the aleurone layers and embryos, which are the storage sites of phytic acid.

Acknowledgments

The authors would like to express their gratitude to Dr. Ikuo Nakamura for the gift of the rice genomic library. In addition, the excellent technical support of Dr. Jun Ito and Ms. Mariko Obara is gratefully acknowledged. This work was supported, in part, by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan to K.T.Y. (No. 13660004).

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