Molecular breeding for transgenic rice with low-phytic-acid phenotype through manipulating *myo*-inositol 3-phosphate synthase gene

Mio Kuwano · Akio Ohyama · Yusuke Tanaka · Tetsuro Mimura · Fumio Takaiwa · Kaoru T. Yoshida

Abstract In most plant seeds, phosphorus is stored primarily as *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate ($InsP_6$; phytic acid). Reducing the phytic acid content of seeds is a major breeding target, both to increase the availability of mineral nutrients and to decrease the environmental load of phosphorus. The first step in phytic acid biosynthesis and inositol metabolism is catalyzed by 1D-*myo*-inositol 3-phosphate ($Ins(3)P_1$) synthase. In this study, we aimed to reduce phytic acid levels in rice seeds by manipulating the expression of the rice $Ins(3)P_1$ synthase gene *RINO1* using transgenic methods. *RINO1* cDNA was transformed into rice plants in the antisense orientation under the control of the rice major storage protein glutelin *GluB-1* promoter. The T_4 generation of a stable transgenic line that contained four copies of the transgene showed little morphological differences compared to non-transgenic rice. In the T_5 seeds of this line, severe reductions in RINO1 protein levels were observed during the late maturing stages of ripening. Most of the T_5 seeds contained higher amounts of inorganic phosphates (Pi), without a reduction in total phosphorus levels, compared to non-transgenic seeds. Ion chromatography analysis suggested that the increase in available Pi is accompanied by a molar-equivalent decrease in phytic acid P. The expression patterns of *RINO1* and *GluB-1* were examined by quantitative real-time reverse transcriptase-polymerase chain-reaction (RT-PCR). Potential strategies for further molecular breeding to reduce phytic acid levels in seeds are discussed.

Keywords Gene expression · Glutelin promoter · *myo*-inositol 3-phosphate synthase · *Oryza sativa* L. · Phytic acid · Transgenic plants

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Abbreviations

DAF	days after flowering
$Ins(3)P_1$	1D-*myo*-inositol 3-phosphate
$InsP_6$	*myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate
IPK	inositol phosphate kinase

lpa   low-phytic-acid
Pi   inorganic phosphate
RT-PCR   reverse transcriptase-polymerase
         chain-reaction

Introduction

Water pollution caused by human activities, such as agricultural runoff and sewage production, is a serious environmental problem. The influx of excessive phosphorus from fertilizers, human and domestic animal wastes, detergents, and industrial sources can result in the eutrophication of lakes and marshes. Animal waste is a leading source of phosphorus pollution from agricultural sources.

In most cereal grains, phosphorous is stored primarily as the molecule *myo*-inositol 1,2,3,4,5,6-hexa*kis*phosphate ($InsP_6$), termed phytic acid. Up to 80% of the total phosphorus in seeds is phytic acid, with the rest consisting of soluble inorganic phosphate (Pi) and the phosphorus present in nucleic acids, proteins, lipids, and sugars. Phytic acid strongly binds to various mineral cations, including potassium, magnesium, calcium, iron, and zinc, to form a mixed salt called phytin (Lott et al. 1995). Monogastric animals utilize phytic acid phosphorus inefficiently because they lack the digestive enzyme phytase, which is required to cleave phosphorus from phytic acid (Cromwell et al. 1993). Mineral phosphate supplements are commonly added to animal diets to prevent phosphorus deficiency, but this practice leads to high phosphorus concentrations in waste matter, contributing to the pollution of water bodies (Sims et al. 2000). Reducing the phytin content of seeds would both increase the availability of mineral nutrients and decrease the environmental phosphorus load.

The first step in phytic acid biosynthesis and inositol metabolism is the formation of 1D-*myo*-inositol 3-phosphate ($Ins(3)P_1$) from D-glucose 6-phosphate, catalyzed by $Ins(3)P_1$ synthase. The biochemical pathways from $Ins(3)P_1$ to $InsP_6$ and the enzymes that catalyze these steps are not fully understood. Yoshida et al. (1999) reported the isolation of a cDNA corresponding to the rice $Ins(3)P_1$ synthase gene *RINO1*. They demonstrated that *RINO1* transcripts accumulate at high levels in the scutellum and aleurone layer, coinciding well with the appearance of phytin. They suggested that $Ins(3)P_1$ synthase plays a key role in phytic acid biosynthesis in developing rice seeds.

Reducing the expression of *RINO1* in developing seeds may lead to a valuable reduction in the phytin content of seeds. Recently, Feng and Yoshida (2004) attempted to reduce the phytic acid content of rice seeds by manipulating *RINO1* expression using transgenic methods. The antisense *RINO1* cDNA fused to either the *RINO1* promoter or the Cauliflower Mosaic Virus 35S promoter was used to create transgenic rice that produced seeds with a low-phytic-acid (lpa) phenotype. Manipulation of *RINO1* gene expression led to increases in levels of available Pi in the transgenic rice seeds but no reduction in the total seed phosphorus levels (Feng and Yoshida 2004). However, the effects of the chimeric transgenes used in the study were not as strong as the effects of the rice *lpa* mutations reported to date (Larson et al. 2000). The less-pronounced effect of the transgenes was thought to be partly caused by the promoters that were used. GUS expression analysis revealed that the *RINO1* promoter, as well as the 35S promoter, was active in vegetative tissues other than developing seeds (Feng and Yoshida 2004). Because RINO1 catalyzes the first step in inositol metabolism, as well as phytic acid biosynthesis, the complete repression of *RINO1* gene expression in tissues other than seeds is strongly detrimental to plant development. The use of a promoter that is active only in developing seeds, the storage site of phytic acid, may be crucial for obtaining seeds with the lowest possible phytic acid content.

The promoter of the gene for the seed storage protein glutelin, the 1302-bp *GluB-1* promoter, is well known to drive extremely high and endosperm-specific expression during rice seed maturation (Wu et al. 1998), and is generally used in transgenic research. Here, we attempted to reduce the phytic acid content of rice seeds by using the *GluB-1* promoter to direct *RINO1* gene expression in transgenic rice plants.

Materials and methods

Plasmid construction and transformation procedures

The antisense *RINO1* cDNA, with a length of 1,844 bp (Yoshida et al. 1999), was fused to the rice seed storage glutelin *GluB-1* promoter fragment of 1,302 bp (Wu et al. 1998). This construction was inserted into the binary vector pGPTV-35S-HPT, which contains the rice *GluB-1* terminator and the hygromycin phosphotransferase gene as a selectable marker (Qu and Takaiwa 2004). The resulting plasmid was transferred into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Transgenic rice plants (*Oryza sativa* cv. Kitaake, an early heading variety) were produced by *Agrobacterium*-mediated transformation. An *Agrobacterium* harboring the plasmid was used to infect scutellum-derived calli of rice. The regenerated seedlings were selected on media containing hygromycin, transplanted, and grown in a greenhouse as described by Goto et al. (1999).

Selection of transgenic lines

Phosphorus levels in T_1 seeds from self-fertilized T_0 plants were analyzed as described below. T_1-generation lines that contained seeds with Pi concentrations more than 3-fold greater than seeds of non-transgenic rice were selected, and the seeds that were harvested from the selected lines were planted and grown to produce T_2 seeds. These lines were selected because seeds with nearly 2-fold greater amounts than the average concentration of non-transgenic seed Pi could be observed in the non-transgenic lines, but seeds that showed more than 3-fold greater levels could only be seen in the transgenic seeds. We decided that the 3-fold greater seed Pi concentrations compared to non-transgenic lines would indicate the effect of the transgenes. The number of transgene copies in T_1 plants was assessed by genomic Southern blot analysis as described below. T_2 seeds were only harvested from T_1 plants that contained one or more copies of the transgenes. The T_2-, T_3-, and T_4-generation seeds were similarly analyzed and selected to identify heritable phenotypes. Plant height, number of panicles on T_4 plants, and weight of 100 dry T_5 seeds were measured and compared with those of non-transgenic plants.

Genomic Southern blot analysis

Genomic DNA was extracted from rice leaves using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNA was digested using the restriction endonuclease *Eco*RI, electrophoresed on a 0.8% agarose gel, and blotted onto Biodyne A membrane (Pall, Melbourne, Australia). The blotted nucleic acids were fixed by baking at 80°C for 2 h.

To produce a template for labeling a DNA probe, a PCR reaction was carried out with the *RINO1* cDNA using the upper primer GTGGTGGTGTTGTGGACTGC (position 782~) and the lower primer AGAATGGGAAAGAC CGAAAA (position ~1773). The PCR product was labeled using DIG DNA Labeling Mix (Roche Diagnostics, Basel, Switzerland) to produce a DIG-labeled cDNA probe. The labeling reactions and hybridization procedures were carried out according to the manufacturer's instructions. Detection was performed using the Luminescent Image Analyzer LAS-3000 (Fuji Film, Tokyo, Japan).

Analysis of seed phosphorus levels

Dehusked whole seeds were dried for 48 h at 60°C. Each grain was crushed using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan) and weighed. The Pi was measured from one half of each crushed seed and the total phosphorus from the other half, after which the percentage of Pi in each seed was calculated. To analyze Pi levels, the crushed powder was extracted in 12.5% (w/v) trichloroacetic acid containing 25 mM $MgCl_2$, and then centrifuged at $20,000 \times g$ for 10 min. Pi in the supernatant was assayed colorimetrically as described by Chen et al. (1956).

Total phosphorus in the seed was determined as described by Raboy et al. (2000). The total phosphorus was extracted from the crushed powder using the wet-ashing method, followed by a colorimetric assay.

Ion chromatography analysis of Pi and phytic acid

Five dehusked whole seeds were gathered and crushed using a Multi-Beads Shocker and weighed. Fifty milligrams of the crushed seeds were ground with a mortar and a pestle and homogenized in 2.4% (w/v) HCl. The homogenates were boiled for 10 min and centrifuged at $20,000 \times g$ for 10 min at 4°C. The supernatant was filtered through a 5.0-μm filter (Millipore, Bedford, USA). The filtrate was filtered through a 10000 NMWL filter (Millipore) and was diluted with deionized water to a final concentration of 0.48% HCl. The HCl-extracts were subjected to ion chromatography as described by Mitsuhashi et al. (2005). This method can separate $InsP_1$ to $InsP_6$ in a single gradient elution.

The amount of Pi or phytic acid in a sample was calculated from a standard curve determined from the peak area of the reference dilution series, from 0 to 3 mg ml^{-1} for Pi (Phosphorus Std. Soln., 160-19241, Wako, Osaka, Japan) and from 0 mg ml^{-1} to 30 mg ml^{-1} for phytic acid (Phytic acid dodecasodium salt from rice, P-3168, Sigma, St. Louis, USA), respectively.

Immunoblot analysis

Total soluble proteins from dehusked whole seeds collected at 7, 10, 14, 21, and 28 days after flowering (DAF) were weighed and extracted in PBST (PBS, pH 7.4, containing 0.5% Triton X-100). The extract was centrifuged at $20,000 \times g$ for 10 min at 4°C, and protein in the supernatant was quantified using the Bio-Rad Protein Assay (Bio-Rad, Chicago, USA) according to the manufacturer's instructions. A 20-μg aliquot of protein was loaded onto a 10% polyacrylamide SDS-PAGE gel, separated by electrophoresis, and transferred to Immun-Blot PVDF Membrane (Bio-Rad).

RINO1 protein produced in *E. coli* was used to raise antibodies in a rabbit (Feng and Yoshida 2004). The blotted membranes were incubated with the rabbit anti-RINO1 antiserum, and the RINO1 protein was visualized using a goat anti-rabbit IgG alkaline phosphatase conjugate using the ProtoBlot II AP System (Promega, Madison,

Wisconsin, USA) according to the manufacturer's instructions. The signals were detected with a LAS-3000 imaging system. Quantitative estimates of the optical densities of the bands were obtained using a Science Lab 2001 Image Gauge (Fuji Film).

Quantitative real-time RT-PCR

Total RNA from rice (cv. Nipponbare) tissues was extracted from 7-day-old roots and leaves, flowers at one day before anthesis, pistils at flowering, embryos at 10 DAF, and dehusked whole seeds at 7, 10, 14, 21, and 28 DAF using the RNeasy Plant Mini Kit (Qiagen). To eliminate residual genomic DNA, the RNA was treated with DNase I using the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions.

To quantify the target RNA, in vitro-transcribed RNA was used as a standard. In vitro transcription was performed using the MEGAscript T3 and T7 kit (Ambion, Austin, USA) for *RINO1* and *GluB-1*, respectively. The template DNA was removed using DNase I treatment, and then the transcribed RNA was purified using the RNeasy Mini Kit. After the RNA concentration was determined by spectrometry, the reference RNA was used to create a 10-fold dilution series, from 100 pg ml^{-1} to 10 ag ml^{-1}. Real-time RT-PCR amplification was carried out using the QuantiTect SYBR Green RT-PCR Kit (Qiagen) and an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, USA). The reaction mixture was adjusted to 20 ng total RNA for *RINO1* and 2 ng total RNA for *GluB-1* in 20-μl reactions. Real-time RT-PCR was performed using the following primer pairs: *RINO1* (5′-CCGTGGCATGTGGCAAAGAG-3′ and 5′-TGCATAGCCCGATAAGAGTC-3′) and *GluB-1* (5′-TATTCTCTCGCCGTTCTGGA-3′ and 5′-TCACGCTCTTGCTTTCTTCAA-3′). The annealing temperature was 54°C, and the PCR reactions were performed in triplicate. Each primer pair generated a single specific RT-PCR product of the expected length. The numbers of RNA molecules were calculated from the linear regression of the standard curve. Data were analyzed using ABI PRISM 7700 Sequence Detection System software (Applied Biosystems).

Results and discussion

Production of stable transgenic rice lines

Thirty-six independent hygromycin-resistant transgenic rice plants were generated by *Agrobacterium*-mediated transformation of a chimeric gene consisting of the rice seed storage protein glutelin *GluB-1* promoter and rice *RINO1* cDNA in the antisense orientation. All seeds of the transgenic lines showed the same germination rate (100%) as the non-transgenic seeds. To examine the storage form of phosphorus in transgenic seeds, we examined available Pi levels in the T_1 seeds of individual transgenic lines and compared them to levels in non-transgenic rice seeds. Pi content and total seed phosphorus in non-transgenic rice seeds were almost constant. Higher seed Pi levels were detected in four of the 36 independent T_0 transgenic lines. Some seeds of these lines had more than three times the level of Pi than non-transgenic seeds. We selected these four transgenic lines (lines 8, 13, 22, and 35) and proceeded to the T_4 generation.

Chimeric transgenes in transgenic plants

The number of transgene copies in the four T_4 transgenic lines was examined by genomic Southern blot analysis. We detected at least five, two, four, and four copies of the transgenes in lines 8, 13, 22, and 35, respectively (Fig. 1). The transgenes in these lines were maintained from the T_1 to the T_4 generation, and the Southern hybridization pattern was the same for all 15 T_4 plants of each transgenic line irrespective of the presence of multiple transgene copies.

Morphological traits of transgenic plants

We compared the morphology of the T_4 plants to that of non-transgenic plants. In juvenile plants, all T_4 and non-transgenic rice plants showed similar morphologies. In mature plants, there was little difference in plant height and number of panicles among plants from T_4 lines 13 and 22 and non-transgenic plants (Fig. 2a, b; $P = 0.05$). However, we found significant differences in plant height and number of panicles of the T_4
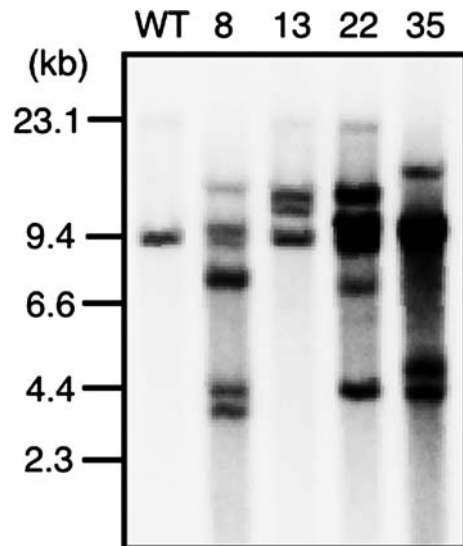

Fig. 1 Southern blot analysis of *RINO1*. Genomic DNA (3 μg per lane) of non-transgenic lines (WT) and transgenic lines 8, 13, 22, and 35 digested with *Eco*RI was separated on an agarose gel, blotted, and hybridized with the *RINO1* probe. The positions and sizes (in kb) of markers are indicated

plants from lines 8 and 35 compared to those of non-transgenic plants. The weight of 100 dry seeds from lines 13, 22, and 35 was not significantly different from that of non-transgenic rice (Fig. 2c; $P = 0.05$). However, there was a significant difference between seeds from line 8 and non-transgenic seeds. Other morphological traits were similar among non-transgenic and transgenic plants.

Phosphorus levels in transgenic plants

We examined the total phosphorus levels in seeds of non-transgenic rice and T_5 seeds of transgenic lines (Fig. 3a). The average total phosphorus content in T_5 seeds from lines 8, 13, 22, and 35 and non-transgenic seeds was 3.2, 3.2, 2.9, 2.7, and 2.8 mg g^{-1}, respectively. No significant difference was observed among lines 22 and 35 and the non-transgenic seeds ($P = 0.05$). To examine the storage form of phosphorus in the seeds, available Pi levels in non-transgenic seeds and T_5 seeds of transgenic lines were determined (Fig. 3b). The Pi content in individual grains was determined. Pi concentrations in non-transgenic seeds were
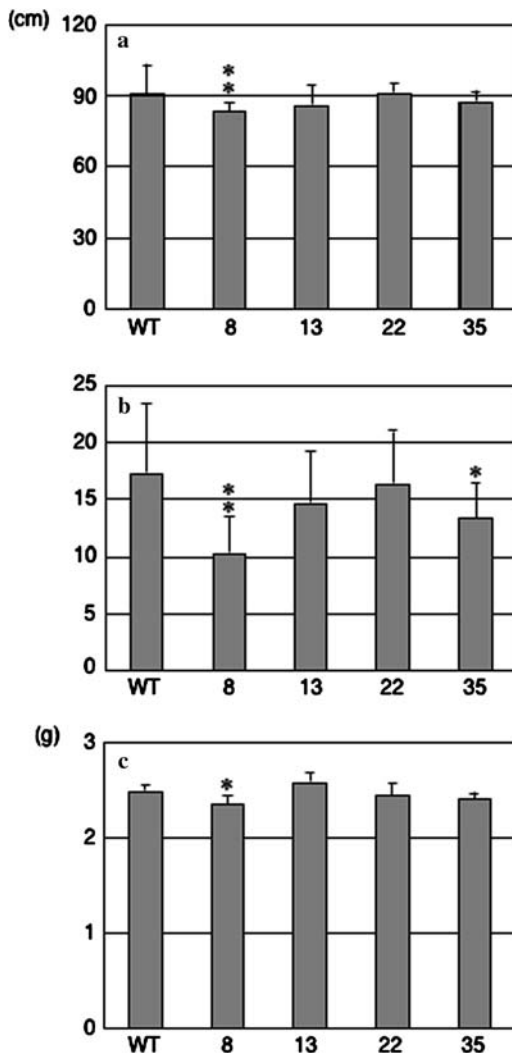
Fig. 2 Plant height (**a**), number of panicles (**b**), and weight of 100 dry seeds (**c**) of non-transgenic rice (WT) and the transgenic rice lines 8, 13, 22, and 35. The values shown are the means ± SE of 15 (for **a** and **b**) or 5 (for **c**) independent measurements. The symbols * and ** indicate significant differences at $P = 0.05$ and 0.01, respectively, when compared to non-transgenic plants

Fig. 3 Total phosphate concentrations (**a**) and Pi fraction of the total phosphorus (**b**) in non-transgenic seeds (WT) and transgenic T_5 seeds of lines 8, 13, 22, and 35. Each total phosphorus value represents the mean ± SE of 15 seeds. The symbols * and ** indicate significant differences at $P = 0.05$ and 0.01, respectively, when compared to non-transgenic plants. The Pi content of each seed is shown as a percentage of the total phosphate content. Horizontal lines indicate mean values

0.065–0.11 mg g^{-1}, and constituted 2.5–3.0% of the total phosphorus in each seed. Seeds of lines 13, 22, and 35 had greater Pi concentrations than non-transgenic seeds, with Pi constituting an average of 8.8, 16, and 9.0% of the total phosphorus, respectively. The highest Pi concentration in this experiment, 1.4 mg g^{-1}, was observed in a seed of line 22. In this seed, the Pi content corresponded to 48% of the total phosphorus (Fig. 3b).
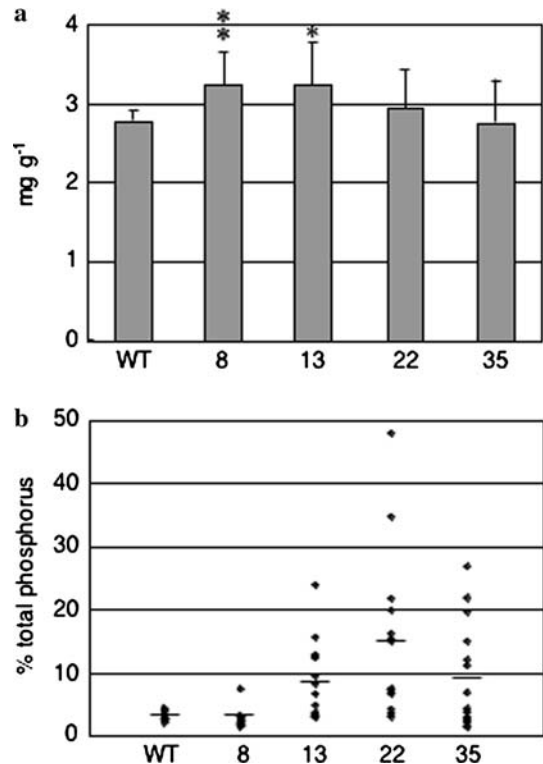
To quantify phytic acid and Pi levels, seed extracts from the transgenic line 22 and the non-transgenic line were analyzed by ion chromatography. Line 22 was chosen for examination in further studies because there were no significant differences in morphological traits (Fig. 2), and the highest Pi level was found in this line (Fig. 3). Larger conductivity of Pi and smaller conductivity of phytic acid was detected for the seeds of line 22 compared to the seeds of the non-transgenic plants (Fig. 4a). Mean phytic acid values was 18 μmol g^{-1} for the non-transgenic seeds, and 15 μmol g^{-1} for the transgenic line (Fig. 4b). This represented an average reduction in seed phytic acid content of 17% for line 22. Ion chromatography analysis also confirmed that no accumulations of other inositol phosphates are
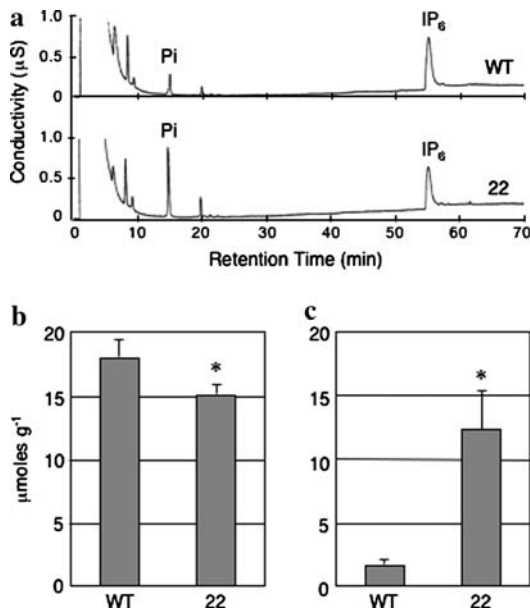
Fig. 4 Ion chromatography analysis of phytic acid and Pi content in non-transgenic seeds and transgenic T_5 seeds of line 22. (**a**) Typical chromatograms of extracts from non-transgenic seeds and transgenic T_5 seeds of line 22. WT, non-transgenic seeds; 22, transgenic T_5 seeds of line 22. Fifty milligrams of tissue from five seeds were extracted, and equal aliquots of filtered supernatant were fractionated. (**b, c**) Concentrations of phytic acid (**b**) and Pi (**c**) in non-transgenic seeds and transgenic T_5 seeds of line 22. Each value represents the mean $\pm$ SE for six replicates. The symbol * indicate significant differences at $P = 0.05$ when compared to non-transgenic plants

observed in the transgenic seeds. Pi content in transgenic seeds were 12 $\mu mol\ g^{-1}$ compared to 1.5 $\mu mol\ g^{-1}$ from non-transgenic plants indicating that expression of transgenes during seed development resulted in an 9-fold increase in Pi (Fig. 4c). In non-transgenic seeds, phosphorus in the phytic acid form was 108 $\mu mol\ g^{-1}$ because phytic acid is equivalent to $InsP_6$ and has six phosphates. Phytic acid form P decreased to 90 $\mu mol\ g^{-1}$ in transgenic seeds, while the available P increased to 12 $\mu mol\ g^{-1}$ compared to 1.5$\mu mol\ g^{-1}$ in non-transgenic plants. There was no significant difference in the amount of seed total P between line 22 and non-transgenic line (Fig. 3a). These results indicate that the reduction in phytic acid P is accompanied by a molar-equivalent increase in Pi.

The results of this study indicate the potential feasibility of using molecular breeding to alter the storage form of phosphorus in seeds through the manipulation of *RINO1* gene expression under the control of a strong seed-specific promoter. In the rice *lpa* mutant, Pi represents 32% of the total seed phosphorus (Larson et al. 2000). Although a large variation in Pi concentration was observed in transgenic seeds (Fig. 3b) and on average the effects of the transgenes used in this study were not so great as the effects of the *lpa* mutations, the grain with the highest Pi level (48%) in line 22 suggests that transgenic lines with much greater rice grain Pi contents could be obtained through molecular breeding.

In some cereal plants, the position in a panicle affects the growth rate of a seed. The timing and the level of expression of some genes encoding starch biosynthesis were affected by the position in a panicle of rice (Ishimaru et al. 2005). In this study, differences in expression patterns of the transgenes at different positions in a panicle may be a major reason for the large variation in Pi concentrations observed in the transgenic plants.

The RINO1 protein in transgenic seeds

Expression of the *RINO1* gene in transgenic plants was assessed using immunoblotting. Total proteins of immature T_5 seeds from line 22 and non-transgenic plants at 7, 10, 14, 21, and 28 DAF were subjected to immunoblotting analysis using anti-RINO1 antiserum. The RINO1 polyclonal antibody reacted with a band of about 56 kDa. In non-transgenic seeds, the RINO1 protein was detected from 7 DAF, and its level increased to a maximum at 10 DAF, after which the level decreased (Fig. 5a). No significant difference was seen in RINO1 protein levels of maturing seeds from 7 to 14 DAF between T_5 seeds from line 22 and non-transgenic seeds (Fig. 5b, c). In contrast, a decreased level of RINO1 protein compared to that in non-transgenic seeds was observed after 14 DAF. The decreased level of RINO1 protein suggests that the expression of the antisense *RINO1* transcripts efficiently represses *RINO1* gene expression later in the ripening period in line 22. The immunoblot analysis demonstrated that
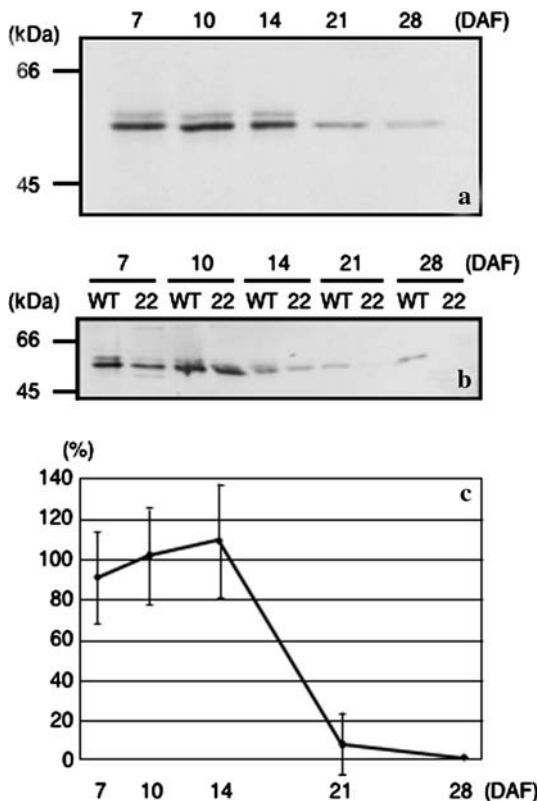
Fig. 5 Immunoblot analysis of RINO1 protein in non-transgenic seeds and transgenic T$_5$ seeds of line 22. (**a**) Immunoblot analysis of non-transgenic seeds. Each lane contains 20 μg of protein from individual seeds at 7, 10, 14, 21, and 28 DAF. The positions and sizes (in kDa) of markers are indicated. (**b**) A typical result of non-transgenic seeds (WT) and transgenic T$_5$ seeds of line 22. Each lane contains 20 μg of protein from an individual seed at 7, 10, 14, 21, and 28 DAF. (**c**) Relative amounts of RINO1 protein in T$_5$ seeds of line 22. Each time point represents the relative image density of RINO1 protein bands on immunoblots as a percentage of that in non-transgenic seeds. Each value represents the mean ± SE for nine replicates

Fig. 6 Quantitative real-time RT-PCR analysis of *RINO1* and *GluB-1* in non-transgenic seeds. (**a**) mRNA levels of *RINO1* (solid bars) and *GluB-1* (open bars) in roots (R) and leaves (L) at 7 days after germination, flowers (F) at 1 day before flowering, pistils (P) at flowering, and embryos (E) and dehusked whole seeds (S) at 10 DAF. (**b, c**) mRNA levels of *RINO1* (**b**) and *GluB-1* (**c**) in dehusked whole seeds at 5, 7, 10, 14, 21, and 28 DAF

increased levels of Pi in the transgenic line may be related to decreased levels of the RINO1 protein during the late stages of ripening.

Expression patterns of RINO1 and GluB-1

To determine the promoter activities and to evaluate a strategy for further increase in the seed available Pi, the relative levels of *RINO1* and *GluB-1* transcripts in non-transgenic rice were estimated by quantitative real-time RT-PCR. High levels of *RINO1* transcripts were observed
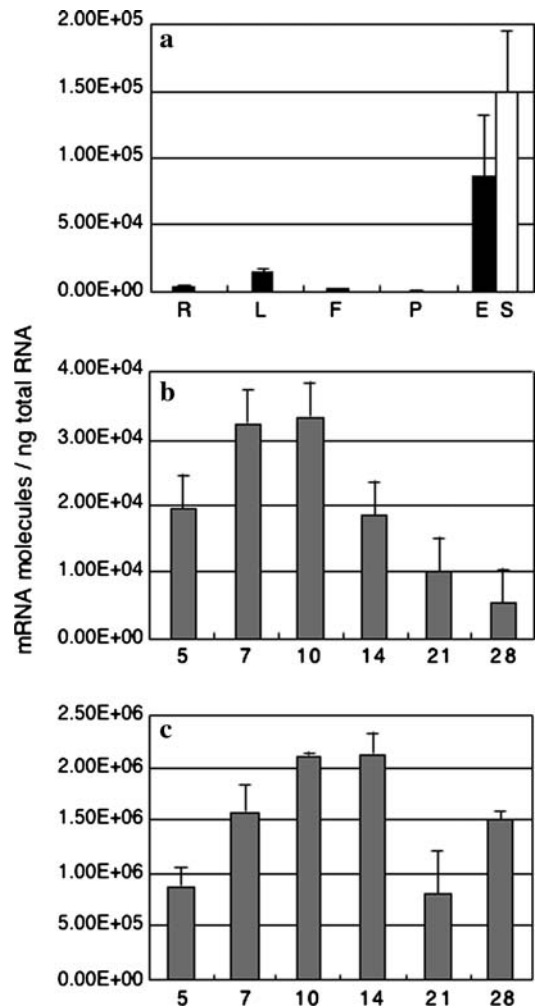
in developing embryos, in contrast to the low levels observed in roots, leaves, flowers, and pistils (Fig. 6a). *Glu B-1* transcripts were exclusively observed in seeds and the expression level was extremely high. This result agrees well with the seed-specific property of the promoter of the seed storage protein gene *GluB-1*, and indicates the potential of this promoter to drive extremely high expression in endosperm (Wu et al. 1998). During seed maturation, *RINO1* mRNA was detected

from 5 DAF and increased to a maximum at 7–10 DAF, after which the level decreased (Fig. 6b). This result corresponds well with immunoblot results for the RINO1 protein in non-transgenic seeds (Fig. 5a). The maximum *GluB-1* transcript occurred from 10 to 14 DAF (Fig. 6c). This difference in the temporal patterns of *RINO1* and *GluB-1* transcript accumulation (mRNA levels) may partially account for the difficulty in the complete suppression of the inherent *RINO1* transcript by the antisense *RINO1* cDNA driven by the *GluB-1* promoter (Fig. 5c). *RINO1* is expressed at high levels within the scutellum and the aleurone layer, where phytic acid is stored (Yoshida et al. 1999), whereas expression of the-*GluB-1* promoter, as revealed by GUS activity, was initially detected in the aleurone and the subaleurone layers, and then in the endosperm (Qu and Takaiwa 2004). This spatial difference in expression may be another reason for the limited repression of the *RINO1* gene in transgenic rice.

In this study, we demonstrated the effect of the seed specific promoter *GluB-1* on producing high-Pi seeds. In transgenic line 22, the Pi level was on average about 5-fold higher than in non-transgenic plants. The highest Pi value of line 22 was about 16-fold greater than that of non-transgenic plants, and was about 1.5-fold greater than in the rice *lpa* mutant. These results indicate the possibility of molecular breeding for rice with an ultra lpa phenotype through the manipulation of *RINO1* gene expression. Stronger effects could be obtained by using a promoter with the same temporal and spatial activity in the seed as *RINO1*, thereby reducing the levels of RINO1 protein earlier in the process of seed maturation.

An alternative approach that has recently been reported to generate grains with lpa phenotype is the seed-specific disruption of inositol phosphate kinases (IPKs). Loss-of-function mutants of *Arabidopsis thaliana* that disrupted either *AtIPK1* or *AtIPK2β* exhibit reductions in seed phytic acid levels (Stevenson-Paulik et al. 2005). We have isolated the rice orthologs of IPK1 and IPK2, and are now working on characterizing them. We are planning to investigate whether rice IPK genes can be used in the genetic engineering of lpa phenotype seeds.

Another strategy that has been reported earlier to achieve the same goal as our research is the expression of *Aspergillus* phytase in transgenic rice grains (Lucca et al. 2001). This approach was aimed to degrade seed phytic acid when the grains were fed to animals, but not *in planta*. To obtain the most effective results, these transgenic cereals are suggested to be ground to powder, and incubated at 37°C with water (Drakakaki et al. 2005). These preparation steps for crop feed would not be necessary when seeds with lpa phenotype are used. Molecular breeding of rice grains with decreased phytic acid content would be a promising strategy to achieve high effects with low cost and labor.

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