Differential Effects of a Transgene to Confer Low Phytic Acid in Caryopses Located at Different Positions in Rice Panicles

Mio Kuwano¹, Fumio Takaiwa² and Kaoru T. Yoshida¹∗

¹Graduate School of Agricultural and Life Sciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo. 113-8657 Japan
²National Institute of Agrobiological Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki. 305-8602 Japan

In previous studies, we attempted to reduce phytic acid in rice seeds by silencing the 1-myoinositol 3-phosphate synthase gene, RINO1, using an antisense sequence under the control of the rice glutelin GluB-1 promoter. The stable transgenic line showed a weak low phytic acid phenotype. In this study, we show that the position of the caryopsis in the panicle might affect the level of gene silencing through a difference in temporal and spatial expression patterns between RINO1 and GluB-1 promoters, resulting in a large variation in Pi levels and a small increase in Pi in the transgenic seeds.

Keywords: Gene silencing • Glutelin promoter • Myo-inositol 3-phosphate synthase • Oryza sativa L. • Phytic acid • Transgenic plants.

Abbreviations: DAF, days after flowering; GUS, β-glucuronidase; lpa, low phytic acid.

In plant seeds, phosphorus is stored mainly as myo-inositol 1,2,3,4,5,6-hexakisphosphate (phytic acid) (Raboy 1997). Phytic acid forms insoluble mixed salt phytate with various minerals and thus reduces the bioavailability of phosphorus and minerals to monogastric animals. Therefore, development of cultivars with a low phytate phenotype is an important breeding objective. In previous studies, we attempted to reduce phytic acid in rice seeds by silencing the 1-myoinositol 3-phosphate synthase gene RINO1 using gene silencing technology (Kuwano et al. 2006). One major explanation for such variation may be the large variations in antisense RINO1 expression levels in the seeds. In some cereals, it is known that the position in the panicle affects gene expression and seed growth (Gao et al. 1992, Ishimaru et al. 2005, Tsai-Mei and Setter 1985). In rice, caryopses with a high grain filling rate are called superior caryopses. In contrast, caryopses that exhibit low growth rates and often abort are termed inferior caryopses. Ishimaru et al. (2005) demonstrated that the timing and levels of expression of some genes encoding carbohydrate-metabolizing enzymes differed between superior and inferior caryopses. It is plausible that genes encoding enzymes involved in the synthesis of storage reserves other than starch may show different expression patterns between superior and inferior caryopses. In this study, we identified that the position in the panicle might affect the expression level of a transgene driven by the GluB-1 promoter, resulting in a large variation in seed Pi levels, and that the difference in the temporal and spatial pattern between RINO1 and GluB-1 promoter activities might be responsible for the limited suppression of the RINO1 gene and for the weak lpa phenotype.

To investigate the differences in phosphorus accumulation between superior and inferior caryopses, we selected caryopses at positions in the panicle that stably exhibited the superior or inferior growth patterns (Supplementary Fig. S1). Although the growth of seeds was retarded in inferior caryopses, the final fresh weights of inferior caryopses were close to those of superior caryopses (Supplementary Fig. S2).
There were no significant differences in available Pi, phytate and total phosphorus contents of seeds between superior and inferior caryopses of non-transgenic rice (Supplementary Figs. S2, S4). To compare the levels of RINO1 gene expression in superior and inferior caryopses of non-transgenic rice, the amount of RINO1 protein was estimated by immunoblotting. During seed development, little difference was observed in RINO1 protein levels between superior and inferior caryopses (Supplementary Fig. S3).

To examine the storage form of phosphorus in transgenic T_{6} seeds from the G-22 line, available Pi levels in each superior and inferior caryopsis were determined (Fig. 1A). The superior caryopses from the G-22 line had significantly greater Pi concentrations than non-transgenic rice (P < 0.05). Inferior caryopses from the G-22 line had much greater Pi concentrations on average; Pi concentrations were higher than those measured in superior or inferior caryopses from non-transgenic plants and higher than those measured in superior caryopses from the G-22 line (P < 0.05). There was no significant difference in the total phosphorus contents of superior or inferior caryopses from non-transgenic or transgenic plants (Supplementary Fig. S4). The expression analysis revealed that there was little difference in RINO1 protein levels from 7 to 14 days after flowering (DAF) in G-22 superior caryopses compared with those of non-transgenic superior caryopses (Fig. 1B). However, a large reduction in RINO1 protein was observed in G-22 superior caryopses from 21 to 28 DAF; RINO1 protein was 35.6 and 9.3% of that in non-transgenic superior caryopses at 21 and 28 DAF, respectively (Fig. 1D). In inferior caryopses from the transgenic line, reduction of RINO1 protein levels began earlier than in transgenic superior caryopses (Fig. 1C). Reduction was observed from 14 DAF, and the protein level was 77.7% of that in non-transgenic inferior caryopses at 14 DAF (Fig. 1D). Notable decreases were observed at 21 and 28 DAF, and RINO1 protein was not observed in the G-22 inferior caryopses at these times.

In this experiment, only the 1,302 bp 5′-flanking region of the GluB-1 gene was used as a promoter in the G-22 transgenic line. To examine the expression pattern of the GluB-1 promoter, immature seeds of GluB-1 promoter:β-glucuronidase (GUS) transgenic plants were subjected to histochemical analysis (Fig. 2). GUS activity was observed in the endosperm from 7 to 28 DAF in all samples examined from both types of caryopses. From 7 to 10 DAF, the GUS expression was, however, restricted in the aleurone and subaleurone layers of the basal region of the seed, i.e. on the embryo side. The strongest GUS expression was observed 14 DAF, which is consistent with the temporal accumulation pattern of GluB-1 transcripts (Supplementary Fig. S5). There was little difference in the temporal or spatial expression patterns of GUS between superior and inferior caryopses. Inferior caryopses, however, showed stronger GUS activities than superior caryopses, especially in the later stages of seed development.

The position of the caryopsis in the panicle may affect the degree of caryopsis superiority and inferiority; therefore, we next examined many caryopses in panicles from the G-22 line. The Pi content of superior caryopses was examined at
three positions in the upper rachis branches and that of inferior caryopses was examined at four positions: two in the upper rachis branches and two in the lower rachis branches (Fig. 3). The Pi content of G-22 seeds changed depending on their position. A large difference in Pi content was observed among inferior caryopses as well as between superior and inferior caryopses. The Pi content of inferior caryopses at position 7 was significantly higher than that of inferior caryopses at positions 4 and 5, as well as that of superior caryopses at positions 1–3 (Fig. 3). The numbers marked in Fig. 3 essentially coincide with the order of flowering time. Thus, the average Pi content of the G-22 line showed a tendency to increase with delay in flowering time in the panicle.

We expressed antisense RINO1 cDNA under the control of the GluB-1 promoter. Although the transgenic rice plants showed the lpa phenotype to a certain extent, the effect of the transgene was not as robust as that found in lpa mutants (Larson et al. 2000, Liu et al. 2007). One explanation for this limited effect is that the GluB-1 promoter does not confer expression in the embryo where active phytic acid synthesis occurs (Yoshida et al. 1999, Qu and Takaiwa 2004). Therefore, the transgene cannot alter the storage form of phosphorus in the embryo. As, in rice, 19% of the total seed phosphorus accumulates in the embryo (Wada and Maeda 1980), phytic acid in this context is probably not affected by the transgene. Furthermore, the GluB-1 expression reached a maximum at 14 DAF and was observed primarily in the basal region of the seed in the early stages (Fig. 2). However, we previously found that high expression of the RINO1 gene occurs in the embryo and the entire aleurone layers 4 DAF, and the highest expression was observed at 7 DAF, gradually decreasing thereafter (Yoshida et al. 1999). The difference in the temporal and spatial expression patterns between the antisense RINO1 driven by the GluB-1 promoter and the target RINO1 mRNA may have been responsible for the less pronounced increase in seed Pi in the G-22 line compared with the lpa mutant. This difference was greater in the early stages of seed development, as confirmed by immunoblot analysis, which showed a reduction in the RINO1 protein only after 14 and 21 DAF in inferior and superior caryopses, respectively.

Even though the G-22 transgenic line is homozygous (Kuwano et al. 2006), our study showed a large variation in Pi concentrations in T₆ seeds from the G-22 line. The Pi content of inferior caryopses was higher than that in superior caryopses (Fig. 3 and Supplementary Fig. S5). This led to a greater suppressive effect of the transgene on RINO1 gene expression in inferior caryopses. Even among inferior caryopses, the Pi content varied according to seed position in the panicle. Relative superiority and inferiority may affect promoter activity of GluB-1, especially among inferior caryopses, and may have led to the large variations in Pi observed in seeds of the G-22 line.

Rice glutelin genes constituting a multigene family are classified into four subfamilies—GlUA, GluB, GluC and GluD (Kawakatsu et al. 2008). In relation to grain positions in the panicle, Iwasaki et al. (1993) investigated the accumulation of glutelin type A1 (GlUA-1) transcripts. The mRNA

![Fig. 2 Expression patterns of GluB1::GUS fusion construct in superior and inferior caryopses at 7 (A, B), 10 (C, D), 14 (E, F), 21 (G, H) and 28 DAF (I, J). Bars indicate 0.5 mm.](https://academic.oup.com/pcp/article-abstract/50/7/1387/1887080)
expression of GluA-1 was retarded in inferior caryopses compared with superior caryopses. In contrast, we report here that both the temporal and spatial patterns of GluB-1 expression were nearly identical in superior and inferior caryopses and that levels of GluB-1 transcripts were higher in inferior caryopses throughout seed development (Supplementary Fig. S5). It is interesting that the total glutelin protein content of inferior caryopses reached the same level as that of superior caryopses at the end of maturation (Iwasaki et al. 1993), although positional expression patterns in the panicle differed among members of the glutelin multigene family.

In this study, the amount of total phosphorus, the Pi and phytic acid concentrations, and RINO1 protein levels were similar in superior and inferior caryopses from non-transgenic rice, strongly suggesting that the level of phytic acid biosynthesis was stable irrespective of panicle position. This is consistent with previous studies that showed no significant difference in phytic acid content among grains at different positions within the panicle (Liu et al. 2005). In contrast, it has been shown that the content of some seed storage reserves is affected by grain position in a panicle. The starch content has been shown to be greater in superior caryopses than in inferior caryopses (Wang et al. 2007). The activities of enzymes relevant to starch synthesis have been found to be much greater in superior caryopses than in inferior caryopses (Liang et al. 2001, Wang et al. 2007). Ishimaru et al. (2005) reported that several genes involved in carbohydrate metabolism were expressed earlier in superior caryopses than in inferior caryopses. This suggests that there are differences in the regulation of expression of genes involved in phytic acid compared with starch biosynthesis.

Our results suggest that selection of a promoter with the same temporal and spatial expression pattern as RINO1 might be important for suppression of the RINO1 gene in transgenic seeds. The native promoter of the RINO1 gene,
However, cannot be used for molecular breeding to produce lpa seeds because RINO1 catalyzes the first step of inositol metabolism and is involved in many important cellular functions (Shears 2004). Therefore, suppression of the RINO1 gene in vegetative tissues using the native RINO1 promoter may be detrimental to the plant (Feng and Yoshida 2004). Recently, Kuwano et al. (2009) reported that strong lpa transgenic rice was generated when the RINO1 antisense construct was expressed under the control of the oleosin promoter, which specifically directs expression in the embryo and the whole aleurone layer from the early stages of seed development. In contrast to transgenic rice directed by the Glu-B-1 promoter, no significant difference in Pi levels was observed between superior and inferior caryopses when transgenic rice was generated using the oleosin promoter (data not shown). These results indicate that the expression patterns of the promoter and target gene strongly affect gene silencing.

In summary, the results suggest that the difference in temporal and spatial expression patterns between sense and antisense RINO1 transcripts driven by the RINO1 and the Glu-B-1 promoter, respectively, are major factors that account for the low level of transcript suppression observed in seeds from the G-22 line. There may be large variations in anti-sense RINO1 expression levels directed by the Glu-B-1 promoter dependent on panicle positions. Thus, suppression of the RINO1 gene varies within caryopses, which results in differences in phytic acid accumulation in transgenic seeds. We demonstrated the gradual change in the physiological state of rice caryopses associated with the superiority and inferiority characteristic, through the analysis of transgenic plants using a non-native promoter. Further experiments are required to elucidate the detailed mechanisms that control storage reserves in rice seeds.

Materials and Methods

The procedures for transformation, selection and growth conditions of the transgenic line G-22 were described by Kuwano et al. (2006). Non-transgenic control plants (Oryza sativa L. var. japonica cv. Kitaake) were grown at the same time under identical conditions. The positions of spikelets exhibiting superior and inferior caryopses in the panicle are illustrated in Supplementary Fig. S1.

Extraction and measurement of Pi, phytic acid and total phosphorus were carried out as described previously (Kuwano et al. 2006, Kuwano et al. 2009).

Immunoblot analysis was performed as described previously (Feng and Yoshida 2004, Kuwano et al. 2006).

The GluB-1::GUS construct and transgenic rice plants were produced as described by Qu and Takaiwa (2004). GUS analysis was performed as described (Qu and Takaiwa 2004).

Supplementary data

Supplementary data are available at PCP online.

Funding

The Integrated Research Project for Plants, Insects and Animals Using Genome Technology (grant No. IP-5007 to K.T.Y.); the Ministry of Agriculture, Forestry, and Fisheries of Japan (No. 15380004 to K.T.Y.).

Acknowledgements

We would like to thank Drs. Y. Nagato, N. Tsutsumi and M. Nakazono for their helpful discussions.

References


(Received April 7, 2009; Accepted May 19, 2009)