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Full Length Research Paper

Breeding elite *japonica*-type soft rice with high protein content through the introduction of the anti-*Waxy* gene

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Low amylose rice (<10%) that lies between glutinous and viscidity rice is called "soft rice". There is an extremely significant negative correlation between amylose contents and protein contents in common rice grains. In an attempt to analyze whether the anti-*Waxy* gene could increase protein content while reducing amylose content in the rice grains, and develop good soft rice varieties with high protein content, an anti-*Waxy* gene was introduced into the high-yield japonica rice strain, Shangshida No. 2. The amylose contents in the T_1 grains were analyzed. The amylose content in the T_1 grains derived from the T_0 plant with two integrated copies of the anti-*Waxy* gene was only 6.8%, just belonging to the range of soft rice. The T_5 transgenic rice seeds were developed, in which amylose content and protein content were 4.3 and 11.2%, respectively, and the corresponding indexes in the wild type were 12.7 and 9.5%, respectively. In conclusion, it was verified that the anti-*Waxy* gene could increase protein contents while reducing amylose contents of the rice grains; a kind of elite *japonica* transgenic rice with low amylose content and high protein content was obtained through the introduction of an anti-*Waxy* gene. In addition, a new rationale and method for breeding marker-free soft rice by *Agrobacterium tumefaciens*-mediated co-transformation is presented.

Key words: Rice, soft rice, amylose content, protein content, anti-*Waxy* gene.

INTRODUCTION

Yunnan, China is home to a special *indica* type of "soft rice" that lies between glutinous rice and viscidity rice and has low amylose content generally less than 10% (Song and Li, 1997; Zhao et al., 1995). This soft rice tastes delicious when eaten hot or cold and is often used as a raw material for processing rice refreshments or pastries and the raw material for making the famous Chinese "rice-flour noodles".

However, the agronomic characteristics of native varieties of soft rice in Yunnan are poor. The plants are excessively tall, the leaves hang down loosely, and the grain yield is low (<3.75 ton/ha). Recently, some new varieties of soft rice have been developed by cross-breeding (Huang et al., 2002; Zhang, 2001). However, because the progeny have both desirable and undesirable traits from the parent strains, breeders must expend much time and care in screening elite lines. Genetic engineering may allow the agronomic improvement of rice quality as a supplement to traditional breeding methods. Using genetic engineering, the gene down-regulating amylose synthesis is introduced into high-yield rice varieties, thus greatly shortening the breeding period and raising the efficiency of developing low-amylose soft rice.

It has been reported that there was an extremely significant negative correlation between the amylase contents and the protein contents in common rice grains (Song and Zhang, 1992; Yang et al., 2004; Zuo et al., 2001). The rice *Waxy* locus encodes the granule-bound starch synthase involved in amylose synthesis (Okagaki and Wessler, 1988). That the anti-*Waxy* gene could reduce amylose content in rice grains by inhibiting the expression of the endogenous Wx protein have been demonstrated by some researchers (Shimada et al. 1993; Terada et al., 2000; Chen et al., 2002; Liu et al., 2003;

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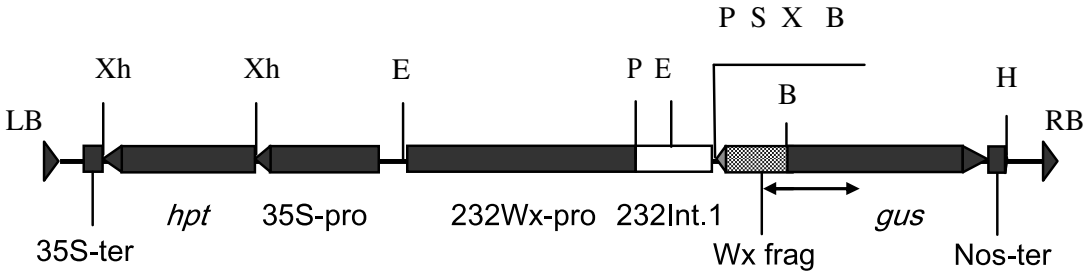

Figure 1. The structure of the T-DNA in the binary vector p13W_4 (Arrows show the location of the PCR product). 232Wx-pro, 232Int.I: promoter and intron 1 of the *Waxy* gene from rice cv.232; Wx frag: antisense 756-bp DNA fragment of *Waxy* gene from rice cv.232; LB, RB: left and right boarder of T-DNA, respectively; Nos-ter: terminator of nos synthase gene; 35S-pro,35S-ter: 35S promoter and 35S terminator of CaMV; *hpt*: hygromicin phosphotransferase gene; *gus*: β-glucuronidase (GUS) gene; X: *Xho*I; E: *Eco*RI; B: *Bam*HI; X*: Xba*I; S: *Sal*I; P: *Pst*I; *H*: *Hin*dIII.

Shen et al., 2004; Li et al., 2005), but in their researches there were not any analysis concerning if the anti-*Waxy* gene could increase protein content of the grains. In our study, an anti-*Waxy* gene was introduced into the high-yield *japonica* rice strain Shangshida No. 2 by *Agrobactium*-mediated methods for attempting to identify whether the anti-*Waxy* gene could increase protein content while reducing amylose content in the rice grains, and to develop good soft rice varieties with high protein content.

MATERIALS AND METHODS

Plant strain and construction of plasmid vector

The high-yield *japonica* rice strain Shangshida No. 2, with a maximum yield of 9.0 ton/ha in the Yangtse Delta, China, was used in this study. The binary vector p13W_4 transfer DNA (T-DNA) (Figure 1) was introduced into *Agrobactium tumefaciens* strain EHA105 and used for rice transformation.

Breeding transgenic rice

Mature embryos of Shangshida No. 2 rice seeds cultivated at the experimental station of Shanghai Normal University, China, were used to induce calli as explants for *Agrobactium*-mediated transformation. After the mature embryos of rice seeds were inoculated on N_6D_2 medium, calli formed in about 7 d. The calli were sub-cultured after two weeks and then used for transformation. The rice calli were infected with *A. tumefaciens* carrying the vector p13W_4 and then cultivated together at 26°C for three days. The selection of hygromycin-resistant calli and regeneration of transgenic plants were performed according to the methods described by Liu et al. (1998). The calli were transferred to selective medium for two rounds of hygromycin selection, after which fresh resistant calli were transferred to differentiation medium. All of the transgenic plants were finally transplanted into flowerpots.

Polymerase chain reaction (PCR) analysis of transgenic plants

PCR using primers W_4P_1 (5'-TGGCAAGAACAAGCATAGACC-3') and W_4P_2 (5'-TAACATACGGCGTGACATCG-3'), located at the anti-*Waxy* gene fragment and the *gus* gene coding region (Figure 1), produces a 626-bp fragment and was used to verify the presence of the inserted gene in p13W_4T-DNA. After 5 min at 94°C for an initial denaturalization, the PCR reactions were subjected to 30 cycles of a 40 s at 94°C, 45 s at 55°C, and 60 s at 72°C, and then an extension step at 72°C for 10 min.

Southern blot analysis of transgenic plants

Genomic DNAs from the T_0 transgenic plants were digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, and transferred to Hybond-N^+ membrane by capillary electrophoresis. The probe was a *gus* gene coding fragment generated by PCR (primers 5'-GGAATCCATCGCAGCGTAATG-3' and 5'-GCCGACAGCAGCAGTTTCATC-3') using p13W_4 as the template and was labeled with HRP by use of the ECL™ Direct Nucleic Acid Labeling Detection System. The blot was developed by enhanced chemiluminescence (Amersham).

Assay of GUS activity

Expression of the *gus* gene in T_1 transgenic seeds was detected as a blue coloration after staining with X-Gluc as described by Jefferson (1987). The segregation ratio of GUS activity was analyzed by the Chi-square (χ^2) test.

Measurement of amylose content in T_1 and T_3 transgenic grains

Thirty rice seeds at the top of the ears of transgenic plants and the wild type were dehulled and floured. The powder was sifted using a sieve with 100 tiny holes and dried at 60°C for 12 h. The amylose content was measured by colorimetry described by Juliano (1971).

Evaluation of quality indexes in T_5 transgenic grains

Four quality indexes, amylose content (AC), protein content (PC), gel consistency (GC) and gel temperature (GT) (usually indicated by Alkali Spreading Value, ASV) were analyzed in T_5 transgenic grains and the wild type by The Quality Supervision, Inspection, and Testing Centre For Rice and Rice Products, Ministry of Agriculture of China. The measurements were repeated three times and the data were analyzed statistically (Tong, 1986).

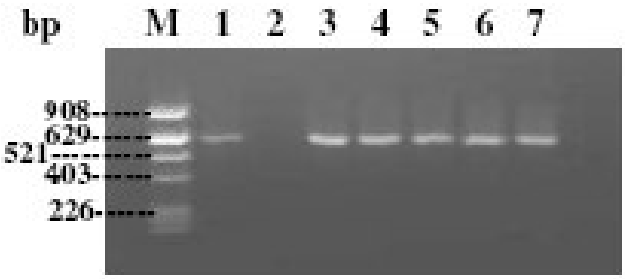

Figure 2. PCR analysis of genomic DNA from transformants and wild type. A 626-bp PCR fragment was amplified from the 5 T_0 transgenic plants and p13W$_4$ plasmid. M: marker DNA, 1: p13W$_4$ plasmid, 2: wild type, 3 - 7: transgenic plants.

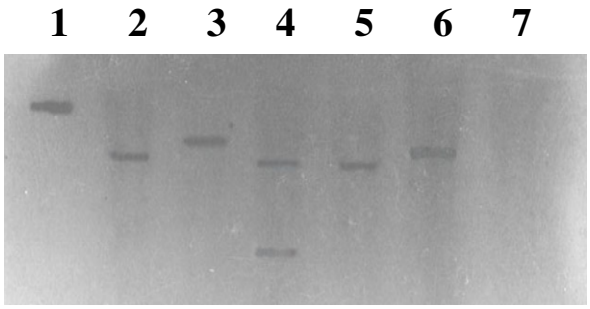

Figure 3. Southern blot analysis of genomic DNA from transformants and wild type. A *gus* sequence from p13W$_4$ plasmid was used as the probe. 1: p13W$_4$ plasmid, 2-6: transgenic plants, 7: wild type

RESULTS

Obtaining transgenic rice plants and molecular confirmation

We successfully obtained five hygromycin-resistant calli that differentiated into seedlings (Sh1-Sh5), with a variable number of transgenic seedlings. After rooting, all of the transgenic young seedlings were transplanted into flowerpots.

To ensure different genetic backgrounds, only one transgenic plant differentiated from each resistant callus was chosen for molecular confirmation. Total DNA from each of the 5 T_0 transgenic plants (Sh1 to Sh5) was analyzed by PCR. The p13W$_4$ and the wild type rice (Shangshida No. 2) were used as positive and negative controls, respectively. A 626-bp PCR fragment was amplified from the 5 T_0 transgenic plants and p13W$_4$ (Figure 2).

To monitor the presence and copy numbers of the introduced T-DNA within the transgenic rice genomes, total DNA prepared from transgenic plants and wild type were digested with *Eco*RI and analyzed by Southern blot using p13W$_4$ as a positive control. The result showed that there were two integrated copies in the Sh3 T_0 plant (Figure 3, lane 4) and only one copy (Figure 3, lane 2, 3, 5 and 6) in the other four transgenic plants (Sh1, Sh2, Sh4 and Sh5).

Assay of GUS activity in the transgenic rice seeds

The T_1 seeds harvested from the T_0 transgenic plants (Sh1 to Sh5) were assayed for GUS activity. The segregation ratio of GUS^+ and GUS^- was 15:1 in the Sh3 T_1 seeds and 3:1 in the other four T_1 transgenic seeds (Table 1). The result of the GUS assay was consistent with the higher number of integrated copies in Sh3 observed by Southern blot analysis.

Amylose content in the T_1 transgenic grains

The amylose contents in T_1 transgenic mature grains (Sh1 to Sh5) were analyzed. The result showed that the amylose content in the T_1 grains derived from the T_0 plant with two anti-*Waxy* gene copies (Sh3) was only 6.8 and 53.4% lower than that in the wild type. The amylose contents in the T_1 grains derived from the T_0 plants with one anti-*Waxy* gene copy (Sh1, Sh2, Sh4, and Sh5) were reduced from 11.6 to 2.7% over the wild type. The degree in reduction of amylose contents in the T_1 grains with two anti-*Waxy* gene copies was more distinct than with one anti-*Waxy* gene copy (Table 2).

Selection of T_5 soft rice line

We observed that the clarity of the T_1 grains derived from the T_0 transgenic plant with two anti-*Waxy* gene copies (Sh3) varied. Some grains were translucent and some were lucent. The remaining GUS-positive, translucent T_1 half-grains obtained from the Sh3 were germinated in 1/2MS culture medium. More than 300 transgenic seedlings were transplanted into the paddy field until maturity. We harvested all of T_2 seeds from the 300 transgenic plants respectively. After evaluating the clarity and assaying GUS activity of T_2 grains from each transgenic plant, we chose the progeny in which grains there were no trait segregations about clarity and GUS activity (it means that all of the seeds harvested from one transgenic T_1 plant were translucent and GUS-positive) continued germinating and obtained T_3 seeds. The average amylose contents in the T_3 grains and the control were 5.0 and 13.9%, respectively.

The T_3 grains had a high-quality appearance after being polished, which were also translucent and looked like pearls (Figure 4). Using the same method, we obtained T_4 and T_5 seeds, respectively, and the T_4 and T_5 grains were also confirmed to have a high-quality appearance by evaluation of clarity.

Table 1. Segregation ratio of GUS activity in transgenic T_1 rice seeds.

Transgenic plants	Total	GUS^+/GUS^-	GUS^+%	Expected ratio	χ^2 Value	P
Sh1	131	96/35	73.3	3:1	0.2062	>0.05
Sh2	95	71/24	74.7	3:1	0.0035	>0.05
Sh3	480	449/31	93.54	15:1	0.0356	>0.05
Sh4	64	54/10	84.4	3:1	3.0000	>0.05
Sh5	64	44/20	68.8	3:1	1.3333	>0.05

Figure 4. Transgenic rice (middle), wild type (left) and glutinous rice purchased from the market (right). Transgenic rice with two anti- *Waxy* gene copies was translucent and looked like pearls, whose clarity lies between the wild type and glutinous rice.

Table 2. Amylose contents (%) in T_1 transgenic grains.

Transgenic and control plants	Amylose contents
Sh1	12.9
Sh2	13.0
Sh3	6.8
Sh4	14.2
Sh5	13.7
Shangshida No. 2 (Wild type)	14.6

Quality indexes in T_5 transgenic grains

Four quality indexes (AC, PC, GC and ASV) of the T_5 transgenic grains from the Sh3 and wild type (Shangshi No. 2) were analyzed and the results are listed in Table 3. The results showed that the AC, PC, GC and ASV in the wild type were 12.7%, 9.5%, 70.0 mm and 6.3 degrees, respectively, while the corresponding indexes in the T_5 transgenic grains with two integrated copies were 4.3%, 11.2%, 94.7 mm and 6.9 degrees, respectively. The differences in the AC, PC and GC between the wild type and the T_5 grains were statistically significant ($P < 0.01$, $P < 0.01$ and $P < 0.05$, respectively). The PC in the T_5 transgenic grains increased while the AC decreased, over 17.89% than that in the wild type, and there was an extremely significant negative correlation between the AC and the PC ($P < 0.01$). The GC in the T_5 transgenic grains increased also while the AC decreased. There was an extremely significant negative correlation between the AC and the GC ($P < 0.01$) and a non-significant negative correlation between the AC and the ASV.

DISCUSSION

Rice is one of the most important cereal crops, which provide the major source of protein for humans, especially in Asian and African countries where the population is still increasing, and storage proteins in rice seeds provide also a source of nitrogen and carbon for germinating seedlings. Compared with other major cereals, rice grains contain small amounts of storage proteins. Therefore, nutritional improvement in protein contents of rice grains is desired. Nowadays, rice nutritional value has been improved by engineering technique (Zhang et al., 1995; Sindhu et al., 1997; Katsube et al., 1999). However, the entire target genes used in their researches was heterologous storage protein genes. We verified the anti-*Waxy* gene from rice own genome is also available in improving rice nutritional quality.

AC, GC, and GT (indicated with alkali spreading value, ASV) are three major indexes affecting rice eating and cooking qualities. A negative relationship between AC and GC (Li et al., 2001; Shun et al., 2005) and a positive relationship between AC and ASV (Shu et al., 1999) has been reported. Our present results show that besides PC,

Table 3. Amylose content (AC), protein content (PC), gel consistency (GC) and alkali spreading value (ASV) in T_5 rice grains and wild type.

Transgenic plants and wild type	AC ± SE (%)	PC ± SE (%)	GC ± SE (mm)	ASV ± SE (degree)
T_5 transgenic grains from the Sh3	4.3 ± 0.3	11.2 ± 0.1	94.7 ± 4.4	6.9 ± 0.1
Shangshida No. 2 (Wild type)	12.7 ± 0.5	9.5 ± 0.2	70.0 ± 4.2	6.3 ± 0.5

SE: Standard error.

a low AC in transgenic rice grains caused by the anti-*Waxy* gene increased both GC and ASV. However, we observed the GC increasing distinctly with AC reduction, suggesting that the linear correlation between AC and GC is more obvious than between the AC and GT. From these results, we conclude that the anti-*Waxy* gene has a greater effect on the GC than the GT apart from its ability to effectively reduce AC in rice.

More and more commercial products of transgenic crops have become available in recent years. The public has attached great importance to biosafety of transgenic food. The application of transgenic crops containing selective marker genes might be confined geographically. Marker-free transgenic plants are very important for the extension application of transgenic crops. We have established a method for breeding marker-free transgenic rice by *A. tumefaciens*-mediated co-transformation and successfully used this method to transfer the anti-*Waxy* gene into rice (Li et al., 2005). Data from our lab (unpublished results) showed that it was difficult to obtain homozygous lines from the marker-free transgenic plants with two integrated T-DNA copies. However, a kind of new marker-free soft rice that might be applied to agriculture production could be rapidly bred by genetic engineering by the following method. Two anti-*Waxy* genes containing their respective promoters are constructed into one vector T-DNA lacking a resistance marker gene, and the rice is co-transformed by two separate strains of *A. tumefaciens* containing a resistance marker gene vector and a vector containing two anti-*Waxy* genes. This program has been already carried out in our laboratory (unpublished results).

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