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Expression of Rice Glutelin Gene *GluA-2* in Wheat Endosperm

CHEN Yu, LI Hui, LI Hong-Jie, LI Yi-Wen, ZHU Yin-Feng, QU Le-Qing*, JIA Xu**

(*State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, The Chinese Academy of Sciences*, Beijing 100101, China)

Abstract: Rice (*Oryza sativa* L.) glutelin accounts for about 80% of total seed storage protein. Glutelin can be easily digested by human and contains high concentrations of lysine and other essential amino acids. To improve the nutritional quality of wheat (*Triticum aestivum* L.), the cDNA sequence of rice glutelin gene *GluA-2* was introduced into common wheat through biolistic bombardment. Six hundreds of immature embryos from wheat cultivar Bobwhite (*T. aestivum* cv. Bobwhite) were bombarded. Four transgenic plants carrying *GluA-2* gene were confirmed by PCR and Southern blotting analyses. Gene *GluA-2* was translated in three of the transgenic plants and their progeny, as indicated by SDS-PAGE analyses. The expression of *GluA-2* gene was not detected in one transgenic plant and the endogenous wheat high molecular weight glutenin subunits Bx7 and By9 contents decreased greatly in both T_0 and T_1 generations.
Key words: rice glutelin gene *GluA-2* ; wheat transformation; nutrient quality improvement

The main composition of endosperm storage proteins in rice (*Oryza sativa*) is glutelins, which constitutes 60%-80% of total endosperm proteins in mature rice kernels. Glutelin can be easily consumed by human and contains high concentrations of lysine (2.6%-3.5%, mol%) and essential amino acids (33.6%-41.4%, mol%). Because of these properties, glutelin has been recognized as one of the most important sources of nutrition for human beings (Villareal and Juliano, 1978).

Wheat (*Triticum aestivum*) is an important cereal crop, which provides a major source of energy and proteins for human being throughout the world. Gliadin and glutenin are dominant parts of the storage proteins in wheat, accounting for about 85% of total grain proteins. However, wheat gliadin and glutenin can not be easily digested by human because of the low solubility and unique structure (Gianibelli *et al.*, 2001). Moreover, lysine and other essential amino acids only account for 0.9% and 13.4% (mol%) of wheat seed storage proteins (Shewry and Tatham, 1990; Gianibelli *et al.*, 2001). This reduces the use of wheat and improvement of wheat nutritional quality is thus an important objective in wheat breeding programs.

The use of cloned genes is an alternative approach to improve nutritional composition of cereal crops to the time-consuming conventional breeding methods. Genes that originate from a crop may express themselves in another crop, which allows prompt improvement of traits of interest. Genes for soybean ferritin and phytoene synthase have been transferred into rice, resulting in high contents of iron and vitamine A in transgenic lines (Burkherdt *et al.*, 1997; Goto *et al.*, 1999). In wheat, much attention has been focused on improving baking-quality-related characteristics. A number of high molecular weight glutenin subunits (HMW-GS) genes isolated from common wheat, such as *1Ax1*, *1Dx5* and *1Dy10* conferring qualitative and/or quantitative effects on baking quality, have been transferred into various wheat cultivars and the baking quality of transgenic lines have been improved greatly in most cases (Altpeter *et al.*, 1996; Blechl and Anderson, 1996; Barro *et al.*, 1997; Rook *et al.*, 1997; Alvarez *et al.*, 2000). However, little information is available for the increase of essential amino acid and digestible protein content through transgenic technique compared to improvement of baking quality.

Rice glutelin gene *GluA-2* belongs to a mini gene family. The coding region of *GluA-2* is 1 500 bp in length, which encodes a 57 kD glutelin precursor. The contents of lysine and essential amino acids are 2.42% and 33.86% (mol %) in glutelin precursor, respectively. Although *GluA-2* was cloned more than ten years ago (Okita *et al.*, 1989), this gene has not been transferred into any crop. Since the peptide encoded by the gene *GluA-2* produces higher contents of essential amino acids, *GluA-2* might be useful to improve wheat seed storage protein composition. In this study, we introduced the cDNA sequence of rice glutelin gene *GluA-2* under the control of wheat endosperm

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* Author for correspondence. E-mail: <lqqu@genetics.ac.cn>.
** Author for correspondence. E-mail: <xjia@genetics.ac.cn>.

specific expression promoter *Glu1-D1* into spring wheat cultivar Bobwhite by biolistic bombardment. The expression of *GluA-2* was confirmed by SDS-PAGE analyses. It is expected that the overexpression of *GluA-2* gene in wheat endosperm will increase the essential amino acid and digestible protein contents.

1 Materials and Methods

1.1 Plant materials

The spring wheat cultivar Bobwhite (*Triticum aestivum* L. cv. Bobwhite) was grown in greenhouse set at 20-28 ºC/15-18 ºC day/night to provide immature embryos for transformation. A wheat cultivar Chinese Spring (*T. aestivum* cv. Chinese Spring) and a Japanese rice cultivar Kinmzae (*Oryza sativa* L. cv. Kinmaze) were used as controls in molecular characterization of transgenic plants.

1.2 Plasmid construction

The 1 645 bp long cDNA sequence of rice glutelin gene *GluA-2* was cloned by Okita (Okita *et al.*, 1989) and cut by *Eco*R Ⅰ, inserted into plasmid pBluscriptKS (Stragene, La Jolla, CA, USA). The fragment released by cutting with *Bam*H Ⅰ and *Sal* Ⅰ was placed at the downstream of wheat endosperm specific expression promoter *Glu1-D1* (Lamacchia *et al.*, 2001) followed by 3' -untranslated region (terminator) of the nopaline synthase (*Nos*) and the plasmid is designated pGluGluA-2 (Fig.1).

1.3 Wheat transformation

Wheat transformation was conducted following the previously published method (Liang *et al.*, 2000) with slight modification. The immature embryos of Bobwhite were collected at 10-14 d after pollination, surface-sterilized with 70% (V/V) ethanol for 1 min and 5% (V/V) sodium hypocholorite for 25 min, and rinsed with sterile-distilled water for four times. The immature embryos were pre-cultured on MS medium (Murashige and Skoog, 1962) supplemented with 2 mg/L 2,4-D (2,4-dichlorophenoxyacetic acid) for 3 d and then placed on high osmolarity medium (MS medium with 0.5 mol/L maltose) for 6 h before bombardment with the DNA-coated gold particles at 1 100 pound-force per square inch (psi) using a BioRad PDS-1000/He biolistic device (BioRad Laboratories Ltd., Hercules, CA, USA). The gold particles were coated with a mixture of the plasmids pGluGluA-2 and pAHC25 at 1∶1 molar ratio. The plasmid pAHC25 contains the marker genes *Bar* (encoding phosphinothricin acetyltransferase) and *UidA* (GUS) (encoding β-glucuronidase), each of which was under the transcriptional control of a separate maize ubiquitin (*Ubi-1*) promoter (Christensen *et al.*, 1992) and terminated by the *Nos* 3' untranslated region. Following bombardment, the immature embryos were maintained on high osmolarety medium for 18 h before being cultured on MS medium with 2 mg/L 2,4-D for 10 d . They were then cultured on selection medium, which was composed of MS salt, 5 mg/L *phosphinotricin* (PPT), 2 mg/L 2,4-D, and 30 g sucrose pH 5.8 at 25 ºC in dark for 2-3 weeks. The embryogenic calli were then transferred onto shoot regeneration medium (MS basal medium except for half-strength of macro-salt and supplemented with 1 mg/L 3-inositol-acetic-acid, 1 mg/L Zeatin, 3 mg/L PPT) and cultured at 25 ºC with 16/8 light/dark photoperiod. After 2-3 weeks growth, green shoots were transferred on rooting medium (MS basal medium except for half strength of macro salt supplemented with 3 mg/L PPT and 8% sucrose). Three weeks later, well-rooted plantlets were transplanted into soil and then grown in greenhouse.

1.4 PCR analysis

Oligonucleotide primers were designed according to the coding sequence of gene *GluA-2* (GenBank accession number M28156). The sequences of the primers were forward 5'-ATTAGGCCAGAGCACTAGTCAATG-3' and reverse 5'-CTTGTATTGGAGGGGAGTGAATG-3'. The amplification was programmed in a PTC-100 thermocycler (MJ Research Inc. Watertown, MA, USA) at 94 ºC for 5 min to allow denature of DNA, followed by 35 cycles of 94 ºC for 1 min, 57 ºC for 1 min, and 72 ºC for 2 min. The final extension step was performed at 72 ºC for 10 min. The PCR products were separated on a 0.8% agarose gel.

1.5 Southern blotting analysis

Genomic DNA was isolated from leaf tissue of transgenic plants by using CTAB method (Rogers and Bendlich, 1994) . Twenty microgrammes of DNA was digested with *Bam*H Ⅰ. Digested DNA was electrophoretically on a 0.8%

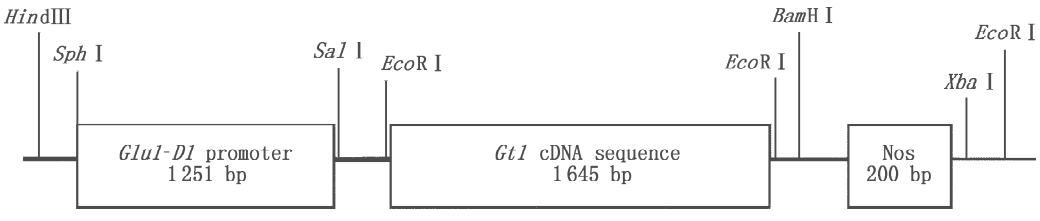

Fig.1. Map of plasmid pGluGluA-2. Nos, nopaline synthase.

agarose gel and transferred onto Hybond N^+ membrane (Amersham, Buckinghamshire, England) according to the instructions of the manufacturer. The 1 645 bp cDNA fragment of gene *GluA-2* was labeled with α-^{32}P-dCTP by random primer labeling method (Primer G labeling system, Promega, Madison, Wisconsin , USA). Hybridization and washing steps were carried out as previously described (Sambrook *et al.*, 1989).

1.6 SDS-PAGE analysis

Half mature grain was grinded by mortar and pestle and extracted by 400 μL of extraction buffer (62.5 mmol/L Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 0.05% bromophenol blue, and 5% mercaptoethanol). Following incubation for at least 3 h at room temperature, the extracts were boiled for 5 min and centrifuged for 5 min at 12 500 r/min. Total proteins were separated by SDS-PAGE method following a method described by Laemmli (1970).

2 Results

2.1 PCR and Southern blotting analysis

Six hundreds of immature embryos from Bobwhite were bombarded and 14 PPT-resistant plantlets were obtained. To examine the presence of gene *GluA-2*, DNA from leaves of all the resistant plants were amplified by PCR reaction, using primers specific for gene *GluA-2*. Four of them (BT-5-2, BT-7-2, BT-9A and BT-11) exhibited the polymorphic band 1 381 bp in length (data not shown). PCR analysis was conducted on all available T_1 progeny plants to determine the presence of gene *GluA-2*. For each sample, PCR reactions were repeated three times. A plant was considered as a transgenic plant when the diagnostic 1 381 bp fragments were amplified at least in two reactions (Table 1, Fig.2).

Genomic DNA was extracted from the four T_0 transgenic plants and digested with *Bam*HⅠ. *Bam*HⅠ has a cleavage site between cDNA sequence of gene *GluA-2* and *Nos* terminator. The products digested with *Bam*HⅠ allow detection of the integration pattern of *GluA-2*. Multiple hybridization fragments similar in size to the plasmid used for transformation (6.0 kb) or longer were detected in DNA

Table 1 PCR analysis of individual transgenic plants of T_1 progenies

T_1 progeny	PCR-positive*	PCR-negative*
BT5-2	8	12
BT7-2	7	8
BT9A	3	2
BT11	6	2

*, a plant that exhibited the polymorphic band 1 381 bp in length was considered as positive to PCR amplification, or negative when this band was not amplified.

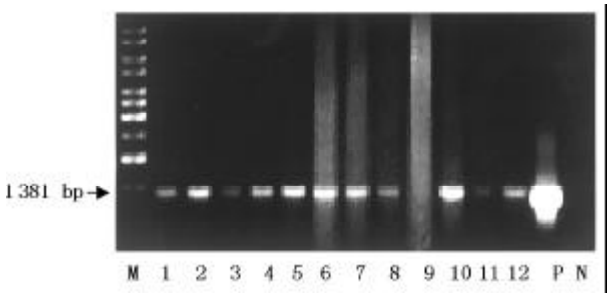

Fig.2. PCR analysis of T_1 generation of transgenic line. Lanes M, 1 kb ladder (MBI Fermentas); N, Bobwhite; P, cDNA of GluA-2; 1-4, T_1 progeny of transgenic plant BT-9A; 5-12 ,T_1 progeny of transgenic plant BT-11.

samples from the T_0 and T_1 transgenic plants, which confirmed that the *GluA-2* gene was integrated into the gonomic of all of the four transgenic plants and their progeny, and the copy number was estimated over five (Fig.3). The smaller hybridization fragments indicated the presence of truncated or rearranged forms of the gene *GluA-2*.

2.2 SDS-PAGE analysis

The SDS-PAGE analysis on the four wheat transgenic plants and their progeny indicated that a major band was present in T_0 and T_1 seeds of the three transgenic plants (BT-5-2, BT-7-2 and BT-11), which was absent in non-transformed Bobwhite and Chinese Spring controls. The molecular weight of this additional band was about 57 kD, which

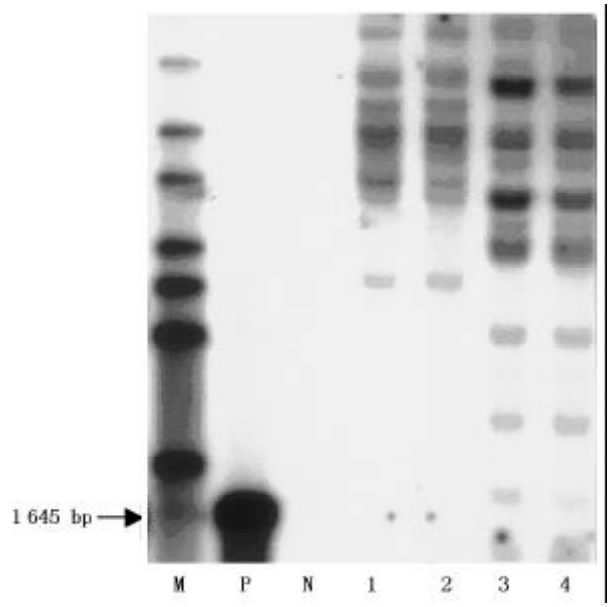

Fig.3. Southern blot of T_1 generation of transgenic line. Lanes M, 1 kb ladder; N, genomic DNA of Bobwhite digested with *Bam*HⅠ; P, cDNA of gene *GluA-2*; 1-2 , genomic DNA of BT9A T_1 transgenic plants digested with *Bam*HⅠ; 3-4, genomic DNA of BT-11 digested with *Bam*HⅠ.

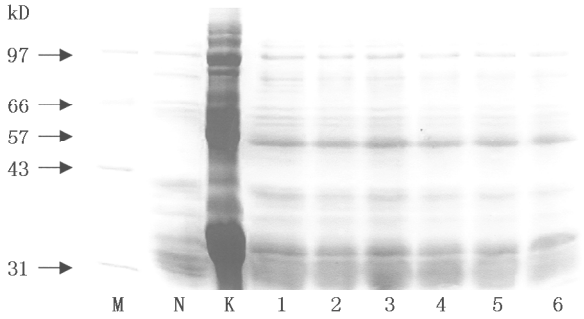

Fig.4. SDS-PAGE analysis of the seeds from transgenic plants. Lanes K, Kinmaze; M, protein marker (Sabc bioengineer Co., Luoyang, China); N, Bobwhite; T_0 and T_1, transgenic plants; 1 and 2, BT5-2; 3 and 4, BT7-2; 5 and 6, BT-11.

was similar to that of the rice glutelin precursor (Fig.4). Based on this result, we can draw a conclusion that glutelin was synthesized in seeds of T_0 and T_1 transgenic plants, and the expression of *GluA-2* gene was almost at the same level (Table 2). The bands of 37-39 kD and 20-22 kD were not observed in wheat seed protein extract. The expression of HMW-GS *1Bx7* and *1By9* genes might be partially or completely suppressed by the introduction of gene *GluA-2* in one transgenic plant (BT-9A) and the suppression can be transmitted to its progeny (Fig.5).

Table 2 Densitometer analysis of relative Glutelin transgene expression in T_0 and T_1 generations of the transgenic plants

Plants	Generation	No. of seeds examined	Glutelin/Bx7*	Glutelin/Dx5*
BT5-2	T_0	5	1.42	2.83
	T_1	9	1.23	2.16
BT7-2	T_0	8	1.33	3.56
	T_1	11	1.20	3.32
BT11	T_0	4	1.06	3.17
	T_1	10	1.01	3.21

*, average from density scan of SDS-PAGE gels. The density value of Glutelin was divided by the density value of HMW-GS and background value.

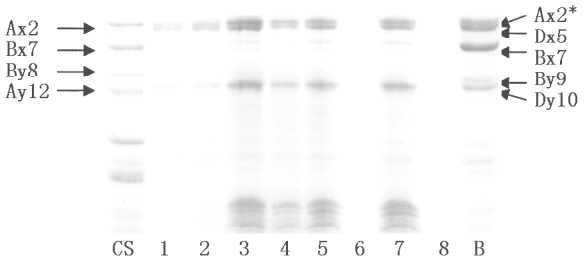

Fig.5. SDS-PAGE analysis of seeds from BT-9A T_0 and T_1 progeny. Lanes B, Bobwhite; CS, Chinese Spring; T_0, transgenic plants; T_1, progeny; 1-2, seeds of BT-9A; 3-8, seeds of BT-9A.

3 Discussion

Gene *GluA-2* for rice glutelin was transferred through biolistic bombardment into wheat cultivar Bobwhite and expressed in wheat endosperm, as indicated by the presence of the glutelin similar in size to the 57 kD rice glutenin precursor (Fig.4). The plants that have been verified to carry gene *GluA-2* are being increased to realize genetically uniform lines. The analysis in later generations will allow understanding whether gene *GluA-2* is helpful to increase the content of essential amino acids in wheat.

In rice, glutelin is synthesized in endoplasmic reticulum as a 57-kD precursor and then processed into two mature subunits with a molecule mass of 37-39 kD and 21-23 kD. Sindhu *et al.* (1997) introduced pea legumin gene into rice. The expression pattern of legumin gene in rice remains the same as that in pea. The 60 kD precursor was synthesized and processed into a 40-kD acid and a 20-kD basic subunit. This might be that the glutelin and legumin share the same ancestor of 11S globulin and similar post-translation processing site. However, the acid and basic subunits were not observed in seed extracts of transgenic wheat plants in present study. This may be caused by the fact that the post-translation process of wheat storage protein is unlike that of rice glutelin. Therefore, the 57 kD precursor can not be divided in wheat endosperm.

Currently, little is known about the precise organization of the HMW-GS and low molecular weight glutenin subunits (LMW-GS) in glutenin polymers. It is widely accepted that HMW subunits form a disulphide bonded network. The LMW-GS acts as branches and possibly provides some cross-links (Shewry and Tatham, 1990). The introduced HMW-GS gene, such as *1Ax1*, dramatically increased the dough strength (Altpeter *et al.*, 1996). However, Popineau *et al.* (2001) reported that the overexpression of HMW-GS *1Dx5* gene in transgenic lines resulted in difficulty for dough formation. Rhelogical analysis of glutens isolated from these lines indicated that the connectivity of gluten network had been greatly increased for 10 to 100 folds. Rice glutelin is hexamers composed of six identical subunits, which are formed by acid and basic subunits. The acid and basic glutelin subunits contain six and two Cysteine residues. The acid subunit has one intramolecular disulfide bond and is linked to the basic subunit with an intermolecular disulfide bond (Huebner *et al.*, 1990) . It is not known whether the same structure would be formed in wheat endosperm because the acid and basic subunits were not observed. However, the glutelin may primarily be

present as small polymer in wheat endosperm. So the glutelin may deposit in the network that was built by wheat HMW-GS other than join the network construction.

Gene silence has been frequently documented in transgenic studies. Alvarez *et al.* (2000) transferred genes *1Ax1* and *1Dx5* conferring HMW-GS into common wheat. In transgenic plants with high copy numbers (20-50) of the transgenes, all endogenous HMW-GS genes were silenced. In two of four plants having low copy numbers (1-3) transgenes, endogenous *1Ax2* gene did not express, and no gene silence was observed in other two transgenic plants. Altpeter *et al.* (1996) transformed genes HMW-GS *1Ax1* and *1Dx5* into wheat cultivar Bobwhite and speculated that the integration of transgene could result in endogenous *1Ax2** gene silence. Since both transgenes and endogenous genes *1Bx7* and *1By9* were suppressed at the same time in one transgenic plant, it resembles co-suppression that mutual inactivation of transgene and homologous gene occurs. Vaucheret (1993) indicated that only partial homology in promoter or coding sequence was needed for co-suppression. However, de Carvalho *et al.* (1995) concluded that the transgene expression level was very high when co-suppression occurred. In the present study, the expression of gene *GluA-2* was not detected in some transgenic plants of T_0 and T_1 generations. The homology between *GluA-2* and HMW-GS *1Bx7* and *1By9* were about 25%, which was measured by DNAMAN software (Lynnon Biosoft Co. USA). In this study, the *Glu1-D1* promoter was amplified from HMW-GS gene *1Dx5* promoter. When gene *UidA* under the control of *Glu1-D1* promoter was introduced into common wheat, *UidA* was specifically expressed in wheat endosperm and no gene silence was observed (Lamacchia *et al.*, 2001). Based on these results, the gene silence in the present study might result from the homology between gene *GluA-2* and genes *Bx7* and *By9* and gene *GluA-2* insertion site in wheat genome. Furthermore, the gene silence can be inherited to T_1 progeny, since genes *1Bx7* and *1By9* were completely silenced.

The transgenic progenies reported in this study provide materials to study relationship between baking-quality and HMW glutenin subunits composition in wheat. Wheat HMW-GS appears to have quantitative and qualitative effects on baking quality. For example, HMW-GS genes *1Ax1*, *1Dx5* and *1Dy10* have been proved to be associated with good bread making quality. While HMW-GS genes *1Ax2* and *1Ay12* are considered to be responsible for poor bread baking quality. However, there is not a clear resolution for the relationships because of lacking enough mutants (Gianibelli *et al.*, 2001). Backcrossing this HMW-GS *1Bx-7* and *1By-9* gene mutants with Bobwhite wheat allows the production of near isogenic lines with different genes for glutenin subunits, with which the relationship between grain baking quality and HMW-GS *Bx-7* and *By-9* subunits can be elucidated clearly.

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