GENETIC TRANSFORMATION OF EGYPTIAN WHEAT WITH 1DX5 HIGH-MOLECULAR-WEIGHT GLUTENIN SUBUNIT GENE

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Wheat (Triticum aestivum L.) seed-storage proteins represent an important source of food and energy (Cooke and Law, 1998). The major endosperm storage proteins of wheat grains are prolamins. It consists of polymeric glutenins and monomeric gliadins. Under reducing conditions, polymeric glutenins are subdivided into high-molecular-weight glutenins (HMW-GS) and low-molecular-weight glutenins (LMW-GS) according to their mobility by SDS-PAGE (Payne \textit{et al.}, 1987 & 1988). The composition of glutenin subunits in wheat is important for determining gluten and dough elasticity of wheat. There is a strong correlation between visco-elasticity and the relative amount of glutenin polymers with highest molecular masses (Payne \textit{et al.}, 1982; Thompson \textit{et al.}, 1983). HMW-GS coding genes in hexaploid bread wheat are located on three \textit{Glu-1} loci at the long arms of chromosomes 1A, 1B and 1D. Each locus is named as \textit{Glu-A1}, \textit{Glu-B1} and \textit{GluD1}, respectively, and contains two closely linked \textit{Glu-1-1} and \textit{Glu-1-2} genes encoding x- and y-types, respectively. The gene products of \textit{Glu-1x} and \textit{Glu-1y} are distinguished from one another primarily on the basis of their size (Lawrence and Shepherd, 1981; Payne, 1987). A number of \textit{Glu-1} genes have been cloned, from bread wheat (Anderson and Greene, 1989; Anderson \textit{et al.}, 1989).

Analyses of wheat cultivars have shown that HMW-GS differ in their impact on bread-making performance with subunits \textit{1Ax1} and \textit{1Ax2} and subunits \textit{1Dx5} and \textit{1Dy10} in particular being associated with high dough strength and good bread-making quality (Payne, 1987; Shewry \textit{et al.}, 2003). HMW-GS have been used to alter wheat grain quality by genetic transformation (Altpeter \textit{et al.}, 1996; Blechl and Anderson 1996; Barro \textit{et al.}, 1997; Fahmy \textit{et al.}, 2006). The HMW-GS \textit{1Dx5} and \textit{1Dy10} give better performances in dough strength and bread-making quality than the homeo-allelic subunits \textit{1Dx2} and \textit{1Dy12} (Payne \textit{et al.}, 1982; Popineau \textit{et al.}, 1994; Payne \textit{et al.}, 1987) which have relatively low-molecular-weights. Therefore, it is possible to improve the gluten quality of wheat by introducing novel copies of HMW-GS genes (Shewry, 1994). Many laboratories have reported the expression of HMW-GS transgenes (Altpeter \textit{et al.}, 1996; Blechl \textit{et al.}, 1997; Barro \textit{et al.}, 1997; Rooke \textit{et al.}, 1999). We have previously reported the transformation of maize, wheat and barley using

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\textit{Egypt. J. Genet. Cytol., 41: 327-335, July, 2012}
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HMW-GS 1Dy10 (Abdallah et al., 2004; Fahmy et al., 2006; Abdalla et al., 2008). Here we report our work on transgenic Egyptian wheat cultivar (Giza 164) expressing HMS-GS 1Dx5 gene using microprojectile bombarded with immature embryos.

MATERIALS AND METHODS

Wheat transformation

Immature embryo-derived calli of the Egyptian wheat cultivar Giza 164 were co-transformed with plasmid pAHC25 (harboring gus and bar genes) and plasmid pK-Dx5 (BlueScript KS plasmid harboring HMW-GS 1DX5 gene driven by its own promoter). The transformation / regeneration procedure was done according to Fahmy et al. (2004 and 2006).

DNA extraction

Genomic DNA was extracted from putative transgenic and control plants (non transgenic). DNA was isolated from leaf samples using DNeasy plant Mini Kit (QIAGEN, Germany).

Protein extraction and separation

Protein was extracted from putative transgenic and control plants. Wheat grains were ground to a fine powder and extracted using 0.25M Tris-HCl buffer (pH 6.8) containing 5% (v/v) β-mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerol and 0.02 (w/v) bromophenol blue. The extracts were heated at 100°C for 2 min and centrifuged for 2 minutes at 15000 rpm. Protein samples were separated by 10% (w/v) polyacrylamide gel electrophoresis in the presence of sodium-dodecyl-sulfate (SDS-PAGE) according to Laemmli buffer system (Laemmli, 1970). The gel was fixed in 5% trichloroacetic acid for 30 min and then stained in Coomassie brilliant blue R250 for 0.5-2 h. Gel was destained with distilled water, until clear protein bands were detected and then dried between sheets of cellophane (Promega, USA).

Histochemical assay

A histochemical GUS assay was conducted as described by Jefferson et al., (1987). Wheat spikelet were incubated overnight at 37°C in X-Gluc solution, containing 0.1 M sodium phosphate buffer, pH 7.0; 50 mM EDTA, 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.1% Triton X-100, 1 mg/ml X-gluc (5-bromo-4-chloro-3-indolyl-b-D-glucuronide, as chromogenic substrate). After staining, grain spikelets were soaked in 70% ethanol for bleaching. Assayed tissues were observed and photographed.

Leaf painting assay

Leaf painting assay was performed to verify the expression of bar gene. The bar gene activity in putative transgenic plants was assayed according to Schroeder et al. (1993). The upper surface of a leaflet was thoroughly wet by painting with an aqueous solution of herbicide Basta (Aventis GmbH, Germany) with a final Phosphinothricin (PPT) concentration of 0.2 mg/L and 0.1% Tween 20. Leaves
were scored for herbicide damage seven days after application.

**PCR analysis**

Genomic PCR was carried out using Taq polymerase (Promega, USA). The detection of the integrated IDX5 gene in the transgenic plants was performed using KS (5’-TCG AGG TCG ACG GTA TC-3’) and DX5R1 (5’-ATC GTC GCC GCC CTT CGT TC-3’) specific primers. The cycling conditions for the reaction were: one cycle at 95°C for 3 min, followed by 35 cycles of 94°C for 40 sec, 60°C for 30 sec, extension at 72°C for 1 min and final extension step at 72°C for 7 min. The primers used to detect the sequences of gus and bar genes were as the following:

GUS F: 5’-AGTGTACGTATCACCCTTGTTGTGAAC-3’;
GUS R: 5’-ATCGCCGCTTTGGACATACCATCCGTA-3’;
BAR F: 5’-CCAGAAAC-CCACGTGATGCC-3’;
BAR R: 5’-GAAGTCCAGCTGCCAGAAA-3’.

The cycling conditions were: one cycle at 95°C for 3 min; followed by 35 cycles of 94°C for 30 sec, 62°C for gus and 57°C for bar at 30 sec, extension at 72°C for 1 min and final extension step at 72°C for 7 min. The PCR products were separated in 1.2% agarose gels.

**RESULTS AND DISCUSSION**

The introduction of foreign genes into wheat is a powerful tool for research and to improve elite wheat cultivars. The successful application of genetic modification depends on an effective transformation system, the availability of genes for target traits and the use of regulatory sequences capable of driving appropriate levels of expression in the tissues and developmental stages required.

This work is a part of collective study on wheat HMW-GS and its effect on bread making quality in Egypt. According to a previous study (Abdalla et al., 2011), we have tested 17 Egyptian wheat cultivars for the distribution of HMW-GS. Accordingly, we selected cultivar Giza 164 to be transformed with IDX5 gene because its genome does not contain such gene.

**Transformation**

Immature embryo-derived calli (1200 calli) were co-transformed with plasmid pAHC25 and plasmid pK-Dx5 using biolistic bombardment. After selection with bilaphose during regeneration process, seven putative transgenic plants were produced in vitro. Plantlets were transferred to pots for acclimatization. The obtained Plants were subjected to molecular and biochemical analysis to confirm the integration of the transgenes in their genome and to study the expression of the inserted genes.

**Molecular analysis**

PCR analysis revealed successful integration of the exogenous genes (bar, gus and IDX5) in the genome of seven
putative transgenic plants. PCR amplifications using primers specific to bar (Fig. 1A), gus (Fig. 1B) and IDX5 (Fig. 1C) genes yielded the expected products of 443 bp, 1050 bp and 529 bp, respectively, in putative transgenic plants (Lanes 3-9). Plasmids pAHC25 (Fig. 1A, 1B, lane 1) and pK-Dx5 (Fig. 1C, lane 1) were used as a positive control templates and they revealed PCR products of the expected sizes. No amplified products were detected in non-transformed control plants (Fig. 1, lane 2).

Expressions of marker genes were examined histochemically for GUS activity and by leaf painting assay for bar gene. The GUS expression of the transgenic wheat plants transformed with pAHC25 was confirmed by GUS staining of wheat spikelet tissues. As shown in Fig. (2), transgenic tissues exhibit GUS expression (blue color) clearly distinguishable from those of the control, indicating stable gus gene integration and expression into the genome of the plants.

The expression of bar gene was confirmed by leaf-painting assay (Lonsdale et al., 1998). Leaf area, putative transformants and control plants were painted using a solution of phosphinothricin (150 mg/L) and 0.1% Tween-20 and examined after seven days. Absence of necrotic damage in putative transgenic plants as compared to controls was taken as evidence for the expression of bar gene (Fig. 3). Several reports showed successful integration and expression of bar gene after transformation using leaf painting assay (Wan and Lemaux, 1994; Cho et al., 1998; Harwood et al., 2000).

After screening with PCR, the plants were subjected to analysis for the expression of HMW-GS IDX5 gene in grains of T0 plants by SDS-PAGE. As indicated in Fig. (4), the total protein extracts from seven putative transgenic plants showed that five of the transgenic lines (samples 3, 4, 5, 6 and 8) contained additional HMW subunit band of the expected mobility for IDx5. However, samples number 2 and 7 did not exhibit such band, even though, these samples were confirmed by PCR for the integration of IDx5 gene. Several authors reported silencing expression of integrated genes (Blechl et al., 1997; Alvarez et al., 2000). Also, DNA methylation may affect the level of transcription of the integrated genes (Muller et al., 1996; Razin, 1988).

Our results are in agreement with there of Barro et al. (1997) who showed the expression of the IDx5 transgene. They were also able to introduce the 1Ax1 transgene alone, or with IDx5 and analyzing the mixing properties of the resulting dough which showed that expression of the transgenes were correlated with increasing dough strength.

In summary, the importance of introducing HMW-GS such as 1Ax1, IDx5 and IDy10 genes in Egyptian wheat cultivars can improve bread-making quality (Altpeter et al., 1996; Blechl and Anderson, 1996; Barro et al., 1997; Shimoni et
Therefore, in this study we produced transgenic Egyptian wheat cultivar (Giza 164) with \textit{Dx5} gene. This might contribute to produce elite wheat cultivars with enhanced bread making quality traits.

**SUMMARY**

Variation in the composition of the high-molecular-weight glutenin subunits (HMW-GS) of wheat is associated with large differences in the bread-making properties. The primary aim of this work was to produce transgenic Egyptian wheat cultivar (Giza 164) with HMW-GS gene (\textit{1DX5}) to improve bread-making quality. Immature embryo-derived calli were cotransformed with pK-DX5 and pACH25 plasmids containing \textit{gus} and \textit{bar} genes using biolistic bombardment. Stable integration and expression of marker genes were detected in seven plants. However, the expression of \textit{1DX5} gene was confirmed in five plants using SDS-PAGE analysis.

**ACKNOWLEDGMENT**

The authors are grateful to Dr. Olin Anderson, USDA, Albany, CA, USA for providing plasmid pK-DX5 harbouring \textit{1DX5} gene.

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Fig. (1): PCR analysis of transgenes. (M: 100 bp ladder for A, B and ΦX147/HaeIII for C), lane 1: positive control, lane 2: negative control, lanes 3-9 are the putative transgenic plants. A: the PCR product for bar gene (443 bp), B: The PCR product for gus gene (1050 bp) and C: The PCR product of 1DX5 gene (529 bp).
Fig. (2): GUS expression in spikelet of transgenic wheat plants. A: GUS staining in spikelet of non transgenic plant B: GUS staining in spikelet of transgenic plant showing blue color.

Fig. (3): Leaf-painting assay, A. transformed plant showing healthy tissues without necrosis, B: non-transformed plant showing necroses.

Fig. (4): SDS-PAGE of total proteins extracted from grains of control (lane 1) and transgenic (lanes 2-8) plants. The arrows indicate Dx5 HMW subunit.
