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 *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 *et al.*, 1982; Thompson *et al*., 1983). HMW-GS coding genes in hexaploid bread wheat are located on three *Glu-1* loci at the long arms of chromosomes 1A, 1B and 1D. Each locus is named as *Glu-A1*, *Glu-B1* and *GluD1*, respectively, and contains two closely linked *Glu-1-1* and *Glu-1-2* genes encoding *x*- and *y*-types, respectively. The gene products of *Glu-1x* and *Glu-1y* are distinguished from one another primarily on the basis of their size (Lawrence and Shepherd, 1981; Payne, 1987). A number of *Glu-1* genes have been cloned, from bread wheat (Anderson and Greene, 1989; Anderson *et al*., 1989).

Analyses of wheat cultivars have shown that HMW-GS differ in their impact on bread-making performance with subunits *1Ax1* and *1Ax2* and subunits *1Dx5* and *1Dy10* in particular being associated with high dough strength and good bread-making quality (Payne, 1987; Shewry *et al*., 2003). HMW-GS have been used to alter wheat grain quality by genetic transformation (Altpeter *et al*., 1996; Blechl and Anderson 1996; Barro *et al*., 1997; Fahmy *et al*., 2006). The HMW-GS *1Dx5* and *1Dy10* give better performances in dough strength and bread-making quality than the homeo-allelic subunits *1Dx2* and *1Dy12* (Payne *et al*., 1982; Popineau *et al*., 1994; Payne *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 *et al*., 1996; Blechl *et al*., 1997; Barro *et al*., 1997; Rooke *et al*., 1999). We have previously reported the transformation of maize, wheat and barley using

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 $K_3Fe(CN)_6$, 0.5 mM $K_4Fe(CN)_6$, 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 *1DX5* 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′-AGTGTACGTATCACCGTTTGTGTGAAC-3′;
GUS R:
5′-ATCGCCGCTTTGGACATACCATCCGTA-3′;
BAR F:
5′-CCAGAAAC-CCACGTGATGCC-3′;
BAR R:
5′-GAAGTCCAGCTGCCAGAAAC-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 *1DX5* 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 *1DX5*) in the genome of seven

putative transgenic plants. PCR amplifications using primers specific to *bar* (Fig. 1A), *gus* (Fig. 1B) and *1DX5* (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 *1DX5* gene in grains of T_0 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 *1Dx5*. However, samples number 2 and 7 did not exhibit such band, even though, these samples were confirmed by PCR for the integration of *1Dx5* 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 *1Dx5* transgene. They were also able to introduce the *1Ax1* transgene alone, or with *1Dx5* 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*, *1Dx5* and *1Dy10* genes in Egyptian wheat cultivars can improve bread-making quality (Altpeter *et al*., 1996; Blechl and Anderson, 1996; Barro *et al*., 1997; Shimoni *et*

al., 1997). Therefore, in this study we produced transgenic Egyptian wheat cultivar (Giza 164) with *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 (*1DX5*) to improve bread-making quality. Immature embryo-derived calli were co-transformed with pK-DX5 and pACH25 plasmids containing *gus* and *bar* genes using biolistic bombardment. Stable integration and expression of marker genes were detected in seven plants. However, the expression of *1DX5* gene was confirmed in five plants using SDS-PAGE analysis.

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REFERENCES

Abdalla, K. S., M. Abdallah and H. F. Ashraf (2008). Development of Egyptian barley with HMW-GS *Dy10* gene of wheat. Arab J. Biotech., 11: 159-168.

Abdalla, K. S., A. H. Fahmy, M. Abdallah and J. Bradova (2011). Allelic variation of high molecular weight glutenin subunits in Egyptian wheat varieties. Arab J. Biotech., 14: 225-234.

Abdallah, M., A. H. Fahmy, K. Abdalla and W. Maaty (2004). Transformation of a high-molecular-weight (HMW) glutenin subunit *Dy10* gene into maize. Arab J. Biotech., 7: 165-172.

Altpeter, F., V. Vasil, V. Srivastava and I. K. Vasil (1996). Integration and expression of the high-molecular-weight glutenin subunit *1Ax1* gene into wheat. Nat. Biotechnol., 14: 1155-1159.

Alvarez, M. L., S. Guelman, N. G. Halford, N. Ryboshkina, P. Shewry, J. Stein, and R. H. Vallejos (2000). Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. Theor. Appl. Genet., 100: 319-327.

Anderson, O. D. and F. C. Greene (1989). The characterization and comparative analysis of high-molecular-weight glutenin genes from genomes A and B of hexaploid bread wheat. Theor. Appl. Genet., 77: 689-700.

Anderson, O. D., F. C. Greene, R. E. Yip, N. G. Halford, P. R. Shewry and J. M. Melpica-Romero (1989). Nucleotide sequences of the two high-molecular-weight glutenin genes from the D-genome of a hexaploid bread wheat, *Triticum aestivum* L. cv Cheyene. Nucleic Acid Res., 17: 461-462.

Barro, F., L. Rooke, F. Bekes, P. Gras, A. S. Tatham, R. Fido, P. A. Lazzeri, P. R. Shewry and P. Barcelo (1997). Transformation of wheat with high molecular- weight subunit genes results in improved functional properties. Nat. Biotechnol., 15: 1295-1299.

Blechl, A. E. and O. D. Anderson (1996). Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nat. Biotechnol., 14: 875-879.

Blechl, A. E., H. Q. Le, F. Bekes, P. W. Gras, Y. Shimoni, G. Galili and O. D. Anderson (1997). Applications of molecular biology in understanding and improving wheat quality. In: Steele J. L., Chung O. K. (eds) Proc. Int. Wheat Quality Conf., Grain Industry Alliance, Manhattan, Kan., p. 205-211.

Cho, M. J., W. Jiang and P. O. Lemaux (1998). Transformation of recalcitrant barley cultivars through improvement of regenerability and decreased albinism. Plant Sci., 138: 229-244.

Cooke, R. J. and J. R. Law (1998). Seed storage protein diversity in wheat varieties. Plant Varieties and Seeds, 11: 159-167.

Fahmy, A. H., Y. H. El-Shafy, O. M. El-Shihy and M. A. Madkour (2004). A highly efficient regeneration system *via* somatic embryogenesis from immature embryos of Egyptian wheat cultivars (*Triticum aestivum* L.) using different growth regulators. Arab J. Biotech., 7: 229-238.

Fahmy, A. H., Y. H. El-Shafy, O. M. El-Shihy and M. A. Madkour (2006). Genetic transformation of Egyptian wheat cultivars (*Triticium aestivum* L.) *via* biolistic bombardment using different constructs. American-Eurasian J. Agric. And Environ. Sci., 1: 58-69.

Harwood, W. A., S. M. Ross, P. Cilento and J. W. Snape (2000). The effect of DNA/gold particle preparation technique and particle bombardment device, on the transformation of barley (*Hordeum vulgare*). Euphytica, 111: 67-76.

Jefferson, R. A., T. A. Kavanagh and M. W. Bevan (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Laemmli, V. K. (1970). Cleavage of structural proteins during assembly of

the head bactriophage. Nature, 227: 680-685.

Lawrence, G. J. and K. W. Shepherd (1981). Chromosomal location of genes controlling seed protein in species related to wheat. Theor. Appl. Genet., 59: 25-31.

Lonsdale, D. M., S. Lindup, L. J. Moisan and A. J. Harvey (1998). Using firefly luciferase to identify the transition from transient to stable expression in bombarded wheat scutellum tissue. Physiol Plant., 102: 447-458.

Muller, E., H. Lorz and S. Lutticke (1996). Variability of transgene expression in clonal cell lines of wheat. Plant Sci., 114: 71-82.

Payne, P. I. (1987). Genetics of wheat storage proteins and the effect of allelic variation on breadmaking quality. Annu. Rev. Plant Physiol., 38: 141-153.

Payne, P. I., L. M. Holt and A. G. Worland (1982). Structure and genetical studies on the HMW-GS: Telocentric mapping of the subunits genes on the long arm of the homoeologous group chromosomes, Thero. Appl. Genet., 63: 129-138.

Payne, P. I., L. M. Holt, A. F. Krattiger and J. M. Carrillo (1988). Relationship between seed quality characteristics and HMW glutenin subunit composition determined using wheats grown in Spain. J. Cereal Sci., 7:229-235.

Payne, P. I., M. A. Nightingale, A. F. Krattiger and L. M. Holt (1987). The relationship between HMW glutenin subunit composition and the breadmaking quality of British grown wheat varieties. J. Sci. Food. Agric., 40: 51-65.

Popineau, Y., M. Cornec and J. Lefebvre (1994). Influence of HMW-GS polymers and rheological properties of near-isogenic lines of wheat. Sicco, J. Cereal Sci., 19: 231-241.

Razin, A. (1988). CpG methylation, chromatin structure and gene silencing, the three way interaction. EMBO J., 7: 4905-4908.

Rooke, L., F. Bekes and R. Fido (1999). Overexpression of a gluten protein in transgenic wheat results in greatly increased dough strength. J. Cereal Sci., 30: 115-120.

Schroeder, H. E., A. H. Schotz, T. Wardley-Richardson, D. Spencer and T. J. V. Higgins (1993). Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). Plant Physiol., 101:751-757.

Shewry, P. R. (1994). Opportunities for manipulating the seed protein composition of wheat and barley in order to improve quality. Transgenic Res., 3: 3-12.

Shewry, P. R., N. G. Halford, A. S. Tatham, Y. Popineau, D. LaWandra and P. S. Belton (2003). The high molecular weight subunits of wheat glutenin and their role in determining wheat processing properties. Adv. Food Nutr. Res., 45: 221-302.

Shimoni, Y., A. E. Blechl, O. D. Anderson and G. Galili (1997). A recombinant protein of two high molecular weight glutenins alters gluten polymer formation in transgenic wheat. J. Biol. Chem., 272: 15488-15495.

Thompson, R., N. P. Harberd and R. B. Flavell (1983). Characterization of the multigene family coding for HMW-GS in wheat using cDNA clones. Thero. Appl. Genet., 67: 87-96.

Wan, Y. and P. G. Lemaux (1994). Generation of large numbers of independently transformed fertile barley plants. Plant Physiol., 104: 37-48.

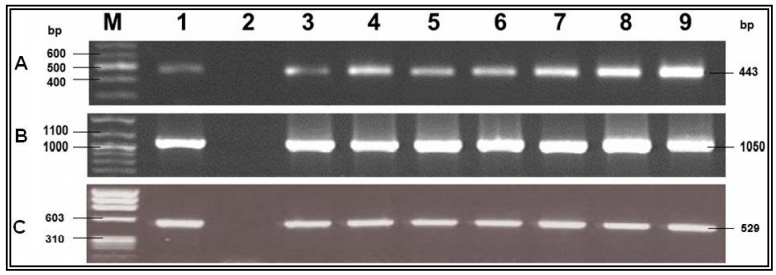

Fig. (1): PCR analysis of transgenes. (M: 100 bp ladder for A, B and ΦX147/HaeIII for C), lane 1: positive control, lane 2: negative control, lanes 3-9 are the putative transgenic plants. A: the PCR product for *bar* gene (443 bp), B: The PCR product for *gus* gene (1050 bp) and C: The PCR product of *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.

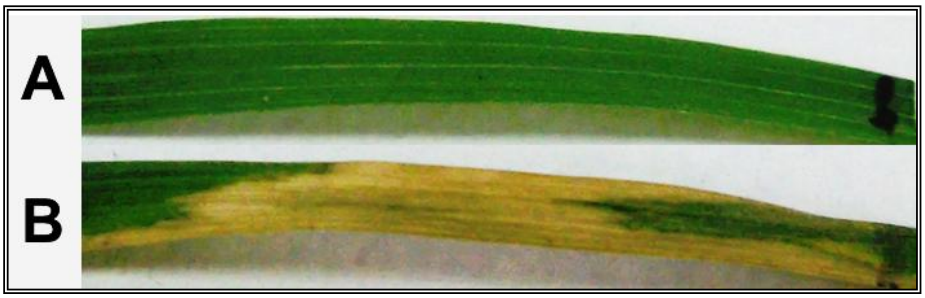

Fig. (3): Leaf-painting assay, A. transformed plant showing healthy tissues without necrosis, B: non-transformed plant showing necroses.

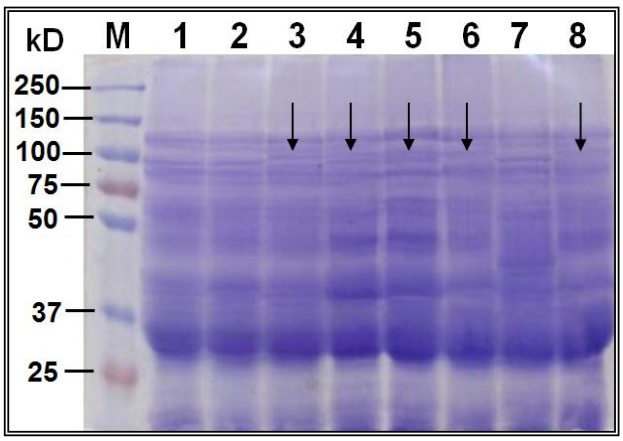

Fig. (4): SDS-PAGE of total proteins extracted from grains of control (lane 1) and transgenic (lanes 2-8) plants. The arrows indicate Dx5 HMW subunit.