Transformation of a high-molecular-weight (HMW) glutenin subunit Dy10 gene into maize

(Received: 10.03.2004; Accepted: 20.03.2004)

Mohamed Abdallah, Ashraf H. Fahmy, Khaled S. Abdalla and Walid S. Maaty
Agricultural Genetic Engineering Research Institute, 9 Gamaa St. Giza, Egypt.

ABSTRACT

Particle bombardment has been used to transform maize genotype Hi-II. Immature embryos were co-transformed with a plasmid containing the selectable and scorable marker genes (bar and uidA, respectively) and a plasmid containing the high-molecular-weight glutenin subunit Dy10 gene. Eight transgenic events (T_0) were recovered from 1000 bombarded scutella (transformation efficiency thus 0.8%). T_1 generation was produced by cross pollination between T_0 plants and non-transgenic plants of the Egyptian inbred line Sd63. Integration of trangenes has been confirmed in the genome of T_0 plants by PCR and Dot blot hybridization analysis. Expression of marker genes was detected in T_0 plants by leaf painting and histochemical staining for bar and uidA genes, respectively.

***Keywords**: glutenin, transformation, maize, uidA, bar,PCR.*

These authors contributed equally to this work
*Corresponding author

INTRODUCTION

Wheat flour is different from other cereal flours, including maize, because it contains gluten that gives it the elasticity and extensity required for bread making (Barro *et al.*, 1997). Gluten consists mainly of two types of seed storage proteins, the glutenins and the gliadins. Glutenins are classified into high-molecular-weight (HMW) subunits and low-molecular-weight (LMW) subunits. Although the HMW glutenins contribute only about 5% of the total protein in mature wheat kernels (Shewry *et al.*, 1989), the elasticity of wheat dough depends mainly on the HMW glutenins, so they are important in bread industry (Payne *et al.*, 1981) but the mechanism by which this is done is still unclear (Payne *et al.*, 1979).

Cloned HMW glutenin genes have been shown to be functional when introduced into *Eschericia coli* (Galili, 1989), tobacco (Roberts *et al.*, 1989), wheat (Alpeter *et al.*, 1996; Blechl and Anderson, 1996; Barro *et al.*, 1997; Alvarez *et al.*, 2000), tritordeum (Rooke *et al.*, 1999) and maize (Sangtong *et al.*, 2002).

Dough made from maize flour lacks elasticity and extensibility (Santong *et al.*, 2002). A probable cause of this is that maize endosperm lacks the proteins responsible for this trait. The HMW glutenin gene could be used to develop maize with novel dough characteristics. Our goal was to transform Egyptian maize with a number of HMW glutenin genes to develop novel maize dough characteristics. These novel dough characteristics could increase the content of

maize flour in bread industry in Egypt and hence reduce the amount of imported wheat.

MATERIALS AND METHODS

Plasmids and maize transformation

Two plasmids were used for co-transformation of maize. The first plasmid, pHC25, contains the selectable marker *bar* gene and the reporter *uidA* gene; both are under the control of the maize ubiquitin promoter (Vasil *et al.*, 1993; Christensen and Quail, 1996). The second plasmid, Blue script KS⁻ (Stratagene, La Jolla, CA), contains the HMW-GS Dy10 of wheat driven by its own endosperm specific promoter. Immature embryos of maize genotype Hi-II (Armstrong *et al.*, 1991) were co-bombarded with both plasmids using biolistic bombardment (PDS 1000/He, Bio-Rad, Hercules, CA) according to Songstad *et al.* (1996).

T_1 generation was produced by mating a T_0 plants and the Egyptian inbred line Sd63. The integration of transgenes was detected by PCR and Dot blot analysis. The expression of transgenes was detected by histochemical staining for *uidA* gene and leaf painting assay for *bar* gene.

DNA analysis

Total genomic DNA was isolated from leaf material of T_0 plants using DNeasy Plant Mini Kit (QIAGEN, Germany). PCR was carried out for the three transgenes for all T_0 plants. The analysis was carried out with 50-200 ng of genomic DNA in a reaction mixture containing 1.25 units HotStarTaq DNA Polymerase (QIAGEN, Germany), 200 µM of each dNTP, 0.4 µM of each primer and 1X buffer containing 1.5 mM $MgCl_2$. PCR amplification was carried out for the *uidA gene* (5`AGTGTACGTATCACCGTTTGTGTGAA C3`,5`ATCGCCGCTTTGGACATACCATCC GTA-3`), annealing temperature 62°C, expected amplified product 1050 kb in size, *bar* gene (5`-GTCTGCACCATCGTCAACC-3`, 5`-GAAGTCCAGCTGCCAGAAAC-3`, annealing temperature 57°C, expected amplified product 443 bp in size and Dy10 gene(5`-ATGGCTAAGCGGCTGGTCCTCT-3`, ATTGTTGCCCTTGTCCTGGTTCT T -3` annealing temperature 55°C, expected amplified product 499 bp in size. The products of PCR amplification were analyzed by electrophoresis in 1% (w/v) agarose gels. Integration of the HMW subunit Dy10 gene was subsequently confirmed by Dot blot hybridization analysis of genomic DNA using DNA labeling and Detection Kit (Boehringer Mannheim, Germany).

Expression analysis of marker genes

GUS activity was examined histochemically using the substrate 5-bromo-4-chloro-3-indolyl β-D-glucoronide (X-Gluc). Plant tissue was cut into small pieces, immersed in X-Gluc buffer (Barcelo and Lazzeri, 1995) and incubated overnight at 37°C. Chlorophyll was extracted from the tissue by incubating in ethanol 98%.

Expression of *bar* gene was examined by local application of 0.5% (v/v) Basta solution (He *et al.*, 1999), to marked regions of fully expanded leaves. Resistance to the herbicide solution was assessed one week after application by scoring the area of leaf necrosis.

RESULTS AND DISCUSSION

Dough made from wheat flour is elastic and extensible, making it suitable for many food products. Dough made from maize flour does not have these properties (Sangtong *et al.*, 2002). The wheat HMW glutenins have a large impact on the physical properties of flour made from wheat, and this class of protein is lacking in maize. Our goal was to develop maize that produces the wheat HMW glutenin

in its kernels. This maize will be valuable for studying the impact of this protein on dough properties and could lead to the development of maize with improved utility for food products, especially bread that represents the major food for the majority of the Egyptian people.

The first step in determining the feasibility of using the wheat HMW glutenin gene in maize was to develop transgenic plants containing this gene, then to determine if the gene is functional. In this report, we focused on transforming one of glutenin genes (Dy10) into maize. In total, 1000 scutella from Hi-II maize inbred line were co-bombarded with HMW subunit (Dy10) and selectable marker and reporter genes, *bar* and *uidA*, respectively. Embryogenic calli derived from immature embryos were used as a substrate for bombardment. Herbicide resistance (Fig. 1) was used as criteria to select callus for regeneration of T_0 plants (Frame *et al.*, 2000). Regeneration frequency of explants undergoing bombardment was 0.8% where 8 callus events regenerated 33 plants (at least one plant from each event) under selection pressure (Table 1). Different regeneration stages of Hi-II maize line are shown in Fig. (1).

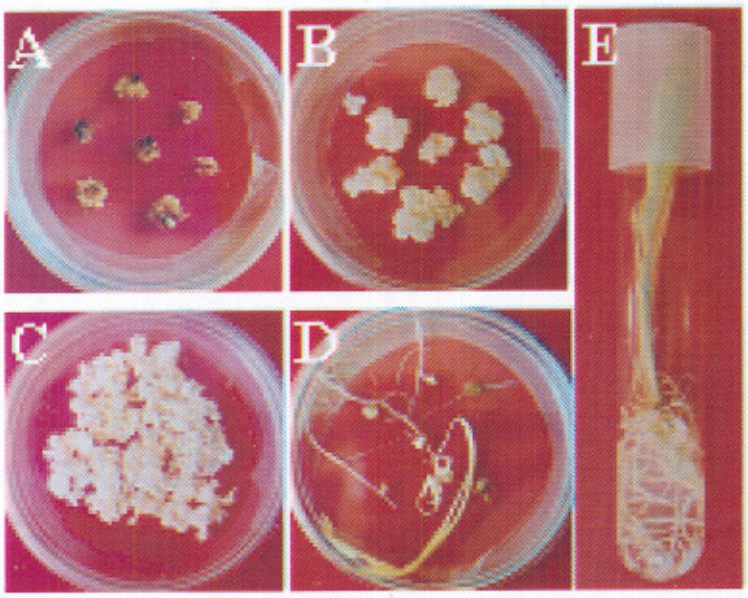

Fig. (1): Regeneration stages of transgenic maize plants. (A) Non-transformed callus colonies (control) on selection medium after 12 weeks showing death or growth inhibition. (B) Callus colonies recovered on selection medium showing healthy growth.. (C) Development of somatic embryos on somatic embryogenesis medium. (D) Shoot formation on regeneration medium. (E) Root formation on rooting medium.

Table (1): Analysis of transgenic maize plants.

Event #. plant #	PCR analysis			GUS *stain*	*Leaf painting*	Fertility
	uidA Gene	*bar* gene	HMW subunit			
1.1	+	+	+	+	+	+
2.1	+	+	–	+	+	+
2.2	+	+	–	+	–	+
2.3	+	–	+	+	–	–
2.4	+	+	+	–	–	–
2.5	+	–	+	+	+	+
3.1	+	+	+	+	+	+
3.2	+	+	+	+	+	–
3.3	+	+	–	–	+	–
3.4	+	+	+	–	–	–
3.5	+	–	+	–	–	–
3.6	+	+	+	+	+	–
4.1	–	+	+	–	+	+
5.1	–	+	–	–	+	–
5.2	–	–	+	+	–	–
5.3	+	–	+	+	–	–
5.4	+	–	+	+	–	+
5.5	+	+	+	+	+	–
5.6	–	+	+	+	+	–
5.7	+	+	+	+	+	–
5.8	+	+	+	+	+	–
5.9	–	+	+	–	+	–
5,10	+	–	–	+	+	+
5.11	+	–	+	–	+	+
5.12	+	+	–	–	+	+
6.1	+	–	–	+	–	+
6.2	+	+	+	+	+	–
6.3	+	–	–	+	+	–
7.1	+	+	+	–	+	–
8.1	+	+	+	+	+	–
8.2	+	–	+	+	+	–
8.3	+	–	+	+	+	–
8.4	+	–	–	+	+	+

PCR analysis was conducted to confirm the integration of the transgenes (*bar*, *uidA* and Dy10) in the genome of all putative transgenic T_0 plants. The result of the reactions showed that the PCR products for all transgenes and the corresponding positive controls were in the expected size (443 bp for *bar* gene, 1050 bp for *uidA* gene and 499 bp for Dy10 gene) and negative controls (non-transgenic plants) showed negative PCR products as shown in Fig. (2). PCR results are scored and presented in Table (1). The data indicated that all putative transgenic plants were positive with at least one transgene. These results not only confirmed the integration of the transgenes in the genome of T_0 plants but also indicated the

high efficiency of the selection system followed in this work where no escapes were recovered. The data indicated also that 20 plants (60.6% of the total number of plants) were positive for the *bar* gene, 28 plants (84.4% of the total number of plants) were positive for the *uidA* gene and 24 plants (72.7% of the total number of plants) for the Dy10 gene. Dot blot hybridization analysis was used to confirm the integration of the Dy10 gene in lines found PCR positive (Fig. 3).

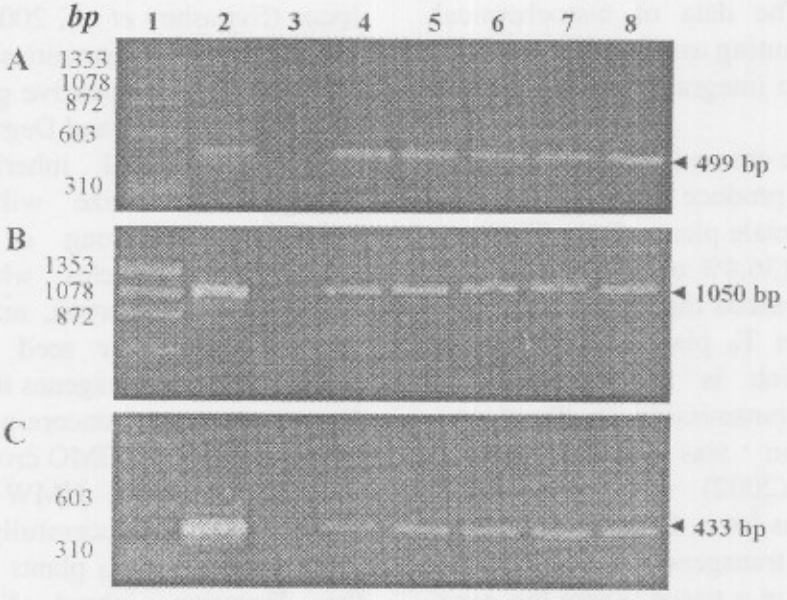

Fig (2): PCR analysis of DNA from representative transgenic maize plants with Dy 10, uidA and bar genes. The amplification products are 499 bp fragment from Dy 10 gene (A), the 1050 bp fragment from uidA gene (B) and the 433 bp fragment from bar gene (C). Lane 1 is DNA marker (φIX 174/HaeIII), lane 2 is plasmid control (positive control), lane 3 is non – transgenic maize (negative control) and lanes 4-8 are representative transgenic maize plants.

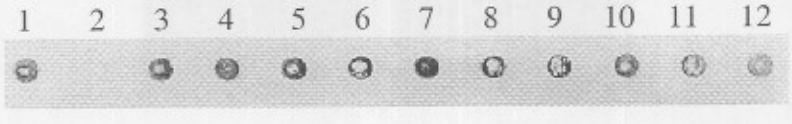

Fig. (3): Dot blot hybridization for transgenic maize plants with Dy10. Dot 1 is plasmid control (positive control), dot 2 is non-transgenic maize (negative control) and dots 3-12 are representative transgenic maize plants.

Histochemical staining and leaf painting assay (Fig. 4) were used to study the expression of the *uidA* and *bar* genes, respectively for T_0 plants. Results of both analysis are presented in Table (1). Histochemical staining results indicated that

the expression of *uidA* gene was detectable in 23 plants (96.7% of the total number of transgenic plants and 82.1% of PCR-positive for *uidA* gene). Leaf painting assay results indicated that 16 plants showed positive expression for *bar* gene (48.5% of the total number of transgenic plants and 80% of PCR-positive plants). The data of histochemical staining and leaf painting assay gave also more confirmation for the integration of the marker genes in T_0 plants

T_0 plants were crossed with the Egyptian inbred line Sd63 to produce T_1. T_0 plants were used as male and female plants. Only 12 plants set seeds (Table 1) (36.4% of the total number of plants). It was noticed that T_1 kernels were obtained only when T_0 plants were used as female plants which is an indication to inefficient pollen transmission in T_0 plants. Similar observation was considered by Sangtong *et al.* (2002) who gave some possible explanations for it. One explanation is that the HMW transgene is ectopically expressed in maize in a tissue where the gene product interferes with pollen development or fertility. A second possible explanation is that the transgene may be inserted into the plastidic or mitochondrial genome. A third explanation is that the transformation process produced chromosomal abnormalities. It has been shown in *Avena* that particle bombardment transformation can generate insertion loci of several megabasis and may cause chromosomal rearrangements at the insertion locus (Svitashev *et al.*, 2000). In maize, some chromosomal abnormalities may result in non-viable or less competitive gametes (Phillips *et al.*, 1971; Rhoades and Dempsy, 1973).

The unusual inheritance of HMW transgene in maize will be particularly interesting (Sangtong *et al.*, 2002). For example, in species where hybrid seed production is important, male sterility system can be useful for seed production. Also, transmission of transgenes through pollen is an important issue concerning the inadvertent pollination of non-GMO crops or weeds.

In summary, HMW glutenin subunit Dy10 gene was successfully transformed into Hi-II maize line. T_0 plants were crossed with the Egyptian inbred Sd63. Inheritance, segregation and expression of this transgene will be studied in details in the following generations.

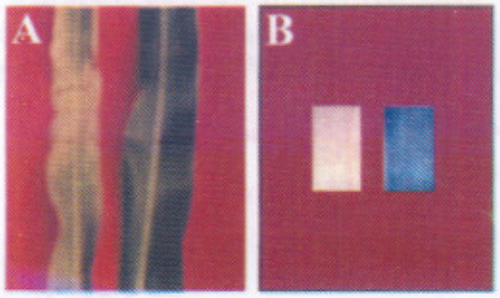

Fig (4): Expression analysis of marker genes in transgenic maize plants. (A) Herbicide leaf painting assay: Leaves of non-transformed plant (left) and transformed plant (right) were painted with 2.0% glufosinate. Non-transgenic plant showed necrosis in the painted area while the transgenic plant showed high resistance to the glufosinate. (B) Histochemical staining of GUS activity: Leaves of non-transformed plant (left) and transformed plant (right) were immersed in in GUS buffer. Transgenic plant showed GUS activity (blue staining) while non-transgenic plant did not show any GUS activity.

ACKNOWLEDGMENT

The authors thank Olin Anderson, UDSA, Albany, CA, USA for the gift of Dy10 plasmid.

REFERENCES

Alpeter, F., Vasil, V., Srivastava, V., Vasil, I.K. (1996). Integration and expression of the high-molecular-weight glutenin subunit AX1 gene into wheat. Nature Biotechnol., 14: 1155-1159.

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

Armstrong, C.L., Green, C.E. and Phillips, R.L. (1991). Development and availability of germplasm with high type culture formation response. Maize Genetics Corporation Newsletter, 65: 92-93.

Barcelo. P. and Lazzeri, P.A. (1995). Transformation of cereals by microprojectile bombardment of immature inflorescence and scutellum tissue. In: H. Jones (Ed.), Methods in Molecular Biology: Plant Gene Transfer and Expression Protocols, vol. 49, Humana Press Totwa NJ, pp., 113-123.

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

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

Christensen, A.H. and Quail, P.H. (1996). Ubiquitin promoter based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgen. Res., 5: 213-218.

Frame, B.R., Zhan, H., Cocciolone, S.M., Sidorenko, L.V., Dietrich, C.R., Pegg, S.E., Zhen, S., Schnable, P.S. and Wang, K. (2000). Production of transgenic maize from bombarded type II callus: effect of gold particle size and callus morphology on transformation effeciency. In Vitro Cell Biol-Plant, 36: 21-29.

Galili, G. (1989). Heterologous expression of wheat high- molecular-weight glutenin gene in *Escherichia coli*. Proc. Natl. Acad. Sci. USA., 86: 7765-7760.

He, G.Y., Rooke,L., Steele, S., Bekes, F., Gras, P., Tatham, A.S., Fido, R., Barcelo, P., Shewry, P.R. and Lazzerti, P.A. (1999). Transformation of pasta wheat (*Triticum turigidum* L.var durum) with high-molecular-weight glutenin subunit genes and modification of dough functionality. Molecular Breeding, 5: 377-386.

Payne, P.I., Corfield, K.G and Blackman, J.A. (1979). Identification of high molecular weight subunit of glutenin whose presence correlate with breadmaking quality in wheats of related pedigrees. Theoret. Appl. Genet., 55: 135-159.

Payne, P.I., Corfield, K.G., Holt, L.M. and Blackman, J.A. (1981). Correlations between the inheritance of certain high molecular weight subunits of glutenin and breadmaking in progenies of six crosses of bread wheat. J Food Sci. Agric., 32: 51-60.

Phillips, R.L., Burnham, C.R., Pattterson, E.B. (1971). Advantages of chromosomal interchanges that generate haplo-viable deficiency-duplications. Crop Science, 11: 525-528.

Roades, M.M. and Dempsy, E. (1973). Cytogenetic studies of transmissible

deficiency in chromosome 3 in maize. J Hered., 64: 125-128.

Roberts, L.S., Thompson, R.D., and Flavell, R.B. (1989). Tissue specific expression of a wheat high-molecular-weight glutenin gene in transgenic tobacco. Plant Cell, 1: 569-578.

Rooke, L., Barro, F., Tatham, A.S. Fido, R., Steele, S., Barcelo, P. (1999). Altered functional properties of tritordeum by transformation with HMW glutenin subunit genes. Theor. Appl. Genet., 99: 851-858.

Sangtong, V., Moran, D.L., Chikwamba, R., Wang, K., Woodman-Clikeman, W., Long, M.J., Lee, M. and Scott, P.M. (2002). Expression and inheritance of the wheat Glu-Dx5 gene in transgenic maize. Theor Appl Genet., 105: 937-945.

Shewry, P.R., Halford, N.G. and Tatham, A.S. (1989). The high molecular weight subunits of wheat barley and rye: genetics, molecular biology, chemistry and role in wheat gluten structure and functionality. Oxford Surv. Plant Mol. Cell Biol., 6: 163-219.

Songstad, D.D., Armstrong, C.L., Peterson, W.L., Hairston, B. and Hinchee, M.A. (1996). Production of transgenic maize plants and progeny by bombardment of Hi-II immature embryos. In Vitro Cell Biol-Plant, 32: 170-183.

Svitashev, S., Anaiev, E., Pawloski, W.P. and Somers, D.A. (2000). Association of transgene integration sites with chromosome rearrangements in hexaploid oat. Theor. Appl. Genet., 100: 872-880.

Vasil, V., Srivastava V, Castello, A.M., Fromm, M.E., and Vasil, I.K. (1993). Rapid production of transgenic wheat plants by direct bombardment of cultured immature embryos. Bio/Technology., 11: 1553-1558.

الملخص العربي

نقل جين Dy10 ذى الوزن الجزيئى العالى للذرة الشامية

محمد عبدالله ، أشرف حسين ، خالد صبرى وليد معاطى
معهد بحوث الهندسة الوراثية الزراعية – مركز البحوث الزراعية - الجيزة-ج.م.ع

تم نقل جين Dyl0 ذى الوزن الجزيئى العالى للذرة الشامية صنف Hi-II وقد تم هذا من خلال نقل بلازميدين لأجنة الذرة غير الناضجة أحدهم يحتوى على جين *uidA* كجين قياسى و جين *bar* كجين إنتخابى بينما يحتوى البلازميدالآخر على جين Dyl0 وهو أحد الجينات المسئولة عن إنتاج بروتين الجلوتين فى نبات القمح وقد أمكن الحصول على ثمانية نسيلات محورة وراثيا بتلك الجينات (T_0) بكفاءة نقل جينى مقدارها 8, % وقد تم تهجين تلك النباتات مع الصنف المصرى Sd63 غير المحور وراثيا بغرض الحصول على الجيل الأول (T_1) وتم التأكد من نجاح عملية النقل الجينى لتلك النباتات وذلك بإستخدام تقنية تفاعل البلمرة المسلسل PCR و تقنية تهجين الـ Dot blot كما تم دراسة التعبير الجينى لكل من جيني الـ *bar* والـ *unidA* بإستخدام تقنيات الدهان الورقى والصبغ النسيجى الكيميائى على التوالى.