Development of Egyptian barley with *HMW-GS Dy10* gene of wheat

 *(Received: 14.03.2008; Accepted: 28.03.2008)*

Khaled S. Abdalla*; Mohamed Abdallah* and Ashraf H. Fahmy*

*Agricultural Genetic Engineering Research Institute, 9 Gamaa St. Giza, Egypt.

**ABSTRACT**

Transgenic barley plants with high-molecular-weight glutenin subunit (HMW-GS) gene from wheat were successfully generated. The HMW-GS Dy10 gene, known to be essential in bread industry, was introduced into the Egyptian barley cv. Giza 123 by biolistic bombardment. The transgenic plants, regenerated from immature embryo-derived callus cultures, were normal and fertile. Stable integration of Dy10 transgene was confirmed by molecular analysis and its expression was studied by protein analysis. Dy10 gene was co-transformed into barley with the plasmid pAHC25 harboring the bar gene for selection and the gus reporter gene. Production of Egyptian barley with wheat HMW-GS genes could be used to develop barley flour with the unique properties of wheat flour for use in bread industry.

**Keywords:** Barley, biolistic bombardment, transgenic, endosperm, protein flour.

*These authors contributed equally to this work, **Corresponding author.

**INTRODUCTION**

The cereal endosperm is of immense economic and nutritional importance, providing the major source of energy and protein for many human populations and for domesticated animals (Lamacchia et al., 2001). Barley, which belongs to the genus *Hordeum*, is a cereal of the grass family *Poaceae*. The genus *Hordeum* comprises different species, but the most common one is *Hordeum vulgare*. It is thought that barley cultivation originated in Egypt over 5000 years ago. Barley is very adaptable to various environments; it is the most adaptable of the cereals. It is more tolerant to soil salinity and drought. It can still thrive in conditions that are too cold or even arid. As a crop, it is used for feed and some food products. In Egypt, barley is qualified to be cultivated in wide areas, especially in the North Cost region and in the new reclaimed lands, because of its tolerance characteristics to biotic and abiotic stresses. But because of the limitation in its commercial use, its cultivation is still limited.

Amongst all cereal crops, wheat has the high-molecular-weight glutenin subunits (HMW-GS) found only in its endosperm (Shewry et al., 1992). These proteins are essential in bread industry (Altpeter et al., 2004) because upon hydration they can interact to form gluten, an insoluble but highly hydrated aggregate. These aggregates create strong dough which traps tiny bubbles of carbon dioxide gas formed naturally by yeast and subsequent rising, thereby enabling the dough to rise forming leavened breads. Dough strength and the ability to contain gas bubbles are known as visco-elasticity (Patnaic and Khurana, 2001).

Barley like all other cereal crops, except wheat, lacks the HMW-GS that limits its use...
in food industry (Alvarez et al., 2000) especially bread. The prospects of using genetic modification to alter the development, structure and composition of cereal’s endosperm are published in several reports (Blechl and Anderson, 1996; Rooke et al., 1999; Sangtong et al. 2002, Barro et al., 2003; Abdallah et al., 2004; Altpeter et al., 2004; Wieser et al., 2005 and Fahmy et al., 2007).

Introduction of HMW-GS genes of wheat into barley genome could result in barley flour with improved backing quality. This goal could be reached using gene transfer technology. This technology provides the opportunity to manipulate crops to enhance the agronomic performance, yield and end use quality (Alvarez et al., 2000). In this paper, we report on the transformation of the Egyptian barley cv. Giza 123 with the HMW-GS Dy10 gene using microprojectile bombardment.

MATERIALS AND METHODS

Plant materials and transformation

Immature embryos were isolated from field grown barley plants (Hordeum vulgare L., cv. Giza 123), and cultured 7 days before bombardment on callus induction medium as described by Abdallah (2007). Transformation was conducted using the helium-driven gene gun and a mixture of two plasmids: pK-Dy10 plasmid containing the HMW-GS Dy10 gene driven by its own endosperm specific promoter (Anderson et al., 1989) and pAHC25 plasmid (Christensen and Quail, 1996) carrying the selectable marker gene bar and the reporter gene gus. Plasmid DNA (an equimolar mixture of the 2 plasmids) was co-precipitated onto gold particles (1.0 µm, BioRad) at a rate of 0.2 µg of DNA per bombardment. After bombardment, calli were cultured in the presence of a selective agent (3 mg/l bialaphos). Plantlets arising from calli were recovered and transferred into rooting medium. Healthy rooted plants were acclimated and grown to maturity in Conviron growth chambers.

DNA isolation and analysis

Total genomic DNA of putative transgenic plants and controls (non-transgenic) were isolated using DNeasy Plant Mini Kit (Qiagen, Germany). DNA was subjected to PCR analysis for Dy10, bar and gus genes as described by Abdallah et al. (2004). Dot-Blot hybridization analysis was conducted to confirm the integration of the Dy10 gene in the transgenic plants using DNA labeling and detection kit (Roche, Germany).

Protein analysis

Protein fractions were extracted by grinding single grains. The resultant flour from each grain was vortexed with 25 µl of total protein extraction buffer (Alvarez et al., 2000) per 1 µg of flour, and allowed to stand for 2 hr at room temperature. The extracts were centrifuged for 15 min at 14000 rpm and the supernatant was boiled for 5 min. The proteins were separated by SDS-PAGE using a Tris-borate buffer system and 10% acrylamide gels (Shewry et al., 1995) and run until the dye front reached the bottom of the gel. The gels were stained with Coomassie blue, the lanes were scanned with GelDoc 2000 scanner and the molecular weight of protein bands was detected using Diversity Database V2 supported by BioRad Corporation.

Analysis of the activities of the marker genes

The activity of gus gene was examined in T₀ plants histochemically according to Jefferson (1987). Leaf painting assay was used to study the expression of the bar gene in T₀ plants according to Cho et al. (1998).
RESULTS AND DISCUSSION

Bread industry is one of the most important industries in Egypt, because bread is the most popular food in this country. However, there is a wide gap between wheat production and consumption in Egypt. The gap is basically filled by importing wheat from abroad and it costs the economy huge amounts of foreign currency. This gap is widening year after year because of the high rate of population increase that reflects negatively on the plans of development. Several strategies are followed to reduce this gap such as production of wheat cultivars with higher productivity and/or tolerant to biotic and abiotic stresses. Another strategy is based on mixing the flour of maize with the flour of wheat in bread processing to reduce the amount of wheat required in bread industry.

This work extends our previous reports (Abdallah et al., 2004; Fahmy et al., 2007) on transferring HMW-GS genes into cereal crops by co-introducing copies of these genes into wheat, maize and barley. Thus, aiming an enhancing the dough strength of the flour of these crops. We anticipate that these unique flours can allow using more corn and barley flours in bread industry so the amount of imported wheat could be reduced. In addition, the nutritious value of these crops would be enhanced because the protein content in the endosperm will be increased.

The approach adopted to reach our goal is as follows: (i) select the most suitable HMW-GS genes related to bread industry, (ii) use genetic transformation to introduce these genes into wheat, maize and barley, (iii) characterize transgenes expression and their inheritance, (iv) study the feasibility of using these new products in commercial applications.

The availability of transformation technology provides an opportunity to manipulate barley for enhancing its agronomic performance, yield and end-use quality. For this reason, the Egyptian barley cv. Giza 123 was chosen for transformation experiments because its regeneration and transformation systems were previously established (Abdallah, 2007).

To alter the composition of seed storage proteins of barley by genetic engineering, the HMW-GS Dy10 gene under the control of its native regulatory sequences was inserted into barley genome using the biolistic approach. The HMW-GS Dy10 gene was chosen in this study because it is associated with superior bread making quality (Altpeter et al., 2004 and Wieser et al., 2005). Embryo-derived calli (1400 calli) were subjected to transformation process in 7 experiments. After selection with bialaphos during callus selection and regeneration phases, four putative transgenic plants, representing 4 putative transgenic callus events, were developed in vitro (Fig. 1). The plantlets were transferred to soil for acclimation and maturation and incubated in Conviron incubators. Generative progenies were produced by self fertilization of plants. These plants were subjected to molecular analysis to confirm the integration of the transgenes in their genome and to study the expression of the inserted genes.
Fig. (1): Development of transgenic barley plants. (A) Calli of non-transformed barley (control) after 3 month on selection medium. (B) Bialaphos tolerant calli (putative transgenic) after 3 months on selection medium. (C) Shoot regeneration. (D) Shoot elongation and root formation.

As an initial characterization of the transgenes in the genome of the putative transgenic plants, genomic DNA was purified and analyzed by PCR for the 3 transgenes. The PCR products were separated on 1.5% agarose gel. As shown in Fig. (2), the PCR products were in the expected size for all transgenes; 499 bp for Dy10 gene, 1050 bp for gus gene and 433 bp for bar. The results of PCR analysis indicated the efficiency of the transformation protocol where the 4 plants were transgenic. DNA blot analysis (Dot-Blot) was also used to confirm the integration of the Dy10 gene (Fig. 3). The data confirmed the integration of Dy10 gene in all T₀ plants. The results of PCR and DNA blot analysis indicated that the frequency of transformation process is 0.3%. It was noticed that all resulted plants were fertile; it indicates that the insertion of the transgenes did not affect the fertility of plants as all of them set seeds. This result agrees with the results obtained by Fahmy et al. (2007) who transformed the same gene into wheat but disagree with the results obtained by Abdallah et al. (2004) who transformed the same gene into maize.

Fig. (2): PCR analysis of T₀ plants. (A) Amplification product of Dy10 gene (499 bp). (B) Amplification product of gus gene (1050 bp). (C) Amplification product of bar gene (433 bp). Lane 1 is DNA marker (ΦX147/HaeIII). Lane 2 is non-transformed barley (negative control). Lane 3 is pAHC25 plasmid (positive control). Lanes 4-7 are the 4 transgenic barley plants.
Expression of the selectable marker gene (bar) was confirmed by tolerance to the bialaphos in tissue culture (Fig. 1) and glufosinate in leaf painting assay in all transgenic plants. The expression of the gus gene was not detectable in leaf tissues of T₀ plants as mentioned earlier in a previous report on transgenic barley production of the same genotype (Abdallah, 2007). The invisible detection of gus gene activity in leaf tissues may result from β-glucuronidase inhibitors found in leaf tissues (Bahieldin et al., 2005).

The endosperm of the grains of T₀ plants were analyzed by SDS-PAGE with Comassie blue staining to detect the expression of HMW-GS transgene (Blechl and Anderson, 1996; He et al., 1999; Alvarez et al., 2000; Barro et al., 2003). Dy10 protein is visualized on SDS-PAGE gel (Fig. 4A) as an independent band. The presence or absence of the SDS-PAGE band was used to identify the phenotype of the transgenic plants, positively or negatively, respectively. The absence of Dy10 subunit from the donor barley line allowed the transgenic products to be clearly identified by SDS-PAGE. Protein extraction was performed from three grains from each transgenic plant and equal portions were fractionated in SDS-PAGE (Shimoni et al., 1997). Non-transfected barley cv. Giza 123 was considered as a negative control, while the wheat cv. Giza 164 was considered as a positive control. The molecular weights of all protein bands were estimated using the digital imaging system and a diagram for such scoring was designed (Fig. 4B) for all bands at 55 kDa and above, where these bands represent the proteins of interest in this study.

Some plants showed no expression to Dy10-GS gene on the SDS-PAGE (plants 1, 2 and 3) although the insertion of this transgene was confirmed. The expression of the transgene may be in a low level and not detectable on the SDS-PAGE. Also, methylation of DNA is expected in some cases to result in reduced gene expression (Muller et al., 1996; Razin, 1988). Another possibility for undetectable expression is gene silencing of the transgene. Genetic engineering of plants sometimes results in transgene silencing after integration into the genome which may relate to a defense mechanism against foreign DNA expression (Kumpatla et al. 1998; Vaucheret et al., 1998). It is a complicated phenomenon as it includes both transcriptional and posttranscriptional gene inactivation (Khurana et al., 1998). Silence may be due to the presence of incomplete copies of the transgenes as a result of the transformation process. Silencing of wheat HMW-GS transgenes was also reported in transgenic wheat (Alvarez et al., 2000). In addition, these plants showed silencing to most endogenous subunits of the HMW of barley and one sample (lane 5, Fig. 4) of the plant number1 showed silencing in all HMW subunits of barley. Similar finding was reported by Alvarez et al. (2000). Altpeter et al. (1996) speculated that integration of HMW transgenes could result in silencing, this speculation was confirmed by Blech et al. (1997).
Fig. (4): SDS-PAGE of total protein from the endosperm of transgenic barley plants. (A) SDS-PAGE stained with Coomasie blue. (B) Diagram of protein bands of the SDS-PAGE above 55 kDa according to gel analysis using Diversity Database V2. Lane 1 is protein marker (protein molecular weight mid/low range). Lane 2 is wheat cv. Giza 164 (positive control). Lane 3 is non-transformed barley (negative control). Lanes 4-6 are 3 grains of transgenic plant number 1. Lanes 7-9 are 3 grains of transgenic plant number 2. Lanes 10-12 are 3 grains of transgenic plant number 3. Lanes 13-15 are 3 grains of transgenic plant number 4.

Plant number 4 showed a novel protein band migrating near the 60 kDa (lanes 14 and 15, Fig.4) or near 65 kDa (lanes 13 and 15, Fig. 4). These bands are smaller than the 72 KDa predicted from the cDNA sequences of Dy10 gene. It is known that sometimes HMW glutenins may have anomalous migration in SDS-PAGE (Shewry et al., 1992). These bands could also result from partial degradation of the protein or the position may undergo slightly different processing in barley than in wheat. These bands may also correspond to a rearranged form of subunit Dy10 arising from unusual crossing over as discussed by D’Ovidio et al. (1996). Similar additional bands were previously observed in transgenic wheat (Barro et al., 1997).

In earlier reports, the introduction of wheat HMW-GS genes into tobacco (Robert et al., 1989), wheat (Blechl and Anderson, 1996; Altpeter et al., 1996; He et al., 1999; Fahmy et al., 2007), tritordeum (Rooke et al., 2000), maize (Sangtong et al., 2002; Abdallah et al., 2004) and rye (Altpeter et al., 2004) has been reported. Here we report for the first time, to our knowledge, the introduction of wheat HMW-GS genes into barley. It is clear from the preliminary results discussed earlier that it is feasible to manipulate the structure and properties of wheat HMW glutenin in barley, although the current knowledge about the expressed transgene is still insufficient to predict the results reliability. Transformation with one or two subunit genes could result in a stepwise increase in dough elasticity (Barro et al., 1997).

In summary, the addition of the HMW Dy10-GS genes of wheat to the genome of the
Egyptian barley cv Giza123, has been confirmed. This could, therefore, be expected to result in improving bread-making performance. Production of Egyptian barley with wheat HMW-GS genes could develop barley flour with the unique properties of wheat flour that could be widely used in bread industry. This may be an important step towards improving bread-making properties of barley whilst conserving its superior stress resistance.

ACKNOWLEDGEMENTS

We thank Dr. Olin Anderson, USDA, Albany, CA, USA for providing HMW-GS Dy10 gene. We also thank Department of barley, Field Crop Research Institute, ARC, Egypt for providing the barley cv. Giza 123 and the wheat cv. Giza 164.

REFERENCES


Conf., Grain Industry Alliance, Manhattan, Kan. pp 205-211.


