PROCEEDINGS OF THE BALKAN SCIENTIFIC CONFERENCE OF BIOLOGY IN PLOVDIV (BULGARIA) FROM 19TH TILL 21ST OF MAY 2005 (EDS B. GRUEV, M. NIKOLOVA AND A. DONEV), 2005 (P. 71–78)

MARKER GENE TRANSFER TO WINTER DURUM WHEAT (*Triticum durum* Desf.) BY MICROPROJECTILE BOMBARDMENT METHOD

Bahar Soğutmaz Özdemir*[1], Sertaç Önde[1], Murat Özgen[2]

[1] Department of Biological Sciences, Faculty of Arts and Sciences, Middle East Technical University, 06531 Ankara – TURKEY
[2] Department of Field Crops, Faculty of Agriculture, Ankara University, 06110 Dışkapı, Ankara – TURKEY
**E-mail: bsozdemir@gmail.com*

ABSTRACT. A marker gene transfer procedure to winter durum wheat (*Triticum durum* Desf.) using microprojectile bombardment method and a regeneration system for the transformed mature embryos have been investigated in this study. Different bombardment pressures and distances were tested using gold particles of 1.0 μm and 1.6 μm average diameters. Efficiency of gene delivery was assessed by scoring transient GUS expression on bombarded embryos. The bombardment pressures of 650 psi or 1100 psi at a 6 cm sample plate distance provided efficient results. The bombarded tissues that showed resistance to the gradual increase of the selective agent, hygromycin-B antibiotic, up to 30 mg/L concentration yielded a total of 15 regenerated plants *in vitro*. However, these plants could not be grown into maturity in soil due to lack of vernalisation that is required for winter durum wheat. Results obtained from this study could aid for further studies of durum wheat transformation and regeneration of fertile transgenic plants.

KEYWORDS. *Triticum durum* Desf., winter durum wheat, gene transfer, microprojectile bombardment, transient GUS expression, regeneration.

INTRODUCTION

Wheat is one of the major cereal crops and while the hexaploid type of wheat (*Triticum aestivum* L.), used for making bread and other baked products, the tetraploid type of wheat (*Triticum durum* Desf.) is used for the raw material of pasta, macaroni and biscuits (Patnaik and Khurana, 2001). Due to their importance in agriculture but the inherent problems related with their growth, wheat species is subjected to many molecular genetic improvement studies.

After the invention of microprojectile bombardment process (Klein *et. al*., 1987), also referred to as biolistics, the first successful production of transgenic bread wheat plants from embryogenic callus (Vasil *et. al*., 1992), many stable transformation studies with scutellar tissue (Becker *et. al*., 1994; Barro *et. al*., 1998) and immature embryos (Zhou *et. al*., 1995; Altpeter *et. al*., 1996; Zhang *et. al*., 2000) were reported. On the other hand, the first successful data recorded with durum wheat is reported by Bommineni *et. al*. (1997). While majority of these studies are on "spring" varieties of bread and durum wheat, little data is available related with "winter" bread and durum wheats.

In the present study, optimization of the bombardment conditions for the transient expression of GUS reporter gene in mature winter durum wheat embryos and a successful selection and regeneration protocol based on the expression of the Hygromycin-B resistance gene in the bombarded embryos are reported.

MATERIAL AND METHODS

Plant Material

Winter durum wheat, *Triticum durum* Desf. cv. Çakmak 79 was used as sources of mature embryos. Seeds were surface sterilized according to a procedure developed in our laboratory previously (Özgen *et al*., 1998). After sterilization, the seeds were imbibed in sterile distilled water for two hours at 33°C in a shaker (160 rpm) and mature embryos were aseptically removed. Embryos were placed scutellum upwards and arranged in a circle of 2.5 cm-diameter at the center of the petri plates containing callus induction MS medium (Murashige, T. and Skoog, F., 1962) supplemented with sucrose (20 g/l), agar (7 g/l) and 2,4-dichlorophenoxyacetic acid (2 mg/l) to prevent excessive germination of the emryos (Fig. 1A). The dishes were kept at 25°C in darkness for 24h prior to bombardment.

Plasmid DNA

The plasmid pBI221.23 (Lonsdale *et al*., 1990) contains the β–glucuronidase (GUS) gene and the *hpt* gene, which confers resistance to the antibiotic Hygromycin-B. Both genes were under the control of Cauliflower mosaic Virus (CaMV) dual'35S' promoter. The plasmid isolation and purification was carried out by using the Large Scale Plasmid Preparation Kit of Promega® (cat. no. A7270) according to the user manual supplied by the manufacturers.

Microprojectile bombardment

For bombardment, Bio-Rad Biolostic® PDS-1000/He particle delivery system was used according to the manufacturer's protocol. Explants were bombarded under a partial vacuum of 25" Hg pressure with 1.0 μm or 1.6 μm average diameter of gold particles coated with 5 μg of plasmid DNA as described in manufacturer's protocol. Five rupture disk pressures (650, 900, 1100, 1350, 1500 psi) and two sample plate distances (6 and 9 cm) were used during the bombardment process.

Assay of gene expression

After bombardment, the explants were incubated further for 48 hours at 25°C in darkness and then subjected to histochemical GUS assay (Jefferson, 1987). Transient gene expression events were counted using a dissecting microscope.

Selection and regeneration of pututative transformants

Those embryos that were not subjected to GUS histochemical staining were kept in callus induction medium at 25°C in darkness for further 4-5 days after bombardment. For the selection, they were transferred to the same medium but this time, further supplemented with 5mg/L Hygromycin-B and incubated again at 25°C in darkness for a week. The antibiotic concentration in the medium was increased to first 10mg/L and then to 20mg/L at the end of a month. For shoot and root initiation, the calli that showed resistance to the Hygromycin-B antibiotic were transferred to hormone-free regeneration medium supplemented with 20mg/L and 30mg/L antibiotic under 16h/8h light (1500 lux)/dark cycle at 25°C. After 3–4 weeks, antibiotic-resistant regenerants were transferred to sterile jars containing regeneration media, without the antibiotic. Established plantlets were transferred directly from regeneration media to pots of a peat-soil mixture (2:1 volume) for further rooting and development under the same photoperiod and temperature conditions with high humidity (90%–70%).

RESULTS

Various bombardment pressures (650 psi, 900 psi, 1100 psi, 1350 psi and 1550 psi) and distances (6 cm and 9 cm) were studied with mature winter durum wheat embryos by employing 1.6 μm average diameter gold particles. In the first experiment, for each bombardment pressure and distance combination, two replicates were done. Fifty mature embryos per petri plate were prepared. Transient GUS expressions were observed on the bombarded mature wheat embryos (Fig. 1B). The results showed us that 650 psi-6 cm and 1100 psi-6 cm gave better results with respect to other pressure-distance combinations (Fig. 2). All the results were statistically analyzed by using Student t-Test. The bombardment pressure of 650 and 1100 psi and 6 cm distance displayed statistically significant differences ($p<0.05$). This also confirmed the higher values (number of blue spots) obtained from 650 psi-6 cm and 1100 psi-6 cm studies.

In order to test the system by employing gold particles of 1.0 μm average diameter, four different pressure settings (650 psi, 900 psi, 1100 psi and 1350 psi) and two distances (6 cm and 9 cm) were applied. For each pressure-distance combination and its replicate 50 embryos were bombarded and 25 of them were exposed to histochemical GUS assay while the remaining 25 were cultured for selection and regeneration purposes as described in Materials and Methods section. Similar to the results obtained in the first experiment, the statistical analysis of this

experiment demonstrated a significant difference between 650 psi and 1100 psi bombardment pressures at 6 cm distance (Fig. 3).

Fifteen regenerants were obtained from this experiment at bombardment pressure-distance combinations of 650 psi-9 cm, 900 psi-6 cm and 1350 psi-9 cm. The regenerants were grown and selected under the conditions described in Materials and Methods section (Fig. 4, Fig. 5, and Fig. 6).

DISCUSSION

So far, majority of the wheat transformation by microprojectile bombardment method was studied especially by using immature embryos, cell suspension cultures and calli (reviewed by Patnaik and Khurana, 2001). However, in this study, a reliable method to use mature embryos for the bombardment process and a successful selection and regeneration protocol is developed.

Explant source

The success of microprojectile bombardment technique relies on physical, chemical and biological factors. The explant source is the principal biological factor affecting the success of this technique. Although immature embryos or the callus obtained from these embryos or suspension cultures derived from these immature embryos are used frequently as the explant source for the wheat transformation studies, the inherent problem of difficulty of obtaining immature embryos whenever they are desired and their fragile nature hampered their suitability. Mature embryos have the advantage of easy isolation, absence of time constraints for isolation, the robust structural forms and finally their easy manipulation.

Microprojectile size and bombardment pressure/distance

The bombardment pressures (650, 900, 1100, 1350, and 1550 psi) and distances (6 and 9 cm) with 1.6 μm average diameter gold of particles were selected according to the literature on wheat transformation studies and also based on our former experience on this matter. As can be seen on Fig. 2, the transient expression of the β-Glucuronidase reporter gene was critically effected by the changes in bombardment pressure and distance. These results when subjected to statistical analysis, the bombardment pressure of 650 and 1100 psi and 6 cm distance displayed statistically significant differences. In order to understand the possible effects of 1.0 μm average diameter of this gold particle size on the transient gene expression events, an experiment is prepared which involves 4 pressure settings and two distances (Fig. 3). The statistical analysis performed on this experiment revealed a significant difference between 650 and 1100 psi pressure at 6 cm distance.

Shot to shot variation

One of the most significant problems of the microprojectile bombardment technique is the variation of gene delivery efficiency between bombardments. This event might be called as “shot-to-shot variation”. The probable reason of this variation is based on only one factor: the experimenter. In order to minimize the

negative effects of this shot-to-shot variation on data evaluation, experiments with replicates were usually performed. Nevertheless the data available on this line of research accompanies large standard error values (Russel, J. A. *et. al.*, 1992; Rasco-Gaunt, S. *et. al.*, 1999). This was also the case in our results.

Plant regeneration

Our first trial to regenerate putative transgenic plants was failed due to the use of Hygromycin-B antibiotic at an extreme concentration of 30 mg/L immediately after bombardment. All of the embryos failed to develop callus and later on died. However, later, we developed a softer approach by increasing the selective agents' concentration step-by-step up to 30 mg/L. This approach has yielded 15 regenerated plants *in vitro*. However, seed formation could not be observed in these plants due to lack of vernalisation.

CONCLUSION

Mature embryos of durum wheat were found to be suitable explant material for transformation studies. For the tested gold particles, 1.6 µm average diameter was found to give better results if used at 650 psi bombardment pressure and at 6 cm target distance. However 1.0 µm average diameter gold particles was found to be more effective at 1100 psi bombardment pressures at 6 cm target distance. Rather than exposing the mature embryos to direct cytotoxic levels (30 mg/L) of the selective antibiotic (hygromycin-B), a step-by-step increase of the concentration was found to be suitable for the regeneration of the transformants. Although 15 hygromycin resistant plantlets were regenerated through *in vitro* selection, seed formation could not be observed in these plants due to lack of vernalisation. However, valuable experience is acquired related with microprojectile bombardment of winter durum wheat mature embryos and regeneration of plants under Hygromycin-B selection. In the future, if the protocols we have followed are applied, regeneration of transgenic plants will become possible.

ACKNOWLEDGEMENTS

This research was supported by the Scientific and Technical Research Council of Turkey and University of Ankara. Turkey, contract no. TOGTAG/1285.

REFERENCES

ALTPETER, F., VASIL, V., SRIVASTAVA, V., STÖGER, E. and VASIL, I.K. 1996a. Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. Plant Cell Reports 16:12-17.

BARRO, F., CANNELL, M.E., LAZZERI, P.A. and BARCELO, P. 1998. The influence of auxins on transformation of wheat and *tritordeum* and analysis of transgene integration patterns in transformants. Theoretical and Applied Genetics 97:684-695.

BECKER, D., BRETTSCHNEIDER, R. and LORZ, H. 1994. Fertile transgenic wheat from microprojectile bombardment of scutellar tissue. Plant Journal 5:299-307.

BOMMINENI, V.R., JAUHAR, P.P. and PETERSON, T.S. 1997. Transgenic durum wheat by microprojectile bombardment of isolated scutella. Journal of Heredity 88:301-313.

JEFFERSON, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5:387-405.

KLEIN, T.M., WOLF, E.D., WU, R. and SANFORD, J.C. 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327:70-73.

LONDSDALE, D., ÖNDE, S. and CUMING, A. 1990. Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment. Journal of Experimental Botany, vol.41, no.230, pp.1161-1165.

MURASHIGE, T. and SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497.

ÖZGEN, M., TÜRET, M., ALTINOK, S. and SANCAK, C. 1998. Efficient callus induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes. Plant Cell Reports 18:331-335.

PATNAIK, D. and KHURANA, P. 2001. Wheat biotechnology: A minireview. EJB Electronic Journal of Biotechnology (online) vol.4 No.2. ISSN: 0717-3458.

RASCO-GAUNT, S., RILEY, A., BARCELO, P. and LAZZERI, P.A. 1999. Analysis of particle bombarment parameters to optimise DNA delivery into wheat tissues. Plant Cell Reports 19:118-127.

RUSSEL, J. A., ROY, M. K. and SANFORD J. C. 1992. Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. Plant Physiol. 98:1050-1056.

VASIL, V., CASTILLO, A. M., FROMM, M. E. and VASIL, I. K. 1992. Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. Biotechnology 10:667-674.

ZHANG, L., RYBCZYNSKI, J.J., LANGENBERG, W.G., MITRA, A. and FRENCH, R. 2000. An efficient wheat transformation procedure: transformed calli with long-term morphogenic potential for plant regeneration. Plant Cell Reports 19:241-250.

ZHOU, H., ARROWSMITH, J.W., FROMM, M.E., HIRONAKA, C.M., TAYLOR, M.L., RODRIGUEZ, D., PAJEAY, M.E., BROWN, S.M., SANTINO, C.G. and FRY, J.E. 1995. Glyphosate-tolerant CP4 and GOX genes as a selectable marker in wheat transformation. Plant Cell Reports 15:159-163.

FIGURES AND CAPTIONS

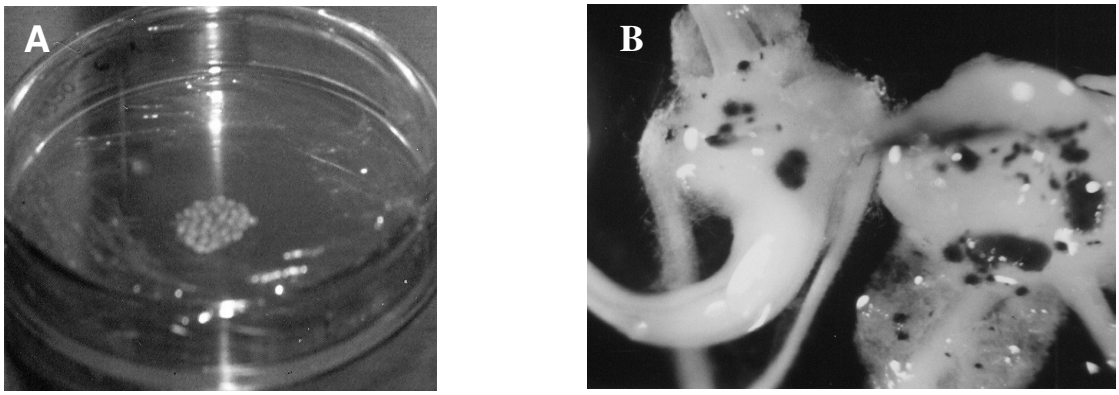

Fig. 1. A-B *Mature winter durum wheat embryos.* ***A*** *In callus induction medium 24h prior to bombardment,* ***B*** *Transient GUS expression 48h after bombardment.*

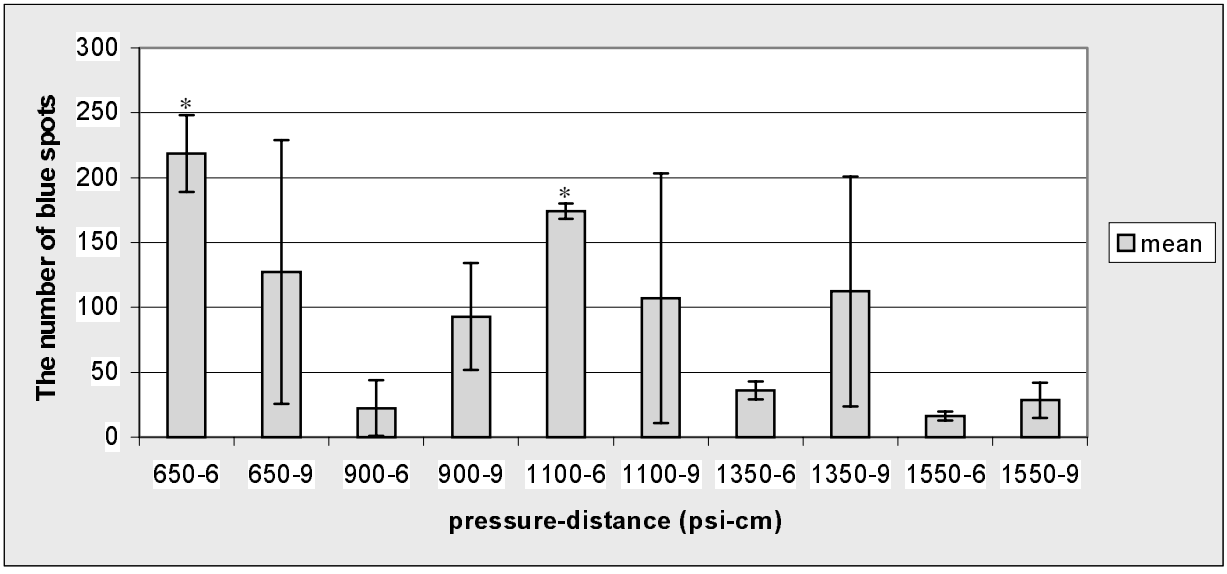

Fig. 2. *The average number of blue spots observed in 2 replicate petri dishes of mature winter durum wheat embryos (50 embryos/petri plate) bombarded with gold particles (1.6 μm) at different pressures and distances. Each data represents the mean±SE values. (*) significant at p<0.05.*

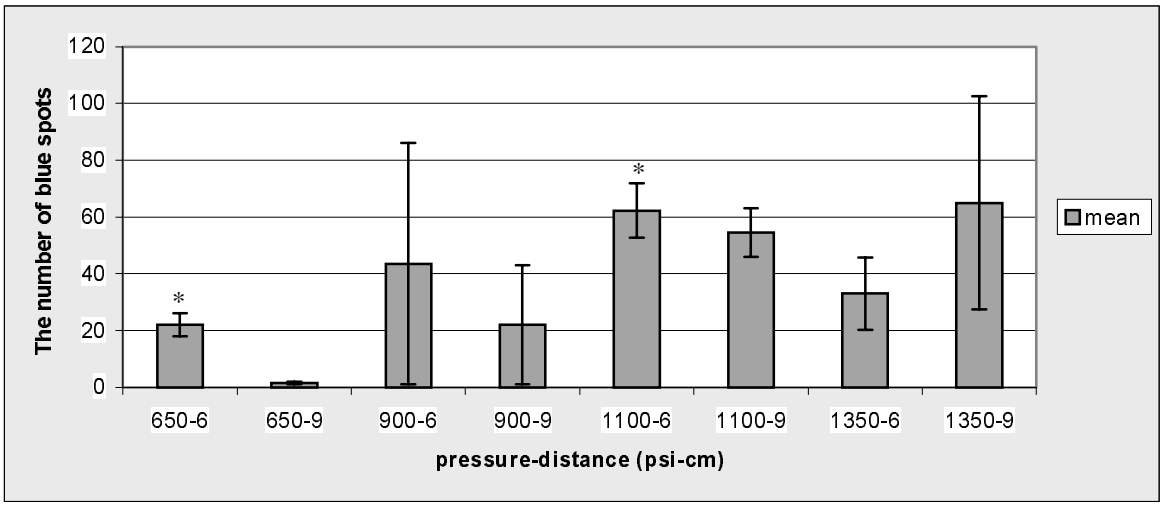

Fig. 3. *The average number of blue spots observed in 2 replicate petri dishes of mature winter durum wheat embryos (25 embryos/petri plate) bombarded with gold particles (1.0 μm) at different pressures and distances. Each data represents the mean±SE values. (*) significant at p<0.05.*

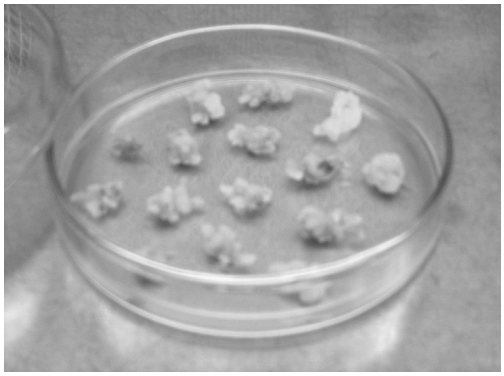

Fig. 4. *Callus formation of mature winter durum wheat embryos under 20 mg/l Hygromycin-B selection 4 weeks after bombardment.*

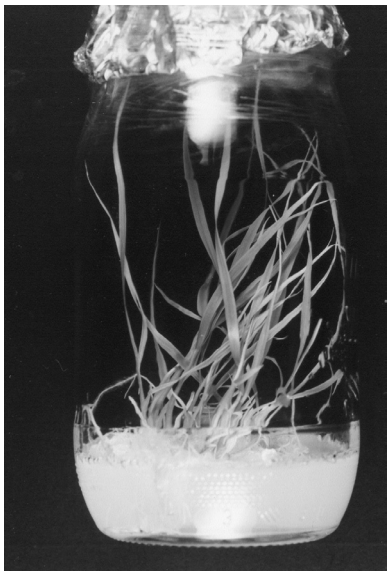

Fig. 5. *The plantlets regenerated after selection from mature durum wheat embryos bombarded at 650 psi pressure*

Fig. 6. *The regenerated plants transplanted to soil under 16h /8h light (500 lux)/ dark cycle at 25 °C.*