Research Article

Marker Gene Delivery to Mature Wheat Embryos Via Particle Bombardment

Hüseyin Avni ÖKTEM, Füsun (İnci) EYİDOĞAN, Fahriye (Setenci) ERTUĞRUL, Meral YÜCEL

Biyoloji Bölümü, Orta Doğu Teknik Üniversitesi, 06531 Ankara-TURKEY

Barnabas JENES, Ottó TOLDİ

Agricultural Biotechnology Center, Gödöllö, HUNGARY

Abstract: The possibility of transferring genes to mature wheat embryos (*Triticum aestivum* L. and *T. durum* Desf.) via accelerated and DNA-coated tungsten particles was investigated. Mature embryos isolated from bread (cv. Atay) and durum (cv. Çakmak) wheat were utilised as targets for bombardment. DNA in the form of circular plasmid (pBSGUSINT) was precipitated on tungsten particles (ca. 2 µm diameter) using the calcium nitrate method. Mature embryos were bombarded by a microprocessor-controlled particle delivery instrument (GENEBOOSTER^{TM}) driven by compressed nitrogen gas. Bombardment was carried out at various gas pressure and in a chamber vacuum. The target material was subjected to histochemical GUS staining 24 hours after bombardment or 7 days after the initial germination of the embryos. Almost 80% of the bombarded embryos expressed the transferred GUS gene observed through blue colour formation on the embryos. Negative controls (non-bombarded embryos or embryos bombarded with bare tungsten particles) did not exhibit GUS activity. Seven-day-old seedlings which had emerged from bombarded material also exhibited patches of GUS staining, indicating the integration and expression of the transferred GUS gene to the genome of some tissue segments. The optimised bombardment method is currently in use to obtain transgenic wheat cultivars.

Key Words: wheat, mature embryo, transformation, particle bombardment, GUS

Olgun Buğday Embryolarına Partikül Bombardımanı ile İşaret Geni Transferi

Özet: Olgun buğday embriyolarına (*T. aestivum* L. ve *T.durum* Desg.) partikül bombardımanı ile gen transfer olanağı araştırıldı. Ülkemizde yetiştirilen ekmeklik (Atay) ve makarnalık (Çakmak) buğday çeşitlerden izole edilen olgun embiryolar bombardıman için hedef olarak kullanıldı. Dairesel formdaki pBSGUSINT plazmidi kalsiyum nitrat metodu kullanılarak tungsten (2mm) partikülleri üzerinde tutturuldu. Olgun embiryolar sıkıştırılmış nitrogen gazı ile çalışan mikroişlemci-kontrollü bir partikül salınım aleti (GENEBOOSTER^{TM}) ile bombardıman edildi. Bombardıman değişik gaz basıncı ve hazne vakumunda uygulandı. Hedef materyal, bombardıman yapıldıktan 24 saat sonra veya embiryoların ilk çimlenmesinden 7 gün sonra histokimyasal GUS boyamasına tabi tutuldu. Embiryoların üzerindeki mavi renk oluşumu izlenerek bombardımanı yapılan embiryoların yaklaşık %80 'ininde transfer edilen GUS geninin ifadesi gözlendi. Negatif kontrollerde (bombardıman yapılmamış embiryolar veya çıplak tungsten partikülleri ile vurulan embiryolar) GUS aktivitesi gözlenmedi. Bombardıman yapılan embiryolardan çıkan 7 günlük fidelerde, bölümler halinde dokusal GUS boyaması gözlendi. Elde edilen bulgular transfer edilen GUS geninin bu dokularda da ifade edildiğini ve genoma integre olduğunu göstermektedir. Halen optimize edilen bombardıman metodu transgenik buğday çeşitlerinin eldesi amacı ile kullanılmaktadır.

Anahtar Sözcükler: buğday, olgun embiryo, transformasyon, partikül bombardımanı, GUS

Introduction

Particle bombardment, also referred to as the biolistic system, is a physical method of gene transfer which employs high velocity metal particles (gold or tungsten) to deliver genetic material to prokaryotic and eukaryotic cells, including plants and animals (1). The method was first described by Standford et al. (2) and later became one of the most effective methods in transferring biologically active DNA molecules into plant cells (3). In plant transformation studies, the particle bombardment method has been accepted as a breakthrough since genetic transformation with this method became almost a routine process in many important crop species, including cereals and legumes, which are known to be recalcitrant for transformation with other techniques.

The genetic transformation of wheat, while it is one of the most important cereals, is still far from being a routine procedure. Although some groups have reported the possible application of the *Agrobacterium*-based transformation system as a useful tool in transforming cereals, this transformation system is not commonly used. Even though it was the first successful method for genetic transformation of wheat, particle or microprojectile bombardment still appears to be the most commonly used procedure for wheat transformation (4).

Several groups have reported successful transformation (5-7) and regeneration (8-10) protocols for wheat (*Triticum aestivum* L.). The majority of the work involved gene delivery via accelerated particle bombardment of regenerable tissues such as embryogenic cell suspension cultures, embryogenic callus and immature embryos. In some other studies, tissues such as apical meristem, which regenerates in situ to fertile plants, were also used as targets for particle bombardment (11, 12).

In our laboratories we employ a new generation particle delivery system (GENEBOOSTER™) for genetic transformation of various crops, including wheat, barley, maize, lentil and chickpea. The GENEBOOSTER™ instrument is a microprocessor-controlled particle delivery system powered by compressed nitrogen gas for acceleration of macrocarriers (13). Compared to other commercially available geneguns, this system has several advantages: automated control of shooting, electronic transfer of experimental data (gas pressure, chamber vacuum, sample distance, stopping plate level etc.) to a personal computer, a built-in vacuum pump and most importantly, low running costs.

The aim of this paper was to investigate the potential of mature wheat embryos as targets for gene delivery via particle bombardment. For this purpose mature embryos isolated from local *Triticum aestivum* (cv. Atay) and *Triticum durum* Desg. (cv. Çakmak) were bombarded with tungsten particles coated with pBSGUSINT plasmid carrying GUS gene as the marker. The results of the experiments were analysed by histochemical GUS activity staining of the bombarded embryos either 24 hours after bombardment or 7 days after the initial germination of the embryos.

Materials and Methods

Seed Material: *Triticum aestivum* cv. Atay and *Triticum durum* cv. Çakmak were obtained from the Geçitkuşağı Research Institute, Eskişehir. The seeds were surface sterilised as follows: 70% ethanol for 1 min, 20% sodium hypochlorite with a few drops of TWEEN-80 for 20 min, and they were then rinsed 4 times with sterile distilled water. The seeds were imbibed in water for 24 hours and mature embryos were isolated and kept overnight in darkness on MS (14) medium (20 embryo/plate) prior to bombardment.

Plasmids and Microprojectile Preparation: Throughout the study, a pBSGUSINT plant transformation vector was used. The vector was obtained by cloning a CaM35S-GUS-CaMV35S-3' Hind-III fragment from a pGUSINT plasmid (15) into a Stratagene pBS vector multiple cloning site. Details of vector construction will be published in another paper. The pBSGUSINT plasmid was propagated in *Escherichia coli* cells and large-scale purification was carried out according to the method of Maniatis et al. (16). Circular plasmid DNA was precipitated on ca. 2 μm tungsten particles using the calcium nitrate method (17). In brief, tungsten particles were sterilised in 70% ethanol overnight, washed 3 times with distilled water and suspended in water at a final concentration of 60 mg/ml. 20 ml of this mixture was mixed with 5 ml plasmid DNA (1mg/ml) and 25 μl 1M calcium nitrate, and incubated on ice for 10 min. After pelleting down, 36 ml of the supernatant was discarded and 5 ml of the remaining suspension was loaded into each macrocarrier.

Particle Bombardment: For bombardment, a microprocessor-controlled GENEBOOSTER™ (13) particle delivery system (ELAK Co., Budapest, Hungary) driven by compressed nitrogen gas was used. The embryos were bombarded twice at a pressure of 25-40 bars and a chamber vacuum of -0.3 or -0.4 bars.

Histochemical GUS Staining: The bombarded embryos were kept in darkness for 24 hours at 24°C. Some of the bombarded embryos were left to germinate (16 hours light-8 hours darkness, 3000 lm) and seedlings were collected. Embryos and/or shoots were analysed by histochemical GUS staining principally according to the procedure of Jefferson (18). In brief, the tissue were kept in X-Gluc (Sigma) staining solution (0.3% W/V X-Gluc, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 100mM sodium phosphate buffer pH 7.0, 0.005% V/V Triton X-100) overnight at 37°C and fixed in 20% ethanol, 10% formaldehyde and 5% acetic acid solution. After fixation, the shoot tissues were rinsed several times in 50% ethanol solution to remove chlorophyll and then photographed under a binocular microscope.

Results and Discussion

In the experiments performed on each cultivar, 200 embryos were bombarded twice with 5 µl of DNA-coated particles. Non-bombarded embryos or embryos bombarded with bare tungsten particles were used as the controls.

For both cultivars, 60 bombarded embryos were analysed after GUS histochemical staining. In both cultivars, more than 80% of the bombarded embryos exhibited GUS positive results after histochemical staining. None of the control embryos showed GUS staining activity. Representative results for Atay and Çakmak cultivars are given in Figures 1 and 2 respectively. In both cultivars, increased gas pressure and chamber vacuum also increased the number of GUS positive sectors. On average, 104±34 and 97±30 (n=10) GUS positive sectors per embryo were observed in Atay and Çakmak cultivars, respectively.
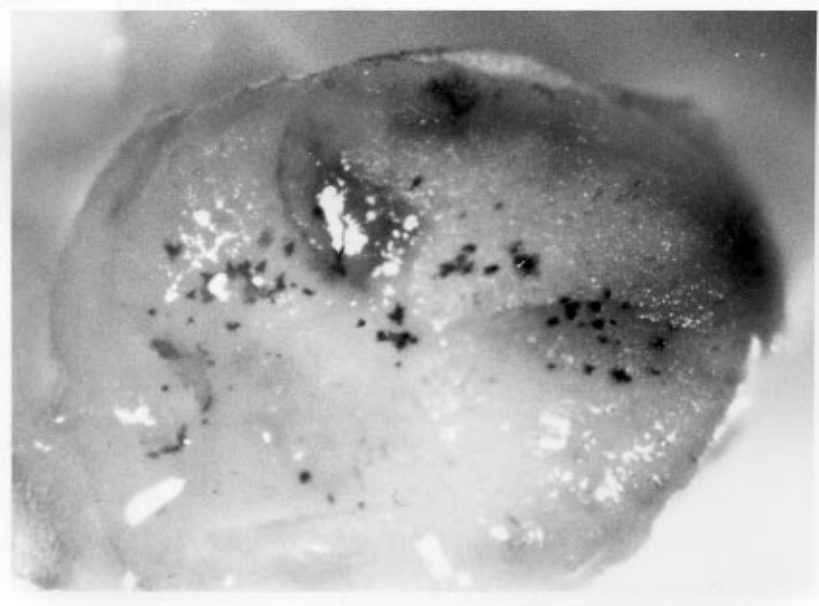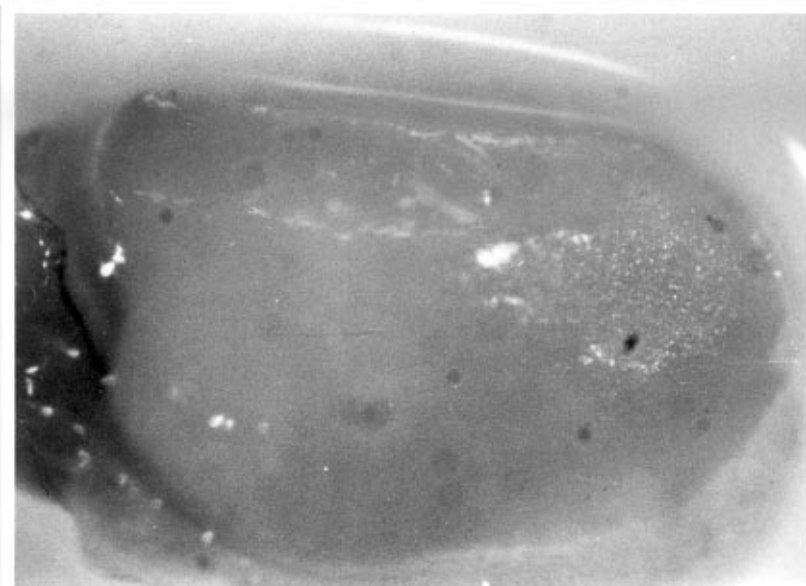

Figure 1. A: Histochemical GUS staining of a mature embryo isolated from cv. Atay and bombarded with pBSGUSINT plasmid. After bombardment, the embryos were kept in darkness for 24 hours and stained for GUS activity for 12 hours at 37°C. Following staining, the embryos were fixed and photographed at 40X magnification. B: GUS staining of a mature embryo bombarded by tungsten particles without DNA coating. After the bombardment, the embryos were treated as explained in A.
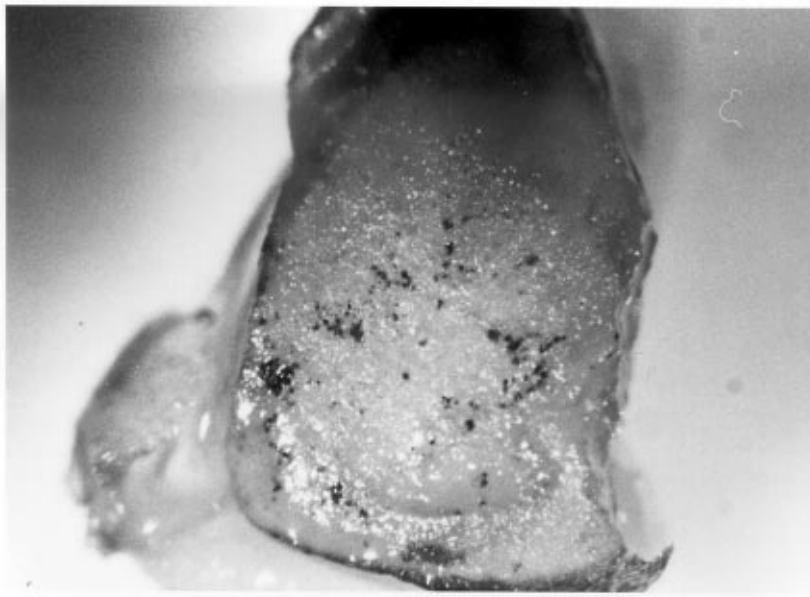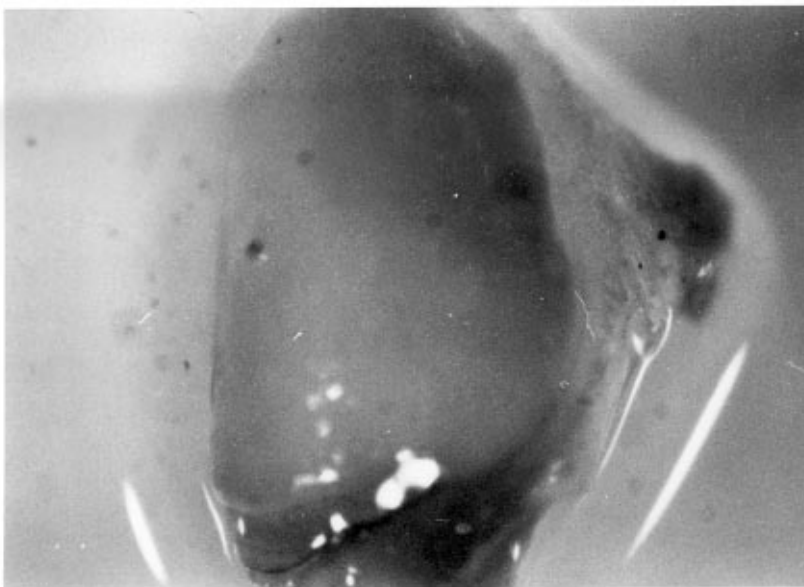
Figure 2. A: Histochemical GUS staining of a mature embryo isolated from cv. Çakmak and bombarded by pBSGUSINT plasmid. The embryos were processed as explained in the legend of Figure 1. The photograph was taken at 35X magnification B: GUS staining of the control embryo.

During the experiments, some of the bombarded embryos were left to germinate. After bombardment of the embryos, the germination frequency was over 90% in both varieties. After seven days of germination, seedlings obtained from 20 bombarded embryos of each cultivar were subjected to histochemical GUS activity staining in order to determine the integration and expression of the GUS gene. Similarly, seedlings obtained from non-bombarded embryos or embryos bombarded with bare tungsten particles were used as the controls. Representative findings for both varieties are given in Figure 3.

In 30% of the analysed seedlings, patches of GUS staining were observed at different locations on the leaves. This observation suggests that in some of the transformed cells the GUS gene was integrated with the genome and showed expression. The control seedlings did not exhibit GUS staining (data not shown). In some
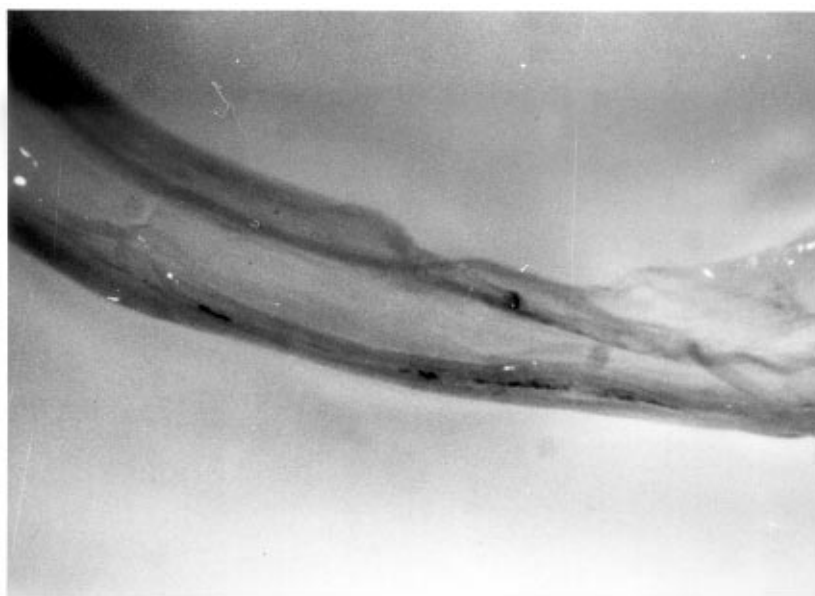
samples, remarkable GUS staining was observed in the shoot apical meristem, which is known to generate the whole green part of the plant body, including the flowers. This observation is of particular importance since one process involves growing the transformed chimeras plants to maturity and to analyse the seeds in order to obtain transgenic progeny. In such a procedure, transformed sectors in the apical meristem may contribute to the gametes, and, thus, to transgenic offspring.

Our preliminary findings demonstrate a comparable transformation frequency, at least at embryo level, with those in the literature. In the study of Klöti et al. (19), in which electroporation-mediated gene delivery to immature wheat embryos was investigated, under optimum conditions, up to a maximum of 60 transformed sectors per embryo were reported. In our experiments, we were able to locate more than 200 individually transformed sectors in embryos bombarded at 40 bars nitrogen gas pressure and 0.4 bar chamber vacuum.

Although we have not yet obtained molecular data, our preliminary findings clearly demonstrate a considerably high frequency of gene delivery at embryo level, which is one of the most important steps in obtaining transgenic wheat cultivars.

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