Factors affecting delivery of *DREB1A* gene in maize B73 split-seeds via biolistic system

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Abstract
Immature embryos as a choice tissue for genetic transformation of maize have a few limitations, such as genotype dependence, time-consuming and requiring a well-equipped greenhouse for access, at any time. In the present study, the split-seed explants were used for genetic transformation of maize, B73 line. The transformation of maize split-seed explants from the inbred line B73, for resistance to abiotic stresses was carried out via particle bombardment using pCA-35S-DREB construct, carrying Arabidopsis transcription factor *DREB1A* under the control of the CaMV 35S promoter and the selectable marker *bar*. For the optimization of gene-gun transformation parameters, we used two distances, 6 and 9 cm, one or double shooting and pre-treatment culturing of explants. The results showed that double shooting in 9 cm under pre -treatment conditions can improve transient expression of GUS gene in split-seed explants. PCR analysis confirmed the presence of *DREB1A* and *bar* genes in one out of 11 transformants. The results showed that split-seeds of B73 line can be used as a suitable explant for maize transformation.

Keywords**:** *DREB1A*, GUS, Maize, Split-seed, Transformation.

INTRODUCTION

Maize (*Zea mays* L.) is one of the most important commercial crops in the world because of its importance as food and feed. In addition to its agronomic importance, maize is an important monocot plant for studying genetics, genomics, and molecular biology (Strable and Scanlon, 2009).

Abiotic stresses such as cold, drought and high salinity influence survival, biomass production and crop yield, seriously. Globally, approximately 22% of the agricultural land is saline (FAO, 2004). The areas under drought are already expanding and it may increase noticeably. Water stress imparted by drought and extreme temperatures are the most prevalent abiotic stresses that limits plants growth and productivity (Burke *et al*., 2006). Plant responses to abiotic stresses require the production of important metabolic proteins such as those involved in the synthesis of osmoprotectants or regulatory proteins such as kinases or transcription factors (TFs). The TFs can regulate the expression of many stress-inducible genes in plants (Vincour and Altman, 2005; Bartels and Sunkar, 2005). They play a critical role in improving abiotic stress tolerance of plants (Chaves and Oliveria, 2004) by interacting with a specific *cis*-acting element (DRE/CRT), which is present in the promoter region of various abiotic stress-related genes (Shinozaki and Yamaguchi-Shinozaki, 1997; Winicov and Bastola, 1997). The TFs that bind to *cis*-actin element were named CRT-binding factors or DRE-binding protein 1 (*CBF*/*DREB1*) (Gilmour *et al*., 1998; Liu *et al*., 1998). *CBF*/*DREB1* gene expression is quickly and transiently induced by cold stress, and in turn *CBF*/*DREB1* TFs activate the expression of more than 40 other genes such as those involved in the production of osmoprotectants and antioxidants (Saibo

et al., 2009). Furthermore, it is reported that the over-expression of *CBF*/*DREB1* in Arabidopsis resulted in plants with improved survival rates when exposed to salt, drought and low temperatures (Jaglo-Ottosen *et al*., 1998; Kasuga *et al*., 1999). Similarly, when *CBF/DREB1* genes from Arabidopsis were over-expressed in other plants, the same results were observed (Kasuga *et al*., 1999; Gilmour *et al*., 2000; Hsieh *et al*., 2002; Pellegrineschi *et al*., 2004). Transformation of maize with *CBF3/DREB1A* via biolistic gun using split-seed explants improved abiotic stresses tolerance in plants (Al-Abed *et al*., 2007). These results indicate that over-expression of DREBs may be a useful approach to enhance significantly drought, salt, and cold stress tolerance in plants.

Due to the fact that high-frequency transformation in maize is beneficial for genetic engineering studies, there is still a potential and need in further improving its transformation frequency. Although it has been proved that immature embryos are the most useful tissues for transgenic maize production, the utility of them have been associated with some restrictions, such as genotype dependence (Frame *et al*., 2000; Tomes and Smith, 1985; Takavar *et al*., 2010) and time-consuming (Ishida *et al*., 1996; Huang and Wei, 2004).Therefore, overcoming these limitations is criticalfor the maximum effects of genetic engineering technologies. There are a variety of maize explants (*e.g*. mature embryos, meristems, leaves and split-seeds) which have been used for the genetic transformation of maize plants (Zhong *et al*., 2003; Dai *et al*., 2001; Ahmadabadi *et al*., 2007; Al-Abed *et al*., 2006; 2007). Most recently, fertile maize plants have been regenerated using a new explant "split-seed" (Al-Abed *et al*., 2006; 2007), which can address these limitations. It is reported that exposing three different types of competent cells which are scutellum, coleoptilar-ring and the shoot apical meristems, may be a key factor in increasing regeneration efficiency (Al-Abed *et al*., 2006).

In this study, several parameters such as osmotic pre-treatment, two distances, 6 and 9 cm and the number of shots were analyzed by using GUS gene assay (48 h after bombardment). Finally, maize transformation by CaMV 35S-DREB cassette was performed using split-seed explants of an inbred line B73.

MATERIALS AND METHODS

Plant materials

Mature dry seeds of the maize line, B73 were sterilized by 70% (v/v) ethanol for 2 min, rinsed four times by sterile water, soaked in 0.5% (v/v) NaOCl for 7 min, rinsed six times by sterile water and finally soaked in sterile water for 24 hours. The seeds were then germinated on the MS (Murashige and Skoog, 1962) basal salts medium, including B_5 vitamins (Gamborge *et al*., 1968) supplemented with 9 µmol/l 2, 4-D. After three to four days, maize embryos axes were positioned so that the seeds were split longitudinally into identical halves using a pair of forceps and a scalpel (Al-Abed *et al*., 2006). Then, the halved explants were cultured facing the wounded sides on the medium.

Plasmids for transformation

The plasmid pCAMBIA3301 (CAMBIA, Australia) (Figure 1) containing an intron-β-glucuronuridase (GUS) was used to monitor transient gene expression and to define the conditions that lead to the maximal gene expression in the early stages of maize plants growth.

For the construction of pCA-35S-*DREB1A*, *Arabidopsis thaliana DREB1A* cDNA (Gifted by Dr Alizadeh, University of Tehran, Iran) was cloned in a binary vector pCAMBIA3300 under the control of Cauliflower Mosaic Virus (CaMV 35S) promoter and *nos* terminator (Figure 1).

Micro-projectile bombardment

The split-seed explants were cultured facing the wounded side on the multiple shoot induction medium (MSI) (Al-Abed *et al*., 2007) for 24 hours. MSI medium contained MS basal salts supplemented with B_5 vitamins, 17.6 µmol/l 6-benzylaminopurine (BAP) and 9.2 µmol/l kinetin (6-furfurylaminopurine), in addition to 1 mg/l glycine, 400 mg/l casein hydrolysate, 30 gr/l sucrose, and the pH was adjusted to 5.8 before adding 8 g/l agar. For the osmotic treatment, after 24 hours in the pre-culturing stage, all 6 explants per plate were arranged on the MSI medium with the cut side upward, left uncovered in the laminar flow hood for 3 hours to allow the split-seeds to air dry before shooting by biolistic (Al-Abed *et al*., 2007). Moreover, split-seeds were bombarded without any culturing on MSI media, as the control treatment.

Plasmid DNA was extracted and its concentration was adjusted to 900 ng/µl. Sixty mg gold particles of 1.0 µm in diameter were coated with 10 µl of plasmid DNA followed by adding 50 µl of 2.5 mol/l $CaCl_2$ and 20 µl of 0.1 mol/l spermidine and were shaken for 20 min at 4°C. After washing three times by absolute

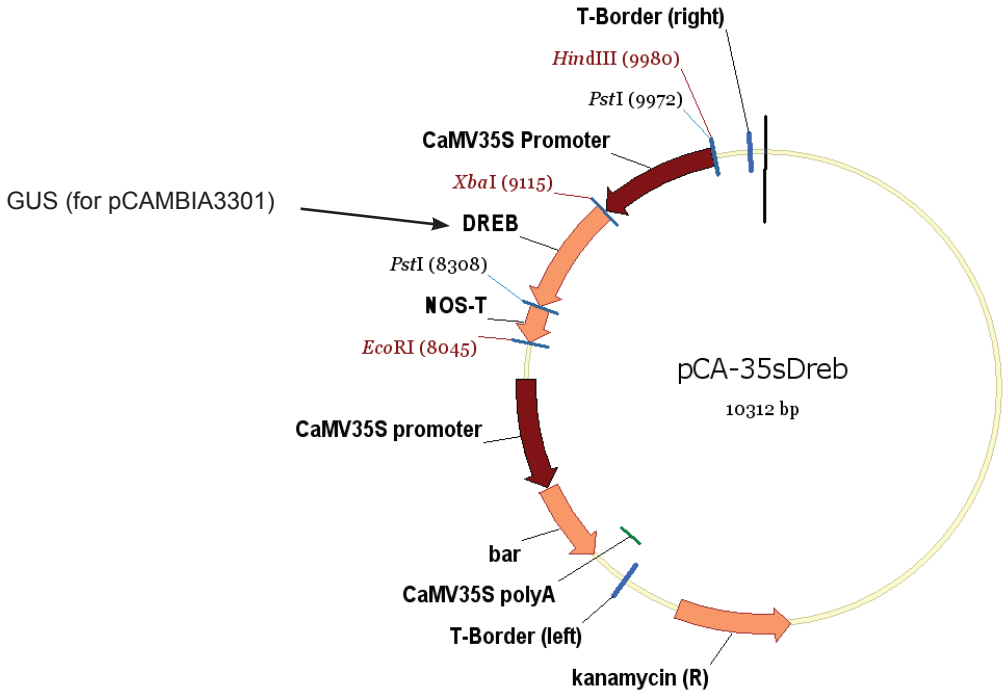

Figure 1. pCA-35S-*DREB1A* and pCAMBIA3301 constructs, used for maize transformation.

ethanol, the particles were re-suspended in 60 µl ethanol. Fifteen µl of the suspension were spread on each macro-carrier and allowed to dry before bombardment. All explants were bombarded at 635 mmHg using the BioRad Biolistic PDS-1000/Helium vacuum system (Bio-Rad Co.).

For the optimization of bombardment parameters, we used a combination of shooting distances (6 and 9 cm), single or double shooting, and the osmotic pretreatments of explants. Thereafter, the bombarded explants by using pCAMBIA3301 binary vector were assayed by GUS staining and the numbers of blue spots were counted per explant. All experiments were conducted in three replications.

For the genetic transformation of maize using pCA-CaMV-*DREB1A*, the explants were positioned at 6 and 9 cm and a combination of both, for bombardment. After bombardment, all plates were kept in the dark at 26°C for 24 hours and used for selection and regeneration steps.

Histochemical GUS assay

The histochemical β-glucuronidase (GUS) assay (Jefferson *et al*., 1987) was used to evaluate early events of transformation by the pCAMBIA3301 plasmid. Transient GUS gene expression was visualized histochemically using X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid) in split-seed explants. The bombarded explants were incubated in the GUS solution for 24 h at 37°C. The number of GUS-positive sectors and their distribution in the scutellum, the coleoptilar ring, and the shoot apical meristems were then scored (Al-Abed *et al*., 2007).

Selection and regeneration

As a recovery period, split-seed explants were then transferred to the fresh MSI medium without herbicide selection for 4 days, in 16/8 light/dark at 26°C. The explants were then sub-cultured on the MSI medium, containing 5 mg/l phosphinothericin (PPT) (Roche Co. Germany) for 2 weeks for the initial herbicide selection. PPT-resistance shoots were transferred to a fresh medium biweekly, three times before root induction. Putative transformants were subsequently transferred to a 150 × 25 mm tube containing the rooting medium, consisting of MS salts, 3.2 µmol/l NAA (1-naphthaleneacetic acid) supplemented with 2.5 mg/l PPT to ensure stringent selection before transferring to soil. Plantlets with fully grown shoots and roots were then transferred to the pots containing soil: perlite: vermiculite and allowed to acclimatize for 2-3 weeks in a growth chamber, under a photoperiod of 16/8 h and a light intensity of 300 µmol/m^2 /s^1 at 24°C) before being

moved to the greenhouse condition.

Molecular analysis

Genomic DNA was extracted from 300 mg of leaf tissues using the CTAB procedure (Doyle and Doyle, 1990). A 750 base pair (bp) PCR fragment containing the *DREB1A* coding region was amplified using primers: forward 5'-AGT CTT CGG TTT CCT CAG G- 3' and reverse 5'-GAT TAT GAT TCC ACT GTA CGG- 3', and a 570 bp PCR fragment containing the *bar* coding region was amplified using primers: forward 5'-CTC GAG TCA AAT CTC GGT GAC GGG- 3' and reverse 5'-CGA GTC TACCAT GAG CCC AGA ACG- 3'. The DNA amplifications were carried out in a thermocycler (Biorad Co., DNA Engine Model) with an initial denaturing step at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min and a final extension of 5 min at 72°C. PCR products were then separated by electrophoresis on a 1% (w/v) agarose gel and stained by gel-red and visualized under a UV light.

RESULTS AND DISCUSSION

Different parameters may be tested to optimize particle bombardment for transformation. In this preliminary study, we mainly focused on finding the best conditions to make the target cells more receptive to DNA uptake and also to combat cell death, which occurs due to wounding of the cell wall, during micro-particles penetration.

The culture of immature maize embryos on the callus induction medium 2-4 days before bombardment or before and after bombardment carried out by Songstad *et al*., (1996) and Brettschneider *et al*., (1997), respectively, both led to an increase in stable transformation frequencies. Al-Abed *et al*., (2007) reported the same results as those of Songstad *et al*., (1996) on split-seed explants. In the present study, we conducted a preliminary experiment for determining the effects of osmotic pretreatments on the transformation frequency of maize split-seed explants, using microprojectile bombardment. Thus, we used the GUS assay to evaluate the efficiency of maize transformation by micro-projectile bombardment methods. We observed a negligible number of GUS blue spots when explants were not cultured on the MSI medium before and after bombardment (Figure 2A). Therefore, the experiments were followed by culturing explants on the MSI medium for 24 h pre and post bombardment steps.

An increase in stable transformation frequency has been observed with partial drying of the target cells (Al-Abed *et al*., 2007) or employing mannitol or sorbitol to the medium as the osmotic inducers (Chen *et al*., 1998; Finer and McMullen, 1990). Finer *et al*., (1999) suggested that the osmotic pre-treatment may be acting to minimize cell death caused by the wounding of the cell wall, during bombardment. In this study, an increase in the average number of GUS blue spots was observed in the explants left uncovered in the air laminar flow hood for 3 hours as an osmotic pre-treatment before bombardment (Figure 2C-E) compared to those which were not exposed to the osmotic pre-treatment (Figure 2B).

The distance between microcarrier and target cells can affect the velocity of microprojectiles and consequently transformation rates. A higher number of transient expression was detected when explants were placed 9 cm than 6 cm away from the microcarrier (Table 1). This may be attributed to the deleterious effects of higher velocity of microprojectiles at 6 cm distance (Zuraida *et al*., 2010). However, Tadesse *et al*., (2003) reported a higher number of transient GUS expression as well as stable transformation events at a distance of 6 cm than at 12 cm in sorghum plant.

Table 1. Effects of pre-treatment, shooting distances and shooting number on transient GUS expression in maize split-seed explants. Shootings were conducted by a single shot at 6 or 9 cm distance and double shots at 6+9 or 9+9 cm.

Treatment	Shooting distance (cm)	Number of blue spots/explants
- Pre-treatment	6	12.3±2.2
	9	16.1±1.8
+ Pre-treatment	6	15.5±1.9
	9	23±2.1
- Pre-treatment	6+9	33±3.1
	9+9	35±3.2
+ Pre-treatment	6+9	38±3.6
	9+9	41±3.8

Zuraida *et al.,* (2010) found that changes in both helium pressure and distance affect the level of transient GUS expression in rice calli. They observed that for 1100 psi, the highest expression was observed at 9 cm. Moreover, Schopke *et al*., (1997) observed similar results by using 1100 Psi and 9 cm combination that gave

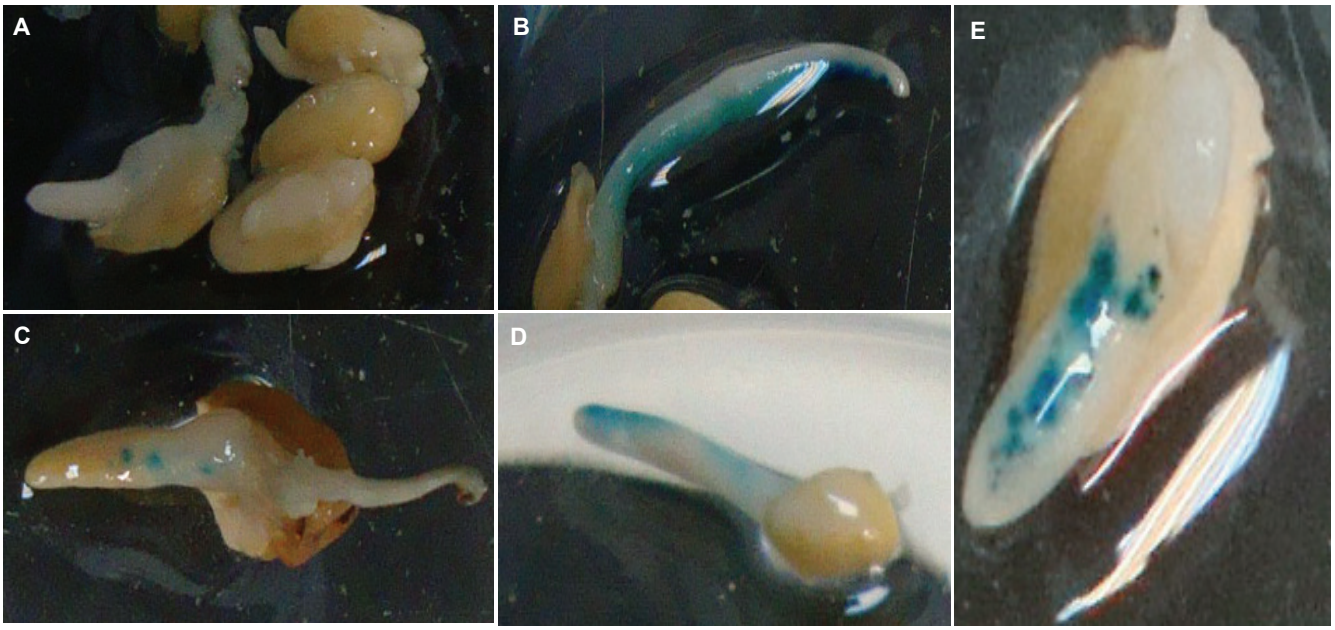

Figure 2. Transient expression of GUS gene under different conditions for the transformation of maize seeds by particle bombardment. **A:** Without culturing, before and after bombardment; **B:** With culturing on the MSI medium for 24 h, but without osmotic pretreatment; **C:** Osmotic pretreatment, 2 shots at 9 cm; **D:** Osmotic pretreatment, 2 shots at 6 and 9 cm; **E:** Osmotic pretreatment, 2 shots at 9 cm.

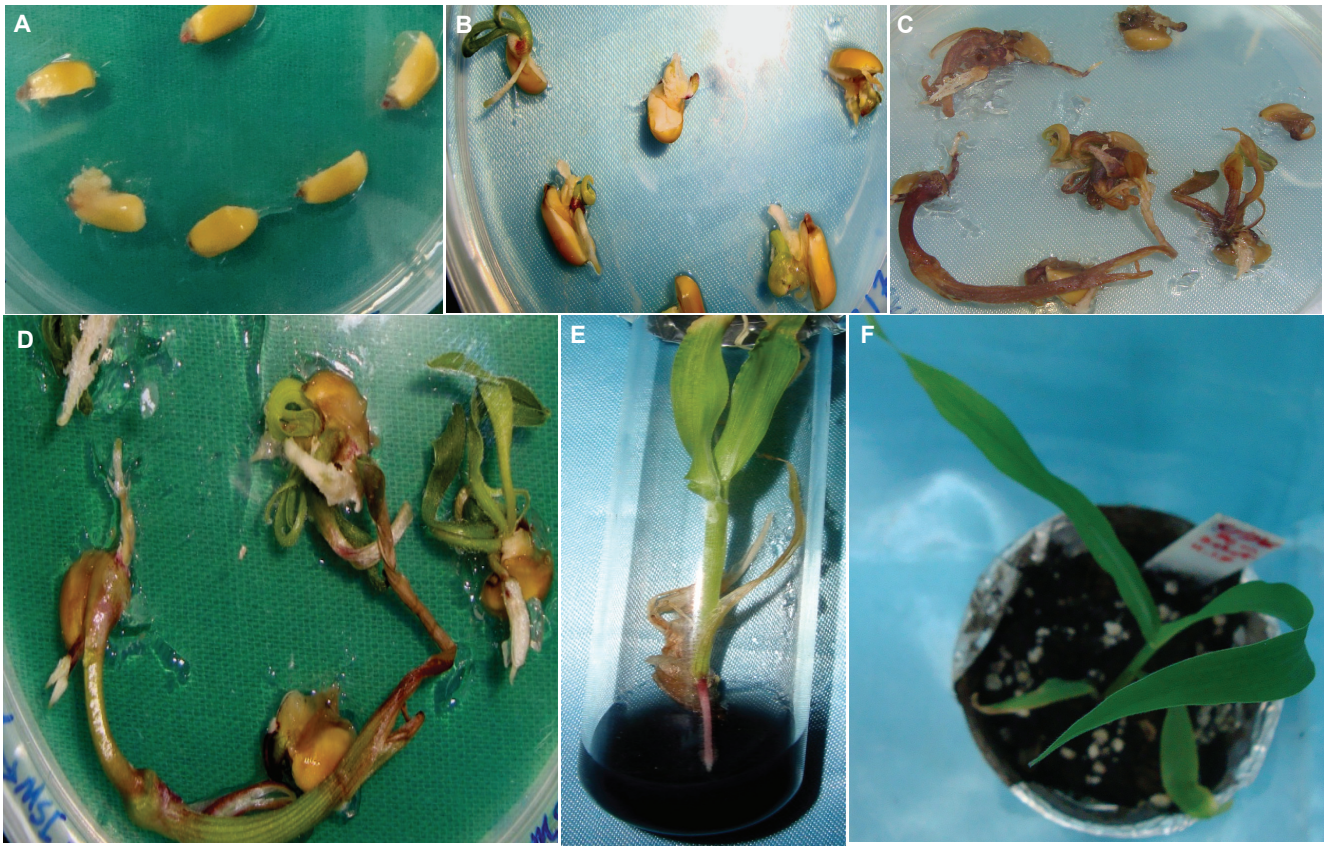

Figure 3. Production of transgenic maize plants using split-seed explants. **A:** Explants after bombardment in a recovery period; **B:** Shoot regenaration through the first stage of selection; **C:** PPT non resistant-shoots in the selection medium; **D:** PPT resistant-shoots; **E:** Rooting of a putative transformant after a second round of selection; **F:** Putative transgenic plants grown in soil.

Table 2. Transformation frequency in the particle bombardment of maize split-seeds by pCA-35S-DREB.

Number of bombarded explants (A)	Number of shooting	Distance (cm)	PPT-resistant shoots	No. of PCR positive plants (B)	Transformation frequency (B/A%)
55	2	9	8	1	1.8%
48	2	9+6	3	-	-

a higher expression in cassava cultures.

In this study, to increase the frequency of transformation, the influence of more than one shot per plate was also tested. When plates were bombarded twice at 9 cm or at 9 and 6 cm at 1100 Psi, a significant increase in the average number of GUS spots was observed in the explants under the osmotic pre-treatment (Table 1; Figure 2). Petrillo *et al*., (2008) reported that two shots per plate can produce 2.3% transgenic plants from the inbred lines, L1345 of maize, while there were no transgenic plants produced when three shots were used. They supposed that three shots per plate probably caused more injuries to the explants, which impaired cell proliferation and regeneration.

Wang *et al*., (1988) observed a direct relationship between an increase in the number of wheat cells, transiently expressing GUS and an increase in the number of shots per plate. However, Reggiardo *et al*., (1991) found that more than one bombardment per plate had lethal effects on barley and maize cells. Zuraida *et al*., (2010) did not find a significant difference between double and triple bombardment in transient GUS expression of rice plants, but they observed that more than one consecutive shot on the same plate resulted in an increase in GUS transient expression. Moreover, Al-Abed *et al*., (2007) reported a significant increase in the average number of GUS blue spots when maize explants were bombarded twice at 750 Psi. Although, they suggested that the pressure and the number of shots are the main factors in transient GUS expression in maize split-seeds, they observed no significant differences when 1100 Psi was used in their treatments (Al-Abed *et al*., 2007). Moreover, they obtained more antibiotic-resistant shoots in cultures bombarded twice at 1100 Psi. Our results indicated that, the level of transient GUS expression is mainly attributed to the application of three parameters: culturing the explants 24 h before and after bombardment, osmotic pre-treatment and two shootings.

With regards to these results, we conducted particle bombardment transformation for split-seed explants, using pCA-35S-*DREB1A*. After bombardment, explants were kept on the MSI medium for a four day recovery period (Figure 3A) and then transferred to the selection medium for a period of six weeks (Figure 3B, C, D). The PPT-resistant shoots were then transferred to the root induction medium (Figure 3E). Two shootings at 6 and 9 cm resulted in 3 regenerated plants from 48 bombarded explants while, eight plants in the selection medium regenerated from 55 bombarded explants by two shootings at 9 cm (Table 2; Figure 3). However, only one regenerated plant was obtained in the selection medium from two shootings at 9 cm produced fully grown shoots and roots by the end of the experiments and showed PCR positive for *DREB1A* and *bar* genes (Figure 4). Therefore, transformation frequency in the inbred line B73 was estimated 1.8%, which is lower than the results (5%) achieved by Al-Abed *et al*., (2007) for the inbred line R23.

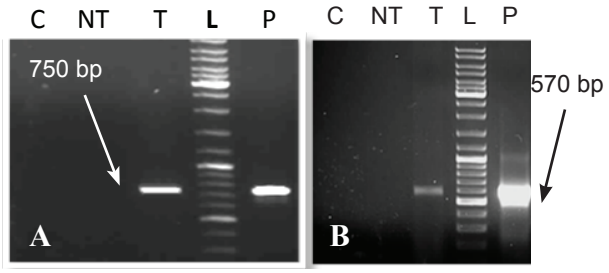

Figure 4. Molecular analysis of transgenic and non-transgenic maize. **A:** PCR analysis for DREB1A gene; **B:** PCR analysis for bar gene; T: Transgenic; NT: non-transgenic; C-negative control (water); P: plasmid (positive control); L, 1 Kb ladder.

In conclusion, split-seeds can be used as substituent

explants for maize transformation. Moreover, we have introduced a rapid and time saving method for the genetic transformation of maize plants.

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