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Transgenic wheat plants resistant to herbicide BASTA obtained by microprojectile bombardment

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Key words: BAR, bombardment, GUS, transformation, *Triticum aestivum*

A**BSTRACT:** Wheat (*Triticum aestivum*) transgenic plants of an important commercial cv (Oasis) was obtained with an efficient and short procedure. First, the optimum conditions for the embriogenic calli generation from inmature embryos with a high regeneration percentage were established. The transformation of calli was performed by high velocity microprojectile bombardment, using the pAHC25 plasmid, which contains the reporter gene β-glucuronidase (GUS) and the selectable BAR gene which confers resistance to the herbicide Basta. The transformations were confirmed by β-glucuronidase assay activity, PCR and Southern blot analysis. The efficiency of this procedure was high and similar to other reports in which "model" cultivar was used.

Introduction

Wheat (*Triticum aestivum* L.) is one of the most important food crops in the world with annual yields exceeding 500MT (FAO, 2000). It is one of the major agricultural products in Argentina; since the seventies the culture surface has steadily increased to approximately 6 million hectares at present (Sagypa, 2001). Biotechnological methods are a complement to breeding programs for the improvement of wheat productivity. The success of plant genetic manipulation requires the ability to deliver functional DNA into the cell, the regeneration of transformant tissue and the evaluation of the transgenic plant. Even though plant transformation using *Agrobacterium tumefaciens* was the first method routinely used in gene transfer, until relatively recent it has been employed successfully only in dicotyledons. Monocotyledons, and particularly cereals, were originally considered out of the host range of *A. tumefaciens*. However, reports of rice or wheat transformation with this method have appeared recently (Hiei *et al*., 1997; Cheng *et al*., 1997). In general, monocot transformation can be achieved by other systems with diverse effectiveness: direct delivery of DNA into protoplasts by electrical (electroporation) or osmotic shock (polyethylene glycol) or by high velocity microprojectile bombardment (Vasil *et al*., 1992; 1993; Weeks *et al*., 1993; Rasco-Gaunt *et al*., 1999) usually termed "biolistic". The most convincing and unambiguous results leading to the recovery of transgenic plants have been obtained by the last strategy. Different constructs and reporter genes have been used in transformations by the biolistic method (Jefferson, 1987; Dupuis and Pace, 1993; Nehra, *et al* 1994; Ortiz *et al*., 1996). Recovery of transgenic plants require a fine tuning of the regeneration strategy and transformation method. In order to increase transformation frequency, most of the attempts have been focused on genotype embryogenic capacity of the explant, bombardment conditions, selectable markers, selection conditions, developmental stage of donor plant and the enviromental conditions in which they are grown. Even though, there have been

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biolistic transformation in wheat, and large differences in regeneration and frequency rates of transformation between varieties (Iser *et al*., 1999; Rasco-Gaunt *et al*., 2001).

In this work we describe a wheat regeneration system from immature embryos calli, and a genetic transformation strategy by high velocity microprojectile particle bombardment using pAHC25 plasmid (Christensen *et al*., 1992), which contains the reporter gene β–glucuronidase (GUS) and the selectable BAR gene which confers resistance to the herbicide Basta. With this system, it was possible to obtain and cultivate transgenic plants of a commercial wheat cultivar with Basta resistance.

Material and Methods

Immature embryo culture and plant regeneration

Immature embryos were isolated from wheat plants (*Triticum aestivum*, cultivar Oasis), and grown in the greenhouse under natural illumination at 22-24°C. To establish callus cultures, 18 to 20 days post-anthesis caryopses were surface-sterilized with: 70% ethanol for 2 min, 2% commercial sodium hypochloride for 20 min, followed by three changes of sterile water.

The immature embryos (0.5- 1.5 mm in length) were aseptically removed under a stereo dissecting microscope and placed, with the scutella exposed, on MS medium (Murashige and Skoog, 1962) with MS vitamins, 30 g l^{-1} sucrose and 9 μM (2 mg l^{-1}) 2,4 dichlorophenoxy acetic acid (2,4D). Twenty immature embryos were cultured per 90 mm Petri dish containing 20 ml of medium. Each replicate was a dish containing explants, and the size of experiments based on the number of scutella isolated ranged from 200-400. All media were solidified with 6 g l^{-1} agar and the pH was adjusted to 5.7 prior to autoclaving. The cultures were incubated in the dark at 22-24°C during 15-20 days. Embryogenic calli were transferred to the light (66 μEm^2 s^{-1}) and cultured in a regeneration medium containing either MS alone or with 1.71 μM (0.3mg l^{-1}) indoleacetic acid and 4.65 μM (1 mg l^{-1}) kinetin until shoots developed. Plantlets (≥ 2 cm in length) were transferred to tubes with MS without plant growth regulators. After 3-4 weeks, plants were transferred to soil and grown in the greenhouse under the growth conditions of donor plants.

Plasmid and leaf tissue plant DNA purification

The plasmid pAHC25 (9.8 Kb) used for wheat transformation was kindly provided by A. Christensen and P. Quail (Plant Gene Expression Center, University of California, Berkeley/U.S. Department of Agriculture, Albany CA). This dual-expression vector consists of the *UidA* gene (Jefferson, 1987) and the selectable BAR gene, each one driven by the maize ubiquitin *Ubi1* promotor (Fig. 1) (Christensen *et al*., 1992). The *UidA* gene encodes the enzyme β-glucuronidase (GUS reporter gene) and the selectable BAR gene encodes the enzyme PAT which inactivates the active ingredient of the herbicide Basta. Plasmid DNA was purified from alkaline-lysed cells according to Sambrook *et al*. (1989), and stored at a concentration of 1 μg/μl. Leaf tissue DNA purification was carried out using the protocol described by Doyle and Doyle (1990).

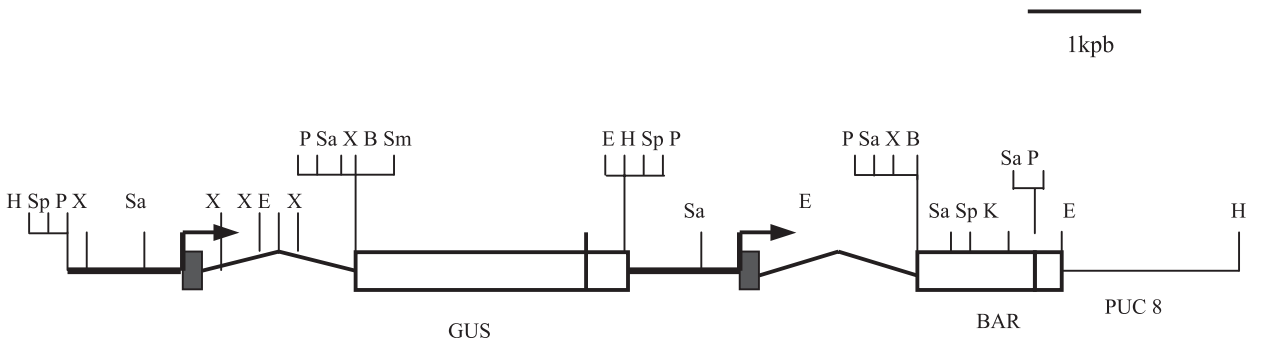

FIGURE 1. Schematic diagrams of pAHC25 vector. The relative sizes of the various segments of the plasmid were drawn to scale. Bold straight line, Ubi-1 promotor sequence; filled box, Ubi-1 exon; angled line, Ubi-1 intron; labelled open, boxes reporter gene sequences; blank open box, nopaline synthases 3' untranslated sequence; thin straight line pUC8 sequence: Arrow at the Ubi-1 exon signifies transcription start site and direction. GUS, β-glucuronidase (Jefferson, 1987); BAR, phosphinothricin acetyltransferase.

Callus treatments and microprojectile bombardment conditions

Embryogenic calli from immature embryos (15-20 days) were incubated in MS medium (solidified with 14 g l^{-1} agar) supplemented with 0 or 0.4 M sorbitol. The calli were kept under these conditions for 4-6 h before bombardment and for 16 h afterwards. Subsequently, the calli were cultured in the regeneration medium without selection for two days and then transferred to a similar medium with 10 mg l^{-1} of filter- sterilized (22 µm Millipore) herbicide Basta.

The bombardment was carried out using a high pressure bombardment device which was built in our laboratory following the model PDS-1000/He® Bio Rad with modifications (Kikkert, 1993). Our device used a

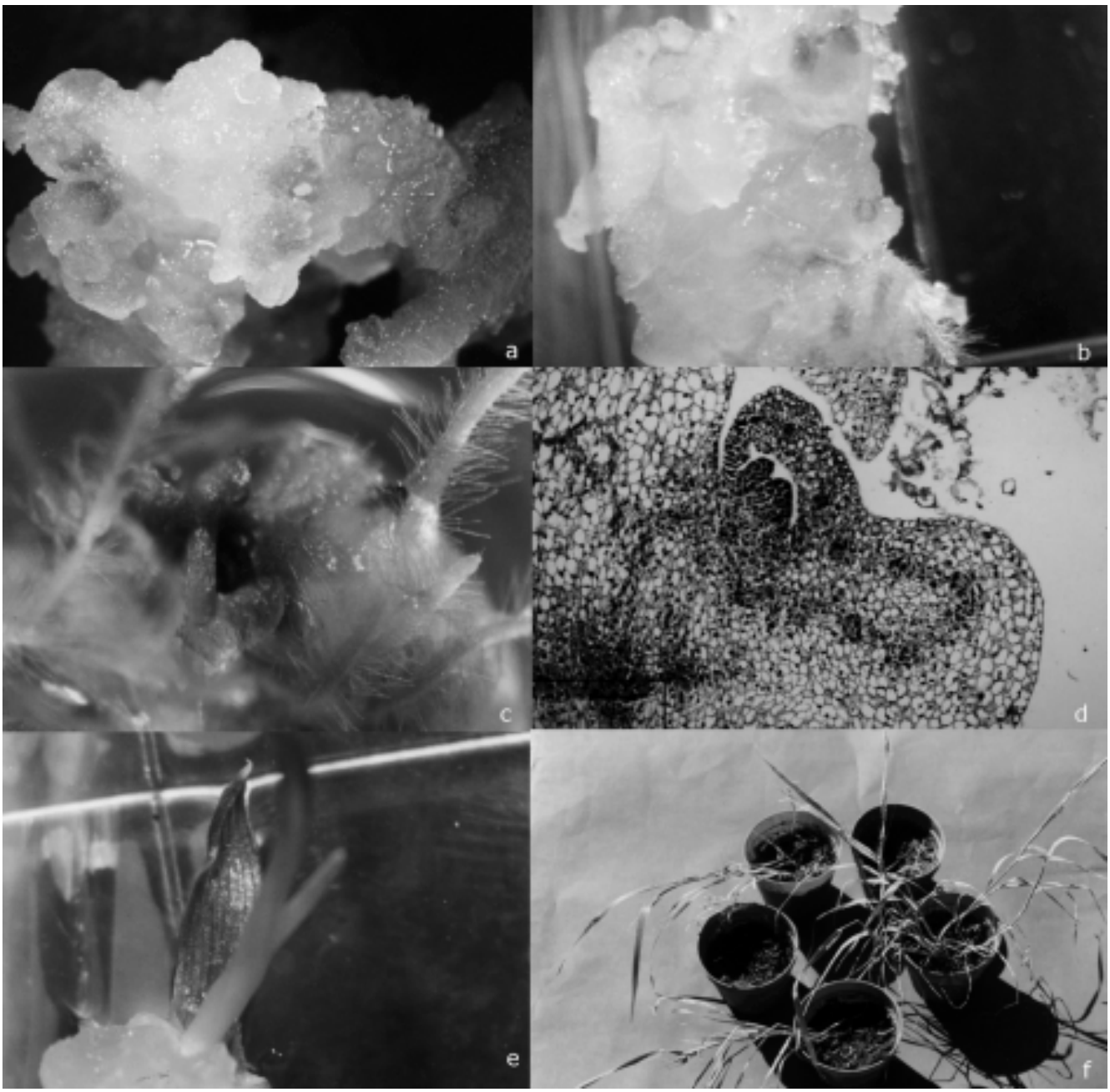

FIGURE 2. (a) Initiation of embryogenic calli from cultured immature embryos, green meristematic spots are zones from which shoots will develop. (b) Embryogenic callus culture after about 4 weeks, arrows indicate non embryogenic callus zone. (c) Shoot development in regeneration media. (d) Longitudinal ultrafine section of embryogenic calli showing shoot developent stained with hematoxiline-eosine. (e) Plantlet growing from embyogenic calli. (f) Wheat (Cv. Oasis) plants transferred to soil.

12 mm-thick Mylar membrane (Du Pont®), instead of Kapton, a rupture disk to temporarily seal the pressure chamber filled with helium (He) gas. When the critical pressure was reached, the rupture disk was perforated by a needle releasing a powerful burst of He shock wave, whereas in the case of PDS-1000/He the rupture disk was ruptured increasing the gas pressure. The helium burst, 600 psi under a vacuum pressure of 24 cm Hg, accelerated a 19 mm-thick Mylar macrocarrier. DNA-coated microcarriers (7 µl), plasmid DNA (10 µg) precipitated and adsorbed onto 50 µl (60 mg/ml) of gold particles suspension (Bio-Rad 1 µm Gold Microcarriers), had been loaded and dried upon the surface of the macrocarrier.

Enzyme assays

GUS activity was histochemically assayed 48 h after bombardment. Blue spots per cm^2 were counted 24 h after incubation in staining buffer at 37°C (Jefferson, 1987) using an image analyzer and the Optimas 6.0 software.

PCR assays

PCR was performed essentially as described by Sambrook *et al*. (1989). The BAR coding region was amplified using the following primers: Fw: 5' TGCACCATCGTCAACCACTA 3' Rv: 5' ACAGCGACCACGCTCTTGAA 3' The reaction was carried out in 12 µl final volume, with Sigma Taq polymerase (0.5 Unit/reaction) and 50-200 ng of DNA. PCR program was: initial denaturation 95°C 5 min, cycles (30) denaturation 95°C 1 min, annealing 54°C 30 s. and primer strand elongation 72°C 1 min.; final elongation 72°C 7 min. Amplification products were separated by agarose (1.2%) gel electrophoresis.

Southern blot analysis

DNA from bombarded and control plants was isolated, as describe above, and were digested with PstI and Eco RI according to supplier instructions (Promega). PstI released BAR and GUS plus Nos cassettes, and Eco RI cut Ubi-1 intron and a internal BAR site (Fig.1). The digestion products were separated by electrophoresis in a 0.8% agarose gel (1V/cm, 4°C) (Sambrook *et al*., 1989), transferred to a nylon membrane by capillarity and fixed to the membrane by UV cross-linking. Prehybridization was carried out at 42°C for 4 h in a solution containing: 5X SSC (NaCl 0.75M, sodium citrate 0.075M pH 7), 50% formamide, 50 mM NaH_2PO_4 pH 7.0, 0.02% SDS, 0.1% N-laurilsarcosine, 2% blocking reagent (Boehringer). The membrane was hybridizated for 16 h with a doble-strand BAR probe (400ng/µl) labelled with digoxigenine. The probe was the BAR coding region amplified by PCR (311 bp) (see

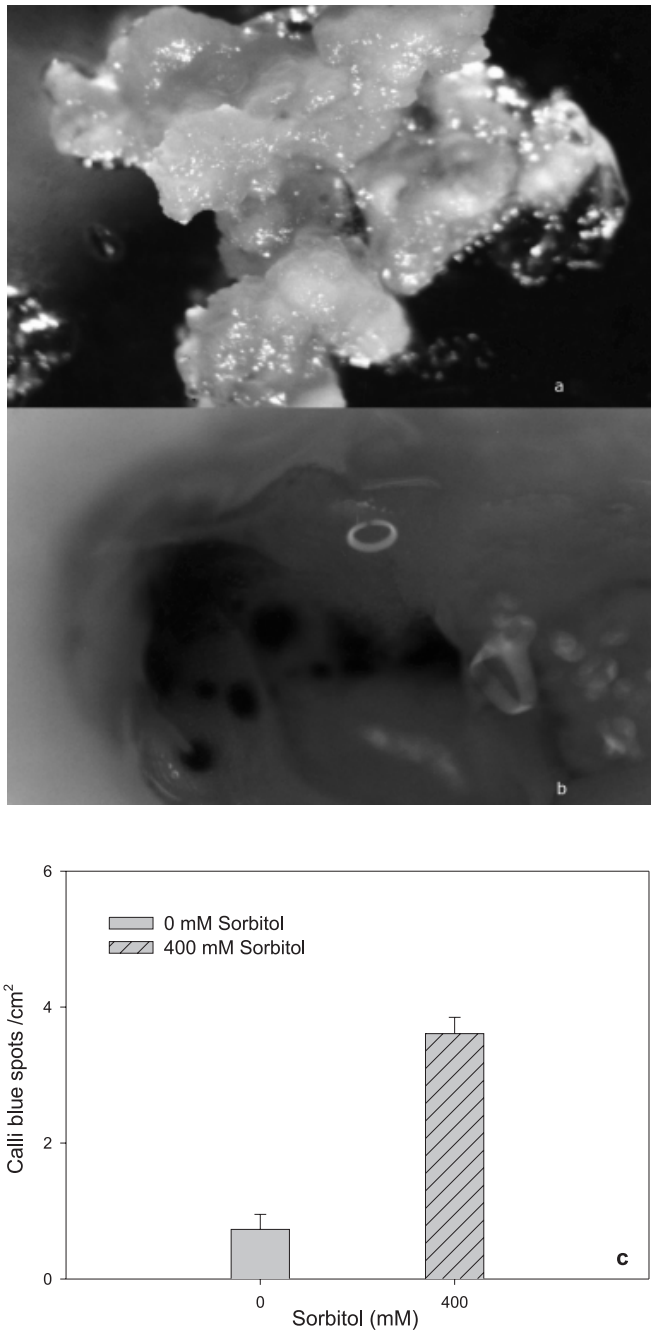

FIGURE 3. (a) Histochemical expresion of GUS activity in callus transformed with pAHC25 plasmid. (b) Detail of GUS expression in meristematic calli zone. (c) GUS activity in osmotically treated calli with 0 and 400mM sorbitol.

PCR assays) and labelled with dig-dNTPs during the PCR reaction. The washing conditions after hybridization were markedly stringent (0.3 mM sodium citrate, 3 mM NaCl, 0.1% SDS and 65ºC). Hybridization signals were visualized with antidigoxigenine alkaline phosphatase conjugate (Boehringer).

Results and Discussion

Immature embryo culture and plant regeneration

Under promoting conditions of callus development, 96-98% of the cultured immature embryos developed calli. The embryos became soft and swollen and formed pockets of calli along the edges of the scutellum. The structures selected as "embryogenic callus" had a dry, compact and nodular appearance (Fig. 2a). A very low percentage of the calli were friable, translucent and with hyperhydric cells. Sometimes both types of structures were observed in the same calli (Fig. 2b), and these parts of the callus were removed and discarded. When these embryogenic calli were placed under regeneration conditions, most of them (93-95%) developed small meristematic regions. These meristematic regions had a dome-like shape, from which shoots were recovered (Fig. 2c). This primary organization was confirmed by histological sections (Fig. 2d). The percentage of plant regeneration was higher in the medium supplemented with hormones (67.3%) than in the medium without them (43.3%). In the latter case, most of the developed structures were roots, probably because cytokinins balance the action of residual 2,4 D in the calli, promoting the development of other structures. After 30-45 days in medium with hormones, shoots were transferred to individual tubes with MS medium alone and kept under light for 2-4 weeks (Fig. 2e). When plantlets reached 5-7 cm in length, they were cultured in Magenta boxes for 4-6 weeks and then transferred to pots containing sterile vermiculite and kept in the greenhouse (Fig. 2f).

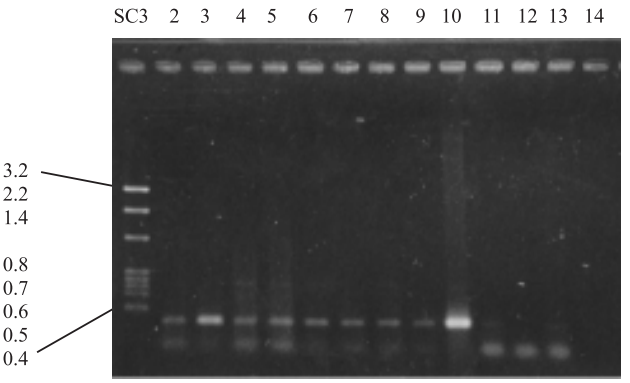

FIGURE 4. PCR analyses of wheat independent Basta resistant plants recovered after transformation with pAHC25. Ethidium bromide stained agarose (1.2%) gel of PCR amplification of the BAR gene. 1: SC3-HindIII MW marker. Lines 2-9, show 310 bp amplification in wheat DNA. 10: 310 pb amplification pAHC25 plasmid purificated 11-13 Control non transformed plant.

Transient transformation

Transient GUS expression in bombarded calli was observed by histochemical β-glucuronidase activity assays (Fig. 3 a-b). In osmotically treated calli (0.4 M sorbitol), GUS expression was 4 to 5 fold higher than in non treated calli (Fig. 3c). Transient expression enhancement generated by the osmotic treatment could be due to the partial plasmolysis of the target cells, because under those conditions cells may be less likely to lose cell sap as a consequence of membrane damage by the penetrating particles (Vain *et al.*, 1993). On the other hand, we also compared the expression of the pAHC25 plasmid with another vector (pGUSNco) which contains the β-glucuronidase gene driven by the CaMV 35S promoter. Using the first construction, GUS expression was much higher than with the last one (data not shown). These results are in agreement with other reports, which show that the Ubi-1 promoter is more active than 35S in monocots (Christensen and Quail, 1996).

Transgenic plants

After bombardment, the calli were maintained under osmotic treatment (16 h) and then transferred to the regeneration medium. Selective conditions (as described in Materials and Methods) were imposed 48 h after transformation and the cultures were maintained under those conditions for 2 to 4 months. During the first two weeks, all calli became yellow-green and meristematic zones were seen as green spots (4-5 per callus) and most of them had incipient leaves (2-4mm). During the following two to four weeks, the embryogenic capabilities of explants declined rapidly, the leaves and shoots became chlorotic and died. At the end of the first month of culture under selective conditions, it was possible to rescue 10-13% of initial plantlets. The survival plantlets (between 2-5 cm in length) were transferred to individual culture tubes and screened for the presence of marker BAR gene sequence by PCR analysis. As expected, the

number of lines that survived initially in Basta, was higher than the number of plantlets with positive PCR for the BAR gene. We found that 64-70% of surviving plantlets had positive PCR reactions, that represented 9% of the initial plantlets. These results indicate that even though selection pressure was very strong, around 30% of Basta-resistant plant were escape events (Fig. 4). In the following weeks, 55% of PCR positive plantlets died, even when subcultured in fresh medium, suggesting that in these plantlets BAR expression was only transient. Other authors eliminate the selection agent from the second month of culture (Ortiz *et al*., 1996) or use lower concentrations at the beginning of the selection step (Rasco-Gaunt *et al*., 2001). These strategies can enhance the percentage of recovered plants, but also enhance the escape events (Altpeter *et al*., 1996). Four months after bombardment, 1.3% of the initial plants were still living under selective conditions. Survival plants were transferred to pots with sterile vermiculite and grown in the greenhouse under the same conditions of donor plants. The survival percentage obtained was similar to other reports (Altpeter *et al.*, 1996; Ortiz *et al.*, 1996).

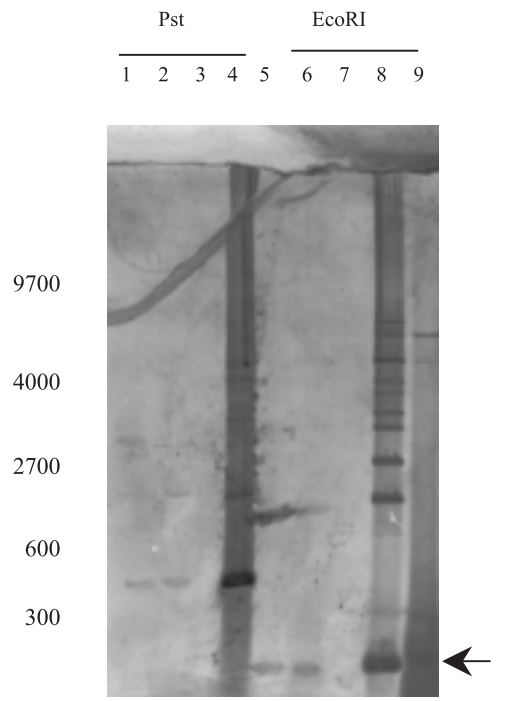

FIGURE 5. Southern hybridization of BAR probe to DNA from regenerants of wheat cv Oasis transformed with pAHC25. 1-4 DNA digested with PstI: 1, 2 transformed lines, 3 negative control, 4 pAHC25 plasmid. 5-8 DNA digested with EcoRI: 5,6 transformed lines, 7 negative control 8 pAHC25 plasmid. 9 hybridization control (311 bp BAR gene amplified by PCR , arrow indicated).

Plant sowing
(~ 90d)

Anthesis
(~ 18d)

Callus culture (dark)
(15-20d)

Prebombardment tratment
(4 h 0.4M sorbitol)

Postbombardment tratment
(16h 0.4Msorbitol)

Shoot formation (light)
(2d) Without selection

Shoot formation (light)
Under selection (10 mg l-1 Basta)
(2-4 weeks)

Shoot elongation
Selection in tubes whitout hormones
(2-4 weeks)

Plantlet growth
Selection in Magenta boxes
(4-6 weeks)

Transfer of plants to pots with
vermiculite

FIGURE 6. Summary of the protocol for the production of transgenic wheat (cv Oasis) plants.

In order to determine if the BAR gene was stably integrated in the wheat genome Southern blot analysis were performed. DNA isolated from leaf tissue of Basta resistant plants were digested with EcoRI and PstI restriction enzymes, separated in 0.8% agarosa gel, transferred to nylon membrane and hybridized with a digoxigenine labelled probe for the BAR gene. As control, we used the pAHC25 plasmid digested with Eco RI and PstI enzimes and the 311 pb PCR product from the pAHC25 BAR gene. Eco RI cut the Ubi-1 intron and an internal BAR site and PstI released BAR and GUS plus Nos cassettes (Fig. 1). In plant DNA digested with EcoRI, we observed two BAR hybridization bands,

one of them of 2,700 bp fragment, and a smaller one of around 300 bp. Plasmid pAHC25 digested with EcoRI and hybridized with the BAR probe also showed the 2,700 and 300 pb bands, the other bigger bands observed could represent partially digested plasmid. Plant DNA digested with PstI exhibited a hybridization band of equal size to that released from the pAHC25 plasmid that corresponded with the BAR gene size. DNA from non transformed plants did not show any hybridization signals (Fig. 5).

Even though, there are previous reports of wheat transformation, the mean efficiency obtained in the present study for Oasis, an important commercial cultivar, was higher or similar to those previously reported for "model" genotypes as Bobwhite (Weeks *et al*., 1993) and Florida (Altpeter *et al*., 1996). The efficiency and the short time frame from regenerative calli culture until the recovery of a transgenic plants (Fig. 6), indicate that this protocol could be used as an adequate strategy to obtain genetically modified wheat plants.

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