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TRANSFORMATION OF AN ARGENTINE SPRING WHEAT GENOTYPE: OPTIMIZATION OF THE PROTOCOLS FOR PARTICLE BOMBARDMENT OF EXCISED IMMATURE EMBRYOS AND RAPID ISOLATION OF TRANSGENIC PLANTS

TRANSFORMACIÓN DE UN GENOTIPO ARGENTINO DE TRIGO PRIMAVERAL: OPTIMIZACIÓN DE LOS PROTOCOLOS PARA EL BOMBARDEO DE PARTÍCULAS DE EMBRIONES INMADUROS EXTRAÍDOS Y AISLAMIENTO RÁPIDO DE PLANTAS TRANSGÉNICAS

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ABSTRACT

Klein Brujo was found to be a promising Argentine spring wheat genotype for transformation studies. In the present work we optimized the biolistic transformation of embryogenic scutellar calli from this genotype. We first identified scutellar callus induction media (SCIM) most promising for *in vitro* embryogenic plant regeneration with *Klein Brujo*. We then co-bombarded embryos of *Klein Brujo* and, for comparison, of *Bobwhite*, a highly transformable wheat line, on these media with 1 µm gold particles coated with two plasmids. One of these contained the marker gene *gfp* (linked to the *CaMV35S*-promoter) and the selection gene *bar* (resistance to phosphinothricin: PPT, linked to the maize *Ubi1*-promoter), whereas the other contained *ipt* (encoding isopentenyltransferase) as a candidate gene under the control of the *HvS40*- or *SAG12*- promoter. Transformation efficiencies of up to 16.4% with *Klein Brujo* and 6% with *Bobwhite* were obtained with embryos pre-cultured on SCIM for 96 h and subjected to pre- and post-bombardment osmotic treatment for 4-5 and 16 h, respectively. Transgenic calli and plants regenerating *in vitro* were identified by screening for GFP expression and PPT resistance. One hundred and three transgenic lines of *Klein Brujo* - far more than of *Bobwhite* (12 lines) - were established on soil, often within only nine weeks. The frequency of co-transformation of *gfp* and/or *bar* and *ipt* exceeded 97% for both genotypes, and the three genes were shown to co-segregate with selected individuals. The *ipt*-gene was structurally stable up to the T3 generation, whereas *gfp* and *bar* were susceptible to silencing.

Key words: Particle bombardment parameters; co-transformation; green fluorescent protein; phosphinothricin resistance; transgene segregation.

RESUMEN

Klein Brujo es un genotipo argentino de trigo de primavera prometedor para estudios de transformación. En el presente trabajo se optimizó la transformación biolística de callos embriogénicos escutelares de este genotipo. Primero se identificaron los medios de inducción de callo escutelar (SCIM) más promisorios para la regeneración embriogénica *in vitro* de *Klein Brujo*. Luego, se co-bombardearon embriones de *Klein Brujo* y de *Bobwhite*, una línea altamente transformable, en medios seleccionados con partículas de oro (1 µm) recubiertas de dos plásmidos. Un plásmido contuvo dos genes, el marcador *gfp* (vinculado a *CaMV35S*) y el de selección *bar* (resistencia a fosfinotricina: PPT, vinculado a *Ubi1*) y el segundo al gen *ipt* (isopenteniltransferasa), controlado por el promotor *HvS40* o *SAG12*. Eficacias de transformación de hasta 16% con *Klein Brujo* y 6% con *Bobwhite,* se obtuvieron con embriones pre-cultivados en SCIM por 96 h y sujetos a tratamiento osmótico pre- (4-5 h) y post-bombardeo (16 h). Los callos transgénicos y las plantas regeneradas *in vitro* se identificaron por la detección de la expresión de GFP y la resistencia a PPT. Se transplantaron a suelo, 103 líneas transgénicas de *Klein Brujo* –mucho más que de *Bobwhite* (12 líneas- con frecuencia en un lapso de sólo nueve semanas. La frecuencia de co-transformación de *gfp* y/o *bar* e *ipt* superó el 97% para ambos genotipos, y los tres genes co-segregaron en individuos seleccionados. El gen *ipt* fue estructuralmente estable hasta en la generación T_3, mientras que *gfp* y *bar* fueron susceptibles al silenciamiento.

Palabras clave: Parámetros de bombardeo de partículas; co-transformación; proteína verde fluorescente; resistencia a fosfinotricina; segregación del transgén.

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INTRODUCTION

Wheat is considered to be the world's most important crop, but it has lagged behind the other major cereal crops in terms of the development of efficient transformation methods (Harwood, 2011). The widely employed method of *Agrobacterium*-mediated gene transfer is useful only with particular cultivars of wheat, whereas biolistic transformation is suitable for a wide range of wheat genotypes (Sparks and Jones, 2009). A more robust technology for the generation of transgenic wheat is required for routine application to commercial cultivars that exhibit different genetic backgrounds of locally adapted genotypes (Bhalla, 2006). Breeding programs, for instance, require large numbers of transformed lines to select those showing stable integration and a high level of expression of the transgene. This requirement can be met either by increasing the efficiency of the gene transfer itself or by shortening the time required for the regeneration of transgenic plants (Altpeter *et al.*, 1996). The efficiency with which wheat can be transformed biolistically depends on the genotype (Takumi and Shimada, 1996; Iser *et al.*, 1999; Lazzeri and Jones, 2009) and on a number of other factors that are still poorly understood (Klein and Jones, 1999). These factors can be categorized into biological and physical parameters. The biological parameters are those associated with the target tissue, which is generally the scutellum of immature embryos, and with the medium employed in the *in vitro* culture of the bombarded embryos (Sahrawat *et al.*, 2003). The physical, or ballistic, parameters comprise the type, size and density of the bombardment particle, together with the propellant force and distance to the target that determine the impact intensity of the particle. Efficient transformation involves balancing the point at which maximum possible DNA introduction with minimum cell death resulting from the bombardment (Klein and Jones, 1999). The various biological and physical factors interact, making their empirical optimization difficult. Particular studies have generally focused on optimizing only one parameter, *e.g.*, acceleration pressure (Becker *et al.*, 1994), pre-culture prior to bombardment (Takumi and Shimada, 1996), type and concentration of auxin in the culture medium (Barro *et al.*, 1998) and exposure to pre- or post-bombardment osmotic treatment (Gil-Humanes *et al.*, 2011). Less frequently, combinations of two parameters, *e.g.*, distance to the target tissue and acceleration pressure (Iser *et al.*, 1999), particle size and distance to the target tissue (Jordan, 2000) and particle size and acceleration pressure (Pellegrineschi *et al.*, 2000) have been analyzed. Variations of different parameters of a standard transformation procedure have also been assessed independently with regard to transient or stable expression and the optimized individual parameter values combined in new transformation protocols (Rasco-Gaunt *et al.*, 1999; 2001; Yao *et al.*, 2007).

The methodology employed for selecting transgenic plants is a key factor in wheat transformation (Fadeev *et al.*, 2006). Efficient, cost-effective transformation systems must both accurately select transgenic plants and prevent non-transgenic plants from "escaping" detection, as identification of the latter entails time-consuming additional analysis (Harwood, 2011). The genes *uidA* (encoding β-glucuronidase) and *bar* (encoding phosphinothricin acetyl transferase, which confers resistance to phosphinothricin: PPT, the active ingredient of the herbicide BASTA) are frequently introduced together into target tissue for selection purposes. A major drawback in the use of this combination is, however, the incidence of numerous escape plants, coupled with the lethal nature of the assay for the product of the scorable *uidA*-gene (Sahrawat *et al.*, 2003). The non-destructive reporter GFP (green fluorescent protein, encoded by the *gfp*-gene) has also been successfully used in wheat transformation (Jordan, 2000; Huber *et al.*, 2002; Fadeev *et al.*, 2006). Transgenic wheat plants have been selected on the basis of GFP expression alone, although the inclusion of an antibiotic resistance gene improved the efficiency of the selection (Jordan, 2000). According to Jordan (2000), transformation with *gfp* and a selection gene enables GFP-expressing plants to be recovered from large masses of shoots and minimizes the likelihood of non-transgenic escape plants avoiding detection. The selection agent generally employed is PPT, which is applied to the transformation target either immediately after bombardment or during callus formation (Altpeter *et al.*, 1996; Becker *et al.*, 1994; Huber *et al.*, 2002) or culture in regeneration medium (Jordan, 2000; Rasco-Gaunt *et al.*, 2001; Pellegrineschi *et al.*, 2002; Gil-Humanes *et al.*, 2011). Efforts have also focused on reducing the time required for the establishment of self-sufficient transgenic plants on soil (Altpeter *et al.*, 1996).

The aim of the present investigation was to optimize the biolistic transformation of immature embryos of

the Argentine spring wheat genotype *Klein Brujo* and the selection and growth of transgenic plants from the bombarded embryos. We had previously found *Klein Brujo* to be the most promising of 22 Argentine spring wheat varieties for transformation studies based on embryogenic scutellar callus production and subsequent plant regeneration (Souza Canada and Beck, 2013). We first identified scutellar callus induction media best suited to the formation of embryogenic callus from immature *Klein Brujo* embryos and the regeneration of plants from it. These media were then used to ascertain the combinations of biological and physical parameter characteristics most amenable to the biolistic co-transformation of the immature embryos with the *gfp*- and *bar*-genes, as well as with *ipt*, which encodes isopentenyltransferase, as a candidate gene (manuscript in preparation). Transformation efficiency and transgenic plant production with *Klein Brujo* were compared with those of the wheat genotype *Bobwhite* SH 9826, which had been found to respond particularly well to biolistic transformation (Pellegrineschi *et al.*, 2002). These investigations produced 103 independent primary transgenic lines of *Klein Brujo* and 12 of *Bobwhite*, which we characterized for the transgene presence and inheritance.

MATERIALS AND METHODS

Plant material

Seeds of the spring wheat cultivars *Klein Brujo* (Souza Canada and Beck, 2013) and *Bobwhite* SH 98 26 (Pellegrineschi *et al.*, 2002) were obtained from Criadero Klein S.A. (Alberti, Buenos Aires province, Argentina) and the International Maize and Wheat Improvement Center (CIMMYT: El Batan, Mexico), respectively. Donor plants were grown from these seeds throughout the year in the greenhouse as described by Souza Canada and Beck (2013).

In vitro culture

Immature zygotic embryos from suitably developed seeds were excised as described by Souza Canada and Beck (2013) and placed on solidified sterilized callus induction medium. The embryos were approximately 1.5 mm in length, with an approximately 1.3 mm long, transparent scutellum; they were at the developmental stage H specified by Souza Canada and Beck (2013), which corresponds to the developmental stage II described by He *et al.* (1986) and the early to middle milking stages 73-76 of the Zadoks´ scale (Zadoks *et al.*, 1974).

Two basic types of scutellar callus induction medium (SCIM) were employed to induce and maintain callus growth of the excised immature embryos. One was ML3 basal medium (Viertel and Hess, 1996), containing 3 % maltose (w/v) and solidified with 0.2 % Gelrite. The second was MSD basal medium (Barcelo and Lazzeri, 1995) with the following modifications: 118 µM $MnSO_4{\cdot}H_2O$, 30 µM $ZnSO_4{\cdot}7\ H_2O$ and 102 µM H_3BO_3, in addition to the double original content of amino acids and maltose instead of sucrose. The maltose content of this medium (termed "MB" in the following), which was solidified with 0.8 % (w/v) agar, was either 3 % (medium "MB3") or 9 % (w/v) (medium "MB9"). The supplementation of ML3, MB3 and MB9 with 1 or 2 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) resulted in six SCIMs for testing (ML3-1 and -2, MB3-1 and -2, MB9-1 and -2).

Five replicates of 25 excised embryos were incubated on solidified callus induction medium in Petri dishes for each individual experiment. After three weeks in darkness at 25° C, callus induction and cell proliferation on the scutellum surface and precocious germination were assessed for each embryo under a stereomicroscope. Embryos with compact, nodular embryogenic calli were transferred to Petri dishes with modified MSB medium and cultivated on this for three weeks for plant regeneration, whereupon the regenerative capacity of the callus was evaluated and the number of plantlets regenerating from it was determined. Regenerating plantlets were transferred directly to autoclaved substrate (70 % compost and 30 % clay) when they possessed a well-developed root system. If the root system was underdeveloped, the plantlets were first cultivated for 2-3 weeks on rooting medium 190-2 (Zhuang *et al.*, 1984). After acclimation to the substrate in a culture room, the plantlets were transferred to a greenhouse for *ex vitro* culture. These procedures are described in detail in Souza Canada and Beck (2013).

DNA plasmid vectors and particle bombardment

The plasmid employed in all experiments was the dual expression vector pGFPBAR (Huber *et al.*, 2002). This contains the *bar*-gene (Thompson *et al.*, 1987) under the control of the maize ubiquitin-promoter and intron-1-complex (*Ubi1*) (Christensen *et al.*, 1992), as well as a chimeric S65T-p*gfp*-intron gene (Pang *et al.*, 1996)

driven by the cauliflower mosaic virus *CaMV35S*-promoter (Odell *et al.*, 1985). It was delivered in co-transformation experiments at an equimolar ratio of 1:1 with one of two different plasmids containing the *ipt*-gene. One of these plasmids was pSG516 (Gan and Amasino, 1995), in which the *ipt*-gene (Li *et al.*, 1992) was linked to the *SAG12*-promoter. The second, pS40-IPT, was constructed from pSG516 and pS40-GUS (kindly donated by Prof. Dr. K. Krupinska, Christian-Albrechts-Universität, Kiel, Germany, containing the *uidA*-gene together with the *HvS40*-promoter) by digesting both with *PaeI* and *NcoI* to remove the inducible *SAG12* and *HvS40* promoters. The *HvS40*-promoter was then coupled to the *ipt*-gene of pSG516 to form the new plasmid pS40-IPT (Souza Canada, 2012). Schematic representations of the three plasmid vectors are shown in Figure 1.

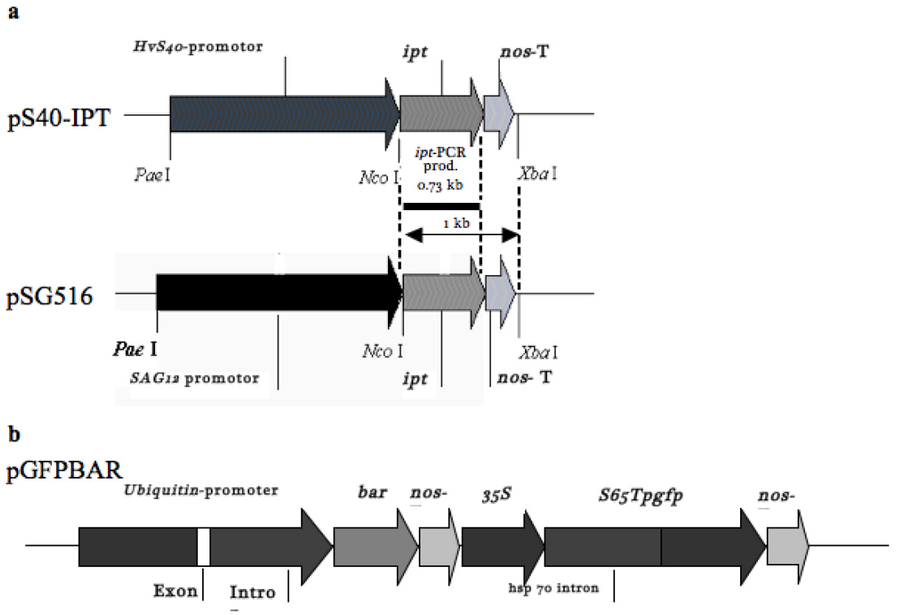

Figure 1. Plasmids used for wheat transformation. a: pS40-IPT (5.7 kb) and pSG516 (5.9 kb) each containing the *ipt* gene (0.7 kb) under the control of the *HvS40* (2 kb) and *SAG12* (2.1 kb) promoters, respectively, and flanked downstream by the 0.3 kb nopaline synthase 3´-terminator sequence (*nos*-T). The PCR-product obtained with *ipt*-primers is designated by a bold bar, and the fragment released by *NcoI/XbaI* digestion is indicated by a double arrow. b: pGFPBAR (8.8 kb). The *CaMV* 35S (35S) promoter governs the *gfp* complex containing the maize heat-shock protein 70 (*hsp* 70) intron and the synthetic *gfp*-gene containing the potato ST-LS1-intron (*S65Tpgfp*). The *bar*-gene was under control of the maize *ubiquitin* promoter followed by exon-1 and intron-1 of the maize *ubiquitin* gene.

Plasmid DNA (5 µg in each case) was precipitated onto a 3 mg suspension of gold particles and the DNA-gold suspension (6 µl) was loaded onto the center of the particle gun macrocarrier as described by Iser *et al.* (1999), whereby the precipitation mixture was finger-vortexed for 6 min instead of mechanically vortexed for 3 min, and then incubated at room temperature for 20 min followed by a brief centrifugation. For each transformation experiment 4-5 replicates of 30-35 immature wheat embryos were arranged in the centers of 90 mm Petri dishes with SCIM within rings of 2.4 cm diameter. They were bombarded with the DNA-coated gold particles using the PDS 1000/He particle gun (BioRad, Germany).

Embryo bombardments were carried out at various settings of a number of variable culture-relevant (termed "biological") and ballistic, or "physical" parameters (Table 1). The basic biological parameter was the particular SCIM on which the embryos were cultured. The excised embryos were pre-cultured on this medium for up to 120 h prior to bombardment and, were in most cases also incubated on their respective SCIM supplemented with 0.5 M mannitol for several hours prior to and subsequent to the bombardment. They were then transferred to fresh SCIM without mannitol. The physical parameters varied with regard to the size of the gold particles, the distance from the microcarrier launch assembly to the target tissue and the acceleration pressure. The actual parameter variant combinations employed in the transformation experiments are specified in the respective sections of the Results.

Table 1. Biological and physical parameters examined in the biolistic transformation of excised wheat embryos; SCIM: Scutellar callus induction medium

Parameter	Variants employed
Biological	
Scutellar callus induction medium (SCIM)	ML3-1, MB3-1, MB9-1
Osmotic treatment- 4-5 h pre-bombardment/16 h post-bombardment	SCIM with or without 0.5 M mannitol
Pre-culture on SCIM prior to bombardment (h)	0, 20, 72, 96, 120
Physical	
Gold particle diameter (µm)	0.6, 1.0
Target distance (cm)	6, 9, 12
Acceleration pressure (psi)	900, 1,350

Selection of transgenic plants

The transformation protocol used is outlined in Figure 2. GFP expression was monitored from 48 h after the bombardment onwards using a MZFLIII fluorescence stereomicroscope (Leica, Germany) with an excitation filter BP 490-550 nm (Leica) and documented with an AxioXam CCD-camera and corresponding computer program (Zeiss, Germany). Scutellar calli growing on SCIM were scored for GFP-expression 10-12 days after bombardment. All viable calli present at the end of the three-week embryogenic scutellar callus formation phase, and any shoots having already developed from them, were divided into three groups. Group A was made up of calli and shoots exhibiting stable GFP expression. Group B included calli and shoots that had initially exhibited GFP expression, but had lost it by the end of the callus formation phase. Group C was composed of calli and shoots that had never exhibited any GFP fluorescence. Calli and shoots of groups A and B were cultivated for shoot regeneration on modified MSB medium (Souza Canada and Beck, 2013) in Petri dishes for three weeks without PPT (round R in Figure 2). Those of group C were cultivated under the same conditions, but with 4 mg/ml PPT in the medium (round P in Figure 2). Calli and shoots or plantlets (shoots in the process of developing roots) were then transferred to fresh shoot regeneration medium in test tubes and cultured for another three weeks. No PPT was included in the medium for shoots and plantlets from group A that still exhibited GFP fluorescence (round S in Figure 2). The medium for the remaining shoots and plantlets from group A, which no longer exhibited GFP fluorescence, as well as for all of the GFP-negative shoots and plantlets from groups B and C was supplemented with 4 mg/l of PPT (rounds T in Figure 2). Plantlets possessing a well-developed root system at the end of the shoot regeneration phase were transferred directly to autoclaved substrate (see above) and transferred to

a greenhouse. Shoots not possessing well-developed roots were first cultivated for 2-3 weeks on rooting medium 190-2 (Zhuang *et al.*, 1984) before being transferred to the substrate. In these cases the medium for GFP-expressing group A plantlets was free from PPT (round V in Figure 2), whereas it contained 4 mg PPT/l for all GFP-negative plantlets (rounds E in Figure 2).

After a few weeks of *ex vitro* culture, a small disk of filter paper imbibed with a 3 mM PPT solution was attached to two or three leaves of the putatively transgenic plants and to non-transformed control plants with a paper clip. This treatment was performed three times with an interval of one week between the treatments. This test confirmed transgenic PPT resistance in the plants when no discoloration of the treated leaf area occurred. The "transformation efficiency" of a particular experiment was defined as the number of identified transgenic plants expressed as percent of the total number of bombarded embryos.

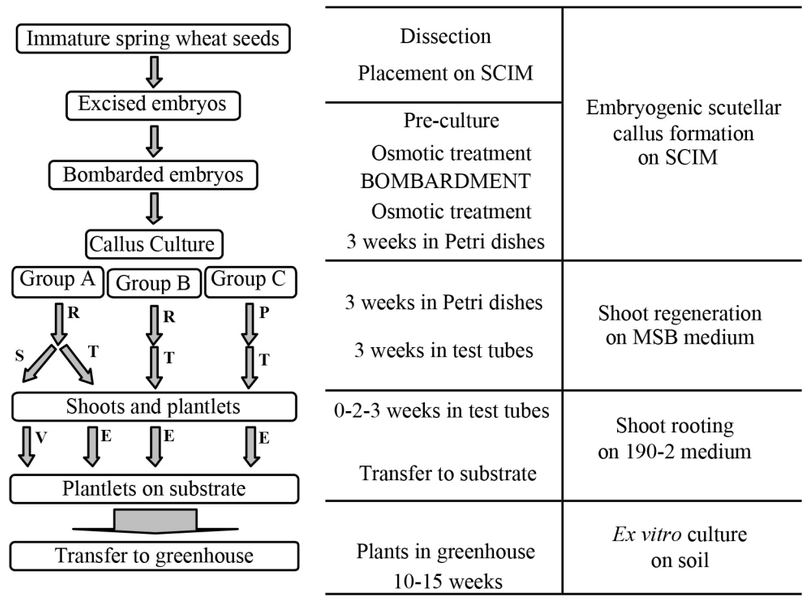

Figure 2. Production of transgenic *Klein Brujo* and *Bobwhite* spring wheat plants. Group A refers to calli and shoots expressing GFP at the end of the scutellar callus formation phase, whereas those of group B no longer showed initially present *gfp* expression and those of group C had never shown any evidence of *gfp* expression. The selection rounds designated by R, S and V were carried out in the absence of PPT, whereas in those indicated by P, T and E the medium contained 4 mg/l PPT. *In vitro* culture was from 63 up to 84 days, depending on the length of the shoot rooting phase.

Transgene analysis

Genomic DNA was extracted from young leaves according to Souza Canada (2007). PCR was carried out at least three times with each extract to determine the presence of the *ipt*-gene in plants exhibiting GFP expression and/or PPT resistance. Controls were performed with 0.2 ng of the plasmids containing this gene. PCR was run in a reaction volume of 30 µl containing 10-50 ng wheat genomic DNA, 3 µl 10x Long PCR buffer (Sambrook and Russell, 2001), 2.5 mM $MgCl_2$, 160 µM of each of the 4 dNTPs, 0.5 µM of the two *ipt*-specific primers and 1 U Taq DNA polymerase (Fermentas, Germany). The *ipt*-gene was amplified by using 5'-ACC CAT GGA CCT GCA TCT A-3' as the forward primer and 5'-GGA GCT CAG GGC TGG CGT AAC C-3' as the reverse primer at an annealing temperature 63° C. DNA was denatured at 95° C for 4 min, followed by amplification cycles of 1 min at 95° C, annealing for 1 min and heating at 72° C for 1 min. After 30 cycles the PCR product (730 bp) was separated by electrophoresis in 0.8 % w/v agarose gels, stained with ethidium bromide and visualized under UV light. Transgene integration was confirmed by *Southern* blot analysis. Genomic DNA (25-30 µg) was digested overnight at 37° C with 0.32 U/µg of each of the restriction enzymes *NcoI* and *XbaI*. For positive controls 10 ng of the *ipt*-bearing plasmids were digested; negative controls were performed with DNA of non-transformed plants. Digested genomic DNA was fractionated by electrophoresis in 0.8 % (w/v) agarose gels, blotted onto a positively charged nylon membrane (B/Plus, Biodyne, Germany) and hybridized with a biotin-11-dUTP-labelled probe at 52° C overnight in hybridization buffer. Non-radioactive labeling of the PCR products was performed with a Fermentas Biotin DecaLabel DNA Labeling Kit according to the manufacturer's instructions. Labelling was detected with streptavidin-alkaline phosphatase conjugate (Streptavidin AP, Novagen, Germany), using the DIG Luminescence Detection Kit from Roche (Germany). Phosphatase activity was visualized by exposure to X-OMAT AR film (Kodak, Germany) for 20 min up to five days, depending on the signal strength.

Transgene inheritance

The inheritance of the transgenes was analyzed by testing for the presence of *ipt* by PCR and for the expression of *gfp* and *bar* by monitoring GFP fluorescence and resistance to PPT in T_1, T_2 and T_3 progeny. Segregation ratios were calculated from these data. T_1 progenies were obtained by selfing T_0 transgenics, excising immature embryos from them at the growth stage 75 according to Zadoks *et al.* (1974), culturing the embryos for five days on WH medium (Wagner and Hess, 1973) supplemented with 3 % (w/v) sucrose and 0.1 mg/l indole acetic acid and solidified with 0.8 % agar and subsequently transferring them to substrate for *ex vitro* culture (see above). The T_2 and T_3 progenies were obtained in an analogous manner.

Statistical analysis

Data were evaluated by the X^2 test of independence (contingency table analysis), variance analysis and calculation of standard deviation. If necessary, the *post hoc* test was carried out between the samples from the six culture conditions, as follows: among the same medium containing different concentrations of 2,4-D and among different media containing the same concentration of 2,4-D. Subsequently, the significance calculated was compared with the manually corrected value (Bonferroni-Correction according to Holm). Segregation of transgene expression in sexual progenies was analyzed by the χ^2 test for statistical deviation from the Mendelian ratio for single locus integration. The statistical software package SPSS 13.0 (SPSS 2004) was used for all analyses.

RESULTS

In vitro culture of immature Klein Brujo embryos

The scutella of embryos excised from immature seeds of the spring wheat cultivar *Klein Brujo* produced both friable, watery and translucent non-embryogenic callus and compact, nodular embryogenic callus on each of the six SCIMs. The embryogenic callus formed on ML3 medium was white to pale yellow (Figure 3a), whereas it was bright yellow on MB3 medium (Figure 3b) and darker yellow on MB9 medium (Figure 3c). The *in vitro* culture performance of the embryos on the six media is documented in Table 2.

The percentages of embryos exhibiting embryogenic scutellar callus formation differed significantly after three weeks of incubation on the six SCIMs (Chi-square-test X^2= 151.2; df= 5; p<0.001). They ranged from 42.2 % on the medium MB3-2 to 98.6 % on MB3-1 (Table 2). The highest rates of callus formation were found on the ML3-, MB3- and MB9- media containing 1 mg/l 2,4-D (ML3-1, MB3-1 and MB9-1). The rate on ML3-2 with 2 mg/l 2,4-D was not significantly different to that on ML3-1, but the rates on MB3-2 and MB9-2 with 2 mg/l of the auxin were significantly lower than those on the corresponding MB-media with the lower hormone concentration (MB3-1 and MB9-1). The extent to which embryogenic callus proliferated on the scutellum also differed significantly with the type of SCIM (Chi-square-test X^2= 54.4; df= 5; p<0.001). The percentage of the scutellum surface covered by embryogenic callus after three weeks of culture ranged from 59.5 % on the medium MB9-1 to 95.7 % on MB3-1 (Table 2).

Immature embryos germinated precociously to significantly different extents during the course of three weeks on all of the six callus induction media (Chi-square-test X^2= 26.9; df= 5; p<0.001). The frequency of precocious germination ranged from 0.61 % on ML3-1 to 10.9 % on MB9-2 (Table 2). However, the respective differences were not always significant and could not be related to different 2,4-D or maltose concentrations.

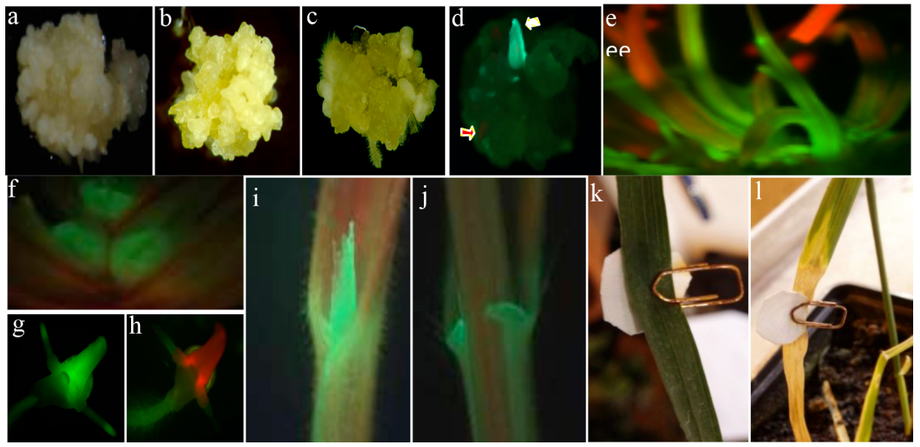

Figure 3. Callus formed on excised immature cv. *Klein Brujo* wheat embryos and transgenic structures and plants developed from callus produced by embryos bombarded with pGFPBAR and pS4o-IPT. a, b and c: Embryogenic calli covering the scutellum surface after three weeks of incubation of the embryos on ML3-1, MB3-1 and MB9-1 medium, respectively. d-j: Expression of the *gfp*-gene in tissues and shoots; d: shoots showing and not showing GFP-expression as indicated by white and red arrows, respectively, three weeks after bombardment, e: plantlets developing on *in vitro* regeneration medium, f: transgenic grains, g and h: immature embryos of transgene (g) and azygous (h) T1 progenies, i: transition from leaf sheaths to the blade wrapping a young leaf, j: auricles and stem. k and l: Sections of mature leaves of T1 wheat plants one week after the last of three applications of 3 mM PPT on a filter paper disc attached to the leaf by a paper clip; k: no leaf discoloration on a homozygous leaf (resistant to PPT), l: leaf yellowing progressing from the paper disk on an azygous leaf (lacking resistance to PPT).

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Table 2. *In vitro* culture performance of excised immature embryos of *Klein Brujo* on six scutellar callus induction media

	SCIM					
	ML3-1	ML3-2	MB3-1	MB3-2	MB9-1	MB9-2
Scutellar callus formation (% of excised embryos) [3]	84.8 ± 12.1^{af}	72.5 ± 12.6^{aj}	98.6 ± 4.3^{bgh}	42.2 ± 51.3^{ckl}	87.7 ± 6.7^{dfi}	54.3 ± 16.5^{ejl}
Callus proliferation on the scutellum surface (% cover)[4]	77.0 ± 18.1^{ae}	71.2 ± 6.4^{ai}	95.7 ± 44.3^{bfg}	61.6 ± 14.9^{cij}	59.5 ± 7.1^{deh}	64.5 ± 10.6^{dij}
Precocious germination (% of embryos)[5]	0.61 ± 2.2^{ad}	2.5 ± 3.3^{ae}	5.5 ± 17.1^{bd}	2.0 ± 5.4^{beg}	2.5 ± 4.4^{cd}	10.9 ± 8.9^{cfg}
Plant regeneration capacity (%)[1, 6]	100^{af}	76.3 ± 22.7^{bj}	100^{cfh}	72.1 ± 7.1^{djl}	55.7 ± 24.5^{egi}	51.3 ± 2.1^{ekl}
Number of regenerated plants per embryo [7]	9.5 ± 6.4^{ae}	5.6 ± 3.5^{bh}	4.5 ± 3.4^{cfg}	3.9 ± 2.5^{chj}	4.4 ± 2.5^{dfg}	2.9 ± 2.1^{dij}
Culture efficiency (%)[2,8]	84.8 ± 6.0^{ag}	55.3 ± 17.5^{bk}	98.6 ± 2.1^{chi}	30.4 ± 29.1^{dlm}	48.8 ± 15.6^{ehj}	27.8 ± 9.3^{flm}

[1] Percentage of callus-forming embryos producing regenerants
[2] Percentage of cultured embryos producing regenerative calli (nodular callus exhibiting green spots)
[3, 4, 5, 6, 7, 8] Post hoc tests (pairwise comparison): Means followed by the same letter are not significantly different at the 5% level

Plantlet regeneration

Embryogenic calli from immature embryo scutella were transferred to shoot regeneration medium. Their ability to regenerate shoots and plantlets (shoots with developing roots) during a further 3 weeks of culture (the "plant regeneration capacity" in Table 2) differed significantly with the medium having been used to induce callus formation (Chi-square-test X^2= 98.1; df= 5; p<0.001). The frequency of plant regeneration ranged from 51.3 % for calli formed on MB9-2 to the maximum possible value of 100 % for calli formed on each of ML3-1 and MB3-1, which both contained 3 % maltose and 1 mg/l 2,4-D. The regenerative capacities of calli on ML3- and MB3-media with 2 mg/l 2,4-D (ML3-2 and MB3-2) were significantly lower than with 1 mg/l of the auxin, and those on the two MB- media containing 9 % maltose (MB9-1 and MB9-2) were the lowest observed, irrespective of the 2,4-D concentration.

The number of plantlets that regenerated from each cultured embryo after 3 weeks on shoot regeneration medium also differed appreciably according to the callus induction medium on which the calli had originally formed (Kruskal-Wallis-test X^2= 48.4; df= 5; p<0.001), ranging from 2.9 on MB9-2 to 9.5 on ML3-1 (Table 2). The highest numbers (9.5 and 5.6 plantlets) corresponded to calli formed on the two ML-media, and these numbers were significantly higher than for the MB-media. The 2,4-D concentration of 1 rather than 2 mg/l in the SCIM led to more plantlets regenerated per embryo only for calli formed on ML3 medium. The number of plantlets regenerated upon callus formation on MB-medium was not significantly influenced by the maltose supply to the medium.

Many of the young plantlets developing from calli possessed well-developed roots following culture on shoot regeneration and rooting media. Random individuals were transferred to soil and cultivated first for 1-2 weeks under *in vitro* culture ambient conditions and thereafter *ex vitro* in a greenhouse. These plants showed normal morphological development and maturation and set viable seeds after approximately three months.

Culture efficiency

The percentage of immature embryos developing regenerative calli (calli from which plantlets regenerated) designates the efficiency of the culture in propagative potential from the embryos. As shown under "culture efficiency" in Table 2, this percentage differed significantly among the six callus induction media (Chi-square-test X^2= 162.4; df= 5; p<0.001). Culture efficiency attained almost the maximum possible value on MB3-1 (98.4 %), and that on ML3-1 (also well over 80 %) was also much higher than on any of the other media. MB medium gave a better result than ML medium at a 2,4-D concentration of 1 mg/l (compare MB3-1 and ML3-1), but ML was better at 2 mg/l of the auxin (compare MB3-2 and ML3-2). Culture efficiency was always significantly better with1 mg/l than at 2 mg/l 2,4-D. A maltose concentration of 3 % in MB medium resulted in significantly better culture efficiency than did 9 % and1 mg/l 2,4-D (compare MB3-1 and MB9-1), but not with 2 mg/l of the auxin (compare MB3-2 and MB9-2).

Choice of medium for biolistic experiments

Overall, media MB3-1 and ML3-1 best induced the formation, and supported the proliferation, of embryogenic scutellar callus with excellent plant regeneration capacity and a high culture efficiency. These two media were accordingly chosen as SCIMs for the biolistic experiments. MB9-1 was also chosen for these experiments despite its considerably poorer culture performance data on account of its greater osmotic strength (due to its maltose concentration of 9 % at the same 2,4-D concentration of 1 mg/l), which had been shown to benefit callus formation on and plant regeneration from bombarded embryos (Rasco-Gaunt *et al.*, 2001).

Transformation by particle bombardment

Immature embryos of the genotype *Klein Brujo* were co-bombarded with the plasmids pGFPBAR and pS40-IPT under combinations of the biological and physical parameter variants listed in Table 1. The embryos were maintained on the SCIM on which they had been bombarded, and scutellar calli, shoots and plantlets formed from them were subsequently cultured on shoot and root regeneration medium and substrate as outlined in Figure 2. Calli and plants regenerated from them were assessed for successful transformation by testing for GFP fluorescence and resistance to PPT. Transformants were obtained only when the embryos were pre-cultured on SCIM for at least 20 h and subjected to pre- and post-bombardment osmotic treatment with 0.5 M mannitol. Using ML3-1 medium transgenic plants were obtained only under a single combination of bombardment parameter settings; pre-culture for 72 h bombardment with 1.0 µm gold particles, acceleration pressure of 1350 psi and a target distance of 9 cm resulted in a culture efficiency of 19.2 % and an overall transformation efficiency of 1.9 % (best value for an individual bombardment 4.5 %). In contrast, a variety of combinations of bombardment parameters led to the production of transgenic plants for embryos cultivated on MB3-1 or MB9-1 medium. Culture efficiencies ranged between 10.8 % and 69.8 % on MB3-1 and between 29.3 % and 67.7 % on MB9-1. Transformation efficiencies ranged up to 10 % (best value for an individual bombardment: 16.4 %) for embryos cultivated on MB3-1 and up to 4.8 % (best individual value: 11.9 %) for those on MB9-1. The highest culture and transformation efficiencies were obtained with the same combination of physical bombardment parameters on both MB3-1 and MB9-1. In general, increasing periods of pre-incubation led to progressively higher culture and transformation efficiencies, with the best results being obtained after 96 or 120 h of pre-culture. Transgenic plants were obtained for 11 of the 12 physical bombardment parameter combinations when MB3-1 was used as the SCIM (96 h of pre-culture; Table 3), but only for five of the 12 combinations tested with MB9-1 (not shown). Both the highest overall and the best individual bombardment efficiencies using MB3-1 as the SCIM were obtained with 1.0 µm gold particles delivered at an acceleration pressure of 1350 psi and a target distance of 9 cm (Table 3). The best efficiencies on MB9-1 were also observed for the 1.0 µm gold particle size and 1350 psi acceleration pressure, but at a target distance of 6 cm following 120 h of pre-culture. Relatively high transformation efficiencies ranging from 4.2 % to 8.6 % were also noted for culture on MB3-1 with the smaller (0.6 µm) gold particles at acceleration pressures of both 900 and 1350 psi and target distances of both 6 and 9 cm (Table 3).

On the basis of these results, excised immature *Bobwhite* embryos were also pre-cultured for 96 h on each of MB3-1 and MB9-1, co-bombarded with pGFPBAR and pSG516 under osmotic treatment with 0.5 M mannitol and combinations of the gold particle sizes, acceleration pressures and target distances listed in Table 1, and cultured further as for *Klein Brujo*. Better culture efficiencies were obtained with the bombarded *Bobwhite* embryos on MB3-1 (1.2-45 %: Table 3) than on MB9-1 (0-32.8 %). Transgenic plants were obtained with four of the

12 combinations of physical bombardment parameters tested with MB3-1 as the SCIM (Table 3), but with only three of the 11 combinations tested with MB9-1. The transformation efficiencies for the successful physical bombardment parameter combinations reached 4.1 % for culture on MB3-1 (Table 3) and 1.6 % respective of MB9-1. As shown in Table 3, both the culture and efficiencies attained with *Bobwhite* were lower than those determined for *Klein Brujo*.

Selection of transgenic plant material

Scutellar calli developing on the bombarded embryos (some already bearing rudimentary shoots; Figure 3d) were assigned to groups A, B or C according to GFP expression at the end of the 3-week scutellar callus formation phase (Figure 2). Further monitoring of GFP fluorescence (in group A) or resistance to PPT led to the isolation of 103 independent primary transgenic lines of *Klein Brujo* and 12 of *Bobwhite*. Eighty-one of the *Klein Brujo* and eight of the *Bobwhite* lines originated from callus culture on MB3-1. The remaining 22 *Klein Brujo* lines stemmed from bombarded embryos cultured on ML3-1 and MB9-1, and the remaining four *Bobwhite* transgenics derived from callus culture on MB9-1. Thirty-two of the 103 transgenic *Klein Brujo* plants derived from group A, 35 from group B and 36 from group C. Five, four and three of the 12 transgenic *Bobwhite* plants stemmed from the groups A, B and C, respectively. Interestingly, 22 of the 103 transgenic *Klein Brujo* plants were first detected as GFP-expressing plantlets emerging from group B and C cultures at the end of either the shoot regeneration or the shoot rooting phase (Figure 2 and 3e), as they had previously been obscured by other tissues. Most of the transgene plants growing *ex vitro* on substrate were phenotypically normal, but the flowers of some spike of some lines were infertile and accordingly showed a reduced grain production.

All but three of the 103 GFP- or PPT- positive *Klein Brujo* plants (Table 3) and all of the 12 transgenic *Bobwhite* plants showed the presence of the *ipt*-gene upon PCR-analysis (Figure 4). This corresponded to a frequency of co-transformation of the *gfp*- and/or *bar*-genes (from pGFPBAR) and the *ipt*-gene (from pS40-IPT or pGS516) of 97.1 % for *Klein Brujo* and 100 % for *Bobwhite*.

In some instances GFP fluorescence observed at 10-12 days after bombardment was lost during the course of scutellar callus formation and plant regeneration. Twenty-seven of the 103 transgenic *Klein Brujo* plants exhibited

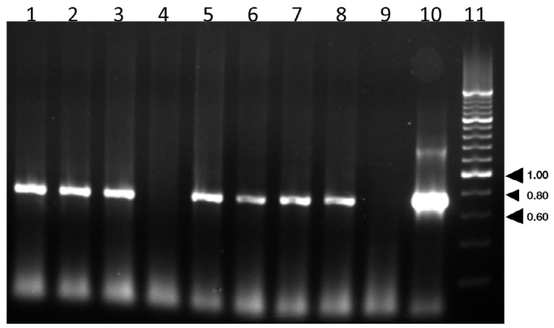

Figure 4. PCR analysis of genomic DNA extracted from control and independent wheat T_o-lines. Amplification of *ipt*-gene was carried out with *ipt*-primers. The *ipt*-PCR product was 730 bp long as shown in Fig 1. Lanes 1-3: cv. *Bobwhite* transformed with pSG516, lane 4: non-transformed cv. *Bobwhite*, lanes 5-8: cv. *Klein Brujo* transformed with pS40-IPT, lane 9: non-transformed cv. *Klein Brujo*, lane 10: plasmid pS40-IPT (positive control), lane 11: O´RangeRuler 200 bp DNA Ladder (Fermentas).

this phenomenon, of which 25 belonged to group B and had thus lost the ability to express GFP during scutellar callus formation. The four group B transgenic *Bobwhite* plants also reflected early loss of GFP expression. The two remaining *Klein Brujo* transgenics were group A plants (Table 3) that had lost *gfp*-gene expression by the end of round R of plantlet regeneration (see Figure 2). Since all surviving *gfp*-negative transgenic plants had tolerated treatment with PPT (see Figure 2), they were considered as stably expressing the *bar*-gene.

Selection on the basis of GFP expression alone without the use of any PPT produced 21 transgenic *Klein Brujo* and three transgenic *Bobwhite* plants after only 9 nine instead of the usual 15 weeks of *in vitro* culture. This rapid regeneration was possible because the transgenic shoots had a well-developed root system at the end of the shoot regeneration phase (round S in Figure 2) and thus could be transferred directly to soil without the need for *in vitro* shoot rooting (round V in Figure 2).

Molecular and biochemical analysis

Six independent transgenic T_0 lines of *Klein Brujo* and two of *Bobwhite* were selected for further investigations under the designations KB-1 to KB-6 and Bw-1 and Bw-2, respectively. Southern blot analyses were carried out on these eight lines and on non-transformed plants for

negative controls. When genomic DNA digested with *NcoI* and *XbaI* (Figure 1) was separated electrophoretically and probed with a biotin-labelled *ipt*-coding region fragment (Figure 1), a 1 kb biotin-labelled product was detected for all eight T_0 plants (Figure 5). This confirmed the transgene integration of at least one intact *ipt*-gene into each target tissue genome, corroborating the positive results of the PCR analyses. Reverse transcription PCR provided further confirmation of the presence and expression of the *ipt*-gene in all eight T_0 plants (not shown).

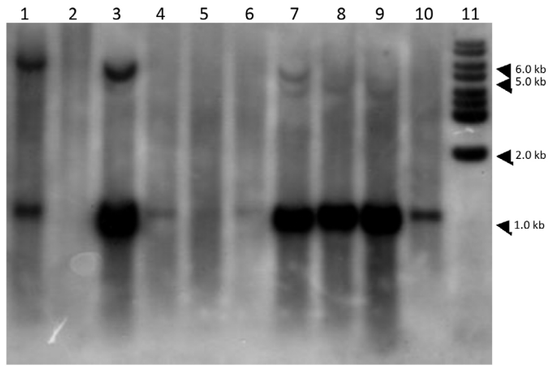

Figure 5. Southern blot hybridization of a biotin-labelled *ipt*-probe to fragmented genomic DNA from six cv. *Klein Brujo* (KB) and two cv. *Bobwhite* (Bw) independent T_o lines transformed by bombardment with the pS40-IPT and pSG516 plasmids, respectively. Genomic DNA was digested with the restriction enzymes *Nco*I and *Xba*I that released a 1.0 kb plasmid fragment containing the *ipt*-gene and the *nos*-terminator (Fig.1). Lane 1: KB-1, lane 2: negative control (non-transformed plant), lane 3: KB-2, lane 4: KB-3, lane 5: KB-4, lane 6: KB-5, lane 7: Bw-1, lane 8: Bw-2, lane 9: KB-6, lane 10: positive control (plasmid pGS516 containing the 1.0 kb fragment released by digestion with *Nco*I and *Xba*I), lane 11: GeneRuler 1 kb DNA Ladder (Fermentas).

The leaves of the selected six *Klein Brujo* and two *Bobwhite* T_0 plants were also resistant to PPT when a 3 mM PPT solution of the herbicide was applied locally to the plants growing *ex vitro* in the greenhouse. Whereas the leaves of the transgenic plants remained green upon the treatment with PPT, those of non-transformed control plants exhibited considerable damage and browning. The *bar*-gene was always stably expressed up to and during *ex vitro* culture, but not the *gfp*-gene. The KB-3 line stemming from group B transgenics had lost GFP expression during *in vitro* callus formation culture, while the KB-6 line was a group C transgenic that had never discernibly expressed GFP (see Figure 2).

Analysis of transgene inheritance

Each of the eight selected T_0 transgenic lines KB-1 to KB-6 and Bw-1 and Bw-2 were self-pollinated and 20 of the resultant T_1 generation seeds were removed from each line (with the exception of KB-3, from which all of the only nine formed seeds were taken). GFP fluorescence was observed in the seeds of the six GFP-positive T_0 lines (Figure 3f). The seeds were planted in soil and the seedlings sprouting from them were grown to fully developed plants in the greenhouse. Each of the 149 T_1 progeny plants was screened for the presence of the *ipt*-gene by PCR, as well as for GFP expression (Figure 3i and 3j) and PPT resistance (Figure 3k and 3l). The results of this analysis are shown in Table 4. The segregation of *ipt* in the T_1 progeny of all eight T_0 plants showed that this gene was inherited at a 3:1 ratio consistent with a single transgene locus, providing further evidence of stable transformation. However, the phenotypic segregation of GFP expression agreed with the Mendelian 3:1 ratio in the T_1 progeny of only four of the six GFP-positive T_0 lines (Bw-1, KB-1, KB-2 and KB-4), but not in Bw-2 and KB-5. GFP fluorescence or the lack of it in T_1 progeny embryos is shown in Figure 3g and 3h. And although the *bar*-gene was expressed in the young leaves of all of the T_0 plants as shown by resistance to PPT, Mendelian segregation was not evident respective of this gene in the T_1 progeny of either of the two T_0 lines that were *gfp*-negative (KB-3 and KB-6). In no instance was there any evidence of independent segregation of the three transferred foreign genes *gfp*, *bar* and *ipt*. Both *ipt* presence and *gfp* expression was observed in all of the T_1 progeny of only KB-1 and KB-4. Some of the T_1 progeny from the T_0 lines Bw-1, Bw-2, KB-2 and KB-5 were PCR-positive for the *ipt*-gene but not for GFP expression, even though Bw-1 and KB-2 exhibited the Mendelian segregation ratio for GFP expression. Similarly, some of the T_1 progeny from the GFP-negative KB-3 and KB-6 T_0 lines were *ipt*-positive plants that were not resistant to PPT and thus did not express the *bar*-gene.

To investigate the inheritance patterns exhibited by the transgenic T_1 plants, T_2 progeny were grown from seeds of all the 149 T_1 plants considered in Table 4, as described above. The number of T_2 progeny plant individuals

stemming from a particular parental T_1 plant varied between 10 and 79. All of these were screened for *gfp* -and *bar*-gene expression by testing for GFP fluorescence and PPT resistance, and for the presence of the *ipt*-gene by PCR analysis. The number of homozygous, hemizygous, and azygous T_1 plants obtained in terms of *ipt* presence, GFP expression and PPT resistance are shown in Table 4. The analysis of T_3 progeny grown from seeds of the T_2 plants revealed that the *ipt*-gene inherited from hemizygous T_2 plants segregated with the same 3:1 ratio that was observed in the inheritance from the T_1 plants (data not shown), again consistent with a single transgene locus.

The progressive loss of expression of transgenes observed in the T_1 plants was also evident in the successive T_2 and T_3 generations as shown in Table 5. Whereas the T_3 progeny of the homozygous T_2 plants KB-1-C-3, KB-4-B-1 and KB-4-B-3 exhibited stable expression of both the *gfp* and *bar*, all other T_3 progeny reflected a loss of this expression to varying degrees. This loss of expression affected either the *gfp*-gene alone or both the *gfp* and *bar* genes. However, the loss of expression of the *bar*-gene tended to be less pronounced than that of the *gfp*-gene. This was particularly well illustrated in the extreme case of KB-5-R-2, where none of the T_3 plants showed any of the GFP expression shown by their parent, whereas they all exhibited resistance to PPT.

DISCUSSION

Choice of a scutellar callus induction medium for in vitro culture

Successful biolistic transformation of wheat depends on an efficient *in vitro* culture system that supports the development and proliferation of embryogenic callus on the scutellum, promotes the development of shoots from these calli and supports rooting of the shoots to produce plantlets for growth on soil. Demands on such a system are high, as biolistic bombardment inevitably results in some damage, even to successfully transformed cells. Such damage must be compensated for by a suitable scutellar callus induction medium (SCIM) on which the bombarded embryos are initially cultured.

The interplay of basal medium composition, hormone and sugar concentration determining the success of a particular SCIM in supporting callus formation and plant regeneration from non-bombarded embryos was not necessarily effective in facilitating the production of transgenic plants from bombarded *Klein Brujo* embryos. The fact that transgenic plants were obtained only when the excised embryos were pre-incubated on the SCIM prior to the bombardment and also subjected to some hours of pre- and post-bombardment osmotic treatment with 0.5 M mannitol demonstrates that the pre-conditioning of the embryos and treatment with high osmotic strength are of paramount importance for successful transformation by particle bombardment. The fact that -given this pre-conditioning and osmotic adjustment- the use of MB3-1 led to the greatest number of transgenic plants subsequent to the widest range of bombardment conditions shows that this SCIM was indeed the best of the three tested for the production of transgenic wheat plants. The regeneration potential of explants and the calli formed from them is crucial for the success of genetic transformation of plants (Varshney and Altpeter, 2001; Altpeter *et al.*, 2005; Danilova *et al.*, 2007), and the superiority of MB3-1 may reside in its ability to preserve much of the 100 % regeneration capacity exhibited on this SCIM by non-bombarded embryos. Nevertheless, the adverse effects of bombardment were still clearly evident in the culture efficiencies shown for MB3-1 in Table 3 that were considerably lower than the 98.6 % observed without bombardment.

ML3-1 proved to be far less successful than MB3-1 in producing transgenic plants, despite the fact that it also gave rise to 100 % regeneration capacity and a higher yield of regenerated plants than MB3-1 upon *in vitro* embryo culture of non-bombarded embryos. This shows that the basal composition of the ML medium must be much less suitable than that of the MB medium for maintaining the integrity –and thus the regenerative potential– of the scutellum tissue. Nevertheless, Huber *et al.* (2002) obtained a transformation efficiency of 4.9 % with a German spring wheat cultivar on ML3-1 medium.

Rasco-Gaunt *et al.* (2001) found a wheat cultivar-specific increase in somatic embryogenesis and a marked improvement in post-bombardment shoot regeneration and stable transformation efficiency with a callus induction medium containing 9 % instead of 3 or 6 % sucrose. In contrast, our use of MB9-1 with 9 % maltose led to significantly poorer embryogenesis and plant regeneration with non-bombarded embryos than did the equivalent medium with 3 % maltose (MB3-1). MB9-1 was nevertheless included in the transformation experiments on the grounds that its higher osmotic strength might be of long-term benefit in stabilizing the water relations of

bombarded embryos. Even if this was the case, it was of no significant advantage for transformation, as this took place much more extensively on MB3-1 than on MB9-1. Nonetheless, even this "inferior" MB medium was distinctly more effective than ML3-1 as a SCIM for the production of transgenic plants.

The present investigation reveals that *in vitro* culture performance data gleaned from preliminary experiments carried out with non-bombarded embryos are of limited value for optimizing the ballistic transformation of spring wheat, since SCIMs resulting in good embryogenesis and plant regeneration with the untreated embryos may be less effective in promoting the repair of damage stemming from bombardment. How particular characteristics of a SCIM make it efficacious in producing transgenic plants subsequent to ballistic transformation cannot easily be deduced in advance, and a suitable medium must be identified experimentally on the basis of previous experience and findings with the particular genotype in question.

The importance of embryo pre-conditioning and osmotic protection

The necessity of both pre-bombardment culture of the excised immature embryos on SCIM and the presence of an osmotic agent prior to and following the bombardment for the production of transgenic plants indicates that both the medium and the osmoticum act in complement to enable the embryonic tissues to recover from bombardment injury.

In agreement with Klein and Jones (1999), we found that the time period of 4 days for which the embryos are incubated on SCIM prior to bombardment is critical for successful transformation. Other studies have reported the benefit of even longer periods of pre-culture (Takumi and Shimada, 1996; Vasil *et al.*, 1993). These studies did not, however, make use of the osmotic treatment that proved necessary in our experiments. A number of other investigators have also successfully employed pre- and post-bombardment osmotic treatment, mainly with 0.4 M sugar alcohols (Altpeter *et al.*, 1996; Jordan, 2000; Gil-Humanes *et al.*, 2011), although Huber *et al.* (2002) reported this treatment to be of no advantage. The 0.5 M mannitol we used as an osmoticum may induce cellular stress responses that prevent the initiation of an apoptotic reaction to particle penetration (Klein and Jones, 1999). In this vein, Pellegrineschi *et al.* (2000; 2002) successfully transferred freshly isolated embryos to MS medium containing 15% maltose directly prior to bombardment without any pre-culture, and Rasco-Gaunt *et al.* (2001) and Sparks and Jones (2009) had success in incubating embryos on a SCIM containing 9 % sucrose for 1-2 days prior to bombardment. These types of osmotic treatment were not successful in our experiments, and may thus be genotype-specific.

The role of physical bombardment parameters

Jordan (2000) observed better biolistic wheat transformation at an acceleration pressure of 1100 psi with 0.6 µm gold particles and target distances of 6 or 9 cm than with 1.0 µm particle size and 3 cm target distance, and Yao *et al.* (2007) achieved a higher rate of transformation with 0.6 µm than with 1.0 µm gold particles. We thus expected that 0.6 µm (rather than 1.0 µm) gold particles delivered at 900 (rather than 1350) psi and target distances of 9 or 12 cm (rather than 6) would bring the best results in our experiments. While the best culture and transformation efficiencies with both *Klein Brujo* and *Bobwhite* were indeed observed with a target distance of 9 cm, they were obtained with the larger 1.0 µm gold particles and higher 1350 psi acceleration pressure.

Souza Canada (2012) described transient transgenic GFP expression taking place mainly in the two surface layers of the scutella of bombarded immature wheat embryos, in agreement with findings of Huber (2002) and Fettig (1999). The penetration potential represented by the 1.0 µm particle size and the 1350 psi acceleration pressure was apparently most suitable for the deposition of the gold particles in the cells of these layers at a distance of 9 cm. This combination of physical parameter variants thus constituted the ballistic conditions giving rise to a most favorable balance between DNA delivery and cell damage with both wheat genotypes. The actual amount of the gold impacting on the target tissue will also influence this balance: the average of 250 µg of gold particles we deployed per bombardment falls within the range used by other investigators (Weeks *et al.*, 1993: 583 µg; Becker *et al.*, 1994: 29-126 µg; Altpeter *et al.*, 1996: 30-100 µg; Iser *et al.*, 1999: 250 µg; Rasco-Gaunt *et al.*, 2001: 60-120; Sparks and Jones, 2009: 58.8 µg; Gil-Humanes *et al.*, 2011: 58 µg).

All but one of the physical parameter combinations led to transformation with *Klein Brujo* to some extent, and the smaller gold particle size in combination with

each of the two shorter target distances and each of the two acceleration pressures resulted in the next best transformation efficiencies. These combinations of physical bombardment parameter values apparently also result in gold particle momentum sufficient to penetrate the scutellum tissue to an extent resulting in relatively good transformation. The failure of the combination of 0.6 µm gold particles, 900 psi acceleration pressure and 12 cm target distance to produce any transgenic plant may be due to failure in producing the necessary particle momentum for cell penetration.

Transformation efficiency

Even though higher transformation efficiency tended to reflect better culture efficiency in our work, there was no clear correlation between these two efficiencies. Iser *et al.* (1999) and Varshney and Altpeter (2001) found no parallelism between regeneration and transformation frequencies among different wheat genotypes, and the latter authors surmised that this reflects the action of independent genotypic factors. In our previous work (Souza Canada and Beck, 2013) we also found no correlation between embryogenic callus formation and regeneration capacity, the two factors that determine culture efficiency, which indicates that these two phenomena are also controlled by different genes or gene combinations. The transformation efficiencies that we observed with both *Klein Brujo* and *Bobwhite* varied considerably, not only in the mean values obtained with different bombardment parameter setting combinations, but also from replicate to replicate under any one particular combination of these settings. High levels of experiment-to-experiment variation in transformation efficiency have also been reported in others studies (Barro *et al.*, 1998; Rasco-Gaunt *et al.*, 2001). The best mean and single experiment transformation efficiencies of 10 % and 16.4 % that we obtained with *Klein Brujo* nevertheless indicate the potential of our methodology with this wheat genotype. The efficiency of biolistic transformation in wheat depends on the genotype (Takumi and Shimada, 1996; Iser *et al.*, 1999; Lazzeri and Jones, 2009). It was thus of interest to compare our transformation efficiency with *Klein Brujo* with that obtained with the model wheat genotype *Bobwhite* SH 98 26, which has been termed a super-transformable wheat line with transformation efficiencies consistently in excess of 70 % (Pellegrineschi *et al.*, 2002). Under our experimental conditions, the best mean and single experiment transformation efficiencies attained with *Bobwhite* were with 4.1 % and 6.9 %, i.e. less than 50 % of the corresponding values obtained with *Klein Brujo* and far lower than the efficiency reported by Pellegrineschi *et al.* (2002). The best transformation efficiencies obtained with *Bobwhite* by other investigators (Altpeter *et al.*, 1996; Nehra *et al.*, 1994; Becker *et al.*, 1994; Zhang *et al.*, 2000; Gil-Humanes *et al.*, 2011) have also not exceeded 7 %. Our present results indicate that *Klein Brujo* is a better wheat genotype than *Bobwhite* for transformation with our methodology.

Selection of transgenic plants

The total of 115 independent primary transgenic lines that we obtained in the present study was due in good part to the two-pronged screening procedure that was employed to identify and select transgenic callus and regenerating plantlets. The monitoring of both GFP expression and PPT resistance throughout the *in vitro* culture of the bombarded embryos permitted a ready identification of *gfp*-transgenics on the one hand and pointed to *bar*-transgenics in the absence of GFP fluorescence on the other. The repeated testing for GFP fluorescence during the *in vitro* culture additionally ensured that the loss of initially present GFP did not result from the loss of the transgene: if expression of the *gfp*-gene proved to be transient in some calli or regenerating plant material, these could still be "rescued" by testing for resistance to PPT indicative of the *bar*-gene. This strategy was invaluable in isolating as many transgenic lines as possible and thus for efficiently realizing the low transformation potential of wheat. Huber *et al.* (2002) also combined GFP screening with PPT selection during callus induction, but their strategy of further culturing only GFP-positive calli and shoots undoubtedly resulted in the loss of numerous transgenics. It is intriguing that numerous GFP-positive transgenics were first identified from apparently only PPT-resistant plantlets at the end of the shoot regeneration or rooting phases when they had become large enough to visually emerge from covering GFP-negative plant material.

We employed PPT for selection purposes at 4 mg/l in the present study, a concentration falling within the range used by other investigators (Rasco-Gaunt *et al.*, 2001: 3 mg/l; Pastori *et al.*, 2001: 4-5 mg/l; Pellegrineschi *et al.*, 2002: 5 mg/l; Huber *et al.*, 2002: 2-5 mg/l; Sparks and Jones, 2009: 2-6 mg/l). We commenced PPT treatment first at the completion of the callus induction phase to give the developing calli the opportunity to fully develop *bar*-

gene expression without premature selection pressure. This agrees with the finding of Rasco-Gaunt *et al.* (2001) that selection with the herbicide first during the later stages of plant regeneration, *i.e.*, after one or two rounds without PPT in regeneration medium, served to maximize the regeneration potential of the calli. In addition, Fadeev *et al.* (2006) reported higher transformation efficiencies when PPT was applied in the plant regeneration phase than when it was already included in the callus formation phase, and Altpeter *et al.* (1996) found that selection with PPT during the shoot regeneration phase rather than during prior selection cycles reduced the time required to obtain rooted transgenic plants.

The successful transfer of transgenic plantlets possessing a vigorous root system at the end of the shoot regeneration phase directly to soil eliminated the need for the time-consuming root regeneration phase.

Analysis of T0-plants

Although the transgenic T_0 plants we obtained from the selection procedure were phenotypically normal, some of them had fully or partially sterile ears. Sterility is a common phenomenon in transgenic cereals (Iser *et al.*, 1998). The fact that almost all of the 115 transgenic T_0 plants we obtained were shown by PCR to contain the *ipt*-gene shows that the rate of co-transformation of *ipt* and the selection genes *gfp* and/or *bar* from the co-integrate plasmid pGFPBAR we obtained with our transgenic T_0 plants was exceptional even in the light of the high values reported for other wheat genotypes. Our rates of 97.4 % co-transformation with *Klein Brujo* and 100 % with *Bobwhite* are markedly higher than those reported by Barro *et al.* (1997: 71.4 %), Leckband and Lörz (1998: 67 %), Stoger *et al.* (1998: 85 %) and Fettig and Hess (1999: 89 %).

The results of the RT-PCR and *Southern* blot analyses of the six *Klein Brujo* and two *Bobwhite* T_0 lines selected for further investigation corroborated the integration of the *ipt*-gene into the target genome of each transgenic plant. Whereas the *Southern* blots demonstrated the integration of at least one intact *ipt*-gene in all cases, the presence of additional positively reacting DNA fragments larger than the one representing the intact gene could represent undigested plasmid and/or truncations and rearrangements of the gene fragment. Rearranged multiple gene copies have been found several times in wheat transformation (Altpeter *et al.*, 1996; Becker *et al.*, 1994; Iser *et al.*, 1999; Nehra *et al.*, 1994; Weeks *et al.*, 1993; Fettig and Hess, 1999; Huber, 2002) and transgenes can undergo rearrangements prior to or during integration into the host genome (Altpeter *et al.*, 2005). The fact that all of the eight transgenic T_0 plants - even those not expressing GFP - evidenced PPT resistance signifies that the *bar*-gene had indeed been incorporated into the genomes of these plants and expressed.

Transgene inheritance

The results of the study of the inheritance of the introduced *gfp*-, *bar*- and *ipt*-genes up to the T_3 generation suggest that all of these genes were arranged in a linkage group and co-segregated as either a single dominant trait locus or as closely associated loci as described by Fettig (1999) and Altpeter *et al.* (2005), and that there was also at least one active copy for each gene. This would hold even if the transgenic plants contained more than one active copy of each of the genes, since multi-copy transgenic plants tend to exhibit all of the transgene copies at the same locus, irrespective of the number of transformation cassettes having been used (Jones, 2005; Altpeter *et al.*, 2005). The *ipt*-gene was present in almost all of the isolated transgenic T_0 lines and in the subsequent generations and was found to be inherited at a Mendelian ratio. On the other hand, a random and progressive loss of GFP expression and PPT resistance was observed in subsequent generations. One explanation for this may be the effect of silencing mechanisms on the *gfp*- and *bar*- transgenes, in which case the silencing of the *gfp*-gene would have been stronger. Anand *et al.* (2003) concluded that gene silencing in wheat is a random, progressive phenomenon that can be associated with a variety of mechanisms (Huber, 2002) including transcriptional and/or post-transcriptional gene silencing (Demeke *et al.*, 1999; Cannell *et al.*, 1999). The differential rate of expression of the *gfp*- and *bar*-genes we observed in our investigation may be rather associated with the different nature of the corresponding *CaMV35S*- and *Ubi1*-promoters, respectively, than with the transformation procedure or the transgene integration pattern. Chen *et al.* (1998; 1999) reported silencing of a *chitinase*-gene driven by the *CaMV35S*-promoter, but not of the similarly present *bar*-gene under the control of the *ubiquitin*-promoter. In other studies in which both a marker and a reporter gene were introduced into wheat, only one (Weeks *et al.*, 1993; Cannell *et al.*, 1999) or both (Fettig, 1999; Huber, 2002) of the genes were inactivated. Christensen *et al.* (1992) reported the *Ubi1*-promoter from maize to be the promoter inducing the highest

and most stable constitutive expression of the *bar*-gene in wheat, but Anand *et al.* (2003) reported that both the *Ubi1*- and *CaMV35S*-promoters are prone to random and progressive gene silencing in transgenic wheat, with the former being somewhat less susceptible to the silencing.

CONCLUSION

Our present investigation resulted in the production of a large number of transgenic lines of the Argentine spring wheat genotype *Klein Brujo* by bombardment of excised immature embryos that exhibited a very high rate of co-transformation of marker/selection genes and a candidate gene that were inherited over several generations with the characteristics of a single dominant trait locus. Our examination of the biological and physical parameters involved in the transformation itself and in the preservation of the original transgenes throughout the regeneration of transgenic plants pointed to an *in vitro* culture medium well suited to the establishment of regenerative transgenic callus, to the necessity of pre-bombardment culture and pre-and post-bombardment osmotic treatment of the embryos and to the advantages of bombardment with large gold particles and a high acceleration pressure for effecting transformation. Our use of a selection procedure based on two different co-transformed selection marker genes and our transfer of regenerants to soil as soon as possible enabled us to establish numerous transgenic plants *ex vitro* within a short time. The results point to the promise of *Klein Brujo* for large-scale transgenic applications, particularly with regard to the fact that this cultivar proved much more amenable to transformation than did the ostensibly super-transformable (Pellegrineschi *et al.*, 2002) wheat genotype *Bobwhite*.

The present study also points to the fact that the success of *in vitro* plant regeneration from non-bombarded wheat embryos cannot necessarily predict the success of transgenic plant regeneration from bombarded embryos, and that there is no really clear explanation of why a particular constellation of bombardment parameter settings leads to the best transformation successes. Finally, the progressive loss of transgene expression we observed in the present study is not yet fully understood and must be taken into account wherever the inheritance of artificially introduced transgenes is an issue.

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