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 callus 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 CaMV 35S-promoter) and the selection gene bar (resistance to phosphinothricin: PPT, linked to the nptI-H1-promoter), whereas the other contained ipt (encoding isopentenyltransferase) as a candidate gene under the control of the Hs40- or SAG12-promoters. 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 biolítica de callos embriogénicos escutelares de este genotipo. Primeiro se identificaron los medios de inducción de callos escutelares (SCIM) más prometedores 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 plasmídos. Un plasmido contiene dos genes, el marcador gfp (vinculado a CaMV 35S) y el de selección bar (resistencia a fosfinotricina: PPT, vinculado a U1) y el segundo al gen ipt (isopenteniltrasferasa), controlado por el promotor Hs40 o SAG12. Eficacia 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-bombardamiento (16 h). Los callos transformados 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 transmíticas 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 estrictamente estable hasta la generación T3, mientras que gfp y bar fueron susceptibles al silenciamiento.

Palabras clave: Parámetros de partículas de partículas; co-transformación; proteína verde fluorescente; resistencia a fosfinotricina; segregación del transgén.
the Argentine spring wheat genotype Klein Brujo and the selection and growth of transgenic plants from the bombarded embryos. We had previously identified 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 compared with those of the wheat genotype Bobwhite SH 98 26 (manuscript in preparation). Transformation efficiency 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 1.5 mm long, transparent scutellum; they were at the developmental stage II 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 malting 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 MnSO₄·H₂O, 30 µM ZnSO₄·7 H₂O and 102 µM H₂BO₃, 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 (Uhi1) (Christensen et al., 1992), as well as a chimeric S65T-pgfp-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 Amaison, 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 HsS40-promoter) by digesting both with Pael and NotI to remove the inducible SAG12 and HsS40 promoters. The HsS40-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 (6.7 kb) and pSG516 (5.9 kb) each containing the ipt gene (0.7 kb) under the control of the HsS40 (1 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 STS-intron (S65T-pgfp). 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 macrorcarrier 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.

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 MZFL III fluorescence stereomicroscope (Leica, Germany) with an excitation filter BP 490-550 nm (Leica) and documented with an AxioCam 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. 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](image-url)

**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 a 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 10× Long PCR buffer (Sambrook and Russell, 2001), 2.5 mM MgCl₂, 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'-GGG CCTCAG 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 gel, 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 XhoI. 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 gel, 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. Labeling was detected with streptavidin-alkaline phosphatase conjugate (Streptavidin AR Novagen, Germany), using the DIG Luminescence Detection Kit from Roche (Germany). Phosphatase activity was visualized by exposure to X-Omat AR AR (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 T₀, T₁ and T₂ progeny. Segregation ratios were calculated from these data. T₀ progeny were obtained by selfing T₀ 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 T₁ and T₂ progeny were obtained in an analogous manner.

Statistical analysis

Data were evaluated by the X² 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 X² 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 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 dark 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 X² = 54.4; 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 p5400-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 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 disc on an azygous leaf (lacking resistance to PPT).
Table 2. In vitro culture performance of excised immature embryos of Klein Brujo on six scutellar callus induction media

<table>
<thead>
<tr>
<th>Plantlet regeneration</th>
<th>SCIM</th>
<th>ML3-1</th>
<th>ML3-2</th>
<th>MB3-1</th>
<th>MB3-2</th>
<th>MB9-1</th>
<th>MB9-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scutellar callus formation (%) of excised embryos</td>
<td>84.8 ± 12.14</td>
<td>72.5 ± 12.68</td>
<td>98.6 ± 4.43</td>
<td>42.2 ± 51.34</td>
<td>87.7 ± 6.79</td>
<td>54.3 ± 16.54</td>
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<tr>
<td>Callus proliferation on the scutellum surface (%)</td>
<td>77.0 ± 18.14</td>
<td>71.2 ± 6.4</td>
<td>95.7 ± 44.39</td>
<td>61.6 ± 14.9</td>
<td>59.5 ± 7.16</td>
<td>64.5 ± 10.64</td>
<td></td>
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<tr>
<td>Precocious germination (%) of embryos</td>
<td>0.61 ± 2.22</td>
<td>2.5 ± 3.32</td>
<td>5.5 ± 17.14</td>
<td>2.0 ± 5.48</td>
<td>2.5 ± 4.42</td>
<td>10.9 ± 8.98</td>
<td></td>
</tr>
<tr>
<td>Plant regeneration capacity (%)</td>
<td>100</td>
<td>76.3 ± 22.79</td>
<td>100</td>
<td>72.1 ± 7.49</td>
<td>55.7 ± 24.5</td>
<td>51.3 ± 2.14</td>
<td></td>
</tr>
<tr>
<td>Number of regenerated plants per embryo</td>
<td>9.5 ± 6.4</td>
<td>5.6 ± 3.5</td>
<td>4.5 ± 3.4</td>
<td>3.9 ± 2.5</td>
<td>4.4 ± 2.9</td>
<td>2.9 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Culture efficiency (%)</td>
<td>84.8 ± 6.08</td>
<td>55.3 ± 17.4</td>
<td>98.6 ± 2.14</td>
<td>30.4 ± 2.9</td>
<td>48.8 ± 15.6</td>
<td>27.8 ± 9.3</td>
<td></td>
</tr>
</tbody>
</table>

1 Percentage of cultured embryos producing regenerants
2 Percentage of cultured embryos producing regenerative calli (nodular callus exhibiting green spots)
3 1, 2, 3 Post hoc tests (parametric comparison) Means followed by the same letter are not significantly different at the 5% level

Plants 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”) differed significantly with the medium having been used to induce callus formation (Chi-square-test X2 = 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 highest numbers (9.5 and 5.6 plantlets) corresponded to calli formed on the two ML-medium, and these numbers were significantly higher than for the MB-medium. 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 X2 = 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 with 1 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 % and 1 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 MB3-1 and MB9-1 medium. Culture 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 (1.2-4.5 %) 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 the 3-week scutellar callus formation phase. 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-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 plantlets 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 Tₐ 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 gfp-coding region fragment (Figure 1), a 1 kb biotin-labelled product was detected for all eight Tₐ plants (Figure 5). This confirmed the transgene integration of at least one intact gfp-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 gfp-gene in all eight Tₐ plants (not shown).

The leaves of the selected six Klein Brujo and two Bobwhite Tₐ 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 ex 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 Tₐ transgenic lines KB-1 to KB-6 and Bw-1 and Bw-2 were self-pollinated and 20 of the resultant T₂ 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 Tₐ lines (Figure 3e). 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 Tₐ progeny plants was screened for the presence of the gfp-gene by PCR, as well as for GUS expression (Figure 3f and g) and PPT resistance (Figure 3h and i). The results of this analysis are shown in Table 4. The results of the gfp in the Tₐ progeny of all eight Tₐ lines 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 Tₐ progeny of only four of the six GFP-positive Tₐ 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 Tₐ progeny embryos is shown in Figure 3g and 3h. And although the bar-gene was expressed in the young leaves of all of the Tₐ plants as shown by resistance to PPT, Mendelian segregation was not evident respective of this gene in the Tₐ progeny of either of the two Tₐ lines that were gfp-negative (KB-3 and KB-6). In this instance there was an evidence of independent segregation of the three transferred foreign genes, gfp, bar and ipt. Both gfp presence and gfp expression was observed in all of the Tₐ progeny of only KB-1 and KB-4. Some of the Tₐ progeny from the Tₐ lines Bw-1, Bw-2, KB-2 and KB-5 were PCR-positive for the gfp-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 Tₐ progeny from the GFP-negative KB-3 and KB-6 Tₐ lines were gfp-positive plants that were resistant to PPT and thus did not express the bar-gene.

To investigate the inheritance patterns exhibited by the transgenic Tₐ plants, Tₐ progeny were grown from seeds of all of the 149 Tₐ progeny plant individuals
stemming from a particular parental T<sub>0</sub> 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 anygous T<sub>1</sub> plants obtained in terms of ipt presence, GFP expression and PPT resistance are shown in Table 4. The analysis of T<sub>1</sub> progeny grown from seeds of the T<sub>1</sub> plants revealed that the ipt-gene inherited from hemizygous T<sub>0</sub> plants segregated with the same 3:1 ratio that was observed in the inheritance from the T<sub>0</sub> plants (data not shown), again consistent with a single transgene locus.

The progressive loss of expression of transgenes observed in the T<sub>1</sub> plants was also evident in the successive T<sub>2</sub> and T<sub>3</sub> generations as shown in Table 5. Whereas the T<sub>1</sub> progeny from the homozygous T<sub>0</sub> plants exhibited resistance to PPT, all exhibited resistance to PPT. 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 MB-3 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 MB-3 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 MB-3 in Table 3 that were considerably lower than the 98.6% observed without bombardment.

ML-3-1 proved to be far less successful than MB-3 in producing transgenic plants, despite the fact that it also gave rise to 100% regeneration capacity and a higher yield of regenerated plants than MB-3-1 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 explants and embryogenic plant tissues that we used. Nevertheless, we have 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 MB-9-1 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; Vaid 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; Iser et al., 1999; Vaid et al., 1993) achieved a higher rate of transformation with 0.6% Sucrose. These studies have reported the benefit of even longer periods of pre-culture (Takumi and Shimada, 1996; Vaid 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; Iser et al., 1999; Vaid et al., 1993).

In our experiments we found that 0.6% (rather than 1.0% sucrose was 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 Brookbo and Bobwhite were indeed observed with a target distance of 9 cm, they were obtained with the larger 1.0 µmol g<sup>−1</sup> and higher 1350 psi acceleration pressure.

Souza (2012) described transient 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 µmol g<sup>−1</sup> and the 1350 psi increase was apparently most suitable for the deposition of the gold particles in the cells of these layers at a distance of 9 cm. The size and shape of the gold particles that were detected in the scutellar layers of bombarded immature wheat embryos. These types of osmotic treatment thus constituted the ballistic conditions giving rise to a more 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 µmol of gold particles we deposited per bombardment falls within the range used by other investigators (Weeks et al., 1993: 583 µmol; Becker et al., 1994: 29-126 µmol; Altpeter et al., 1996: 30-100 µmol; Iser et al., 1999: 250 µmol; Rasco-Gaunt et al., 2001: 60-120; Sparks and Jones, 2009: 58-8 µmol; Gil-Humanes et al., 2011: 58 µmol).

All but one of the physical parameter combinations led to transformation with Klein Brookbo 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 but also in the variances. The best mean and one particular combination of these settings. High levels of transformation efficiency varied considerably, not only in the mean values but also in the variances. Klein Brujo and 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., 2001; 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 plants. The monitoring of both GFP expression and PPT resistance throughout the in vitro culture of the bombarded embryos permitted a rapid 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; Pastor 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, Fabiyi 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 T0 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 T0 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 GFPBAR we obtained with our transgenic T0 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 Barno et al. (1997: 71.4%), Leckhard and Lörz (1998: 67%), Stoeger (1998: 88%) and Fettig and Hess (1999: 89%).

The results of the RT-PCR and Southern blot analyses of the six Klein Brujo and two Bobwhite T0 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 T1 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 T1 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 events having been used (Jones, 2000, Altpeter et al., 2005). The ipt-gene was present in almost all of the isolated transgenic T1 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). 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 Bujo* 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-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 Bujo* 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 (*Pellegrinoesch et al., 2002*) wheat genotype *Bohbatte*.

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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ACKNOWLEDGEMENTS

We are grateful to Dr. M. Huber, Dr. R. Amasino and Dr. K. Krupinska for providing the pGFPBAR, pS6G16 and pS6G-GUS plasmids, respectively. We wish to thank Heike Deinlein and Reiner Krug for growing the plants, Heidi Jäger for skilful technical assistance, and Dr. Christiane Reinbothe for valuable discussions. Part of this work was supported by the Katholischer Akademischer Ausländer Dienst (KAAD), Bonn, Germany, by the Vater und Sohn Eiselen Stiftung.