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Full Length Research Paper

Optimization of genetic transformation protocol mediated by biolistic method in some elite genotypes of wheat (*Triticum aestivum* L.)

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We report here an efficient genotype-independent genetic transformation system in wheat. Highly regenerable embryogenic calli obtained from mature seeds were employed as the target tissue for the genetic transformation of three bread wheat varieties *viz* C306, HDR77 and PBW343 representing diverse genetic background. The plasmid pDM803 containing *GUS* and *BAR* genes driven by rice *Act1* and maize *Ubiqutin* promoter, respectively was transferred into a month-old calli employing particle delivery system. The bombarded calli were transferred to medium supplemented with phosphinothricin at 4 mg L^{-1} for the selection of the transformed calli. The transgenic calli were confirmed for the expression of *GUS* by histochemical analysis of *β-glucuronidase.* Transformation efficiency of the genotypes was calculated based on the number of calli bombarded and the number of plants that were resistant to Basta. Among the three genotypes studied, C306 had a higher efficiency of 0.56% followed by HDR77 with 0.5% and PBW343 with 0.22%. The transformation system developed in this study may facilitate studies on functional genomics and crop improvement *via* transgenic development.

Key words: Wheat, biolistic, transgenics, bar, phosphinothricin, transformation efficiency.

INTRODUCTION

Cereals are the major contributors to food grain production in India. Wheat is the second most important food cereal crop of the country, and contributes nearly one third to the total food grain production. While conventional breeding methods continue to provide avenues for wheat improvement, transgenic development is gaining importance for incorporating traits such as herbicide tolerance, diseases resistance and quality improvement. However, wheat is a highly recalcitrant crop species. Several research groups have attempted to develop regeneration and genetic transformation in wheat and successes have been been achieved (Abebe et al., 2003; Permingeat et al., 2003; Pellrgrineschi et al., 2004). The efficiency of stable transformation of wheat strongly depends on genotype, explant source, medium composition and transformation method (Abdul et al., 2010). Initially, wheat transformation was obtained by direct gene transfer methods into protoplast from cultured embryonic cells (Lröz et al., 1985) with low transformation efficiency. Difficulties involved in establishing and maintaining long-term culture conditions accompanied with a little plant regeneration limited transformation of wheat.

Microprojectile bombardment is currently the most widely applied technique used to transfer genes to some of the most recalcitrant cereal species (Christou et al., 1996). Micro projectile bombardment has the advantage of uni-

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universal delivery system and transformation of intact organized tissue. Recovery of the transformed plants depends on the ability to regenerate plants from the bombarded cells. Most of the wheat transformation protocols are based on immature embryos as the explants. Initial successes in wheat transformation were achieved using established long-term embryogenic cultures (Vasil et al., 1992). Additional works have since been demonstrated with consistent results using freshly initiated cultures from immature embryos (Vasil et al., 1993; Weeks et al., 1993; Becker et al., 1994; Nehra et al., 1994). Patnaik and Khurana (2003) utilized mature embryo-derived calli of bread wheat (*Triticum aestivum*, cv. CPAN1676) *Triticum dicoccum* (Chugh and Khurana, 2003), and durum wheat (*Triticum durum*, cv. PDW215) for genetic transformation and more over the transformation efficiency reported by previous workers generally varies from 0.1 to 5.7% (Patnaik and Khurana, 2001; Vasil and Vasil, 1999). Though, genetic transformation in wheat has been reported in genotypes responding to regeneration from immature, as well as, mature embryos as explants, there is still a need to extend it to diverse genotypes with a scope of crop improvement or for facilitating functional genomics in the present day genomic era. We report here a transformation system that is functional in genetically diverse genotypes based on gene transferred to mature embryo derived calli.

MATERIALS AND METHODS

Plant materials

Seeds of *T. aestivum* cvs. PBW343, HDR77 and C306 were obtained from Division of Genetics, IARI, New Delhi.

Embryo culture

Mature seeds of three genotypes PBW343, HDR77 and C306 were used as source of mature embryos. Mature seeds were surface sterilized with 2% sodium hypochlorite for 20 min and washed several times with sterile distilled water. The seeds were then imbibed in sterile water for 16 to 18 h at room temperature. For callus induction mature embryos were aseptically excised with a sterile scalpel and forceps from the imbibed seeds. The embryos were placed scutellum side upward in the callus induction medium containing MS mineral salts sucrose 30 gL^{-1}, agar 0.8%, 2 mg L^{-1} 2, 4-D, 1 mgL^{-1} ABA and 0.5 mgL^{-1} kinetin and Cultures were kept at 25 ± 1°C in total darkness for 4 weeks. The developed calli were pooled at the centre of the petridish containing osmotic medium (MS mineral salts with sucrose 30 g L^{-1} agar 0.8% and 0.2 M mannitol for 4 h before bombardment).

Expression vector

Plasmid pDM803 contains a chimeric *BAR* gene from *Streptomyces hygroscopicus* (Thompson et al., 1987) under the control of the maize Ubi promoter (Christensen et al., 1992) and *Agrobacterium tumifaciences* nopaline synthase (nos) terminator (Bevan et al., 1983) and the *Escherichia coli* uidA (β–glucuronidase) gene (Jefferson et al., 1987) under control of the rice *Act1* promoter (McElory et al., 1995) and rice rbcs terminator (Xie et al., 1987). Plasmid DNA was isolated by Qiagen plasmid midi kit method.

Preparation of tungsten particles

Tungsten particle were prepared by washing thrice with absolute ethanol and finally dissolved in 1 ml sterile distilled water. DNA was precipitated onto 50 µL of tungsten (50 mgL^{-1}) by adding 10 µL of plasmid pDM803 (1.5 µg $µL^{-1}$), 50 µL $CaCl_2$ (2.5M) and 20 µL spermidine (λ free base, 0.1 M Sigma), vortexing well after each addition. The supernatant was discarded and the pellet washed with 250 µL ethanol, and resuspended in 30 µL ethanol.

Bombardment

Target tissue prepared as described above was bombarded using BioRad helium driven particle delivery system. Tungsten particle suspension in ethanol (6 µL) was loaded on to the macrocarrier and allowed to dry before use with 1100 psi rupture disks.

Culture conditions and recovery of transformed plants

After bombardment, embryo cultures were incubated in the dark for 24 h before transfer to fresh medium containing 1 mgL^{-1} of indole-3-acetic acid (IAA) and 0.5 mgL^{-1} of kinetin. Two days after bombardment, two to four embryos were removed from each dish and stained for *β-glucuornidase* activity using a solution containing 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Jefferson et al., 1987). Cultures, five to seven days following bombardment were transferred intact to selection medium containing 0.5 mgL^{-1} of IAA, 1 mgL^{-1} of kinetin and 4 mgL^{-1} of Basta. Surviving shoots were transferred to half strength of Murashige and Skoog (MS) without any selection medium for root development. After development of root system, the resulting plantlets were transferred to light soil mixture in peat pots.

β-Glucuronidase (GUS) assay

The *gus* reporter gene activity was localized histochemically according to the protocol described by Jefferson et al. (1987). Histochemical localization of *gus* was carried out by incubating the tissue samples overnight at 37°C in histochemical buffer (0.1 M sodium phosphate buffer, pH7.0, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.5 m M K_3 Fe $(CN)_6$, 0.5 mM K_4 Fe $(CN)_6$, 0.1 triton X 100 and 1 mg mL^{-1} X-gluc (Amresco Inc. Ohio., USA). The explants were thoroughly washed with 70% ethanol prior to observation.

Polymerase chain reaction (PCR)-Southern

PCR products from the putative tranformants were electrophoresed through 1% agarose gel and transferred to nitrocellulose membrane and then hybridized with radioactive probe of size 496 bp, which was labeled by following the manufacturer's instructions of Hexa Label DNA labeling kit (MBI Fermentas, Lithuania) with α^{32}P dCTP.

PCR analysis of genomic DNA

PCR analysis of genomic DNA was carried out using 200 to 300 ng

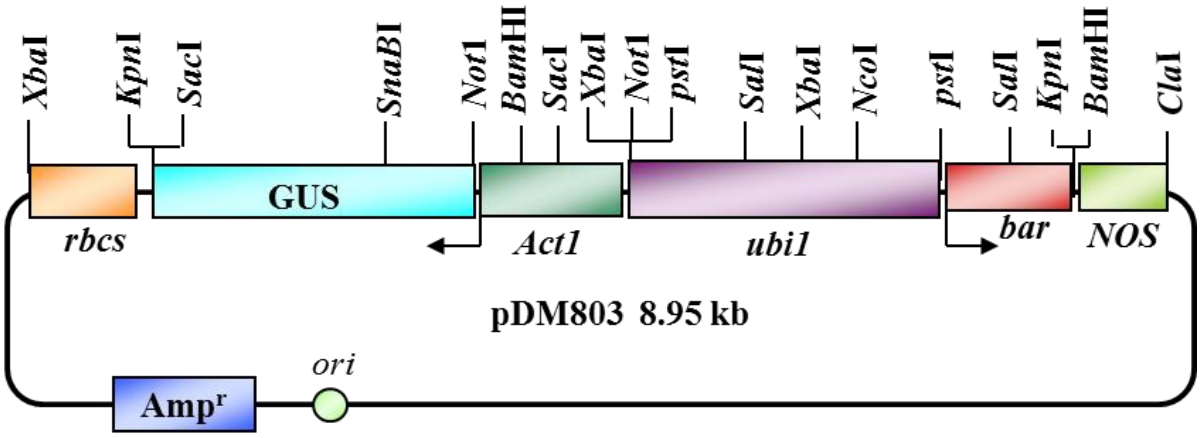

Figure 1. Restriction map of the plasmid pDM803 (8.95 kb). *Act1* promoter, first exon and intron of the rice actin1 gene; *gus*, coding region of the *E. coli* β-glucuronidase *uidA* gene; *bar,* coding region of *streptomyces hygroscopicus phosphinothricin acetyltransferase* gene*: NOS*, 3' transcript termination region of the *Agrobacterium tumifaciences* nopaline synthase gene; Amp^r , ampicillin resistance gene of pSP72 (Witrzens et al., 1998).

of wheat genomic DNA employing reagents from MBI fermentas (USA) in a 25 µL reaction volume as per manufacturer's instructions. The PCR amplification was performed by initial denaturation at 94°C (5 min hold), followed by 35 cycles at 94°C (30 s), annealing (30 s) and 72°C (30 s) and finally holding at 72°C (7 min) for extension employing a perkin-Elmer Gene Amp PCR system 2400.

RESULTS

Recovery of transgenic plants from cultures selected with bialaphos

The different steps involved in transformation of wheat *T. aestivum* for herbicide tolerance with pDM803 is shown in Figure 2. The calli were regenerated in medium with 1 mg L^{-1} IAA and 0.5 mg L^{-1} kinetin, and the cultures were maintained at 25°C with 55 to 60% relative humidity in white light (200 μmol^{-1} m^{-2} s^{-1}) for 16 h a day. After three weeks, the calli were transferred to MS medium supplemented with 1 mg L^{-1} IAA and 2 mg L^{-1} kinetin for shoot formation in jam bottles. After two weeks, regenerated shoots were transferred on ½-MS medium without any plant growth regulators (½-MS_0) in jam bottles for rooting. After two weeks the plantlets with well-developed roots and shoots were transferred in liquid ½-MS_0 in culture tubes for one week and then transferred to pots containing sterile soil rite for hardening.

In initial experiments with the plasmid pDM803 (Figure 1), transgenic wheat plants of C306, HDR77, and PBW343 were recovered following bombardment of cultured calli derived from matured embryos, with a Bio-Rad delivery system. The plants grown on the selection medium were transferred to National Phytotron Facility, New Delhi and the presence of the *bar* transgene was confirmed by polymerase chain reaction with *BAR* specific primers. Transformation efficiency of the genotypes PBW343, HDR77 and C306 was calculated based on the number of calli bombarded and the number of plants obtained that were resistant to Basta. Among the three genotypes studied, C306 had a higher efficiency of 0.56% followed by HDR77 with 0.5% and PBW343 with 0.22% (Table 1).

A total of 24 independently transformed lines were obtained at frequencies of less than one independent transgenic line for every 100 bombarded embryos. While the plasmid pDM803 carries a *uidA* gene that was functional in assays for transient expression. When the calli were stained for GUS expression, blue spots were visible, but the leaf sample taken from these transformed plants showed no β-glucuronidase activity when treated with x-gluc histochemical stain. The PCR products of 24 putative transformants were transferred to the nylon membrane and hybridized with 492 bp amplicon as probe for the confirmation of the presence of the selection marker gene *BAR* (Figure 3). The variety C306 had better transformation frequency, but the hybridization signals were found weak in many of the C306 transformants in comparison to the other varieties PBW343 and HDR77.

Progeny analysis

To study the segregation pattern of the transgene *bar*, 5 T_0 PCR-Southern positive plants (two of C306, two of HDR77 and one of PBW343) were selected. These plants were normal in growth and had a seed set of 80 to 450. DNA was isolated from a small leaf segment and PCR amplification of genomic DNA samples of T1

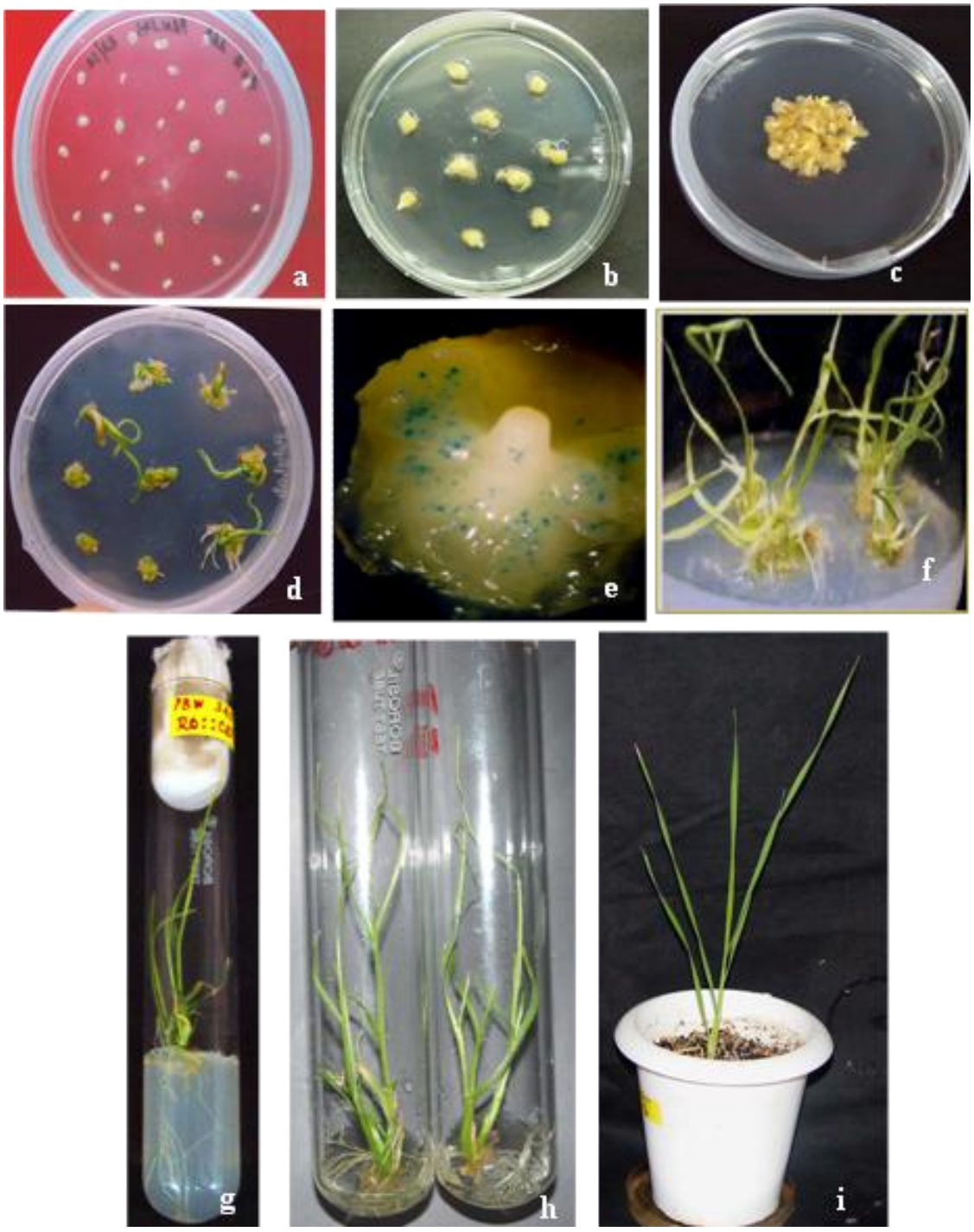

Figure 2. Steps involved in genetic transformation of wheat with pDM803. **a)** Embryos placed in callusing medium MS medium supplemented with 2 mgL^{-1} 2, 4 D, 1 mgL^{-1} ABA, 0.5 mgL^{-1} Kinetin. **b)** Calli formed from the embryos after a period a month of incubation in dark. **c)** Calli pooled to the centre of the petriplate to facilitate bombardment at a distance of 6 cm. **d)** Calli after bombardment transferred to selected MS medium supplemented with 0.5 mgL^{-1} IAA, 1 mgL^{-1} kinetin and 4 mgL^{-1} Basta. **e)** Calli showing GUS histochemical localization at 48 h post-bombardment. **f)** Transformants growing under the second phase of selection. **g)** Shoots transferred to half strength MS medium without growth hormones for root development. **h)** Developed plants transferred to liquid MS Medium for hardening. **i)** Hardened plants transferred to soilrite for further development.

Table 1. Transformation efficiency of *BAR* gene into different genotypes of *Triticum aestivum* via particle bombardment

Genotypes transformed	Number of calli bombarded	Number of plants regenerated on selection medium (Basta at 4 mg L^{-1})	Number of plants established in soil	No. of PCR positive plants	Transformation efficiency (%)
PBW343	4785	22	18	11	0.22
HDR77	595	15	9	3	0.50
C306	1775	28	16	10	0.56

To check the response of different wheat genotypes to the selection pressure, 300 150 and 250 calli from cvs. PBW343, HDR77 and C306, respectively were kept for regeneration as selection media (Basta at 4 mg L^{-1}). However none regenerated into whole plants

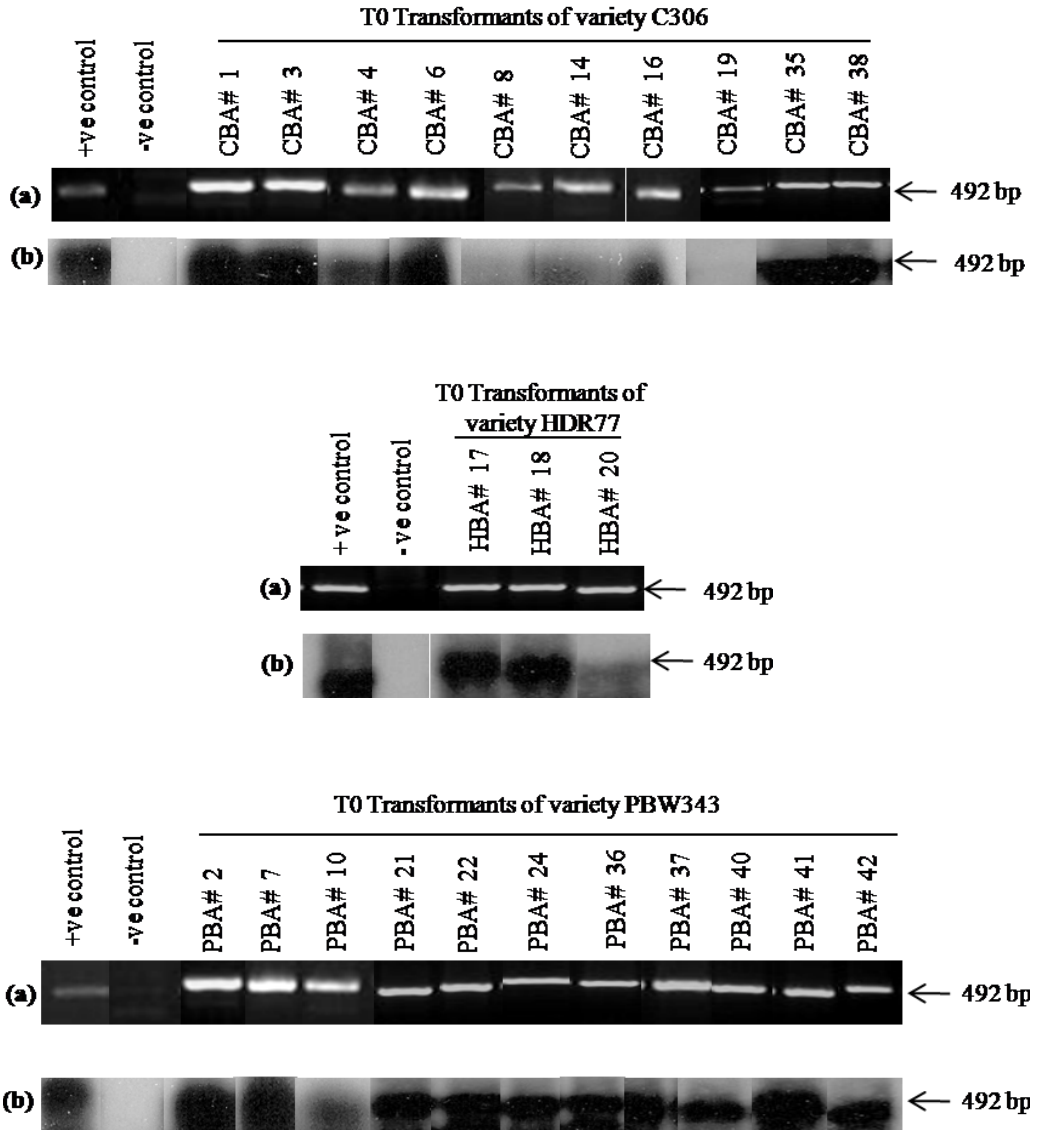

Figure 3. PCR analyses of the T0 wheat transformants with *BAR*-specific primers. **(a)** The PCR product was separated on 1% agarose gel for gel documentation. The expected size of the amplicon (492 bp), the coding region of the gene was amplified from 100 ng of wheat genomic DNA, and no amplification was seen in the wild type genomic DNA. **(b)** For further confirmation of the PCR, products were hybridized against the probe, the coding region of the *BAR* gene.

Table 2. Segregation analysis of T_1 plants from the wheat transformants.

Plant Number	Number of T1 plants analyzed	No of T1 plants positive for the selection marker gene *BAR*	Chi-square test
CBA#1	36	36	2.4 (15:1)
CBA#4	56	51	0.68 (15:1)
PHA#22	38	21	7.87 (3:1)
HBA#17	90	71	0.72 (3:1)
HBA#18	95	64	2.94 (3:1)

progeny using primers specific to *BAR* gene, revealed the presence of the transgene in the T1 progenies (Table 2). CBA#1 plant gave 448 seeds out of which 65 seeds were sown in soilrite for screening. Only 36 seeds germinated and all were PCR positive for the selection marker gene *BAR.* In CBA#4 which gave 335 seeds out of which 56 seeds taken for screening and 51 plants shown amplification, proved positive for the selection marker gene *BAR.* For HBA#17 and HBA#18 plant 106, 134 seeds were sown out of which 70 and 95 only germinated, for which 71 and 64 were positive in the presence of the transgene *BAR.* In the case of PBA#22 plant, 40 seeds were taken for T1 segregation analysis, out which 15 were positive for the marker gene BAR.

Inheritance of *BAR* selectable marker

Segregation pattern for the presence of *Bar* gene in the T1 plants were analyzed by PCR amplification. All the 36 T1 plants of CBA#1 and 51 T1 plants out of 56 showed the expected 492 bp amplicon and therefore this gene did not fit to 3:1 ratio. But it was much closer to 15:1 ratio proving an integration of two copies of *BAR* gene in the genome (Table 2). In the case of HBA#17 and HBA#18, 71 out of 90 and 64 out of 95 T1 plants showed a χ^2 value 0.72 and 2.94, respectively, which is lesser than the tabulated χ^2 value (P=0.05). This proved that it fits in the ratio 3:1 and has single copy integration in the genome. But in the case of PBA#22 the χ^2 value which is higher than the tabulated value and therefore it does not fit in the 3:1 ratio.

DISCUSSION

Wheat was the last of the three major cereals to be transformed due to low success rate in obtaining stably transformed plants. The technology of genetic transformation of wheat has been least successful because of many technical difficulties like, low transformation frequencies ranging from 0.1 to 2.5% (Vasil et al., 1992; Weeks et al., 1993; Altpeter et al., 1996; Zhou et al., 1995); very few genotype with high regeneration in tissue culture, such as “bobwhite”; shortage of explants source like immature embryos, which is not available throughout the year. However, several research groups have performed various attempts to establish the stable and high frequency of transformation protocol. While most of the published work on transgenic wheat has used tissue culture responsive varieties such as ‘Bobwhite’ (Pellegrineschi et al., 2004; Hu et al., 2003) and ‘Florida’ (Barro et al., 1997). Efforts are being made to extend the technology to elite genotypes, which are either agronomically important breeding lines or currently commercial varieties (Machii et al., 1998). Very few reports are available on the transformation of some Indian wheat genotypes (Gopalakrishnan et al., 2000; Patnaik and Khurana, 2003).

The aim of the present study was to optimize a genotype independent transformation system in elite wheat cultivars irrespective of their tissue culture potential. Using the biolistic approach to develop transgenic wheat, mature embryos of three elite genotypes (PBW343, HDR77 and C306) were chosen as the study material. Among the genotypes studied, C306 had a higher efficiency of 0.56% followed by HDR77 and PBW343 with 0.5 and 0.22%, respectively. Recent studies on wheat transformation using biolistic approach have reported efficiencies of 0.13 to 8.6%. Low transformation efficiency (0.22 to 0.56) in the present work was presumably due to the recalcitrant nature. Transformation efficiencies of 0.1 to 2.5% have been reported in genotypes including PBW343 (Gopalakrishnan et al., 2003). The number of plants regenerated on selection medium was 22, 15 and 28 of PBW343, HDR77 and C306 respectively, out of which 11, 3 and 10 were PCR positive for the selection marker gene BAR. Plants that survived selection but tested negative for the presence of the selection marker gene BAR were considered escapes. More than 50% escapes were observed among the genotypes studied. Unfortunately, wheat transformation suffers high escape frequencies (50 to 95%) as reported by several workers (Becker et al., 1994; Nehra et al., 1994).

The *GUS* gene was primarily used to monitor the bombardment process through GUS expression assay (Jeff-

erson et al., 1987). The transient GUS assay helped in ensuring that the plasmid DNA delivery was well distributed on the scutellar calli and the bombardment process was successful (Figure 2e). The presence of the BAR gene in the vector pDM803 helped in selection of the transformed calli as the *BAR* gene confers resistance to Basta herbicide added to the selection medium. A total of 7000 wheat scutellar calli were bombarded with pDM803, out of which 63 putative T_0 transformants were recovered from the selection medium. However, due to the difficulties during hardening of the transformants, only 43 transformants survived. The segregation pattern of the T_1 plants was analysed for all the transformants developed; however, it did not fit into mendalian ratio. This might be due to several reasons:

i. T_0 transformants were not selfed by bagging, which would have resulted into a considerable amount of cross pollination,
ii. Varying number of the transgene copy integration into the genome,
iii. More than 50% of the seeds harvested from CLCB3#1 did not germinate thus resulting into segregation distortion.

It may be concluded that a transformation system that is functional in genetically diverse genotypes based on gene transferred to mature embryo derived calli and the potential application of this transformation system developed in this study may facilitate studies on functional genomics and crop improvement via transgenic development.

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