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Genetic transformation of Indian bread (*T. aestivum*) and pasta (*T. durum*) wheat by particle bombardment of mature embryo-derived calli

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Abstract

Background: Particle bombardment has been successfully employed for obtaining transgenics in cereals in general and wheat in particular. Most of these procedures employ immature embryos which are not available throughout the year. The present investigation utilizes mature seeds as the starting material and the calli raised from the hexaploid Triticum aestivum and tetraploid Triticum durum display a high regeneration response and were therefore used as the target tissue for genetic transformation by the biolistic approach.

Results: Mature embryo-derived calli of bread wheat (Triticum aestivum, cv. CPAN1676) and durum wheat (T. durum, cv. PDW215) were double bombarded with 1.1 gold microprojectiles coated with pDM302 and pAct1-F at a target distance of 6 cm. Southern analysis using the bar gene as a probe revealed the integration of transgenes in the T0 transformants. The bar gene was active in both T0 and T1 generations as evidenced by phosphinothricin leaf paint assay. Approximately 30% and 33% primary transformants of T. aestivum and T. durum, respectively, were fertile. The transmission of bar gene to T1 progeny was demonstrated by PCR analysis of germinated seedlings with primers specific to the bar gene.

Conclusions: The transformation frequency obtained was 8.56% with T. aestivum and 10% with T. durum. The optimized protocol was subsequently used for the introduction of the barley gene encoding a late embryogenesis abundant protein (HVA1) in T. aestivum and T. durum. The presence of the HVA1 transgene was confirmed by Southern analysis in the T0 generation in case of Triticum aestivum, and T0 and T1 generation in Triticum durum.

Background

Wheat is one of the most abundant sources of energy and nourishment for mankind. Ninety-five percent of the cultivated wheat is of the hexaploid type used for the preparation of bread and other baked products and the remaining 5% is durum (tetraploid) wheat, which is used essentially for making pasta and macaroni (*see* [4]). Although stable transformation of wheat has been achieved with either co-cultivation with the natural plant genetic engineer *Agrobacterium tumefaciens* or particle bombardment, particle bombardment is emerging as the method of choice for introduction of agronomically important genes for quality improvement, molecular pharming, engineering of nuclear male sterility, transposon tagging, resistance to drought stress, fungal pathogens, and insect pests (for details *see*, [27]).

Till date, most of the studies on wheat transformation have focussed on few model cultivars and the procedures employed require a constant supply of immature embryos which is difficult to achieve throughout the year unless greenhouse grown plants are utilized. Mature embryos are thus an alternative explant for production of calli which can be used as target tissue for genetic transformation studies [13]. Mature embryo-derived calli also show a regeneration response comparable to that achieved from the calli initiated from immature embryos. The present work was thus initiated to develop an efficient method of transformation for Indian cultivars of bread wheat and durum wheat. Calli derived from mature embryos of the cultivars CPAN1676 of *T. aestivum* and PDW215 of *T. durum* were chosen for genetic transformation experiments based on their high regeneration potential. A common protocol was developed for the introduction of marker gene constructs into both *T. aestivum* and *T. durum*. The optimised protocol was subsequently used for the introduction of a barley gene coding for a late embryogenesis abundant protein (*HVA1*) known to confer tolerance to drought stress in *T. aestivum* and *T. durum*.

Results and Discussion

Wheat has been earlier transformed by particle bombardment of immature embryos but, the transformation frequencies have remained low (usually 0.1–2.5 %) and various attempts have been made to enhance the frequency [1,7,8,10,21,22,33–36,38,39]. The low success rate in wheat transformation experiments thus necessiates the use of a large number of explants throughout the year which is difficult to achieve with immature embryos as the target tissue. Mature embryos which show a regeneration response comparable to that of immature embryos [17,23–25] were thus chosen as the starting explants for transformation studies during the present investigation. The suitability of mature embryo as a starting explant for wheat transformation has also been demonstrated earlier from our laboratory, for cellular permeabilization of mechanically isolated embryos with membrane permeabilizing agents [18] and for *Agrobacterium*-mediated transformation [28].

Identifying explants /cultivars with a relatively high embryogenic response is one of the most critical factors for any transformation endeavour. Based on the higher regeneration response, the cultivar CPAN1676 of *T. aestivum* and PDW215 of *T. durum* were chosen for genetic transformation experiments (data not presented).

Introduction of bar as the selectable marker and gus as the reporter gene

Towards achieving herbicide resistance in *T. aestivum* and *T. durum*, the plasmid constructs pDM302 (*Act1-bar-nos*) and pAct1-F (Fig. 1) were co-transformed in mature embryo-derived calli on MSE2 by particle bombardment. The untransformed calli did not show any growth on $MSERP_{2.5}$ (Fig. 2B) as against the bombarded calli (Fig. 2C). To eliminate the possibility of escapes and also for shoot elongation, the regenerated plantlets were grown on half-strength MS medium supplemented with 2.5 mg/l phosphinothricin (Fig. 2D&2E). Out of 747 and 350 explants of *Triticum aestivum* and *T. durum* cocultivated, 64 and 35 plantlets, respectively, were recovered. The putative transformants obtained were analyzed by the phosphinothricin leaf paint assay (Fig. 2F). Plants with a functional *bar* gene were regarded as transformants. The transformation efficiency was calculated based on the results of several experiments and was 8.56% and 10% for *T. aestivum* cv. CPAN1676 and *T. durum* cv. PDW215, respectively. The regenerated plantlets were transferred to pots for maturity and hardening.

Screening of transformants

To confirm the presence of transgenes in the primary transformants, a number of transformants were tested by PCR amplification of genomic DNA using primers specific to *gus*, *npt*II and *bar* gene. No amplified product was detected in the samples containing genomic DNA from an untransformed plant. The results of PCR analysis were also found to be consistent with that observed with the phosphinothricin leaf paint assay. The *gus* positive plantlets were identified by the presence of an amplified product of ~1711 bp (Fig 3A). PCR analysis of transformants with *npt*II specific primers detected an amplified product of ~721 bp (data not shown). The *npt*II positive status of the transformants was also confirmed by *nptII* dot blot assay (Fig 3B) and by spraying the leaves with paromomycin (Fig 3C). The plants detected positive by PCR suffered little to no damage upon spraying the leaves with phosphinothricin thus demonstrating the functional activity of *npt*II gene, whereas the leaves of untransformed control and a few of the putative transformants developed yellow spots.

The present results demonstrate the importance of an efficient selection regime. The use of phosphinothricin as the selection agent did not interfere in the regeneration process. To eliminate the escapes, the putative transformants were grown for shoot elongation on half strength MS medium supplemented with 2.5 mg/l phosphinothricin (Fig 2E). The reason for a high transformation efficiency may be attributed to optimized regeneration protocol and the effective selection procedure employed for the recovery of transformants (8–10 weeks following particle bombardment). Our results support the observations of Altpeter et al. (1996a), who reported an enhanced transformation efficiency by reducing the total time taken for production of transgenic plants. Using immature embryos as the target explant, Takumi and Shimada (1996) have

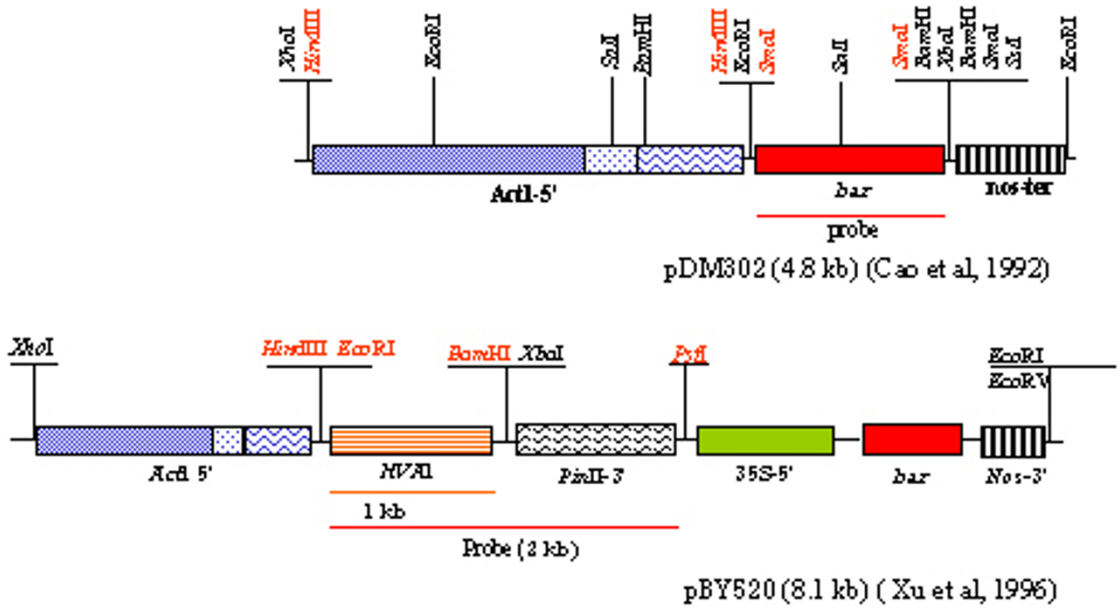

Figure 1
Line diagram of constructs used for transformation.

also reported the positive influence of culture duration of the target tissue prior to bombardment on stable transformation efficiency. In the present study, two-week-old mature embryo-derived calli were cultured on fresh MSE2 medium for one week prior to bombardment resulting in a high transformation efficiency (up to 10 %) as depicted in terms of transgenic plantlets regenerated per hundred resistant calli.

Southern analysis of T_0 transformants

The transgenic status of T_0 transformants of *T. aestivum* and *T. durum* cultivars transformed with pDM302 was confirmed by Southern analysis with *bar* specific probes. Digestion of genomic DNA samples with *Hin*dIII, resulted in the detection of a ~3.3 kb hybridizing band, which corresponds to the vector backbone with *bar* coding region and *nos* terminator. Digestion of genomic DNA of T_0 transformants of CPAN1676 with *Xho*I, which cuts only once in pDM302 and hybridized with 0.6 kb *Sma*I fragment of pDM302. Of the five independent lines tested, four lines were found to contain two bands, one expected band of ~4.8 kb and another band above the 6.5 kb range, and the line CPB8 was seen to contain only one band of around ~4.8 kb (Fig. 4A). Of the various lines tested, the *bar* gene was thus found to be present in two copies in four lines CP2, CP5, CP4 and CPB1 and one copy in CPB8. The similar sized band in three transformants is intriguing. It can only be speculated at this stage that they may have arisen from a common clone. Further investigations are thus warranted in this regard. Similarly, genomic DNA was isolated from 12 independent lines of *T. durum* cv. PDW215 and digested with *Hin*dIII and probed with 0.6 kb *bar* fragment. All the transgenic lines contained the expected 3.3 kb band, which gets released upon digestion of pDM302 with *Hin*dIII (data not presented). Several bands ranging from less than 2 kb in size to 6.5 kb range were also visible upon autoradiography, which may probably be due to multiple integration.

Progeny analysis

A representative T_0 transgenic line of *T. aestivum* set 14 seeds and was chosen (CP1) for progeny analysis. Nine out of the 14 seeds obtained appeared normal and the other were poorly formed. The seeds were germinated on selection free half-strength MS medium. After four days, six seedlings (A-F) germinated, which were transferred to transfer plugs for establishment of root system. Since the growth of seedlings was slower as compared to that from the control seeds, no selection pressure was applied at this stage. Prior to phosphinothricin leaf paint assay, genomic DNA was isolated from a small leaf segment of a 10-day-old T_1 plant. PCR amplification of genomic DNA samples

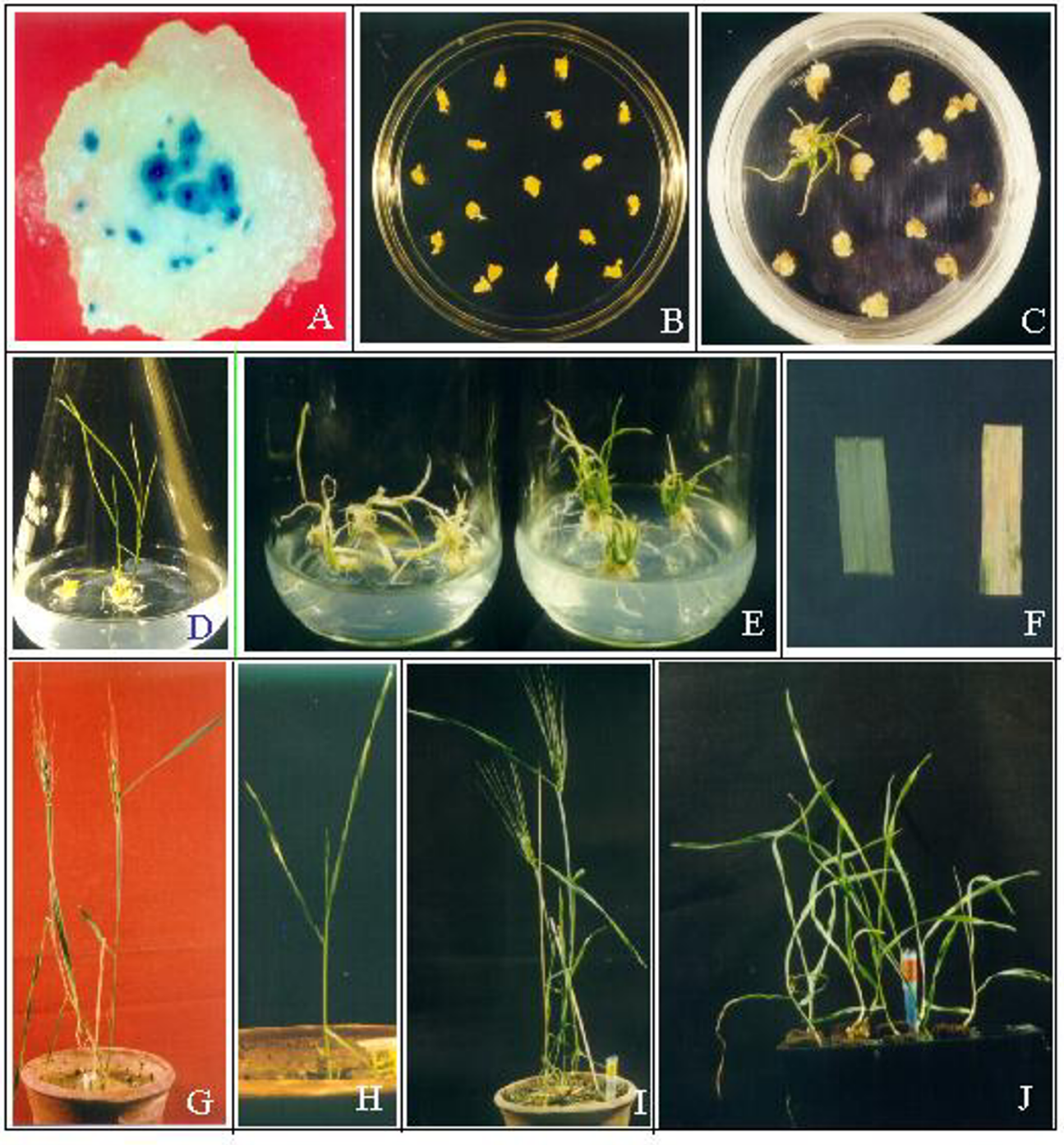

Figure 2
Genetic transformation of *T. aestivum* and *T. durum* by particle bombardment employing mature embryo-derived calli as the target tissue. The calli were bombarded with either pDM302/pAct1-F or pBY520 or pBI101::Act1. **A**. Mature embryo-derived callus of *T. durum* showing histochemical localization of the *gus* gene activity two days after bombardment with pAct1-F/pDM302. **B & C**. Treated and control explants, respectively, on regeneration medium supplemented with 2.5 mg/l phosphinothricin. **D & E**. Putative transformants of *T. aestivum* and *T. durum,* respectively, growing on half-strength MS medium supplemented with 2.5 mg/l phosphinothricin. **F**. Phosphinothricin leaf paint assay; transformant (left), control (right). **G & H**. Putative transformants of *T. aestivum* and *T. durum*, respectively. **I**. A T_0 transgenic plant of *T. durum* obtained after bombardment with pBY520. **J**. T1 progeny plants of *T. durum* bombarded with pBY520.

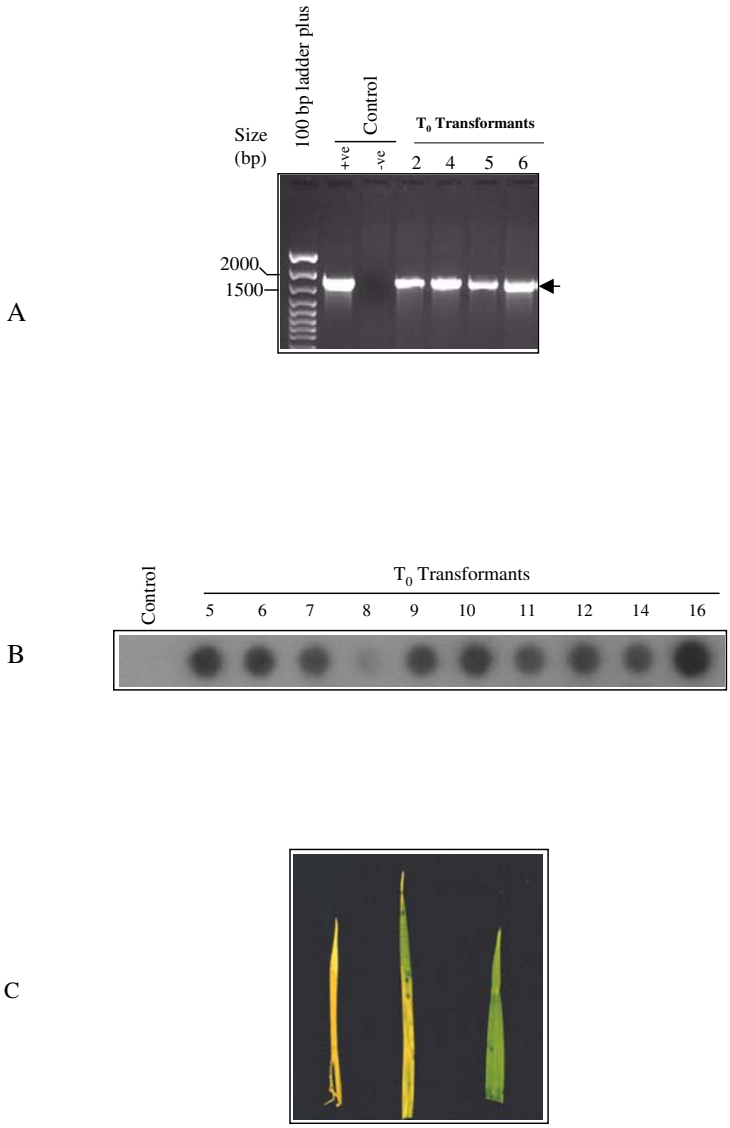

Figure 3
Introduction of *gus* and *npt*II gene in *T. aestivum* cv. CPAN1676. **A**. PCR screening of genomic DNA samples of putative transformants of *Triticum aestivum* cv CPAN1676 using primers specific to *gus* gene. The transformants were obtained after bombardment of mature embryo-derived calli with pBI101::Act1. The plasmid pBI101::Act1 and the genomic DNA isolated from untransformed plant were used as the positive and the negative controls, respectively. **B**. Autoradiograph showing NPTII activity in dot blots of some of the T_0 transformants, C. Result of paromomycin leaf spray on T_0 transformants showing the functional activity of *npt*II gene (Extreme left: control)

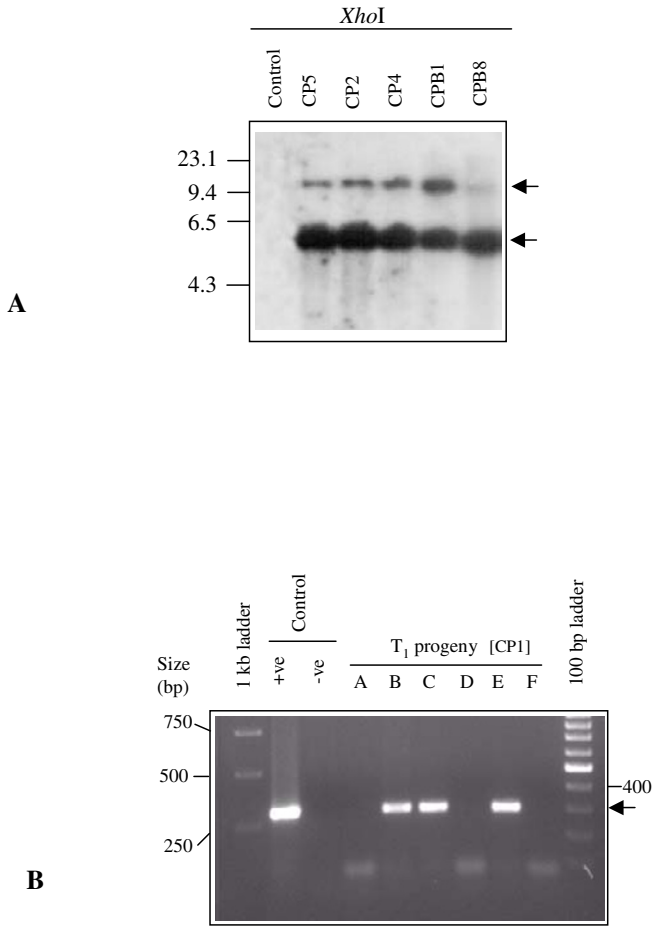

Figure 4

A. Southern analysis of some T_0 transformants of *Triticum aestivum* cv. CPAN1676 cotransformed with *gus* (pAct1-*F*) and *bar* (pDM302) by particle bombardment using mature embryo-derived calli. The genomic DNA of control and putative transformants (identities mentioned above) were digested with *Xho*I. Other details were similar to that described above. **B**. PCR screening of genomic DNA samples of T_1 progeny of *Triticum aestivum* cv CPAN1676 line CP1 using primers specific to *bar* gene. The plasmid pDM302 and DNA isolated from an untransformed plant were used as positive and negative controls, respectively.

of T_1 progeny using primers specific to *bar* gene, revealed the presence of transgene in three of the six progeny plants tested (Fig 4B). The results of PCR screening (after seven days) were found to be consistent with that observed with the leaf paint assay.

Introduction of HVA1 gene in T. aestivum and T. durum

The optimised transformation protocol was used for the introduction of a barley late embryogenesis abundant protein (*HVA*1), which is known to confer tolerance to water deficiency [32]. Mature embryo-derived calli from *T. aestivum* cv CPAN1676 and *T. durum* cv PDW215 were bombarded with plasmid pBY520. By employing previously optimized methodology for particle bombardment putative transformants were obtained in both *T. aestivum* and *T. durum* (Fig 2G&2H). Of the 258 and 287 calli cocultivated, 20 and 30 plantlets were obtained corresponding to a frequency of 7.7 and 10 %, respectively. Integration of *HVAI* gene into wheat genome was confirmed by using the *HVA1* coding region as the probe.

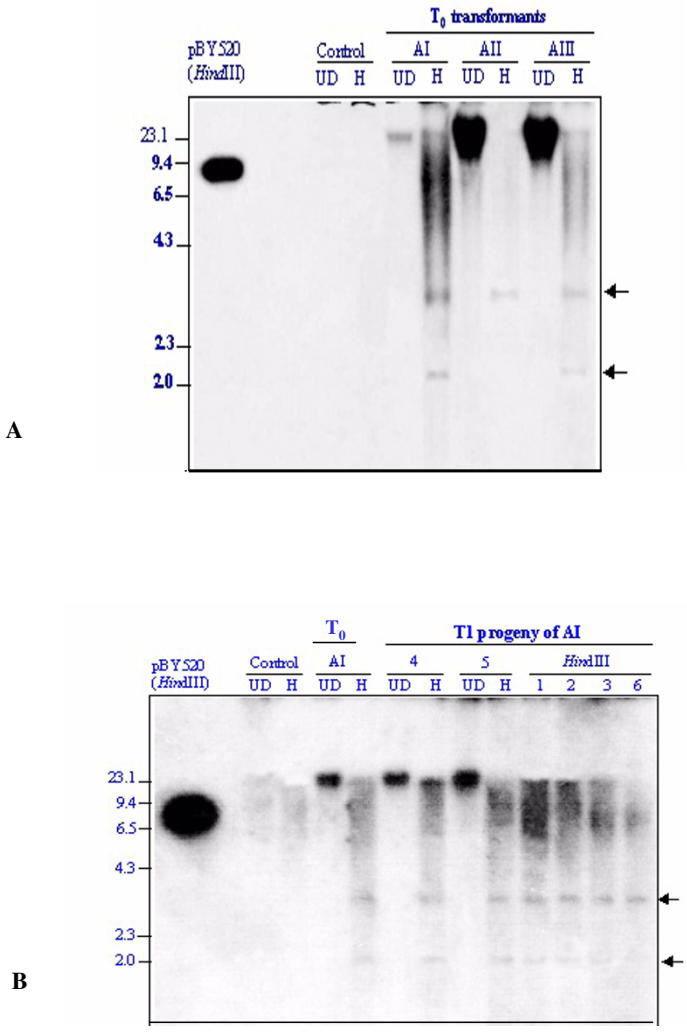

Figure 5

A & B. Southern analysis of T_0 and T_1 progeny of *Triticum durum* cv. PDW215 transformed with *HVA1* gene (pBY520) by particle bombardment using mature embryo derived calli as explants. The numerals on the top of the lanes indicate the designation of progeny plants. Hybridization was performed with 2 kb *HVA1* coding region-*pin2* terminator fragment (2 kb fragment obtained after digestion of pBY520 with *Hin*dIII and *Pst*I). UD: undigested, H: *Hin*dIII.

Genomic DNA of two representative plants of *T. aestivum* cv. CPAN1676 were digested with *Hin*dIII. The DNA blot of primary transformants of *T. durum* cv PDW215 was probed with a 2 kb fragment (pBY520) *HVA1* coding region-*pin2*-3' region terminator to confirm the integration of the transgene. The T_0 transformant AI (*T. durum*), was chosen for progeny analysis as it produced 16 seeds which appeared normal and comparable with the control seed samples. Genomic DNA of these plants was digested with *Hin*dIII and probed with P^{32} labelled *HVA1-pin2*-3' fragment. The hybridization pattern of most of these plants observed in the autoradiogram was similar to that of its T_0 parent (Fig 5A &5B) indicating the stability of the integrated genes.

Table 1: Abbreviation of various media used for wheat regeneration and transformation

Name	Composition
MSE	MS medium supplemented with 200 mg/l casein hydrolysate and 100 mg/l inositol
MSE2	MSE supplemented with 2 mg/l 2,4-D
MSER	MSE supplemented with 0.5 mg/l BA and 0.02 mg/l NAA
$MSE2P_5$	MSE supplemented with 2 mg/l 2,4-D and 5 mg/l phosphinothricin
$MSE2PM_{100}$	MSE supplemented with 2 mg/l 2,4-D and 100 mg/l paromomycin
$MSERP_{2.5}$	MSER supplemented with 2.5 mg/l phosphinothricin
$MSERPm_{50}$	MSER supplemented with 50 mg/l paromomycin

The overexpression of genes encoding for group 3 LEA proteins in transgenic plants has the potential to improve plant survival when subjected to environmental stresses. *HVA1* is a ABA-responsive gene isolated from *Hordeum vulgare* [12] which encodes a group 3 LEA protein and has been successfully used for engineering of rice [37] and wheat [32] for drought tolerance. The later group achieved high levels of expression of *HVA1* gene, regulated by maize ubiquitin promoter in leaves and roots of independent transgenic wheat plants and which were inherited to the offsprings. The T_3 generation when tested for tolerance to soil water deficit displayed improved growth characters under soil water deficit conditions. With respect to the tolerance conferred against drought conditions, in the present instance, experiments would be conducted in near future subsequent to a detailed molecular analysis of various transformants.

Conclusions

Durum wheat is considered to be more recalcitrant than bread wheat and so far only two reports of its transformation exist [2,11]. These two reports have also employed immature embryo-derived explants as the target tissue for delivery of marker genes [2], and high-molecular weight glutenin subunit (HMW-GS) genes [11]. In the present investigation, we have been able to successfully introduce marker genes into bread wheat and durum wheat with a high transformation frequency. The present study has also achieved the introduction of *bar* and *HVA*1 gene into *T. durum* by particle bombardment. All the transformant lines obtained in the present investigation displayed functional activity of selectable marker genes (*bar* and *npt*II genes). The genetic transformation methodologies employed are identical for bread and durum wheat, thus opening the possibility of extending this system to other genotypes as well. The present efforts are thus encouraging and further indepth analysis of the integration and segregation patterns for both *T. aestivum* as well as *T. durum* will pave way for the possibilities of engineering Indian bread and macaroni wheat with genes of agronomic importance.

Methods

Materials and Methods

Plant Materials and Culture Conditions

Seeds of Triticum aestivum cv. CPAN1676 and Triticum durum cv. PDW215 were obtained from Directorate of Wheat Research, Karnal, Haryana, India. Based on their in vitro differentiation response these cultivars were chosen for genetic transformation experiments. Mature embryos were excised from the surface sterilized seeds by a sterile blade and inoculated in petriplates containing MSE2 medium (Table 1). The explants were cultured for two weeks in dark at 26 ± 2°C, and maintained at 26 ± 20C under 16 h photoperiod with a light intensity of 100–125 mmoles $m^{-2}s^{-1}$ provided by fluorescent tube light (Philips India Ltd.). The regenerated calli were separated from the hardened scutellum for further subculture. The two-week-old calli were subcultured for one week on MSE2 and arranged in the centre of 90 mm petriplates prior to bombardment. The various media employed during subsequent phases of experiment are listed in Table 1.

Vectors

The plasmid vectors pDM302 [5] and pAct1-F [19] were employed for the delivery of *bar* and *gus* genes as selectable and scorable markers, respectively (Fig. 1). The plasmid vector pBY520 [37] has the barley *HVA*1 gene under the control of rice *Act1*-5' promoter and *pin2*-terminator; and *bar* gene as selectable marker under the control of 35S promoter and *nos* terminator. For construction of the binary vector pBI101::Act, the rice *Act1*-5' region was excised from the plasmid pDM302 as a 1.5 kb *Hind*III fragment and cloned in the vector pBI101 (Clontech). Plasmids were isolated on a large scale and purified by CsCl-EtBr density gradient centrifugation following standard protocols [30].

Particle Bombardment, Selection and Regeneration of Transformants

Microcarriers (1.1 μ gold particles) were prepared and coated with plasmid DNA according to the protocol by Sanford et al., [31]. The explants were bombarded twice at 1100 psi helium pressure and at a target distance of 6 cms with appropriate plasmid DNA coated microprojectiles by

employing the Biolistic PDS-1000/He Particle Delivering System (Biorad, USA) according to manufacturer's instructions. After 24 h of bombardment, the calli were transferred to $MSE2P_5$ or $MSE2Pm_{100}$ (see Table 1) and subcultured after two weeks. The calli were maintained for three weeks on 2,4-D containing selection medium ($MSE2P_5$ or $MSE2Pm_{100}$), and then transferred to regeneration medium ($MSERP_{2.5}$ and $MSERPm_{50}$) for another two weeks and to a selection free medium for another 7–10 days. The regenerating plantlets were finally transferred to half strength MS medium [20] supplemented with 2.5 mg/l phosphinothricin or 25 mg/l paromomycin. The regenerating plantlets were ultimately transferred to transfer plugs in seedling trays (Sigma). Rooted plantlets were transferred to pots containing a mixture of soilrite (Kel Perlite, Bangalore, India) and garden soil (1:1) and grown to maturity in growth chambers (Conviron, Control Environments Limited, Winnipeg, Canada) operating at 21/18°C at 16/8 h light/dark cycle. The plants were supplied with a liquid medium [15] recommended for growth of wheat plantlets.

GUS Histochemical Assay

The reporter gene activity was histochemically localized in the bombarded explants according to the protocol described by Jefferson et al, [14]. 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, pH 7.0; 50 mM EDTA, pH 7.0; 0.5 mM $K_3Fe(CN)_6$, 0. 5 mM $K_4Fe(CN)_6$, 0. 1 % Triton X-100, 1 mg/ml X-gluc (Amresco Inc., Ohio, USA). The explants were thoroughly washed with 70% ethanol prior to taking the observations by using a stereo zoom microscope (SMZU, Nikon).

nptII Assay

nptII functional assay

The functional assay of *nptII* gene was performed as described by Cheng et al., [6]. Wheat seedlings at the 3-leaf stage were sprayed with a solution of 2% (w/v) paromomycin and 0.1% Tween-20. Alternatively, a 1–2 cm section of leaf tips were painted by the paromomycin solution using a cotton bud. The response was observed after 7 days of paromomycin application.

nptII dot blot assay

The *npt*II dot blot assay was performed according to the protocol of Roy and Sahasrabuddhe [29]. To study the expression of *npt*II in leaf tissue, frozen samples (~50 mg) were homogenized in a microcentrifuge tube using liquid nitrogen and 200 µl extraction buffer (100 mM Tris-Cl, pH 7.0, 10 mM EDTA, pH 7.0; 0.1% Triton X-100). Protein quantification was done according to Bradford [3]. An aliquot of the crude extract containing 10 µg of protein was made up to 50 µl with extraction buffer and incubated with 100 µl of assay buffer [100 mM Tris-Cl, pH 7.4; 10 mM $MgCl_2$; 400 µg/ml kanamycin sulfate, 10 µM ATP, 10 µCi/ml (γ-^{32}P)ATP (3000 Ci/mmol specific activity, BRIT, India)] for 30 min at 20°C, after which they were blotted onto P81 phosphocellulose paper (Whatman Ltd, England) using a dot-blot apparatus (Schleicher and Schuell, Germany). The blot was wrapped in cling film and exposed to a X-ray film (Kodak, India) in Hypercassettes (Amersham, UK) at -20°C for a suitable duration.

Phosphinothricin Leaf Paint Assay

The progeny of transgenic plants with *bar* gene as the selectable marker were analysed by leaf paint assay. Leaf painting was performed as described by Lonsdale et al. [16]. A solution of phosphinothricin (150 mg/l) and 0.1% Tween-20 were applied to leaf sections for three times a week at a two day interval. Absence of necrotic damage as compared to controls was taken as evidence for the expression of *bar* transgene.

DNA Isolation and Southern Analysis

Total genomic DNA was isolated from wheat leaves according to Dellaporta et al. [9]. Ten to fifteen microgram of genomic DNA was digested with appropriate restriction enzyme (s) and resolved on a 1% agarose gel and blotted onto a nylon membrane (Hybond N, Amersham, UK). The blot was probed for the presence of *gus* and *bar* gene. The *gus* probe was excised as a *Bam*HI-*Sac*I fragment of pAct1F which spans the *gus* coding region, and the *bar* gene probe was derived from pDM302 as a ~0.6 kb *SmaI* fragment which spans the *bar* coding region. The probes were radiolabelled using Megaprime DNA Labelling kit (Amersham International Inc, UK) and [α-^{32}P)ATP (BRIT, Hyderabad, India) as per manufacturer's specifications. Hybridization was carried out for 16–24 h at 37°C with shaking at 40 rpm. The blot was washed in sequence, with the following solutions for 10 min each (i) 50% formamide, 5X SSC, 0.1% SDS; (ii) 2X SSC, 0.1% SDS; (iii) 1X SSC, 0.1% SDS; (iv) 0.5 XSSC, 0.1% SDS.

PCR Analysis of Genomic DNA

PCR analysis of genomic DNA was carried out using 200–300 ng 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 25 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. The forward and reverse primers employed for amplification of *bar* gene were 5'-ACC ATC GTC AAC CAC TAC ATC G-3' (*bar*5) and 5'-TCT TGA AGC CCT GTG CCT C-3' (*bar*3). The primers used for the detection of *gus* gene in the transformants were 5'-ATC AGC GTT GGT GGG

AAA GC-3' (*gus*5) and 5'-CAT TGT TTG CCT CCC TGC TG-3' (*gus* 3); and for the amplification of *npt*II gene were 5'-TCG GCT ATG ACT GGG CAC AAC AGA-3' (*npt*F) and 5'-AAG AAG GCG ATA GAA GGC GAT GCG-3' (*npt*R), respectively. The annealing temperatures for the amplification of *bar, gus* and *npt*II genes were 50°C, 53°C and 57°C respectively. The PCR products were run on 1.6% agarose gel in 1X TAE alongwith size markers (GeneRuler™ 1 kb ladder and GeneRuler™ 100 bp ladder plus, MBI Fermentas, USA).

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