Herbicide-resistant transgenics of bread wheat (*T. aestivum*) and emmer wheat (*T. dicoccum*) by particle bombardment and *Agrobacterium*-mediated approaches

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Hexaploid bread wheat (*Triticum aestivum*) and tetraploid emmer wheat (*T. dicoccum*) hold immense agricultural and economical importance as their end products have varied utilities depending upon the visco-elasticity and other properties of the flour. In the present study, highly regenerable basal segment calli have been employed as the target tissue for genetic transformation of Indian varieties of bread wheat (CPAN1676, PBW343) and emmer wheat (DDK1001). The *bar* gene conferring herbicide resistance was introduced in one-month-old calli employing both particle bombardment and *Agrobacterium*-mediated transformation strategies. Transgenic calli were selected on phosphinothricin-containing regeneration medium and putative transformants were raised to maturity. Though the plants exhibited reduced vigour in terms of height and tillering, nonetheless, seed set was normal. The presence of the transgene (*bar*) was confirmed by PCR and Southern hybridization. In general, the transformation efficiency was found better with *Agrobacterium,* even though the construct carried CaMV*35S* driven *bar* gene, whereas in the case of biolistics *Ubi1* (a monocot promoter)-driven *bar* gene was employed. Transformation efficiency in the range of 4% was obtained with particle bombardment, whereas it was 7.5% using *Agrobacterium*-mediated co-cultivation. The different varieties of bread and emmer wheat investigated did not show any marked difference in their transformation ability and could be attributed to a well-established regeneration system in these varieties.

AMONGST various cereal grains, wheat is an important ingredient of the human diet. Different varieties of wheat, depending upon their dough quality and other visco-elastic characteristics, are used for making a range of food products. Modern-day hexaploid wheat (*Triticum aestivum*) is used generally for bread-making, but lacks certain agronomically important features such as drought or disease resistance. Tetraploid emmer wheat (*Triticum dicoccum*), in contrast, shows high adaptability to dry, parched lands and exhibits drought and salinity resistance; but its poor visco-elastic properties and hardness

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of the grain reduce its consumer acceptability. Thus both bread and emmer wheat possess mutually exclusive but economically desirable qualities. With the advent of transgene technology and practical amenability of cereals it should be possible to introduce advantageous traits in different wheats to augment their nutritive value and consumer acceptance.

Tissue-culture procedures in wheat have been generally developed for embryogenic explants such as mature embryos, immature embryos and scutella. These explants and their calli are now regularly employed as the target tissue for various transformation strategies due to their high regeneration efficiency[1,2]. In the present investigation, basal segment calli raised from five-day-old seedlings, previously standardized for efficient regeneration, have been employed for the introduction of herbicide resistance in both hexaploid and tetraploid Indian wheat (CPAN1676, PBW343 and DDK1001 respectively). Resistance to L-phosphinothricin, glyphosinate and bialaphos has been successfully used earlier to select transgenic calli and plants in wheat[1,2]. Also use of *bar* gene, besides providing an efficient selection strategy, renders transformed plants with a useful agronomic trait. In the present study, the regenerative basal segment calli have been employed for introduction of herbicide resistance in the selected Indian cultivars based on their regeneration potential. A comparison has been attempted by particle bombardment and *Agrobacterium*-mediated transformation protocols and presence of transgene confirmed by PCR and Southern hybridization in the primary transformants.

T. aestivum var. CPAN1676, PBW343 and *T. dicoccum* var. DDK1001 were procured from Directorate of Wheat Research, Karnal, India. Seeds were surface-sterilized with 4% v/v hypochlorite and washed with water thrice prior to use. Five-day-old seedlings were raised on callusing medium [MS + 2,4-D (2 mgl^{-1}) + kinetin (0.4 mgl^{-1})] under discontinuous light (18 : 6 h light/dark cycle, 26 ± 1°C). Primary callus, roots along with the seeds were removed carefully and basal segments (~ 2 mm) of the shoot were excised and re-inoculated on callusing medium. One-month-old basal segment calli were employed for all the transformation studies.

One-month-old calli were bombarded with pAHC25 (ref. 3; the 9.7 kb plasmid carries *Ubi 1* promoter-driven *bar* gene and *GUS* reporter gene) coated gold particles of 1 micron size. Particle preparation was carried out according to the manufacturer's instructions (Biorad, USA). Calli were precultured on medium supplemented with 0.2 M mannitol and 0.2 M sorbitol for 4 h prior to shooting. They were double bombarded at 6 cm barrel distance with 1100-psi rupture disks. Control cultures were the calli bombarded under similar conditions, but without DNA-coated macrocarriers. The calli were transferred to callusing medium without osmoticum after 24 h. Transient GUS histochemical assay was performed after 48 h of bombardment according to Jefferson[4].

The bombarded cultures were subjected to the following stages of selection. In the first selection phase, the calli, after 48 h of bombardment, were transferred to regeneration medium [MS + $AgNO_3$ (10 mgl^{-1}) + TIBA (1 mgl^{-1}) + kinetin (0.4 mgl^{-1})] containing 3.5 mgl^{-1} L-phosphinothricin for at least two weeks. Calli surviving the first phase of selection were transferred further on regeneration medium supplemented with 1.5 mgl^{-1} phosphinothricin for a week. The final round of selection was optional and plantlets were kept on 2 mgl^{-1} phosphinothricin for one week. The regenerating calli, after these stages of selection, were transferred to selection free regeneration medium for further growth. The cultures were maintained at 26 ± 1°C, discontinuous light throughout the selection procedure.

Among the various CAMBIA vectors[5] available, pCAMBIA3301 conferring herbicide resistance to the plants was chosen for *Agrobacterium*-mediated transformation of the basal segment calli. The construct carries *bar* and *GUS* reporter genes driven by CaMV*35S* promoter. This binary vector was mobilized in the *Agrobacterium* host strain LBA4404 by triparental mating. Bacterial culture of LBA4404 (pCAMBIA3301) was initiated by inoculating 50 µl of glycerol stock in 25 ml YEP medium supplemented with 100 µg/ml rifampicin, 50 µg/ml kanamycin sulphate and 200 µM acetosyringone. The cultures were kept at 28°C, 200 rpm for 24–48 h. For callus transformation, 100 µl of freshly-grown culture was inoculated in Erlenmeyer flasks containing 25 ml YEP medium supplemented with appropriate antibiotics and 200 µM acetosyringone. The cultures in the log phase with OD_{600} 0.25 to 1.00 were pelleted in SS-34 tubes at 4000 rpm, 4°C for 10 min. Bacterial density was calculated and cell density was adjusted to 5×10^8 cells/ml with liquid MS medium supplemented with 200 µM acetosyringone. Basal segment calli were incubated in the bacterial suspension for 1 h. After blotting on Whatman No. 1, the calli were placed on callusing medium supplemented with 200 µM acetosyringone for two days (co-cultivation period) in dark at 28°C.

After the co-cultivation period, explants were transferred to 100 ml conical flasks and washed in liquid MS medium 3–4 times. The final wash was given with liquid medium supplemented with bacteriostatic 125 mgl^{-1} cefotaxime. Explants were blotted on sterile Whatman No. 1 and then transferred to callusing medium containing 125 mgl^{-1} cefotaxime medium. After three days, the explants were tested for GUS expression by the histochemical assay[4]. The proliferating calli were transferred to regeneration medium containing 1.5 mgl^{-1} phosphinothricin for a week. In the second phase of selection (for two weeks), the concentration of phosphinothricin was raised to 3.0 mgl^{-1}. During the third phase of selection (optional), the regenerating explants were subjected to selection with 1.5 mgl^{-1} phosphinothricin for ten days. The surviving

calli were transferred to selection-free regeneration medium for another one week.

The regenerated plantlets obtained via particle bombardment or *Agrobacterium*-mediated approach were finally transferred to transfer plugs (Sigma Chemicals Co, USA) and placed in seedling trays containing wheat growth medium[6] for 10–12 days for a well-developed root system. Rooted plantlets were transferred to pots containing 1 : 1 mixture of soilrite and garden soil and grown till maturity in a growth chamber maintained at 21°C at 16/8 h light/dark cycle and 75% relative humidity.

PCR amplification of genomic DNA samples (isolated according to Dellaporta *et al.*[7]) of the putative transformants was performed using PCR kit (MBI Fermentas, USA) and *bar*-specific primers (bar5-5′-ACCATCGTC-AACCACTACATCG-3′, bar3-5′-TCTTGAAGCCCTGT-GCCTC-3′). The thermal cycler (PCR system 2400, Perkin–Elmer) was programmed for initial denaturation at 94°C for 5 min followed by 25 cycles of amplification. A cycle consisted of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 7 min. The length of the product was checked on an agarose gel.

For Southern hybridization; genomic DNA was isolated from 100 to 200 mg leaf material of the putative transformants following the protocol of Dellaporta *et al.*[7]. Then 20–22 μg of genomic DNA of putative transformants was either double digested with *Eco*RI and *Bam*HI (in case of biolistically obtained plants) or digested with *Xho*I (*Agrobacterium*-mediated transformation) and fractionated on 1% agarose gel. The DNA was capillary-blotted onto Hybond-N membrane (Amersham International Inc., UK) and incubated in prehybridization solution (50% formamide, 5X Denhardt's reagent, 5% dextran sulphate, 5X SSC, 200 μg/ml Herring sperm DNA) for one day at 37°C with shaking at 40 rpm. The probe was purified *Pst*I fragment of pAHC20 (ref. 3) (carries *Ubi*1-driven *bar* gene) excised from low-melting agarose gel and radiolabelled using Megaprime labelling kit (Amersham International Inc., UK) and (α^{32} P) ATP (BRIT, India), according to manufacturer's specifications. The radiolabelled probe was added to the hybridization solution (same composition as prehybridization solution but with 10% dextran sulphate and 250 μg/ml of Herring sperm DNA) for two days at 37°C and at 40 rpm. The blot was washed and exposed to X-ray film in hypercassettes at – 80°C for the required time. The autoradiogram was developed and fixed using Kodak Affiliate Products, Mumbai, India.

Despite the extensive use of herbicides, productivity losses in wheat due to weeds amount to nearly 12.3%. Weeds commonly found in wheat fields are known for their high degree of resistance to the commonly used herbicides. Therefore, Basta and Glyphosate have been suggested as more suitable herbicides for weed control[8]. Resistance of wheat towards Basta has the potential to provide substantial relief from yield losses caused by weeds. In the earlier reports, *bar*-resistant plantlets have been obtained by employing either immature embryos or the scutellar tissue[1,2]. The present investigation tests the amenability of the basal segment cultures as a recipient explant for herbicide resistance encoded by the *bar* gene and compares transformation efficiency by particle bombardment and *Agrobacterium*-mediated transformation approaches.

One-month-old basal segment calli were bombarded with pAHC25 (ref. 3) using the parameters mentioned earlier. The bombarded calli were subjected to two or three rounds of selection, initially at 3 mgl^{-1} PPT followed by 1.5 mgl^{-1} PPT for two weeks and one week respectively (Figure 1 *c*, *d*), and finally on a selection-free medium. At maturity (Figure 1), although the number of tillers produced by the transformants was similar to the normal untransformed control plants, they showed reduced height and the number of seeds produced per inflorescence was also less (5–7 seeds). The transformation efficiency (Table 1) of the two genotypes was recorded as the number of regenerating plantlets/total number of explants bom-

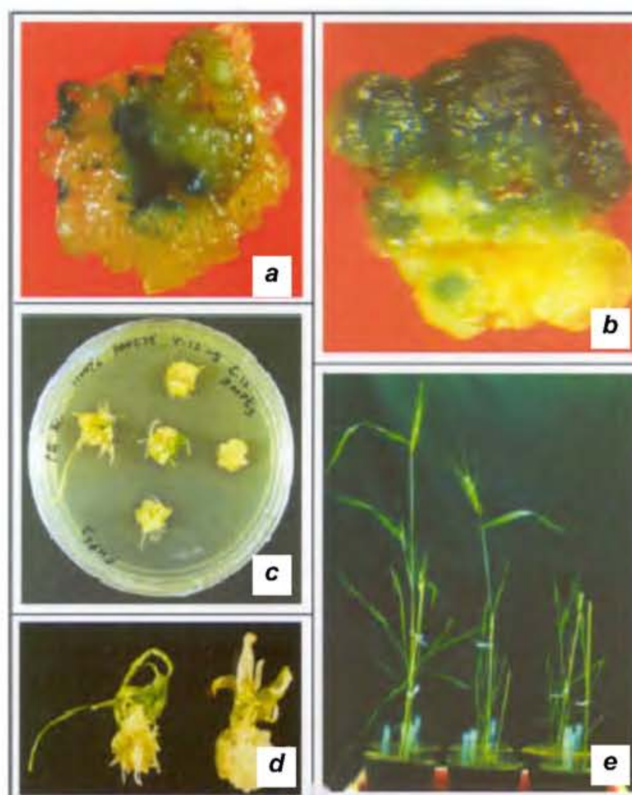

Figure 1. Transformation of wheat (*T. aestivum* var. CPAN1676) basal segment calli for herbicide resistance. ***a***, GUS histochemical localization in the callus at 48 h post-bombardment. Calli were bombarded with pAHC25 that carries *bar* gene as the selection marker; ***b***, Callus showing GUS expression after two days of co-cultivation with *Agrobacterium* LBA4404 (pCAMBIA3301) followed by washings and transfer of cultures to MS medium supplemented with 125 mgl^{-1} cefotaxime for three days; ***c***, Explants kept under first stage selection (1.5 or 3 mgl^{-1} phosphinothricin); ***d***, Transformants growing in second stage of selection (1.5 or 3 mgl^{-1} phosphinothricin); ***e***, Mature, putative transformants at seed-set stage.

barded, according to Takumi and Shimada[9]. Although CPAN1676 (*T. aestivum*) exhibited maximum transformation efficiency (4.4%), 80% of the transformed plants of PBW343 were fertile. In case of *T. dicoccum*, 4.3% transformation efficiency was noted with 33.33% fertility (Table 1).

Presence of the transgene '*bar*' in the primary transformants was confirmed by PCR amplification of genomic DNA samples using *bar*-specific primers. Plasmid pAHC25 was used as a positive control and the genomic DNA of an untransformed plant served as the negative control. Along with the positive control, a 296 bp amplicon was observed in all the transgenic lines tested but not in the untransformed, negative control (Figure 2 *a*).

For Southern analysis of T_0 transformants of *T. aestivum* and *T. dicoccum*, genomic DNA was double-digested with *Eco*RI and *Bam*HI and probed with *Pst*I-fragment of pAHC20 (~ 603 bp) spanning the *bar* coding region. Southern analysis revealed the presence of a 860 bp band (*bar* coding region and the *nos* 3′ terminator which is excised upon double digestion with *Eco*RI and *Bam*HI) in all the independent transformants (Figure 2 *b*). The transformation efficiency reported by previous workers generally varies from 0.1 to 5.7% (refs 1, 2 and 10). Uze and co-workers[11] have, however, reported 14% transformation efficiency of the wheat embryonic tissue via biolistics using a single-stranded linearized DNA. In the present study, a transformation efficiency of approx. 4% has been achieved, which is comparable to that obtained by earlier workers, and is significant since the recipient tissue is of non-embryogenic origin.

Methods have been developed for efficient *Agrobacterium*-mediated transformation of rice[12], maize[13], barley[14] and sorghum[15]. Cheng and co-workers[16] described the production of normal, fertile transgenic wheat plants following incubation of immature embryo and embryogenic calli with *Agrobacterium tumefaciens*. Though few reports are available on *Agrobacterium*-mediated stable wheat transformation, preliminary reports have demonstrated the interaction of the bacterium with wheat tissues, as observed in the scanning electron micrographs[17,18]. The suitability of various explants for *Agrobacterium*-mediated gene delivery and factors influencing *Agrobacterium*-mediated introduction of transgene have been extensively investigated[19,20]. Although rapidly dividing tissues are more amenable to *Agrobacterium* invasion[21] paradoxically, meristematic basal segment cultures with actively dividing cells have been rarely exploited for their transformation potential. In the present study, the amenability of the basal segment calli as competent explants for *Agrobacterium*-mediated wheat transformation has been investigated by introducing the herbicide resistance gene '*bar*'.

The basal segment calli were thus incubated with LBA4404 (pCAMBIA3301) for 1 h along with the phenolic inducer, acetosyringone (200 μM). A cell density of 5×10^8 bacterial cells/ml and co-cultivation period of two days were found optimum in terms of transient GUS expression (Figure 1 *b*) as well as callus survival. At higher concentrations or co-cultivation periods, GUS expression was high in all the varieties, but due to overgrowth of the bacteria the callus showed necrosis and ultimately death.

Initially, low concentration of PPT (1.5 mgl^{-1}) was supplied in the regeneration medium. In the second phase the concentration of PPT was raised to 3 mgl^{-1}. A third round of selection was optional before final transfer to garden pots for maturity (Figure 1). Interestingly, PBW343, a high-yielding Indian variety of *T. aestivum* showed maximum transformation efficiency (8.7%) as well as 100% fertility (Table 1). The other varieties, CPAN1676 of *T. aestivum* and DDK1001 of *T. dicoccum* exhibited a similar range of transformation efficiency (6.7 and 6.9% respectively). However, in general, the transformation efficiencies were comparable to the transgenics obtained

Table 1. Introduction of *bar* gene in *T. aestivum* and *T. dicoccum* via particle bombardment and *Agrobacterium*-mediated transformation

Plant material	No. of explants bombarded	No. of plants regenerated	No. of plants established in soil	No. of fertile plants	Transformation efficiency (%)
*Particle bombardment**					
T. aestivum var. CPAN1676	181	8	7	3	4.4
T. aestivum var. PBW343	137	5	5	4	3.6
T. dicoccum var. DDK1001	208	9	6	2	4.3
*Agrobacterium-mediated***					
T. aestivum var. CPAN1676	222	15	3	2	6.7
T. aestivum var. PBW343	251	21	4	4	8.7
T. dicoccum var. DDK1001	187	13	4	1	6.9

*Calli were bombarded at 1100 psi and 6 mm barrel distance with pAHC25 coated on gold particles (1 micron).
**Calli were co-cultivated with LBA4404 (pCAMBIA3301) for two days followed by washings and transfer to regeneration medium supplemented with 125 mgl^{-1} cefotaxime. After three days, calli were subjected to selection (1.5–3 mgl^{-1} phosphinothricin).

employing mature embryos as explants for co-cultivation, and better than the immature embryos and embryogenic calli (4%)[16].

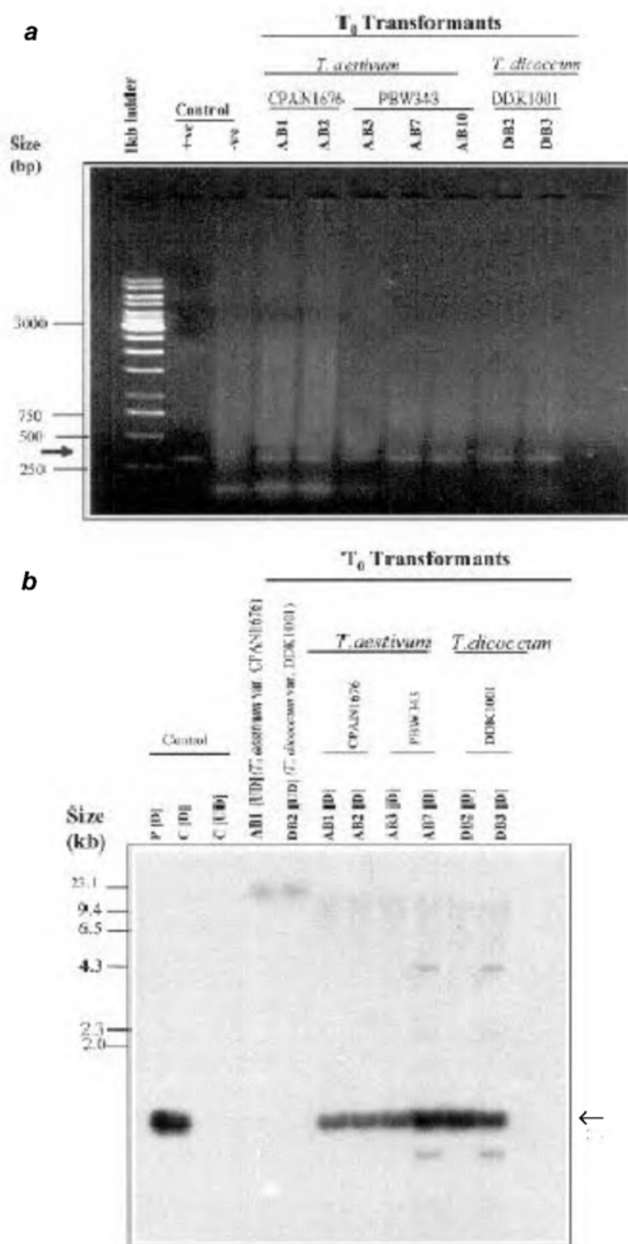

Figure 2. ***a***, PCR analysis of T_0 transformants of *T. aestivum* var. CPAN1676, PBW343 and *T. dicoccum* var. DDK1001 using primers specific to *bar* gene. Transformants were obtained by particle bombardment of the basal segment calli with pAHC25. Plasmid and genomic DNA isolated from untransformed plant were used as positive and negative controls respectively. ***b***, Southern analysis of T_0 transformants of *T. aestivum* var. CPAN1676, PBW343 and *T. dicoccum* var. DDK1001 obtained by particle bombardment with pAHC25. Lane 1 (from left), Positive control, P [D]; lanes 2 and 3, Digested [D] and undigested [UD] genomic DNA of a control *T. aestivum* var. CPAN1676 untransformed plant; lanes 4 and 5, Undigested genomic DNA of putative transformants of *T. aestivum* and *T. dicoccum* respectively; lanes 6–11, Double-digested (*Eco*RI + *Bam*HI) genomic DNA of transformed lines. Southern blot was probed with 600 bp '*bar*' fragment obtained by *Pst*I digestion of pAHC20. Size (kb) represents the fragment size of λ (*Hin*dIII digest) marker. A 840 bp band was obtained in all the transformants.

Presence of the *bar* gene was confirmed by Southern hybridization of the putative transformants digested with *Xho*I and probed with *bar* fragment (600 bp) of pAHC25 obtained by *Pst*I digestion. The autoradiogram revealed an expected band of ~ 560 bp in all the independently transformed transgenic lines (Figure 3).

Comparison of the transformation frequencies obtained in the present study for *bar* gene introduction by *Agrobacterium*-mediated transformation with that of biolistics-mediated gene transfer indicates the utility of the agrobacterial approach over the biolistics one. This is not unexpected, but highly desirable in crop plants. Not only are the transformation efficiencies higher with *Agrobacterium* (6.7–8.3%) than biolistics (3.6–4.4%), the fertility of the transformants is also higher (7/11 = 63.6% vs 9/18 = 50%, respectively), even though *bar* gene was dri-

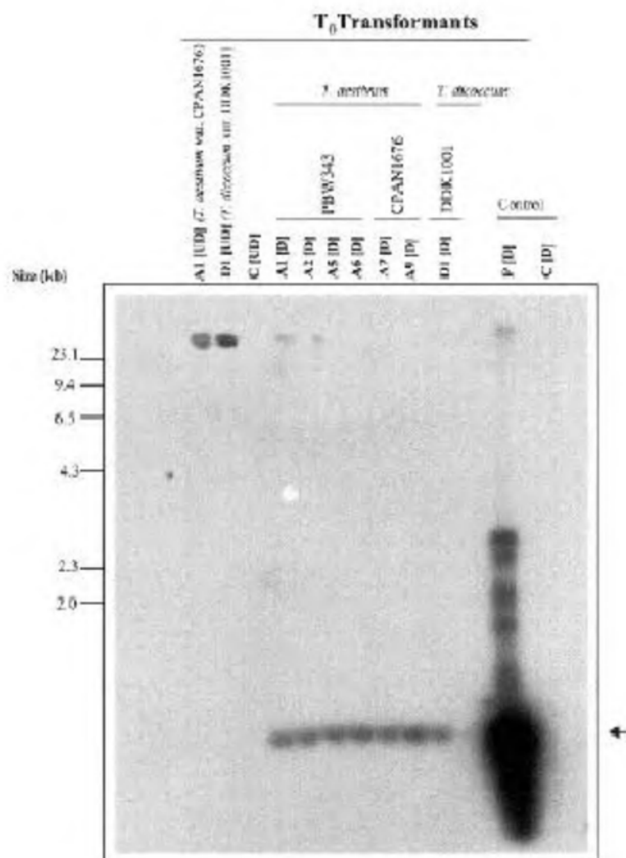

Figure 3. Southern analysis of T_0 transformants obtained by *Agrobacterium*-mediated transformation of one-month-old basal segment calli co-cultivated with LBA4404 (pCAMBIA3301). Lane 1 (from right), *Xho*I digested [D] genomic DNA from a control, untransformed plant; lane 2, Digested positive control; lanes 4–10, *Xho*I digested genomic DNA of *T. dicoccum* and *T. aestivum* with the varieties mentioned at the top; lane 11, Undigested [UD] genomic DNA of control (*T. aestivum* var. CPAN1676), untransformed plant; lanes 12 and 13, undigested genomic DNA sample of *T. dicoccum* and *T. aestivum* respectively. The Southern blot was probed with 600 bp fragment coding for *bar* gene obtained on *Pst*I digestion of pAHC20. Autoradiogram shows presence of ~ 560 bp band coding for *bar* gene in the transformed plants. Size (kb) represents the fragment size of λ (*Hin*dIII digest) marker.

ven by CaMV*35S* promoter during *Agrobacterium* co-cultivation, whereas for particle bombardment the gene was driven by the monocot promoter *Ubi1*. The difference in the ploidy level of bread and emmer wheat did not appear to interfere with the transformation efficiency as both varieties exhibited similar transformation efficiency with microprojectile or *Agrobacterium*-mediated transformation approaches. Differences in transformation efficiencies between the two different varieties of *T. aestivum* obtained by either of the strategies are not significant. There are few reports describing the transformation of Indian wheat cultivars, some of which have been demonstrated to be highly regeneration-dependent[22,23]. Thus, high transformation frequencies obtained in the present study may be attributed to a well-standardized regeneration system for these species and varieties. The choice of a vegetative tissue is also advantageous and intentional due to its year-round, non-seasonal availability. The present work thus paves the way for introduction of other agriculturally desirable traits in commercially popular Indian wheat types.

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