

Agrobacterium-mediated transformation of mature embryos of *Triticum aestivum* and *Triticum durum*

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Plant regeneration studies in cereals have been undertaken in immature embryos, scutellum and also in immature inflorescence tissue. The wheat mature embryos can also be employed for callusing and regeneration, as they are available throughout the year and have presently been employed for transformation studies. An efficient and reproducible method for *Agrobacterium*-mediated transformation of mature embryos of hexaploid bread wheat (*Triticum aestivum*) and tetraploid pasta wheat (*Triticum durum*) is reported. Presence of acetosyringone at 200 μ M concentration in the bacterial growth medium, inoculation medium and co-cultivation medium was essential for achieving a 1.5–2.0 fold increase in transient expression of the introduced *gus* gene. Successful generation of *T. aestivum* and *T. durum* transgenic plants at a transformation frequency ranging from 1.28 to 1.77% has been achieved following 2–3 days co-cultivation using mature embryos and also mature embryo-derived calluses with binary *Agrobacterium* strain LBA4404 (pBII101:: *Act1*) and LBA4404 (p35SGUSINT) respectively. Paromomycin and phosphinothricin served as effective selection agents as they did not adversely affect plantlet regeneration. Successful integration as well as inheritance of the transgene was confirmed by Southern hybridization and PCR amplification in T₀ as well as T₁ generation. Optimization of this method facilitated the introduction of *bar* gene as a selectable marker conferring herbicide resistance as well as potato proteinase inhibitor gene (*pin2*) for insect resistance into wheat.

Keywords: *Agrobacterium*, embryos, transformation, *Triticum aestivum*, *Triticum durum*, wheat.

GENETIC transformation of crop plants by *Agrobacterium*-mediated co-cultivation is an efficient and cost-effective method for gene delivery. Monocotyledonous plants, including important cereals were earlier thought to be recalcitrant to *Agrobacterium*-mediated gene transfer^{1,2}, but the scenario has changed in the last few years. Consistent efforts by researchers on cereal crop plants have resulted in the development of protocols for efficient gene delivery via *Agrobacterium* into rice^{3,4}, maize⁵, barley⁶ and wheat⁷.

One of the key points in these protocols has been the use of actively dividing cells/tissues such as immature embryos and immature embryo-derived calluses that were further co-cultivated with *Agrobacterium* in the presence of potent inducers of virulence genes⁸. Mooney and coworkers⁹ were the first to demonstrate the wound-independent *in vitro* attachment of *Agrobacterium* to wheat embryos. Subsequently, Chen and Dale¹⁰ reported a higher frequency of infection by incubation of exposed apical meristems of dry wheat seeds with *Agrobacterium*. Previous work from this laboratory also reported the transient expression of *gus* gene in meristematic leaf bases, calluses, mature seeds and mesocotyl punctured seedlings following co-cultivation with different strains and vectors of *Agrobacterium tumefaciens*¹¹. Stable *Agrobacterium*-mediated transformation of wheat and transmission of the transgenes to subsequent generations have now been reported by many workers^{7,12–15}. Nonetheless, the widescale application of this methodology in diverse genotypes is still restricted.

The present study thus focuses on the use of excised mature embryos and mature embryo-derived calluses as primary explants for *Agrobacterium*-mediated transformation of bread wheat (*Triticum aestivum*) and also the macaroni wheat (*Triticum durum*). The optimized protocol was subsequently used for the introduction of potato proteinase inhibitor (*pin2*) gene into *T. aestivum* and *T. durum*, the successful use of which had conferred insect resistance in *japonica* rice¹⁶. The use of proteinase inhibitors for genetic engineering of insect resistance in wheat has also been reported¹⁷. Here we report *Agrobacterium*-mediated transformation of *T. aestivum* and *T. durum* using mature embryos as recipient explants.

Materials and methods

Plant materials and culture conditions

Seeds of *T. aestivum* cvs HD2329, CPAN1676, PBW343 and *T. durum* cvs PDW215, PDW233 and WH896 were obtained from IARI, New Delhi as well as the Directorate of Wheat Research, Karnal, Haryana, India. Seeds were initially washed with a liquid detergent (Teepol, Reckitt & Coleman of India) for 1 min with running tap water, and

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Table 1. Abbreviations of various media used for wheat regeneration and transformation

Medium	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 2.22 μ M BA and 0.1 μ M NAA
MSE2As	MSE2 supplemented with 200 μ M acetosyringone
MSE2Cef ₂₅₀	MSE2 supplemented with 250 μ g/ μ l cefotaxime
MSE2P ₅	MSE supplemented with 2 mg/l 2,4-D and 5 mg/l phosphinothricin
MSE2P ₅ Cef ₂₅₀	MSE-2P ₅ supplemented with 250 mg/l cefotaxime
MSE2Pm ₁₀₀	MSE supplemented with 2 mg/l 2,4-D and 100 mg/l paromomycin
MSE2Pm ₁₀₀ Cef ₂₅₀	MSE-2Pm ₁₀₀ supplemented with 250 mg/l cefotaxime
MSERP _{2.5}	MSER supplemented with 2.5 mg/l phosphinothricin
MSERPm ₅₀	MSER supplemented with 50 mg/l paromomycin
MS _{1/2} P _{2.5}	MS half supplemented with 2.5 mg/l phosphinothricin

surface-sterilized with absolute ethanol for 30 s followed by 4% (v/v) sodium hypochlorite for 30 min. The seeds were thoroughly washed in sterile water prior to mature embryo excision in a laminar flow hood. Mature embryos were excised by removing the endosperm part from the surface-sterilized caryopses with a sterile blade.

MS medium¹⁸ supplemented with 200 mg/l casein hydrolysate and 100 mg/l inositol designated as MSE¹⁹, was used in this investigation. The pH of the culture medium was adjusted to 5.8 using 1.0 M NaOH/HCl. After adjustment of pH, Phytigel (0.4%) was added as the gelling agent (Sigma, USA) and autoclaved at 121°C and 1.08 kg/cm² pressure for 15 min. Mature embryos along with the attached scutellum were placed on MSE2 medium with the embryo axis side facing downwards, as precocious germination of mature embryos was drastically reduced when the embryos were placed so. The explants were cultured on MSE2 for three weeks in dark at 26 \pm 1°C with regular subculturing at weekly intervals. The different media used are listed in Table 1.

Bacterial strain and plasmid vectors

A. tumefaciens strain²⁰ LBA4404 and the binary vectors (p35SGUSINT, pBI101::Act, and pCAMBIA3301::pin2) were employed for *Agrobacterium*-mediated transformations. For evaluating the parameters involved in *Agrobacterium*-mediated transformations, the constructs pCAMBIA1301, pCAMBIA2301 and pCAMBIA3301 were also used. For construction of the binary vector pBI101::Act1, the rice Act1-5' region was excised from the plasmid²¹ pDM302 as a 1.5 kb HindIII fragment and cloned in the vector pBI101 (Clontech). For the construction of pCAMBIA3301::pin2, the expression cassette of the potato proteinase inhibitor (pin2) gene was excised as a 3.0 kb PstI fragment from the vector pTWa 16 and cloned in pCAMBIA3301 (ref. 22). The binary vector²³ p35SGUSINT was isolated from the *Agrobacterium* strain GV2260 and mobilized by triparental mating^{24,25} into *A. tumefaciens* strain LBA4404.

Transformation

The bacterial cultures were initiated by inoculating 50 μ l of glycerol stock in 30 ml LB + medium (LB medium supplemented with 0.5% glucose) with 100 μ g/ml rifampicin, 100 μ g/ml kanamycin sulphate and 200 μ M acetosyringone. The cultures were incubated at 28°C/200 rpm. For plant transformation, bacterial cultures with A₆₀₀ (absorbance at 600 nm) value of 0.5 to 1.00 were chosen. The excised mature embryos or three-week-old mature embryo-derived calluses were inoculated by immersion in bacterial suspension and incubating for 1 h after which the explants were transferred to MSE2As medium. Co-cultivation was performed by incubating the petri plates at 28°C in dark for 2–3 days.

Selection and regeneration of transformants

After 2–3 days of co-cultivation, the explants were washed in liquid MSE2Cef₂₅₀ to remove *Agrobacterium* and after several washes, placed on sterile blotting sheets to remove excess moisture and transferred to MSE2Cef₂₅₀ medium supplemented with 100 mg/l paromomycin (for LBA4404 (pBI101:Act1) and LBA4404 (p35SGUSINT)) or 5 mg/l phosphinothricin for LBA4404 (pCAMBIA3301::pin2). The mature embryo explants were cultured on MSE2Pm₁₀₀Cef₂₅₀ or MSE2P₅Cef₂₅₀ for two weeks. Explants showing callusing on the selection medium were transferred to fresh MSE2Pm₁₀₀Cef₂₅₀ or MSE2P₅Cef₂₅₀ medium and kept for ten days. The mature embryo-derived calluses were also cultured on MSE2Pm₁₀₀Cef₂₅₀ or MSE2P₅Cef₂₅₀ for three weeks with one subculture in between.

For regeneration, the explants were transferred to MSER medium supplemented with 2.5 mg/l phosphinothricin or 50 mg/l paromomycin. After 10 days on MSERP_{2.5} or MSERPm₅₀, the explants were transferred to selection-free regeneration medium and after another 10 days, the regenerated plantlets were transferred to MS_{1/2} supplemented with 2.5 mg/l phosphinothricin or 50 mg/l paromomycin for shoot elongation. The regenerated plantlets were

transferred to transfer plugs (Sigma) and maintained for nearly ten days till the root system established firmly. 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 (Convicon, Control Environments Limited, Winnipeg, Canada) operating at 21–18°C at 16/8 h light/dark cycle. The plants were supplied with a liquid medium²⁶ recommended for growth of wheat plantlets.

Enzyme assay

GUS assay: The *gus* reporter gene activity was either localized histochemically in the explants or quantitatively determined by spectrofluorometric method according to the protocol described by Jefferson *et al.*²⁷. 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; 0.5 mM K₃Fe(CN)₆, 0.5 mM K₄Fe(CN)₆, 0.1% Triton X-100, 1 mg/ml X-gluc (Amresco Inc., Ohio, USA)). The explants were washed thoroughly with 70% ethanol prior to observations using a stereo-microscope (Nikon, SMZU).

For quantitative estimation of *gus* activity, samples were incubated with 300 µl of GUS assay buffer (1.0 mM MUG in GUS extraction buffer) at 37°C for 15 h and 900 µl of stop buffer (0.2 M Na₂CO₃) added. Relative fluorescence of the samples was measured in Silica quartz cuvettes, employing a RF540 spectrofluorophotometer (Shimadzu, Japan) at an excitation wavelength of 365 nm and emission wavelength of 455 nm. The fluorometric units were converted into amount of 4-MU using a calibration curve. Specific activity was expressed in terms of pmol or nmol of 4-MU mg protein/h. Any GUS activity observed in controls was deducted to obtain the final values.

***NptII* dot blot assay:** The *nptII* dot blot assay was performed according to the protocol of Roy and Sahasrabudhe²⁹. The blot was wrapped in cling film and exposed to an X-ray film (Kodak, India) in Hypercassettes (Amersham, UK) at –20°C for 2–3 days depending on the counts. The X-ray film was developed to check for *nptII* activity in the form of intensity of the dots.

***NptII* functional assay:** The functional assay of *nptII* gene was performed²¹ on wheat seedlings at the three-leaf stage by spraying with a solution of 2% (w/v) paromomycin and 0.1% Tween-20. Alternatively, 1–2 cm sections of leaf tips were painted with paromomycin solution using a cotton bud. The response was observed after seven days of paromomycin application. Plants with a functional *nptII* gene showed little or no damage. However, plants lacking a functional *nptII* gene were identified by the presence of bleached spots throughout the leaf, which subsequently affected their survival.

Phosphinothricin leaf-paint assay: The progeny of transgenic plants with *bar* gene as the selectable marker was analysed by leaf-painting assay. Leaf painting was performed as described by Lonsdale *et al.*³⁰ A solution of phosphinothricin (150 mg/l) and 0.1% Tween-20 was applied to leaf sections, three times a week at two-day intervals. 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.*³¹. The probe was radio-labelled using Megaprime DNA Labelling kit (Amersham International Inc. UK) and (α-³²P) ATP (BRIT, Hyderabad, India) according to 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 and (iv) 0.5 X SSC, 0.1% SDS.

PCR analysis

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 according to manufacturer's instructions. 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 the amplification of *nptII* gene were 5'-TCG GCT ATG ACT GGG CAC AAC AGA-3' (*nptF*) and 5'-AAG AAG GCG ATA GAA GGC GAT GCG-3' (*nptR*) respectively. Primers used for the detection of *bar* gene are 5'-ACC ATC GTC AAC CAC TAC ATC G-3' (*bar5*) and 5'-TCT TGA AGC CCT GTG CCT C-3' (*bar3*). Annealing tempe-

Table 2. Per cent callusing and regeneration response of mature embryos isolated from different varieties of *T. aestivum* and *T. durum* on MSE2 and MSER media respectively

Genotype	Callusing (%)	Regeneration (%)
<i>T. aestivum</i> cultivar		
HD2329	92.00 ± 1.73	54.93 ± 3.03
CPAN1676	78.33 ± 2.60	68.47 ± 2.25
PBW343	58.00 ± 2.30	58.00 ± 2.30
<i>T. durum</i> cultivar		
PDW215	59.67 ± 2.02	58.17 ± 1.96
PDW233	60.33 ± 1.76	67.10 ± 2.26
WH896	51.67 ± 1.45	63.83 ± 2.57

ratures for amplification of *nptII* and *bar* genes are 57 and 50°C respectively. PCR products were run on 1.6% agarose gel in 1X TAE along with size markers (GeneRuler™ 1 kb ladder and GeneRuler™ 100 bp ladder, MBI Fermentas).

Results

Regeneration studies in wheat have been mostly confined to leaf bases, immature embryos, scutellum and to immature inflorescence tissue^{32,33}. Although mature embryos have been the explants of choice for regeneration and transformation experiments³⁴⁻³⁷, their use is not prevalent. In the present investigation, mature embryos (ME) have been used for evaluating the regeneration and transformation response in three cultivars each of *T. aestivum* and *T. durum*, employing various media (Table 2). In general, all cultivars of *T. aestivum* and *T. durum* experimented with displayed reasonable regeneration response on MSER, and this has been the medium of choice for regeneration in the present study. However, some differences in callusing and regeneration of different cultivars were evident, e.g. *T. aestivum* cv. HD2329 displayed the highest callusing but lowest regeneration. In contrast, in *T. durum* cv WH896 displayed poor callusing but a relatively higher regeneration percentage. Highest regeneration of

about 67–68% was observed in *T. aestivum* cv. CPAN1676 and *T. durum* cv. PDW233 (Table 2).

Factors affecting gene delivery

The present investigation explores the suitability of excised mature embryos as well as the mature embryo derived calluses towards agrobacterial infection. Excised mature embryos were chosen for *Agrobacterium*-mediated transformation, as the regeneration response of this explant was found to be comparable to that of immature embryos in culture. Mature embryos were excised aseptically from surface-sterilized seeds and co-cultivated with *Agrobacterium tumefaciens* LBA4404 harbouring the binary vectors (pBI101 :: *Act1* and pCAMBIA3301 :: *pin2*). In general, two significant observations were recorded. First, the co-cultivation duration of 2–3 days was more effective than others, as evidenced by the quantitative measurement of GUS-specific activity (Table 3). Second, the *T. aestivum* cultivar CPAN1676 was more receptive to *Agrobacterium*-mediated gene transfer in comparison with *T. durum* cultivar PDW215, especially in the presence of *vir* inducers such as acetosyringone (Figure 1). The GUS activity of an individual explant (mature embryo) of *T. aestivum* was always observed to be more than that of *T. durum* following co-cultivation with *Agrobacterium* (Figure 2).

Table 3. *Gus*-specific activity (nmol MU/mg protein/h) of mature embryo-derived calluses of *T. aestivum* and *T. durum* varieties using various constructs

Cultivar	Constructs			
	p35SGUSINT	pCAMBIA1301	pCAMBIA2301	pCAMBIA3301
<i>T. aestivum</i>				
HD2329	26 ± 3.0	50 ± 3.1	58 ± 5.5	44 ± 4.2
CPAN1676	46 ± 4.8	48 ± 3.6	45 ± 3.5	18 ± 3.0
PBW343	38 ± 4.0	49 ± 5.6	30 ± 3.0	42 ± 5.0
<i>T. durum</i>				
PDW215	52 ± 4.3	38 ± 4.3	58 ± 5.2	31 ± 4.8
PDW233	44 ± 6.0	52 ± 7.3	69 ± 6.0	56 ± 5.8
WH896	34 ± 5.2	43 ± 4.7	60 ± 5.7	52 ± 3.8

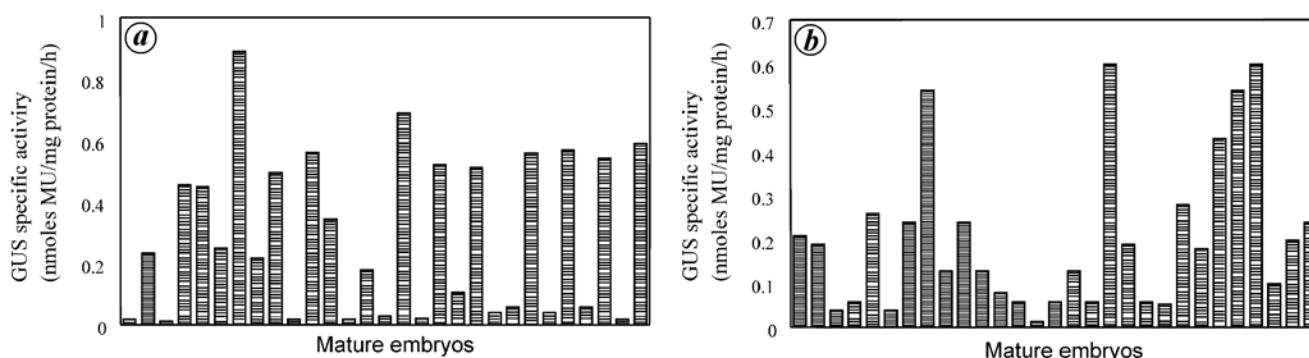


Figure 1. GUS-specific activity of individual mature embryos after three days of co-cultivation with *Agrobacterium tumefaciens* LBA4404 (pBI101 :: *Act1*). **a**, *T. aestivum* cv. CPAN1676 and **b**, *T. durum* cv. PDW215.

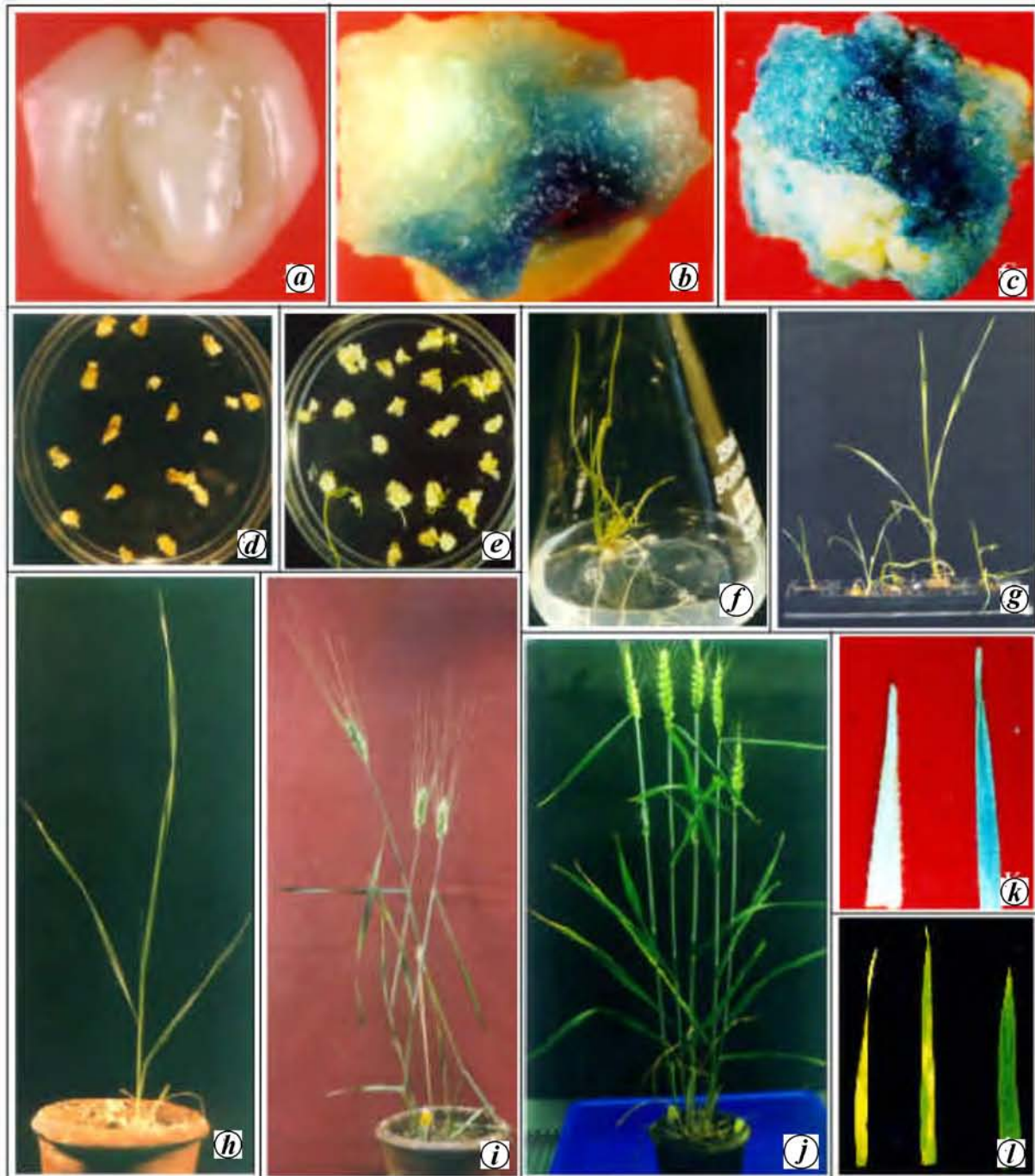


Figure 2. *Agrobacterium*-mediated transformation of wheat. Excised mature embryos of *T. aestivum* cv. CPAN1676 and *T. durum* cv. PDW215 were co-cultivated with LBA4404(pBI101 :: *Act1*), while mature embryo-derived calluses of *T. aestivum* cv. HD2329 were co-cultivated with LBA4404 (p35SGUSINT). *a*, Mature embryo of *T. aestivum* (control); *b*, Histochemical localization of GUS expression in excised mature embryos of *T. durum* after ten days of co-cultivation; *c*, Histochemical *gus* expression in mature embryo-derived callus of *T. aestivum* after three weeks on MSE2Pm₁₀₀; *d*, *e*, Control and transformed explants respectively, of *T. durum* on selection medium (MSERPm₅₀Cef₂₅₀); *f*, Transformants on selection-free half-strength MS medium; *g* and *h*, Transformants in seedling tray and pot respectively; *i*, Fertile *T. durum* transgenic plant growing in pot; *j*, Fertile T1 transgenic of *T. aestivum* CPAN1676; *k*, *GUS* histochemical assay in *T. durum* T1 transgenics leaf whole mounts (left – control, right – transgenic respectively); *l*, Result of paromomycin leaf spray on T₁ progeny of *T. aestivum* var. CPAN1676, Control (left).

Acetosyringone was also supplemented in the bacterial growth medium, resuspension medium and co-cultivation medium as an inducer. This is consistent with the earlier

observations in case of rice and wheat^{8,10} transformation. In the presence of acetosyringone, different plasmid constructs show different preferences for different genotypes

(Table 3). Such genotypic preferences are well documented in the literature. The suitability of mature embryo-derived calluses as explants for *Agrobacterium*-mediated transformation was evaluated after 2–3 days of co-cultivation with *Agrobacterium*. Preliminary experiments detected the activity of *gus* gene up to 45 days after co-cultivation of mature embryo-derived calluses of *T. aestivum* cv. HD2329 with LBA4404 (p35SGUSINT). Therefore, this vector–genotype combination was employed for generation of transgenic plants. The genetic transformation methodologies and conditions were similar to those optimized for mature embryos except that more caution was observed to minimize damage at the cefotaxime wash step. Thus, three days of co-cultivation was found to be ideal for successful gene transfer as evidenced by histochemical localization of GUS expression and was also reconfirmed by GUS fluorometric assay of mature embryo-derived callus of three cultivars each of *T. aestivum* and *T. durum* using four different constructs (Table 3).

Selection and regeneration of transformants

Mature embryos of *T. aestivum* cv. CPAN1676 and *T. durum* cv. PDW215 were aseptically excised from the seeds and co-cultivated with *A. tumefaciens* LBA4404 (pBI101 :: *Act1*). After three days of co-cultivation followed by thorough washing, the mature embryos of *T. aestivum* var CPAN1676 were transferred to MSE2Pm₁₀₀Cef₂₅₀ for callus induction for approximately 2–3 weeks. Callus formation was observed on MSE2Pm₁₀₀Cef₂₅₀ in 35 and 16% of co-cultivated explants and untransformed explants respectively. Approximately 80% of mature embryos (control) formed calluses on MSE2. In *T. durum* cv. PDW215, callus formation on MSE2Pm₁₀₀Cef₂₅₀ was observed in 32 and 12% of co-cultivated and untransformed mature embryos respectively (Figure 2d and e), and callus induction was about 78% in control explants on MSE2. Growth of untransformed explants was curtailed when the calluses were subsequently transferred to MSE2Pm₁₀₀ for further multiplication. Untransformed calluses showed no growth on MSE2Pm₅₀; however, calluses derived from co-cultivated mature embryos showed growth on MSE2Pm₁₀₀. After four weeks of co-cultivation, both proliferating and non-proliferating calluses were transferred to MSERPm₅₀ for regeneration. After 2–3 weeks, the regenerated plantlets were further transferred to MS_{1/2} for shoot elongation (Figure 2f) and subsequently relocated to transfer plugs for reinforcement of the root system (Figure 2g).

Mature embryo-derived calluses are among the preferred explants with a regeneration response (~60%) comparable to that observed from calluses derived from the immature embryos. However, this explant demands greater care during handling and physical damage to calluses is reflected in the reduced regeneration response and transformation efficiency as well. Mature embryo-derived calluses are

particularly prone to damage during the antibiotic washing step, which is required for elimination of *Agrobacterium*. While washing resulted in loss of competent cells, insufficient washing with cefotaxime usually resulted in necrosis and in non-recovery of regenerating calluses from co-cultivated explants. Thus in several experiments despite the calluses proliferating on the selection medium, no regeneration was obtained from the mature embryo-derived calluses. Regeneration of plantlets was obtained from the mature embryos co-cultivated in several independent experiments; however, data presented are from two successful experiments indicating a transformation efficiency of 5.57% (16 putative transformants out of 287 co-cultivated explants) with *T. aestivum* cv. HD2329, calculated on the basis of paromomycin leaf spraying of T₀ transformants.

PCR analysis

PCR amplification was undertaken to screen for the presence of *bar* gene in the transformants transformed with LBA4404(pCAMBIA3301 :: *pin2*). Leaf samples were collected from putative transformants immediately after regeneration, but prior to transferring the plantlets to half-strength MS medium supplemented with 2.5 mg/l phosphinothricin. Plants scored as positive (Figure 3a) on the basis of PCR analysis (presence of an amplified product of ~296 bp) also survived on MS_{1/2}P_{2.5}, whereas plants scored as negative by PCR analysis were unable to survive (25%), thus confirming the practical authenticity of PCR and the phosphinothricin selection (Figure 3a, where three-fourths of the plants are PCR-positive). Based on the result of phosphinothricin leaf paint assay and PCR analysis, transformation efficiency by co-cultivation of mature embryos of *T. aestivum* cv. HD2329 with *Agrobacterium* strain LBA4404 (pCAMBIA3301 :: *pin2*) was nearly 1.77%. PCR amplification was further undertaken to screen for the presence as well as segregation of *nptII* gene in the T₁ progeny of *T. aestivum* CPAN1676 transformants transformed with LBA4404(pBI101 :: *Act1*; Figure 3b).

The genomic DNA of putative transformants of *T. aestivum* HD2329 (LBA4404(p35SGUSINT)) obtained by co-cultivation of mature embryo-derived calluses was screened by PCR employing primers specific to *nptII*. PCR amplification of the *nptII* gene in genomic DNA samples of T₀ transformants indicated a successful transformation event (Figure 3c), which is evidenced by the presence of an amplified product of expected size (~721 bp).

NPTII assay

The transformed plantlets (8–10 cm long) growing in seedling trays were sprayed with a solution of 2% w/v paromomycin

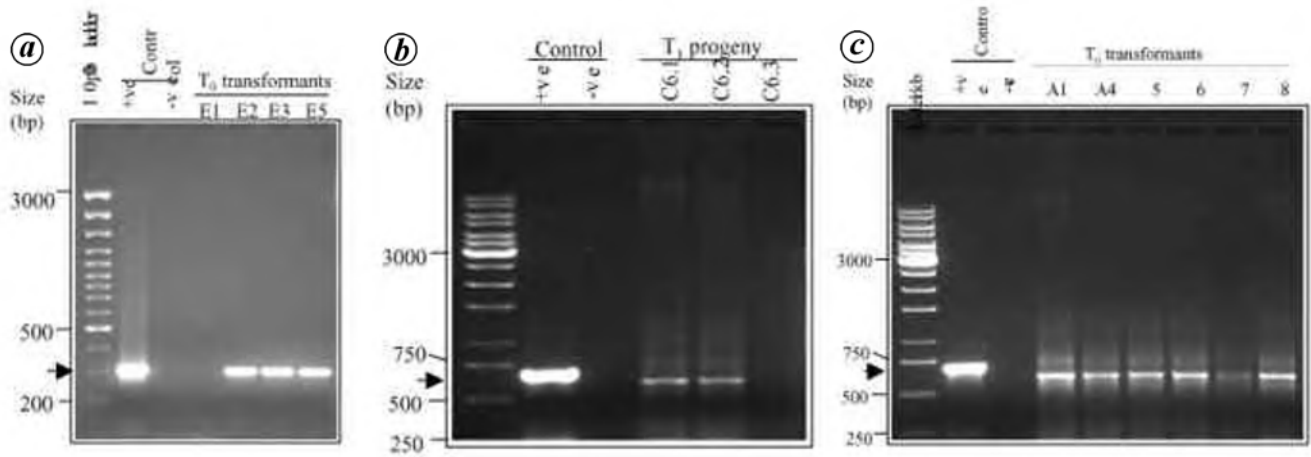


Figure 3. *a*, PCR analysis of genomic DNA samples of T_0 transformants of *T. aestivum* cv. HD2329 using primers specific to *bar* gene. The T_0 transformants were obtained by co-cultivation of mature embryos with *A. tumefaciens* strain LBA4404(pCAMBIA3301::pin2). The plasmid pCAMBIA3301::pin2 and genomic DNA from an untransformed plant were used as positive and negative control respectively. *b*, T_1 progeny: PCR analysis of *T. aestivum* cv. CPAN1676 transformed with LBA4404(pBI101::Act1) using primers specific to *nptII* gene. The plasmid pBI101::Act1 and genomic DNA isolated from an untransformed plant were used as positive and negative controls respectively. *c*, PCR analysis of genomic DNA samples of T_0 transformants of *T. aestivum* cv. HD2329 using primers specific to *nptII* gene. The T_0 transformants were obtained by co-cultivation of mature embryo-derived calluses with *A. tumefaciens* strain LBA4404 (p35SGUSINT). The plasmid p35SGUSINT and genomic DNA from an untransformed plant were used as positive and negative control respectively.

sulphate and 0.1% Tween-20. After seven days, transformed plants displayed little to no damage following paromomycin spraying. Untransformed control plants developed bleached spots and subsequently displayed significant growth inhibition (Figure 2*l*). The activity of *nptII* gene was further confirmed by dot-blot assay in the transformants of *T. aestivum* var CPAN1676 and *T. durum* cv. PDW215 tested (Figure 4*b*), which were selected on the basis of paromomycin leaf-spraying results. Transformation frequencies observed in case of *T. aestivum* and *T. durum* were 1.6 and 1.28% respectively, using the construct LBA4404 (pBI101::Act1). When the construct LBA4404 (pCAMBIA::pin2) was used, transformation efficiency of 1.77 and 1.54% was observed in *T. aestivum* and *T. durum* respectively. However, the growth response of T_0 transformants was poor compared to the untransformed control plants. Seed setting was observed in only 40 and 33.33% of transformants of *T. aestivum* and *T. durum* respectively, and even the quality of seeds was not satisfactory. This could probably be due to the non-availability of optimal growth conditions.

Southern analysis of T_0 transformants

Genomic DNA of T_0 transformants of *T. durum* obtained after co-cultivation of mature embryos with LBA4404 (pBI101::Act1) was digested with *EcoRI* and blotted onto nylon membrane and hybridized with a *gus*-specific probe. Digestion of pBI101::Act1 releases a ~3.3 kb fragment which has the *gus* coding region, a portion of rice *Act1*-5' region and *nos* terminator. A specific fragment hybridizing to GUS was observed in the transgenic lines tested. This observation confirms the presence of the *gus* gene in the screened transformants (Figure 4*a*).

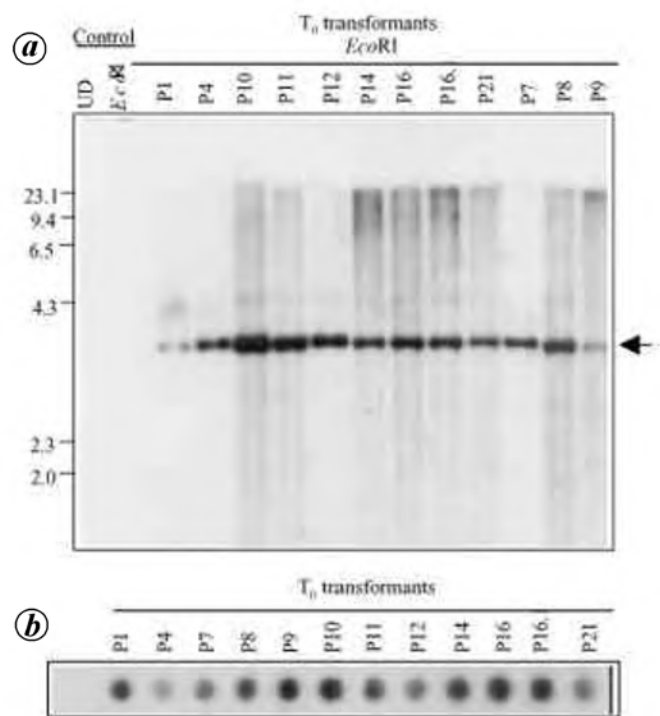


Figure 4. *a*, Southern analysis of T_0 transformants of *T. durum* cv. PDW215 co-cultivated with *A. tumefaciens* LBA4404(pBI101::Act1). Lanes 1 and 2, Untransformed control (undigested and digested respectively); Lanes 3–14, Genomic DNA of putative transformants digested with *EcoRI*. Digestion of pBI101::Act1 with *EcoRI* releases a ~3.3 kb fragment containing *gus*-coding region, *nos* terminator and part of *Act1*-5' region. Numerals on the left indicate size of the DNA fragments of the length standard in kb (λ DNA, *HindIII* digest). Hybridization was performed by *Bam*HI–*Sac*I fragment of *pAct1-F*, which spans the *gus* coding region. *b*, Autoradiograph showing NPTII activity in dot blots of T_0 transformants obtained by co-cultivation with *A. tumefaciens* LBA4404(pBI101::Act1).

DNA blot of primary transformants of *T. aestivum* cv. HD2329 transformed with LBA4404 (pCAMBIA3301 :: *pin2*) was probed with a 1.5 kb *Bam*HI fragment of pCAMBIA3301 :: *pin2* which contains the *pin2* coding region and also a *pin2*-3' terminator. Southern analysis of *Bam*HI-digested samples revealed the presence of hybridization signals in the 1.5 kb range in the T₀ transformants tested. *Eco*RI-digested genomic DNA samples show the presence of hybridizing bands (Figure 5), demonstrating the presence of *pin2* gene in the T₀ regenerants.

Progeny analysis

As mentioned earlier, the *T. aestivum* T₀ transformants produced few seeds and most of the seeds were poorly developed. From one representative T₀ plant, three out of ten seeds were successfully germinated on half-strength MS. After paromomycin spraying of seven-day-old seedlings,

one of the T₁ plants developed extensive yellow patches on the leaves, while the other two plants suffered little or no damage (Figure 2*l*). Presence of *nptII* gene in two of the T₁ plants was also confirmed by PCR amplification of genomic DNA samples using *nptII*-specific primers (Figure 3*b*). T₁ plants also displayed better growth than T₀ plants (Figure 2*j*), on pots containing a mix of garden soil and soilrite.

Discussion

Cereals, especially wheat, were once considered recalcitrant to *Agrobacterium*-mediated transformation. However, since the first successful report of fertile transgenic wheat plants⁷, there has been considerable progress. Various factors influencing successful *Agrobacterium*-mediated genetic transformation of wheat have been explored by several workers in a quest to achieve higher transformation effi-

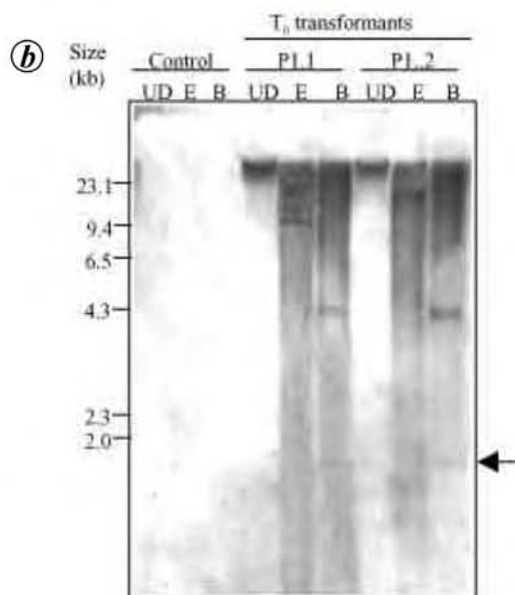
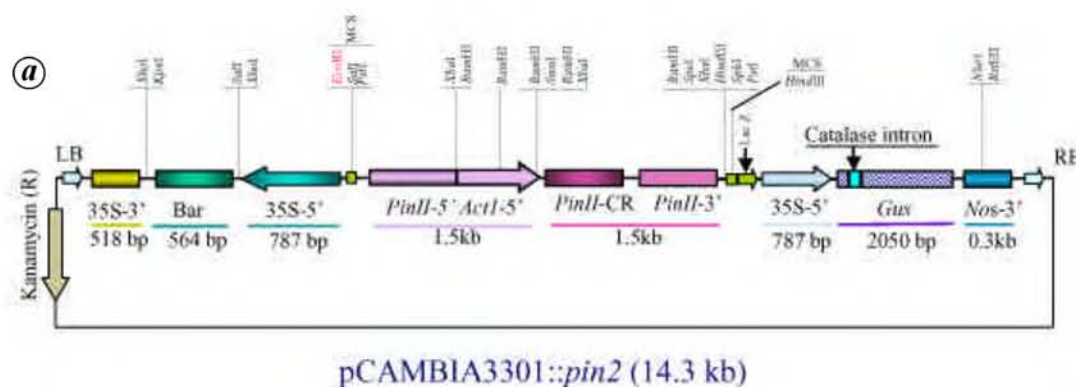


Figure 5. Southern analysis of T₀ transformants of *Triticum aestivum* cv. HD2329 co-cultivated with *A. tumefaciens* LBA4404 (pCAMBIA3301 :: *pin2*). UD, Undigested; E, *Eco*RI; B, *Bam*HI. Numerals on the left indicate size of the DNA fragments of the length standard in kb (λ DNA, *Hind*III digest). Hybridization was performed by 1.5 kb *Bam*HI fragment of pCAMBIA3301 :: *pin2*, which spans the *pin2* coding-*pin2* terminator region.

ciency^{9,13,12,35}. The advantages of using the *Agrobacterium*-mediated approach involve the ability to transfer large segments of DNA with minimal rearrangement, with fewer copies at a higher efficiency incurred with minimal cost. Thereby, such an approach results in simple segregation pattern, better transformation frequency and also the absence of vector backbone sequences. Furthermore, in *Agrobacterium*-mediated transformation DNA generally inserts into transcriptionally active regions³⁸ via illegitimate recombination, thereby ensuring the active transcription/expression of the transgenes.

Transformation efficiency in wheat may be influenced by many factors such as genotype, type of explant, optimized conditions of inoculation and co-cultivation, optimized callusing and plant regeneration, selection media, vector and *Agrobacterium* strain, etc.¹³. The present investigation was initiated to develop an efficient gene delivery protocol for bread wheat and durum wheat. There have been limited studies on genetic transformation of wheat via *Agrobacterium*-mediated co-cultivation and few reports demonstrate the successful production of transgenic wheat plants. Most *Agrobacterium*-mediated research has employed immature embryos, precultured immature embryos, and their embryogenic calluses for co-cultivation^{7,16,35,36}. For efficient introduction of T-DNA genes into recalcitrant explants, several groups have employed different approaches such as enhancing the *vir* gene expression (by wounding) or promoting the adherence of *Agrobacterium* to the plant tissues. Various approaches include mechanical abrasion of wheat seeds¹¹, use of surfactants⁷, agrolistics³⁹, use of silicon carbide fibres for wounding of immature wheat embryos⁴⁰, and subjecting the plant tissues to brief periods of ultrasound in the presence of *Agrobacterium* (sonication-assisted *Agrobacterium*-mediated transformation)⁴¹. The procedure reported here involves excision of mature embryos: it is simple and fulfils the basic wounding requirement and does not require special media. In the present investigation, our transformation efficiencies (1.28–1.77%) are comparable with those reported earlier^{7,12,13,42,43}, though we have not been able to characterize in detail the segregation patterns due to paucity of transgenic events. *Agrobacterium*-mediated transformation has been reported to achieve a maximal of 4.4% transformation efficiency using the construct *aroA* :: *CP4* and immature embryos as explants¹².

The key factor in the successful generation of transgenic plants is the optimization of conditions for regeneration of plantlets after several rounds of selection with herbicide/antibiotic, which is also necessary for elimination of escapes. Our work also emphasizes on the prospects of paromomycin and phosphinothricin as effective selection agents for wheat transformation, and this is consistent with the observations of earlier investigators^{2,17}. Strategies towards enhancing the transformation frequency involve optimization of parameters such as the use of suitable explants along with optimizing factors that promote *Agro-*

bacterium infection. Since there were two limiting steps, agroinfection and regeneration from the transformed calluses, approaches such as transformation of immature/mature embryos instead of immature/mature embryo-derived calluses limit the time duration without hampering the regeneration ability of the explants. In the present investigation, callusing and regeneration were optimized to attain reproducible, standardized transformations, as loss of regeneration potential over time has been a major limitation in most wheat-transformation experiments. This loss of regeneration potential, especially during prolonged culture, has been overcome by supplementing the regeneration media with polyamine spermidine to improve recovery of regenerants from transformed calluses¹⁴. However, in the present approach direct transformation of the mature embryos was undertaken to minimize the loss of regeneration potential due to prolonged culture.

In the present investigation, LBA4404 was employed as the *Agrobacterium* strain of choice. Successful production of transgenic plants was achieved by co-cultivation of mature embryos and mature embryo-derived calluses. Our investigations support the observations of other researchers, who recommend the use of acetosyringone and glucose for inclusion in the inoculation and co-culture medium⁷. Acetosyringone was employed in both the inoculation as well as the co-cultivation medium, resulting in 1.5-2-fold increase in transient expression of *gus* activity, thereby validating the observation of other workers^{42,43}. Durum wheat transformation has been accomplished successfully using the biolistics approach^{37,44-48}, but *Agrobacterium*-mediated transformation is yet to be accomplished.

The vectors successfully employed in this study are conventional binary vectors, while the supervirulent vectors (having extra copies of *virB*, *virC* and *virG* of A281) that have been extensively used for transformation of rice and maize^{4,5} do not appear to be essential for wheat transformation, as this observation is also shared by other co-workers⁷. In the binary vector pBI101 : *Act1*, the *gus* gene is under the control of a monocot promoter (rice *Act1*) for specific expression in plant tissues and the *gus* gene contains an intron in the vectors pCAMBIA3301 :: *pin2* and p35SGUSINT, which efficiently blocks its expression in bacteria, thus excluding false positives. The antibiotic/herbicide-resistant nature of transgenic tissues proves that the selectable marker genes are active in wheat. The results of antibiotic and herbicide resistance assays were observed to be consistent with those of molecular analysis. The transformants that scored positive on the basis of assays for antibiotic and herbicide resistance were also detected positive by Southern and PCR analysis with the respective genes. A direct correlation was observed (data not presented) between expression of *pin2* in *T. durum* and resistance against the cereal cyst nematode, *Heterodera avenae*⁴⁹.

In our laboratory, the suitability of mature embryo as a starting explant for wheat transformation has been demon-

strated also for cellular permeabilization experiments involving mechanically isolated embryos with membrane permeabilizing agents³⁵ and for particle bombardment of basal embryo-derived calluses⁴⁸. Therefore, it appears that mature embryos are an excellent system for *Agrobacterium*-mediated transformation, as they are not only convenient due to their ease of availability and but also for handling large numbers within a reasonable time, thus aiding recovery of transgenics. In the present study, most of the regenerated plants reached maturity and set seeds, and the further analysis of the transformants indicated absence of vector backbone sequences and confirmed T-DNA integration in low copies, thereby resulting in simple segregation patterns. Genetic transformation techniques have often been associated with aberrations in morphology and fertility of plants. The T₀ transformants obtained in the present study displayed poor growth compared to plantlets regenerated from untransformed calluses on selection-free medium. However, the T₁ progeny appeared to be normal phenotypically and similar in morphology to seed-derived control plants.

Conclusion

A reproducible method for efficient production of transgenic wheat plants using *A. tumefaciens* with a frequency of nearly 1.28–1.77% has been achieved in both bread wheat as well as durum wheat. Although no significant difference was observed in the transformation efficiencies under similar conditions, the level of transient *gus* gene expression was always observed to be higher in *T. aestivum* varieties compared to *T. durum* varieties. The reason for this is difficult to ascertain and probably resides in the better optimized regeneration capabilities of *T. aestivum* compared to *T. durum*. *Agrobacterium*-mediated transformation efficiency can be enhanced by factors that activate the *vir* genes and enhance the susceptibility of plant cells against *Agrobacterium* such as acetosyringone, monosaccharides (including phytohormonal, CaCl₂ and osmotic treatments). The present study focuses on the hitherto unexplored usage of mature embryos as suitable explants for *Agrobacterium*-mediated transformation. The factors that impact T-DNA delivery and regeneration were similar to the ones observed when immature embryos are employed, such as duration of pre-culture and also inoculation and co-cultivation parameters¹³. Nonetheless, we have developed a method for production of transgenic wheat by *A. tumefaciens* applicable for both bread wheat and durum wheat, thus demonstrating considerable genotypic independence.

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