Journal of Phytology 2012, 4(2): 22-29
ISSN: 2075-6240
Available Online: http://journal-phytology.com/

JOURNAL OF PHYTOLOGY

A Novel approach for *Agrobacterium*-mediated germ line transformation of Indian Bread wheat (*Triticum aestivum*) and Pasta wheat (*Triticum durum*)

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Abstract
Recalcitrance of wheat towards tissue culture procedures has hampered the wide use of conventional transformation techniques for its improvement. In the present study, a novel, non-tissue culture, cost effective approach has been established for the introduction of transgenes in wheat. Dry, mature seeds of two Indian varieties of wheat, *Triticum aestivum* cv. HD2329 (bread wheat), and *Triticum durum* cv. PDW215 (pasta wheat), were co-cultivated with *Agrobacterium* strain GV2260 (p35SGUSINT) and LBA4404 (pCAMBIA 3301), respectively, in the presence of 200 μM acetosyringone. The plantlets testing *gus* positive were raised till maturity in garden pots. T0 lines were screened by PCR for presence of selectable markers in the transformed plants followed by confirmation with Southern hybridization. In bread wheat, *npt*II was detected in five primary transformed lines (T0) (ws1, ws2, ws3, ws4, ws5) and the *bar* gene in three putatively transformed durum wheat lines (wsb1, wsb2, wsb3). The transformation efficiency was calculated as 1.16%, and 0.84% for *T. aestivum* and *T. durum*, respectively.

Keywords: *Agrobacterium*, bread wheat, durum wheat, transformation, whole seed.

INTRODUCTION

Wheat is the second largest staple crop of the world, thereby making it an attractive target for genetic engineering for the improvement of its qualitative and quantitative traits. On the other hand, genetic transformation of wheat has been a challenging task for various reasons. Recalcitrance of wheat towards tissue culture procedures, genotype dependency and high genome complexity are some of the major factors that have strongly impeded the establishment of an optimized protocol for wheat transformation [1]. Although, the major transformation strategies -- particle bombardment [2-15] and *Agrobacterium* co-cultivation mediated transgenics [16-18, 12, 19-25] have been reported in wheat, however, these strategies rely entirely on tissue culture procedures and require a suitable recipient target tissue with high regeneration efficiency.

Reports on alternate approaches employing *Agrobacterium*-mediated wheat transformation such as pollen tube pathway method [26-29], silicon carbide whiskers-mediated transformation [30-32], cellular permeabilization mediated transformation [33] and sonication assisted *Agrobacterium*-mediated transformation [SAAT; 34] have emerged in the past [35]. However, these alternative techniques are cumbersome (pollen tube pathway method), or employ hazardous chemicals (silicon carbide fibers) or require sophisticated equipments (SAAT). Additionally, these protocols depend substantially upon cultured explants, thus making the procedure time consuming and laborious. In the present study, an alternative, simple and cost effective method, amalgamating the advantages of a non-tissue culture based approach and *Agrobacterium*-mediated transformation has been developed. Whole dry seeds of two different wheat genotypes; bread wheat (a hexaploid) and durum wheat (a tetraploid), were co-cultivated with *Agrobacterium* for stable expression of transgenes rendering kanamycin and herbicide resistance, respectively.

Received: Nov 10, 2011; Revised: Jan 13, 2012; Accepted: Feb 05, 2012.

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MATERIALS AND METHODS

Co-cultivation of Whole Seeds

Step 1- *Agrobacterium* strains GV2260 (p35SGUSINT) and LBA4404 (pCAMBIA3301) were freshly grown from the glycerol stocks on YEB medium (1 g/l yeast extract, 5 g/l beef extract, 5 g/l bacto-peptone, 5 g/l sucrose, 0.5 mg/l MgSO4, pH 7.0) for 48 h with shaking at 200 rpm, 28^0C.

Step 2- For plant transformation, 100 μl of freshly grown culture was inoculated in Erlenmeyer flasks containing 25 ml YEB medium supplemented with appropriate antibiotics and 200 μM acetosyringone (AS).

Step 3- When the culture was in the log phase with OD600 0.25 to 1.00, the cells were pelleted in SS-34 Sorvall tubes at 2000 g, 4^0C for 10 min.

Step 4- Bacterial cell density was calculated (1 absorbance unit at 600 nm = 8 x 10^8 cells/ml) and cell density was adjusted to 5 x 10^8 cells/ml with liquid MS medium supplemented with 200 μM acetosyringone.

Step 5- Dry mature seeds were pre-treated by gentle abrasion of the embryonal end on a sand/flint paper no. 80, individually, to expose the embryonal region (Fig. 1a & b).

Step 6- The abrased seeds were surface sterilized and air-dried in a laminar flow (Kartos Inc., India) for 2-3 h.

Step 7- Dry abrased seeds (145-150 seeds) were incubated with 25 ml of *Agrobacterium* suspension (from step 4) for 1 h.

Seeds of bread wheat *T. aestivum* cv. HD2329 were co-cultivated with GV2260 (p35SGUSINT) conferring kanamycin resistance, whereas, seeds of durum wheat *T. durum* cv. PDW215 were co-cultivated with LBA4404 (pCAMBIA3301) for herbicide (bar) resistance. Attachment of bacteria to the embryonal region of the seed was confirmed by scanning electron microscopy (All India Institute of Medical Sciences, New Delhi, India).

Step 8- Incubated seeds were placed on absorbent cotton soaked with MS liquid medium (without sucrose), in trays for two-three days for co-cultivation.

Step 9- *Agrobacterium* cells were washed off with sterile MS liquid medium (3 washes); the final wash was supplemented with 500 µg/ml cefotaxime and given for 20 minutes to completely remove excessive *Agrobacteria*.

Step 10- Germinating seeds were placed on MS liquid medium soaked cotton-lined plastic trays in the culture room without selection pressure and progeny seeds were harvested.

Analysis of Transgenic Plants

GUS histochemical assay and PCR were employed as the preliminary screening methods for detecting putatively transformed plants from a large population of plants raised after *Agrobacterium* treatment of dry, abrased seeds of bread and pasta wheat.

GUS Histochemical Assay -- Activity of the *gus* reporter gene was histochemically detected in the leaf tips according to the protocol described by Jefferson [36]. Background activity in the plant tissue (if any) was eliminated by addition of 20% methanol to the buffer. Excess of the staining and chlorophyll pigmentation from the explants was removed by giving four washes with absolute alcohol: acetone (3:1) before observing the explants under stereo zoom microscope (SMZU Nikon, Japan).

Transgene Detection Using PCR – Genomic DNA was extracted by modified protocol of Dellaporta et al. [37]. Non-transformed wheat DNA was used as a negative control. Putative transformants were detected by PCR screening. Primers for the selectable markers *nptII* and *bar* gene were designed by using Gene RunnerTM software (*nptF* 5'TCG GCT ATG ACT GGG CAC AAC AGA3', *nptR* 5'AAG AAG GCG ATA GAA GGC GAT GCG3'; *barF* 5'ACC ATC GTC AAC CAC TAC ATC G3', *barR* 5'TCT TGA AGC CCT GTG CCT C3'). PCR amplification was performed as per manufacturer's instructions (MBI Fermentas, Lithuania) by initial denaturation at 94^{0}C (5 min hold), followed by 25 cycles at 94^{0}C (30s), annealing (30s) and extension at 72^{0}C (30s) with a final holding at 72^{0}C (7 min) for extension employing a Perkin-Elmer Gene Amp PCR system 2400. PCR products were checked on 1.2% agarose gel by ethidium bromide staining.

Southern Hybridization – Preliminary detection of the transgenes by PCR was further validated for stable expression by Southern analysis. Genomic DNA (30 µg) was digested with *Eco*RI and fractionated on 1% agarose gel. DNA was capillary blotted on to Hybond–N membrane (Amersham International Inc., UK) and probed with radiolabelled (Megaprime labelling kit, Amersham International Inc, UK) fragments coding for *nptII*, *gus* or *bar* gene.

RESULTS AND DISCUSSION

Considerable efforts have been made for optimization of *Agrobacterium*-mediated transformation of mature embryos and the development of direct *Agrobacterium* mediated transformation of germ line cells in seeds, shoot meristem, developing inflorescences and immature embryos with limited success [38-42]. Modification of different genetic and environmental aspects of transformation method may lead to high efficiency transformation [43].

In the present investigation, we have been able to successfully establish a novel, non-tissue culture based approach for wheat transformation. Dry, whole seeds of two different genotypes *T. aestivum* cv. HD2329 and *T. durum* cv. PDW215 were co-cultivated with *Agrobacterium tumefaciens* to confer antibiotic (kanamycin) and herbicide (*bar*) resistance, respectively.

Scanning Electron Microscopy

Use of seed material as the target tissue for *Agrobacterium*-mediated co-cultivation offers two significant advantages, firstly, it directly gives rise to an adult plant and, secondly, seeds contain germ cells which get exposed to *Agrobacterium,* thereby increasing the chance of infectivity. Thus, in an event of transformation the probability of obtaining transgene expression with chances of its inheritance increases strongly. Also, since the exposed apical meristems of the dry wheat seeds are more susceptible to *Agrobacterium* than the intact seeds [44], in the present investigation, gentle scraping of the embryonal region of the seed with a sand/flint paper was performed prior to co- cultivation (Fig. 1A, B). This exercise though did not result in enhanced *gus* expression, nonetheless, led to a higher incidence of exposure of the apical meristem and other deeply embedded cells to the bacteria by removal of the seed coat, enhancing the prospects of transformation.

Attachment of *Agrobacterium* to the target tissue is the first step for an effective host-microbe interaction and was seen to occur within one hour, as evidenced by scanning electron microscopy (Fig. 1C). Multiplication of *Agrobacterium* on the seed was visible in the next two days of co-cultivation as a halo around the seeds comprised of smooth, slimy layer of agrobacterial growth. These observations together support the proposition that attachment of the bacteria is not a limiting step for transformation in wheat tissues [45, 46].

General morphology of the putatively transformed plants and progeny seeds

Plants grown from abrased seeds treated with *Agrobacterium* showed normal growth (Fig. 1G). The seed set (T_1 and T_2) showed large variation in the number of seeds (2- 18; Table 1 and 2), nonetheless, the seeds formed were healthy with similar texture,

colour and grain size as the control seeds for both bread and pasta wheat transformants (Fig. 1 H).

GUS Histochemical Assay – Preliminary Screening for Putative Transformants

Subsequent to *Agrobacterium* co-cultivation, seeds of *T. aestivum* [GV2260 *(p35SGUSINT)*] and *T. durum* [LBA4404 *(pCAMBIA3301)*] were germinated and screened at various growth stages for *gus* gene expression in the leaf tips (Fig. 1 D, E, F). Control plantlets raised from non-treated seeds did not show *gus* expression at any stage, however, *gus* gene activity was also observed in the coleoptile sheath in *Agrobacterium* treated plants (Fig. 1 D, E). It was also noted that as the plants matured, *gus* gene activity was not observed by the histochemical assay and was completely undetectable in the progeny. It is possible that the levels of *gus* gene activity lowered to a great extent and could be detected, if any, only by the fluorimetery analysis [23]. Also, transgene silencing is common in wheat, this has been observed in our lab in tree species (mulberry) as well [47]. We speculate that GUS histochemical assay is a reliable method of screening of the transformed plants only till the early growth stages of the treated plants. A recent report also indicates presence of non-proteinaceous inhibitor(s) of GUS enzyme in wheat leaf and root tissues [48].

Table 1A. *Agrobacterium*-mediated whole seed transformation of *T. aestivum* cv. HD2329. The seeds were co-cultivated with GV2260 (p35SGUSINT) for two days.

Experiment No.	No. of Seeds	Total no. of germinated seeds	Number of Plants carrying "*nptII'* gene detected by PCR and confirmed by Southern blot
I	150	143	2
II	150	147	2
III	145	139	1

% Transformation Efficiency- 5/429= 1.16%

Table 1B. PCR and Southern analysis of the T_1 progeny of *T. aestivum* cv. HD2329 transgenic lines obtained by whole seed transformation by *Agrobacterium*- mediated approach.

Transgenic line	Number of seeds obtained (T1 progeny)	Number of seeds that germinated and grew till maturity	No. of Plants carrying *'nptII'* gene detected by PCR and confirmed by Southern blot
ws2	18	12	3
ws3	7	5	0
ws4	10	10	7
ws5	2	1	0
ws8	5	4	0

Table 2A. *Agrobacterium*-mediated whole seed transformation of *T. durum* cv. PDW215. The seeds were co-cultivated with LBA4404 (pCAMBIA3301) for two days.

Experiment No	No. of Seeds	Total No. of Germinated Seeds	No. of Plants carrying *'bar'* gene detected by PCR and confirmed by Southern blot
I	150	115	2
II	150	140	1
III	150	100	-

% Transformation efficiency-3/355 = 0.84%

Table 2B. PCR and Southern analysis of the T_1 progeny of *T. durum* cv. PDW215 transgenic lines obtained by whole seed transformation by *Agrobacterium*- mediated approach.

Transgenic line	Number of Seeds obtained (T1 Progeny)	Number of seeds that germinated and grew till maturity	No. of Plants carrying *'bar'* gene detected by PCR and confirmed by Southern blot
wsb1	3	3	2
wsb2	5	5	4
wsb3	2	2	1

Molecular Analysis

PCR screening

Putatively transformed plants of *T. aestivum* cv. HD2329 were screened for the presence of *nptII* gene and *T. durum* cv. PDW215 for *bar* gene using PCR amplification. PCR based screening was also carried out for the T_1 progeny in both the wheat genotypes.

Detection of nptII and bar gene

PCR analysis revealed presence of *nptII* gene in the plants that had tested positive for GUS histochemical assay (Table 1A and 1B; ws2, ws3, ws4, ws5, ws8). Presence of *nptII* gene was also detected in T_1 plants of ws2 (3/12) and ws4 (7/10). However, no PCR amplification of *nptII* gene was detected in the T_1 progeny of ws3, ws5 and ws8 transgenic lines (Table 1B). This may be due to the gradual loss of the foreign genes in the progeny or as the seed set was low in these lines it may not have segregated in the surviving plants. However, the presence of *gus* gene was observed in T_1

progeny of both the wheat genotypes as described in the section for Southern analysis.

Presence of *bar* gene was also detected by PCR in three T_0 plants of durum wheat (Table 2A and 2B; wsb1, wsb2, wsb3). Among the T_1 plants raised, seven plants out of ten showed presence of *bar* gene (Table 2B).

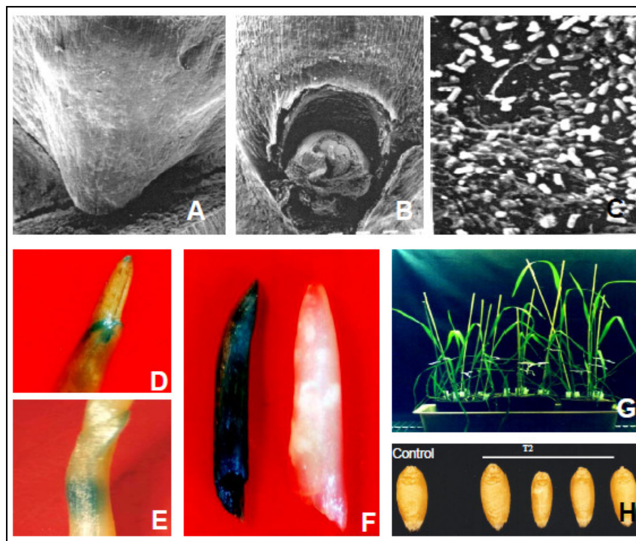

Fig 1. *Agrobacterium*-mediated whole seed transformation in *Triticum aestivum* cv. HD2329. Scanning electron micrographs of wheat described in:

A. Surface view of the embryonal axis of mature seed (x42), B. View of the embryo after scrapping with a sand paper (x42), C. Bacterial adherence after an incubation period of one hour (x5250), D. GUS histochemical localization in the leaf at various stages of seedling growth; seven-day-old seedling, E. Fourteen-day-old seedling, F. Twenty one-day-old seedling, G. Progeny (T_1) raised from primary transformants, H. Progeny seeds (T_2)

Southern Analysis

Presence and transmission of nptII and gus gene in *T. aestivum*

Southern analysis of the putatively transformed (T_0) plants of bread wheat showed presence of a 13 kb band and higher, in ws2, ws4, ws5, ws8 transgenic lines (Fig. 2). The negative control and other lines that were not PCR positive did not show any signal during Southern hybridization, confirming the results obtained by PCR analysis.

Presence of *gus* reporter gene was confirmed in T_1 progeny of ws2 transformant line. As expected, integration of *gus* gene was confirmed in seven out of ten plants that also tested PCR positive for *nptII* gene (Fig. 3 A, B). An expected band of ~2.8 kb corresponding to the *gus* gene was obtained as a result of *Pst*I digestion of the genomic DNA. However, an additional band (~ 4.2 kb) in four lines, i.e. ws 4.4, ws 4.6, ws 4.8 and ws 4.10, was intriguing. It is possible that additional restriction sites for *PstI* in the vector p35SGUSINT resulted in partial digestion of the genomic DNA leading to these signals.

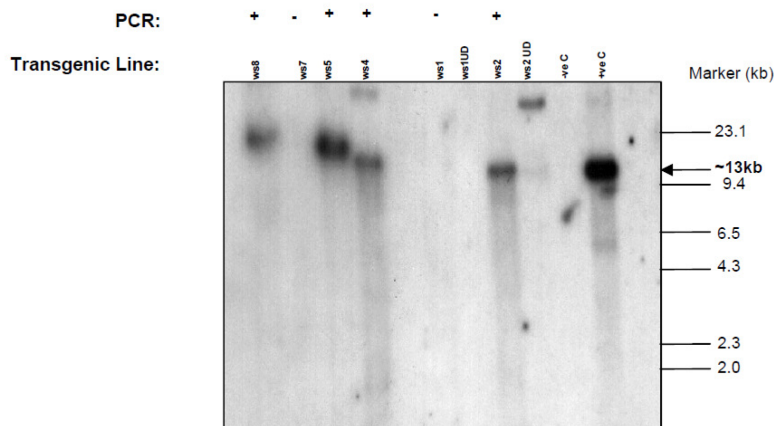

Fig 2. A representative autoradiogram of Southern hybridization showing the presence of *nptII* gene in four primary transformants (T_0) obtained by *Agrobacterium*-mediated whole seed transformation of *T. aestivum* cv. HD2329. Abrased, dry seeds were co-cultivated with GV2260 (p35SGUSINT) for two days. The genomic DNA (30µg) of primary transformants was digested with *Eco*RI. The Southern blot was probed with PCR amplified *nptII* fragment (729 bp). +ve C; positive control, linearised p35SGUSINT, -ve C, negative control, *Eco*RI digested DNA from non- treated plants. UD, undigested DNA. The transformants not testing PCR positive for *nptII* gene, also did not show any signals in the autoradiogram (ws1, ws7).

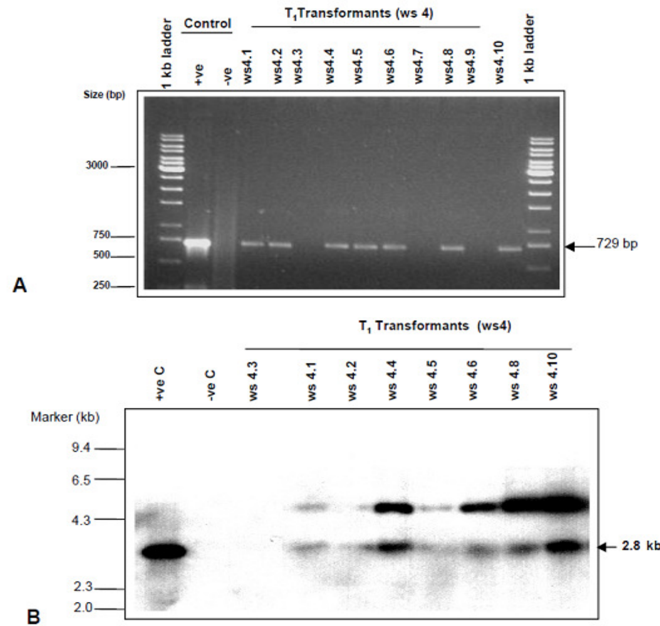

Fig 3A. A representative PCR screening of T_1 progeny of *T. aestivum* cv.HD2329 seeds co-cultivated with GV2260 (p35SGUSINT). DNA from ten T_1 plants of ws4 line (T_0) was isolated. PCR amplification was carried out using *nptII* specific primers.

Fig 3B. Southern analysis confirming the presence of *gus* gene in T_1 plants of ws4 line that tested PCR positive for *nptII* gene. The genomic DNA (30 µg) was digested with *Pst*I and probed with 2.2kb *Eco*RI-*Bam*HI fragment of pBI221 spanning the *gus* coding region. Positive control (+ve C), *Pst*I digested p35SGUSINT; Negative control (-ve C) *Pst*I digested DNA of non- transformed plants.

Presence and Transmission of bar and gus gene in *T. durum*

The positive results obtained with bread wheat encouraged us to extend the utility of this non-tissue culture based transformation approach to pasta wheat. In *durum* wheat *bar* gene was introduced keeping in view its agronomic importance, besides being a popular selection marker.

The presence of *bar* gene was confirmed by Southern analysis in the primary transformants of T. *durum* (Table 2A). Presence of *bar* gene was also confirmed in seven T_1 transformants (Table 2B; Fig. 4A) and a specific band corresponding to 11.3 kb was detected in wsb1.1, wsb 1.2, wsb2.2, wsb2.4 and wsb 3.1. A smaller band of 3.5 kb size was detected in four lines (Fig. 4A).

The same Southern blot was reprobed for *gus* gene. Interestingly, the signal was not detected in lines ws2.1 and ws2.3 which also showed weak presence of the *bar* gene (Fig. 4A and B). We speculate gradual loss of the two foreign genes in these two lines as these may be chimeric in nature.

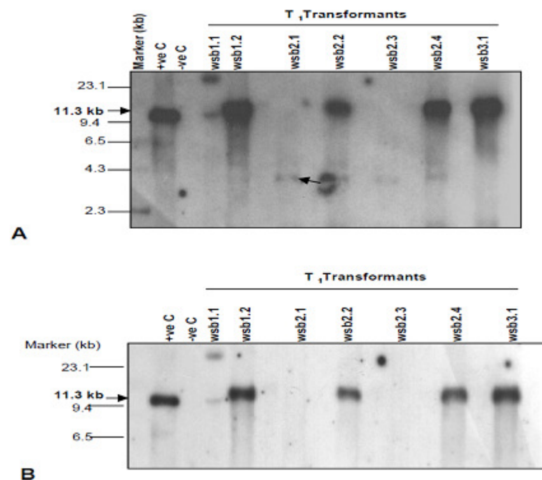

Fig 4A. Southern analysis of the T_1 plants of *T. durum* cv. PDW 215, obtained by whole seed co-cultivation with *Agrobacterium* LBA4404 (pCAMBIA3301) for the confirmation of '*bar'* gene. The genomic DNA of the transformants and negative control (-ve control) was digested with *Eco*RI. The Southern blot was probed with *Pst*I fragment of pAHC20 coding for *bar* gene (600 bp). Positive control (+ve C), linearised pCAMBIA3301.

Fig 4B. Southern analysis of T_1 plants of *T. durum* for the confirmation of *gus* gene. The Southern blot prepared for Figure 4B was stripped and reprobed for *gus* gene. The probe was 2.2 kb *Eco*R1-*Bam*HI fragment of pBI221 spanning the *gus* coding region.

Transformation Efficiency

Transgenes were successfully introduced in two different wheat genotypes. The transformation efficiency was calculated as number of primary transformants confirmed by Southern analysis/ total number of seeds germinated [49]. In *T. aestivum*, 1.16% plants were transformed for kanamycin resistance and in *T. durum*, 0.85% transformation efficiency was obtained for *bar* gene. The transformation efficiency is appreciable considering no selection pressure was given at any stage. Stable transgene expression is reported in wheat without any selection pressure [50].

Also, the use of non-destructive markers such as anthocyanin, green fluorescent protein markers has partially eliminated the need for selection pressure. The transformation efficiency obtained in the present study is evidently comparable to the transformation efficiencies reported earlier for wheat transgenics obtained *via* particle bombardment. It is reported 1.3% transformation efficiency by bombardment of the immature embryo callus [2]. Similarly, transgenesis obtained transgenic wheat plants at a transformation frequency ranging from 0.1-1.3% [5, 4, 51]. A slightly higher efficiency was reported [3] by bombarding the isolated embryo scutella (0.5-2.5%). Similarly, employing glyphosate selection, [52] reported 0.15% transformation frequency and [6] reported 2% transformation frequency. Higher frequencies (upto 14%) have also been reported by [8] in wheat embryonic tissue employing a single stranded linearized DNA but is not popular. In contrast, *Agrobacterium*-mediated co-cultivation of immature embryos and embryogenic calli have yielded 3-9.82% efficiency [16, 23, 25] and receiving wider attention.

A non-*in vitro* based strategy for transforming germinating seeds of *Arabidopsis thaliana* with an *Agrobacterium* strain carrying a Ti plasmid coding for *nptII* gene was reported [53]. Plants resulting from the treated seeds (T_1) gave rise to progeny (T_2) resistant to kanamycin. The presence of *nptII* was confirmed by Southern analysis. This method has virtually revolutionized the field of *Arabidopsis* based functional genomics.

In the present investigation we employed dry and abrased seeds as the target tissue for *Agrobacterium*-mediated transformation and eliminated use of tissue culture procedures at all stages of the experiment. Although the T_0 transformation efficiency is quite low, nonetheless, the percentage transformation is significant considering the stability and transmission of the markers to T_1 progeny. Although *durum* wheat had a low seed set (10 seeds) and transformation efficiency, but it showed higher rate of transmission of the marker gene (70%) than bread wheat (31.25%). Higher ploidy level of the bread wheat may partially explain the low inheritance level of the marker genes. Further experiments may be, however, designed to study the level of inheritance and segregation pattern of the introduced genes.

Of late, a similar study involves the establishment of a protocol for genetic transformation of wheat without involving tissue culture procedures with limited success. Wheat seeds were soaked in different concentrations of 2,4-D for various durations as a pre-treatment procedure for the transformation strategy. Imbibed seeds of wheat were sterilized, incised through apical meristem, wounded and inoculated with *Agrobacterium tumefaciens* containing *GUS* and NPT-II genes in its plasmid [54].

The simple technique of transformation in bread wheat could be extended to *durum* wheat implying that the protocol is relatively variety and genotype independent. We also show that different strains of *Agrobacterium* carrying different genes of interest are capable of transferring DNA to the wheat seeds. Further, integration of two different selection markers (*npt*II and *bar*) demonstrates possibility of introducing a variety of genes of interest.

CONCLUSION

Thus, a simple, alternate, cost effective, skill and labor independent procedure for wheat transformation has been developed, which in the near future has the potential of being used as a routine method for transforming various other crop species. In the era of functional genomics, the simplicity of the described novel protocol can supersede the time-consuming tissue culture based transformation strategies such as particle bombardment for investigating gene functions in a short time span.

ACKNOWLEDGEMENTS

We thank Dr B S Malick, IARI, India, for providing the seed material and Prof Lothar Willmitzer, Germany, for the construct p35SGUSINT. This work was financially supported by the Department of Biotechnology, Govt of India, New Delhi. AC and AM acknowledge the Fellowships awarded by UGC, Govt of India, New Delhi.

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