assumed to be normal may not be truly normal, since it is known that the 'normal' appearing region surrounding the cancerous tumour may have biochemical changes occurring due to the field-effect of the malignancy[10]. Indeed, when the discrimination analysis was carried out on the basis of the spectra averaged over all cancerous sites and the spectra averaged over all normal sites from a patient, a sensitivity and specificity towards cancer of 100% was obtained. The remarkably good results obtained on site-averaged spectra may suggest that a few of the normal sites had signatures very different from the other sites of the group.

This pilot study shows that LIF can discriminate SCC of oral tissues from normal squamous tissues. However, since the objective of the LIF-based system is the early diagnosis of cancer, in the next stage of the study, a qualified doctor will locate the suspected region of the oral cavity using visual assessment or other conventional means. Before the biopsy is taken, autofluorescence spectra will be recorded from the suspected sites as well as several other surrounding normal sites. The biopsy specimens from the suspected regions will then be subjected to histopathological examination and the spectral information will be correlated with the tissue histopathology to evaluate the true clinical potential of the LIF-based approach.

To conclude, a pilot study to evaluate the clinical potential of autofluorescence spectroscopy for diagnosis of cancer of oral cavity has been carried out using a system developed at the Centre for Advanced Technology (CAT), Indore. In this study involving 25 patients with histopathologically confirmed cancer of oral cavity, a sensitivity and specificity of 86% and 63%, respectively, towards cancer was obtained. The relatively lower specificity value appears to be due to the advanced stage of cancer in the patients investigated, due to which uninvolved regions treated as normal may not be truly normal. It will be desirable to carry out these studies in patients with early stages of cancer, to truly evaluate the diagnostic potential of the technique.

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Herbicide-tolerant transgenic plants in high yielding commercial wheat cultivars obtained by microprojectile bombardment and selection on Basta

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Most of the published work on transgenic wheat has used tissue culture responsive varieties such as 'Bobwhite'. The aim of the present study was to produce transgenic lines in modern high-yielding wheat cultivars irrespective of their tissue culture potential, using 'Biolistic' approach. Three of the highest yielding Indian wheat cultivars, namely CPAN 3004, Sonalika and UP 2338 were chosen for transformation. Stable transgenic plants with *bar* (conferring resistance to herbicide Basta) and *gus* genes were produced in each of these cultivars. Earlier developed transformation and regeneration protocols for tissue culture responsive lines were modified for obtaining transformation frequencies ranging from 0.14 to 0.83%. These frequencies are comparable with the published reports using the most responsive lines. Analysis of co-segregation patterns for *bar* and *gus* genes in the R1 progeny of a CPAN 3004 transformant indicated integration at two different sites in the genome. While both the sites have functional *gus* gene, only one of these has a functional *bar* gene, giving 15:1 and 3:1 segregation ratios for the two genes, respectively. Expression of *bar* gene under the maize ubiquitin promoter was high enough to provide protection against topical application of 250 mg/l gluphosinate ammonium, a concentration that killed most of the weeds including *Phalaris minor*.

PHALARIS minor commonly known as *Gulli Danda* or *Gehun Ka Mama* has established itself as the most predominant weed in the wheat fields along the Indo-Gangetic plains. As a control measure, Isoproturon has

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been extensively used as the post-emergence herbicide. The continuous use of Isoproturon over the years has resulted in emergence of several biotypes of *Phalaris minor* that are resistant to Isoproturon, resulting in losses up to 80% in the wheat-growing belts of Haryana, Punjab, Uttar Pradesh and Bihar. To check the menace caused by *Phalaris minor* and its resistant biotypes in wheat fields, an ecofriendly, biodegradable and broad-spectrum contact herbicide 'Basta' can be used to control this weed. Therefore, development of herbicide-tolerant transgenic wheat plants could provide effective control against *Phalaris minor* through the use of herbicide Basta.

The use of a particle gun to deliver DNA into plant cells was shown by John Sanford[1]. This 'Biolistic' approach has been used extensively for the genetic transformation of monocot species[2]. Wheat was the last of three major cereals, the other two being rice and maize, to be transformed due to low success rate in obtaining stably transformed plants, with reported transformation frequencies ranging from 0.1 to 2.5% (ref. 3, 4). Most of the transgenics were obtained in wheat genotypes that showed high regeneration in tissue culture. Apart from the 'Biolistic' method, electroporation-mediated and polyethylene glycol-mediated uptake of DNA into protoplasts has allowed genetic transformation of wheat, but such instances are limited as regeneration of plants from protoplasts is extremely cumbersome[5,6].

Immature embryo-derived callus is the favourite explant for obtaining regeneration of shoots in wheat. Several research groups have been able to obtain stable transgenic wheat plants by targeting the foreign DNA in scutellar tissues of wheat by the 'Biolistic' approach[7-14]. In most of these studies, either *bar* or *pat* gene, conferring resistance to phosphinothricin (PPT), has been used as selectable marker, though *nptII* gene conferring resistance to geneticin or paromomycin, *CP4* and *GOX* genes for glyphosate selection and *hpt* gene for hygromycin selection have also been used[10,14]. PPT, the active ingredient of gluphosinate (trade name Basta, Finale, Liberty, Ignite) is a non-selective herbicide that acts by irreversibly inhibiting glutamine synthetase (GS), an essential enzyme in nitrogen metabolism. The inhibition of GS accelerates ammonia accumulation to levels up to 100 fold higher than control plants causing death in two to five days[15,16]. PPT tolerant transgenic lines offer a good weed control in wheat, especially under minimum or zero tillage systems.

The present strategy for creating transgenic plants involves selecting modern high-yielding genotypes with moderate *in vitro* regeneration efficiency for transformation. This would allow introduction of useful transgenes directly into well-adapted cultivars. Based on the above perception, we attempted production of herbicide-tolerant fertile transgenic plants in three of the highest yielding Indian wheat cultivars, namely CPAN 3004, Sonalika and UP 2338.

Immature spikes of bread wheat cultivars Sonalika, CPAN 3004 and UP 2338 were collected from the field. Plasmid pDM 803 containing a chimeric *bar* gene from S*treptomyces hygroscopicus* under the control of maize u*bi1* promoter with *nos* terminator, and a *gus* gene from *E. coli* under the control of rice *act1* promoter with rice *rbcS* terminator was obtained from CSIRO, Division of Plant Industry, Canberra[17-20].

Green seeds isolated from freshly collected spikes, 10–15 days after anthesis, were sterilized by soaking in sodium hypochlorite (1.25%) for 30 min and then rinsed four times with sterile distilled water. Immature embryos of 1–1.5 mm size were aseptically excised and placed scutellar side up on a BEG-2 callus induction medium with 2 mg/l 2,4-dichlorophenoxyacetic acid (2, 4-D)[20]. The embryos were pre-cultured in dark for 5–7 days at 28°C. Four to six hours prior to bombardment, embryos showing signs of callus induction were transferred to new petri plates containing BEG-2 medium with 0.2 M sorbitol and 0.2 M mannitol. Thirty-five embryos were arranged at the center of each petri plate and bombarded with the DNA-coated gold particles.

The preparation of gold particles and transformation protocol were modified based on the results of our optimization experiments with the size and amount of gold particles on transient expression of GUS and anthocyanin reporter genes (unpublished work). The detailed protocol is as follows: Three-milligram aliquots of 1 μm gold particles were weighed in eppendorf tubes, washed twice with sterile distilled water, and then suspended in 50 μl of 40% sterile glycerol and stored at −20°C until use. On the day of transformation, the tubes containing gold particles were transferred to an ice box. Ten microlitre of plasmid solution (1 μg/μl) was added directly to this suspension followed by addition of 50 μl of 2.5 M CaCl$_2$ and 20 μl of freshly prepared 0.1 M spermidine free base. The mixture was vortexed vigorously after each addition and kept on ice in between the operations, and finally after a brief centrifugation of 2 s the supernatant was discarded. The DNA-coated gold particles were washed twice with absolute ethanol and finally resuspended in 100 μl of the absolute ethanol, the eppendorf tube was kept on ice until use on the same day (2–3 h). Immediately after vortexing, a 10 μl of suspension (300 μg gold) was spread uniformly on the macro-carrier disc in a laminar flow cabinet, allowed to air dry and delivered to the cultured embryos using Biolistic ® PDS 1000/He device at 1100 psi in a chamber with 26 inch Hg vacuum.

After bombardment, the cultures were incubated at 28°C for 12–16 h in dark for recovery and then transferred back to fresh BEG-2 medium without osmoticum and any selection and cultured in dark. Forty-eight hours after the bombardment, 4–5 embryos were removed from each plate and analysed for GUS expression by histochemical staining with X-Gluc[18]. Blue

spots on the scutellar surface were scored after 24 h of staining. After two weeks of culture in dark the calluses were transferred to fresh plates with BEG-2 medium containing herbicide 'Basta' (3 mg/l PPT) and kept in diffused light (30 μE m^2 s^{-1}, 16 h) for another 15–20 days (Selection I). Thereafter only those surviving calluses that produced somatic embryos with green spots on top were transferred to regeneration medium (BEG with 10 mg/l zeatin and also containing 5 mg/l PPT), (Selection II). After 15–20 days of culture, caution was taken to select healthy green shoots for transfer to shoot elongation medium containing half strength BEG with 10 mg/l zeatin and 5 mg/l PPT (Selection III). The shoots surviving after 15–20 days of culture were transferred to hormone free, half strength BEG medium with 3 mg/l PPT for rooting in full light (200 μE m^2 s^{-1}, 16 h), (Selection IV). Rooted plantlets obtained after the four step selection in Basta were transferred to four-inch plastic pots and kept for hardening under high humidity conditions. After one week of hardening, the plants were transferred to 12-inch terracotta pots, and grown to maturity in containment glass house.

The expression of GUS was analysed in the scutellum of immature embryo explants following bombardment and also in the roots and young leaves of R0 and R1 transgenic plants. Histochemical GUS assay with X-Gluc as substrate was performed according to the protocol described earlier[18].

Microtitre plate assay, with 4-methylumbelliferyl-β-D-glucuronide (MUG) as substrate, was performed on crude extracts prepared from the young leaves of R0 and R1 generation transformed plants along with controls, according to procedure described earlier[21]. Protein estimation in the extract was done by dye binding method[22]. Aliquots of extracts containing 4 μg of protein from each of the R0 and R1 plants were used in the individual wells of the microtitre plate for GUS assay. The reaction was initiated in a 100 μl assay solution (50 mM NaHPO$_4$, pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na$_2$EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) by the addition of MUG to 1 mM followed by incubation at 37°C. The reaction was stopped after 30 min by addition of 150 μl of 0.2 M Na$_2$CO$_3$ stop buffer. The microtitre plates were visualized under an overhead long wavelength (365 nm) ultraviolet light and photographed using CCD camera in a photodocumentation system.

The presence of GUS protein was also detected by immunoblotting of GUS protein in the plant leaves. Equal amount of protein (25 μg) extracted from the transformed and control plants were used along with 2.5 μg of purified GUS enzyme (Clonetech, USA) as positive control. After SDS–PAGE, proteins were transferred to a nitrocellulose membrane by electroblotting and probed according to the method described earlier[23]. Anti β-glucuronidase antibodies were obtained from

Clonetech and their binding to the GUS protein present in the leaf extract was detected with goat anti-rabbit IgG-alkaline phosphate conjugate and NBT-BCIP staining system.

To study the presence of the *bar* gene, genomic DNAs from the transformed and control plants were extracted by the CTAB method[24]. The PCR reactions (50 μl) were set up using 150 ng of wheat genomic DNA template, 1.5 mM MgCl$_2$, 100 μM each of the four dNTPs, 1.25 units of Taq DNA Polymerase (Genei, Bangalore, India), and 2 μM each of the forward and reverse primers for *bar* gene 39F (5'-GAA CGA CGC CCG GCC GAC ATC C –3') and 531R (5'-GTC CAG CTG CCA GAA ACC CAC-3')[20]. For positive control, 2 ng of the pDM-803 vector DNA was used as template in place of genomic DNA. Cycling conditions involved initial denaturation at 95°C for 4 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 2 min and extension at 74°C for 1 min. Amplified DNAs were visualized by electrophoresis in a 1.5% agarose gel and ethidium bromide staining. After electrophoresis the DNA was transferred to nylon membrane, and identity of the amplicons was tested by probing with labelled *bar* gene fragments using ECL direct labelling and detection system (Amersham, UK). A *bar* gene fragment of 500 bp was amplified from the vector pDM803 and purified using PCR purification kit (Qiagen,USA). Labelling and detection was done as per the manufacturer's instructions for the ECL kit.

Genomic DNAs (25 μg each) were digested overnight by restriction enzyme *Nco*I, and electrophoresed in a 1% agarose gel, and capillary blotted on to a nylon membrane (Hybond N$^+$). Labeling and detection was performed using ECL direct nucleic acid labeling and detection system as mentioned above.

An aqueous solution (250 mg/l PPT) of herbicide Basta (50% PPT) was used for testing the tolerance of transgenic plants. Sixty-four R1 progeny of a single transgenic plant of CPAN 3004, thirty non-transformed control plants of CPAN 3004 and ten weed plants (*Phalaris minor*) were sprayed once at mid tillering stage (30 days after planting). Assessment of damage was done 14 days after herbicide application.

Eight separate transformation experiments were carried out using a total of 3195 embryos from three high-yielding commercial cultivars, viz. Sonalika, CPAN 3004, and UP 2338 (Table 1). After bombardment one out of every seven bombarded embryos was picked up randomly and analysed for transient GUS expression by histochemical staining using X-Gluc. Overall, the average number of blue spots per embryo was highest in Sonalika (88), followed by UP 2338 (45) and CPAN 3004 (31), showing a clear genotype effect[11]. Similarly, the size and amount of microcarrier gold particles used per shot also significantly affected the number of transient transformation events (unpublished results). Use

Table 1. Summary of the transformation experiments with three high-yielding wheat cultivars

Expt. no.	Cultivar	Number of embryos bombarded	No. of blue spots per embryo (embryos stained)	Transformants selected	gus H	gus M	gus I	bar P	bar S	Transformation efficiency* (%)
1	CPAN 3004	820	32 (117)	C1	+	+	+	+	++#	0.14
2	CPAN 3004	210	26 (30)	–						0.00
1-2	*Total*	*1030*	*31(147)*	*1 Plant*						*0.11*
3	Sonalika	840	94 (120)	S1	+	++	+	+	++	0.14
4	Sonalika	105	52 (21)	–						0.00
3-4	*Total*	*945*	*88 (141)*	*1 Plant*						*0.11*
5	UP 2338	525	44 (75)	U1	+	++	+	+	+	
				U2	+	+	+	+	+	0.67
				U4	+	+	+	–	–	
6	UP 2338	175	57 (25)	–						0.00
7	UP 2338	280	58 (40)	U3	+	+	+	+	++	
				U5	+	+	+	+	+	0.83
8	UP2338	240	54 (34)	U6	++	++	+	+	++	0.49
5-8	*Total*	*1220*	*45 (174)*	*6 Plant*						*0.57*
1-8	Total	3195	(462)	8 plants						0.29

*Excluding embryos sacrificed for GUS staining. H, Histochemical assay. M, MUG assay. I, Immunoblotting. P, PCR. S, Southern. +, Presence (++, higher intensity). –, Absence. #An R1 plant of CPAN-3004 was analysed.

of 300 µg of 1 µm gold particles was found to be optimal. Thus, 1 µm gold particles produced more number of transient spots than the same amount of 1.6 µm gold particles. Increasing the amount of gold particles used per shot from 150 to 600 µg resulted in the higher number of transient spots up to 300 µg. After this there was a decline. Use of 1.6 µm particles and 600 µg amount resulted in more tissue damage and less number of transient expression spots.

Selection was avoided during initial two weeks to allow recovery and unhindered proliferation of the transformed cells, which get damaged during the process of delivering DNA[25]. Subsequently, selection for the putative transformants was carried out on plates containing Basta using a four-step strategy. During the first phase of selection all the calluses showed slow growth, subsequently green spots and then multiple shoots were observed in the resistant calluses on the same culture medium. Further exposure to a higher concentration of Basta (5 mg/l PPT) both in shoot regeneration and shoot elongation media allowed only healthy, green shoots to survive (Selection-II and III). Lowering the concentration of PPT to 3 mg/l in BEG medium allowed better development of root system in the surviving shoots (Selection IV). Total duration from embryo culture to transfer of putative transgenic plants to soil took approximately 14 weeks for cultivars CPAN 3004 and UP 2338, and 13 weeks for Sonalika.

Two separate experiments were carried out for each of the cultivars CPAN 3004 and Sonalika. An overall transformation efficiency of 0.11% was obtained for CPAN 3004 from 883 embryos (excluding the embryos used for staining) that were carried forward for selection. A similar efficiency was obtained with Sonalika from 804 embryos (excluding the embryos used for staining). However, in four experiments with cultivar UP 2338, the transformation efficiency was higher (0.57%) giving six confirmed transgenic plants from a total of 1046 bombarded embryos that were taken through the selection process. The transformation efficiency in individual experiments ranged from 0.14 to 0.83% with an average of 0.29% for all the three varieties (Table 1). There was a clear genotypic effect but it shows that stable transformants can be achieved in a wide range of cultivars if sufficient number of explants are bombarded.

One putative R0 transgenic plant obtained from cultivar Sonalika and six R0 plants from UP-2338 were analysed for stable integration and expression of the *gus* and *bar* transgenes. PCR analysis and Southern blotting of PCR products as well as genomic DNAs were used to assess the presence of *bar* gene. Forward and reverse primers internal to the *bar* gene amplified a DNA fragment of ~500 bp (ref. 20). One R0 plant of cultivar Sonalika (designated S1) and five R0 plants of cultivar UP 2338 (designated U1, U2, U3, U5 and U6) showed the expected 500 bp amplicon (Figure 1 *a*), only one of the regenerants from UP 2338 (U4) did not show the *bar* gene amplification. The identity of the 500 bp band as a *bar* gene product was confirmed by Southern blotting of the PCR products with labelled *bar* gene amplicon of the transformation vector pDM803 (Figure 1 *b*).
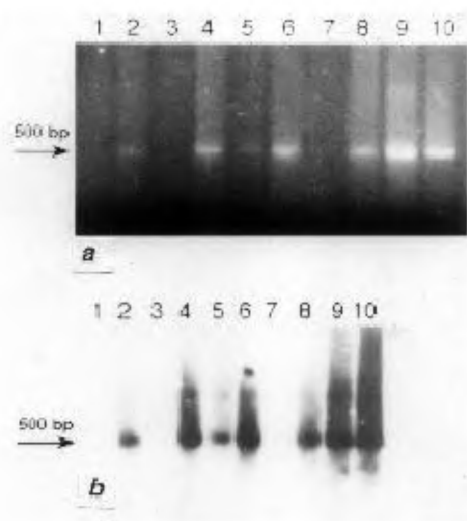
Figure 1. Molecular analysis of Basta resistant R0 plants. *a*, PCR amplification of 500 bp *bar* gene fragment from leaf DNA; *b*, Southern blot of the PCR products probed with ECL-labelled *bar* DNA. Lane 1, normal Sonalika; lane 2, transformed Sonalika (S1); lane 3, normal UP 2338, lanes 4–9, putative transformants U1-U6 in UP-2338; lane 10, pDM803 control.
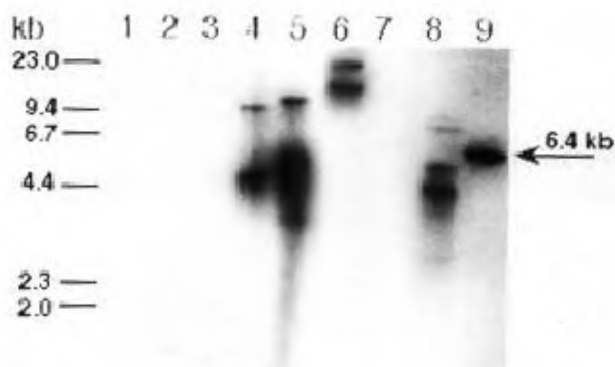

Figure 2. Southern blot analysis of transgenic wheat plants. Genomic DNAs from the transformants and vector pDM803 were digested with *Nco*1, electrophoresed and after transfer to nylon membrane, probed with ECL labelled *bar* DNA. Lanes 1–3, non-transformed CPAN 3004, Sonalika, and UP 2338, respectively; lane 4, a *bar* and *gus* positive R1 plant of CPAN 3004 (C1); lane 5, R0 transformant of Sonalika (S1); lanes 6-8, R0 transformants of UP 2338 (U3, U4, U6, respectively); lane 9, pDM 803 as positive control with *bar* gene located on a 6.4 kb *Nco*I fragment.

Genomic DNAs from R0 plants S1, U1, U2, U3, U4, U5 and U6 were analysed in Southern blots probed with *bar* gene. In addition, one of the herbicide-tolerant R1 progeny of the transformant in CPAN 3004 (designated C1) was also analysed by Southern blotting. Four of the transformants (namely C1, S1, U3, and U6) showed multiple hybridizing fragments of different size (Figure 2). Each band can be interpreted as the result of a single

Table 2. Analysis of segregating R1 progeny from transformed CPAN-3004

| | Frequency | | |
Test	Positive	Negative	χ^2 (Ratio)
Histochemical GUS assay*	41	4	0.357 (15:1)
Fluorometric MUG assay	58	6	1.066 (15:1)
PCR for *bar* gene	40	24	5.330 (3:1)
Herbicide tolerance	45	19	1.155 (3:1)

*Tested on root tips from a separate set of 45 seeds. Other three tests were done on the same set of 64 plants.

integration event. Size of the hybridizing DNA fragments in the genomic Southern were different, thus indicating that transgene with different length of vector DNA has integrated in the host genomic DNA at different locations (Figure 2). Furthermore, in only one of the transformants namely S1, a band of 6.4 kb was detected (equal to the vector DNA) by the *bar* probe (Figure 2, lanes 5, 9). This is most likely due to integration of incomplete vector DNA in most cases. Unlike the *Agrobacterium* system, there is no fixed T-DNA boundaries in the Biolistic transformation. Hence, different parts and size of the vector DNA are known to integrate[26]. Similar to the PCR results shown above, the transformant U4 did not hybridize with the *bar* probe. For transgenics U1, U2 and U5, weak hybridizing signals were detected on longer exposure of the film (not shown).

Expression of *gus* gene in the R0 plants was tested qualitatively and quantitatively by microtitre plate-based MUG assay, Western blotting and histochemical GUS assays (Table 1). The β-glucuronidase activity in the leaf extracts of transformed plants was tested with MUG as substrate showing blue fluorescence in all the transformants of cultivar UP 2338, including U4 which was negative for the *bar* gene, with varying levels of light emission (Table 1), intense fluorescence was also observed with the transformed Sonalika (S1) and CPAN 3004 (C1). No background fluorescence was observed in the control plants of these cultivars.

Total proteins extracted from the leaves of R0 transformants of cultivar UP 2338 were also probed with anti β-glucuronidase antibodies in immunoblots. All the R0 plants (U1, U2, U3, U4, U5, U6) of UP 2338 showed a 68 kDa band reacting with the antibodies, similar to purified GUS protein (Table 1), though U4 and U5 reacted only weakly. All of these plants were also positive in fluorometric MUG assay, where the intensity of signal was highest in U1 and U6. Histochemical assay with the X-Gluc substrate also showed strong blue colour in the leaf samples of U1 and U6 (Figure 3), while other R0 plants showed very light staining. Interestingly, the leaves of R0 plant CPAN 3004 also showed weak reac-

tion in histochemical GUS assay, but their R1 progenies showed strong colour intensity in the root tips as described below.

Seeds harvested from the single R0 transformant of CPAN 3004, were tested in R1 generation to study the segregation patterns of *bar* and *gus* genes. Forty-five R1 seeds germinated on filter paper were analysed first by histochemical GUS assay on the root tips. After forty-eight hours of staining, clear segregation of blue and cream colour root tips was seen in a 15:1 ratio (Table 2). Although there were different shades of blue due to gene dosage effect, root tips from the GUS negative R1 seeds and the control CPAN 3004 were clearly yellowish cream (Figure 4). Leaf proteins and DNAs were extracted from individual R1 plants and non-transformed control plants for analyses of tansgenes at the protein and DNA level. The same plants were used for herbicide spray later to establish a correlation between the presence of *bar* gene and its expression. In visual fluorometric assay of leaf proteins with MUG, 58 plants were positive for GUS activity with varying levels of fluorescence intensity, once again confirming a digenic segregation ratio ($\chi^2 = 1.066$), similar to the results of histochemical assay on root tips (Table 2).

Segregation pattern for the presence of *bar* gene in the same R1 plants was analysed by PCR amplification in duplicates. Forty out of the 64 plants tested showed expected 500 bp amplicon which also hybridized to the labelled *bar* gene probe in Southern blots similar to that shown in Figure 1. However, unlike the *gus* gene, this frequency did not fit with a 15:1 ratio, and was much closer to a monogenic 3:1 ratio (Table 2). The χ^2 value was significantly higher even for a 3:1 ratio ($P = 0.02$–0.01), but this is the simplest ratio expected as any higher number of the gene would give still higher number of *bar* positive R1 progeny.
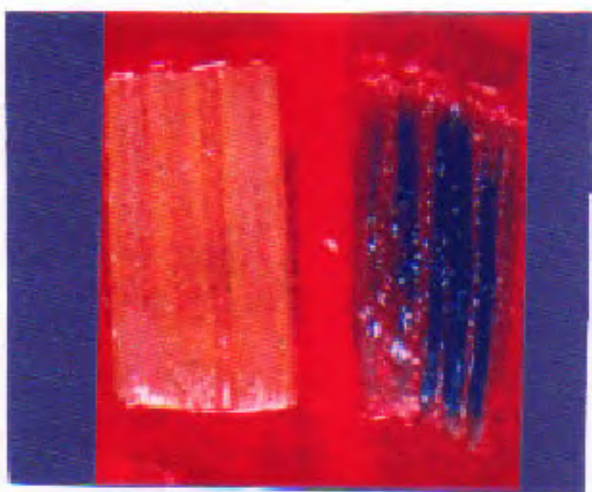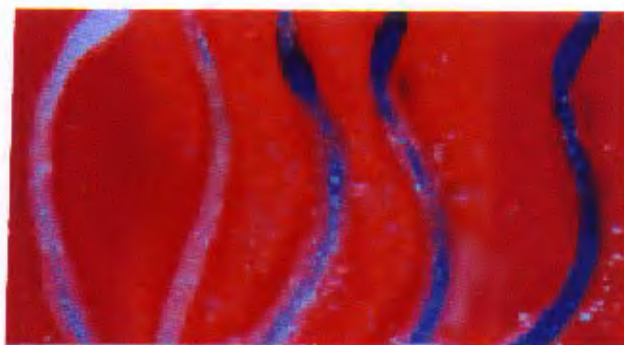

Figure 4. Different levels of GUS expression in the roots of segregating R1 progeny of transgenic CAPN 3004, representing different dosage of the *gus* gene.
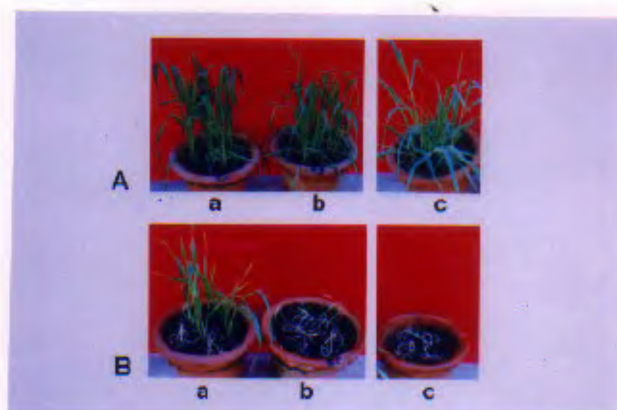

Figure 5. Herbicide tolerance in the R1 progeny of transgenic CPAN 3004. A, Plants without herbicide application. B, Plants sprayed with Basta (250 mg/l PPT); *a*, segregating R1 progeny of transformed CPAN 3004; *b*, non-transformed controls; *c*, weed *Phalaris minor*.

After one month of growth, these 64 R1 plants were sprayed with herbicide Basta (250 mg/l PPT). *Phalaris minor* was also challenged with the same dose of Basta. Plants of *Phalaris minor* and control wheat were completely killed whereas the transgenic R1 plants segregated for tolerant and susceptible in a 3:1 ratio, $\chi^2 = 1.155$ (Table 2, Figure 5). Two thirds of the herbicide-tolerant plants showed partial damage and subsequent recovery, suggesting a dosage effect of the segregating *bar* gene in a 1:2:1 ratio. The dose of the herbicide Basta was critical in discriminating between transgenic and normal wheat plants, as a higher dose (500 mg/l) resulted in appreciable damage to the transgenic plants. However, PCR amplification was not obtained in five of the herbicide-tolerant R1 plants,

Figure 3. Strong GUS expression in the leaf of a R0 transgenic UP 2338 (Plant U6). left, Control; right, transgenic.

suggesting that PCR data may not give sufficient information to determine the Mendelian segregation pattern in transgenic population due to inconsistency. The segregation patterns indicate that the original R0 transformant of CPAN 3004 had two functional *gus* genes but only one functional *bar* gene. Since both the genes are located on a single vector DNA, this indicates integration of different lengths of the vector DNA in the genome. This is also evident from the occurrence of two different NcoI fragments hybridizing in the Southern blots (Figure 2, lane 4). Out of the two restriction fragments hybridizing with the *bar* gene probe, only one may bear a functional *bar* gene as indicated by the 3:1 segregation ratio in the R1 progeny. A 15:1 segregation ratio with the *gus* gene suggests that this gene is functional at both the integration sites.

Of the eight putative transformants that were selected on PPT-containing medium, all were found to be positive for *gus* gene expression. One transformant (U4) of cultivar UP 2338 tested negative for the *bar* gene, and this could be a possible escape during selection. Low number of escapes could be due to judicious four-step selection strategy, including high selection pressure applied during the shoot regeneration period. Up to 95% escapes have been reported in some studies with bialaphos as selection agent[8–12]. Low transformation efficiency in the present work may be due to use of high-yielding commercial cultivars irrespective of their *in vitro* regeneration capacity. Transformation efficiencies of 0.1% to 2.5% have been reported by other research groups[4,8–10,12,13,20]. We could recover transgenic plantlets within a period of 13 to 14 weeks from the date of culture of immature embryos from these cultivars.

In conclusion, we report production of herbicide tolerant transgenic plants in three high-yielding Indian wheat cultivars namely CPAN 3004, Sonalika and UP 2338, resulting from several independent integration events. Use of a modified transformation protocol and a careful selection strategy has resulted in an overall transformation efficiency of 0.29%. Even this low frequency is quite useful, as the agronomically important genes have to be transferred directly to best commercial cultivars to avoid time loss in backcrossing. Biosafety of PPT-tolerant crops has been evaluated in detail based on a transgenic approach, describing both PPT and its metabolites as well as the *bar* gene and its products fully biosafe[27]. Transgenics developed in this study could find a major use in tackling the problem weed *Phalaris minor* in the wheat fields, particularly under low-tillage and zero-tillage conditions. Since in some areas this weed has developed resistance against commercially-used herbicides, e.g. Isoproturon, the new transgenic lines provide an attractive alternative for weed management in the affected areas.

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