cacy of curcumin for its probable action through its active metabolite such as tetrahydrocurcumin even after its metabolism.


ACKNOWLEDGEMENTS. This work was supported by a grant from the Council of Scientific and Industrial Research, New Delhi. We thank Sanj Chemicals and Extracts (Bangalore) for the gift sample of tetrahydrocurcumin.

Received 10 June 2002; revised accepted 24 October 2002

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

Archana Chugh and Paramjit Khurana*
Department of Plant Molecular Biology, University of Delhi South Campus, Benito Juarez Road, New Delhi 110 021, India

Hexaploid bread wheat (Triticum aestivum) and tetraploid emmer wheat (T. dicoccum) hold immense agricultural and economical importance as their end products have varied utilities depending upon the viscoelasticity and other properties of the flour. In the present study, highly regenerable basal segment calli have been employed as the target tissue for genetic transformation of Indian varieties of bread wheat (CPAN1676, PBW343) and emmer wheat (DDK1001). The bar gene conferring herbicide resistance was introduced in one-month-old calli employing both particle bombardment and Agrobacterium-mediated transformation strategies. Transgenic calli were selected on phosphinothricin-containing regeneration medium and putative transformants were raised to maturity. Though the plants exhibited reduced vigour in terms of height and tillering, nonetheless, seed set was normal. The presence of the transgene (bar) was confirmed by PCR and Southern hybridization. In general, the transformation efficiency was found better with Agrobacterium, even though the construct carried CaMV35S driven bar gene, whereas in the case of biolistics Ubi1 (a monocot promoter)-driven bar gene was employed. Transformation efficiency in the range of 4% was obtained with particle bombardment, whereas it was 7.5% using Agrobacterium-mediated co-cultivation. The different varieties of bread and emmer wheat investigated did not show any marked difference in their transformation ability and could be attributed to a well-established regeneration system in these varieties.

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

*For correspondence. (e-mail: paramjitkhurana@hotmail.com)
of the grain reduce its consumer acceptability. Thus both bread and emmer wheat possess mutually exclusive but economically desirable qualities. With the advent of transgene technology and practical amenability of cereals it should be possible to introduce advantageous traits in different wheats to augment their nutritive value and consumer acceptance.

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

	extit{T. aestivum} var. CPAN1676, PBW343 and \textit{T. dicoccum} var. DDK1001 were procured from Directorate of Wheat Research, Karnal, India. Seeds were surface-sterilized with 4\% v/v hypochlorite and washed with water thrice prior to use. Five-day-old seedlings were raised on callusing medium [\(\text{MS} + \text{AgNO}_3 (10 \text{mg} \text{l}^{-1}) + \text{TIBA} (1 \text{mg} \text{l}^{-1}) + \text{kinetin} (0.4 \text{mg} \text{l}^{-1})\)] containing 3.5 \text{mg} \text{l}^{-1} L-phosphinothricin for at least two weeks. Cali surviving the first phase of selection were transferred further on regeneration medium supplemented with 1.5 \text{mg} \text{l}^{-1} phosphinothricin for a week. The final round of selection was optional and plantlets were kept on 2 \text{mg} \text{l}^{-1} phosphinothricin for one week. The regenerating cali, after these stages of selection, were transferred to selection free regeneration medium for further growth. The cultures were maintained at 26 ± 1°C, discontinuous light throughout the selection procedure.

Among the various CAMBIA vectors\(^3\) available, pCAMBIA3301 conferring herbicide resistance to the plants was chosen for \textit{Agrobacterium}-mediated transformation of the basal segment calli. The construct carries \textit{bar} and \textit{GUS} reporter genes driven by CaMV35S promoter. This binary vector was mobilized in the \textit{Agrobacterium} host strain LBA4404 by triparental mating. Bacterial culture of LBA4404 (pCAMBIA3301) was initiated by inoculating 50 \text{µl} of glycerol stock in 25 ml YEP medium supplemented with 100 \text{µg} \text{ml}^{-1} rifampicin, 50 \text{µg} \text{ml}^{-1} kanamycin sulphate and 200 \text{µM} acetosyringone. The cultures were kept at 28°C, 200 rpm for 24–48 h. For callus transformation, 100 \text{µl} of freshly-grown culture was inoculated in Erlenmeyer flasks containing 25 ml YEP medium supplemented with appropriate antibiotics and 200 \text{µM} acetosyringone. The cultures in the log phase with OD\(_{600}\) 0.25 to 1.00 were pelleted in SS-34 tubes at 4000 rpm, 4°C for 10 min. Bacterial density was calculated and cell density was adjusted to 5 × 10^5 cells/ml with liquid MS medium supplemented with 200 \text{µM} acetosyringone. Basal segment calli were incubated in the bacterial suspension for 1 h. After blotting on Whatman No. 1, the calli were placed on callusing medium supplemented with 200 \text{µM} acetosyringone for two days (co-cultivation period) in dark at 28°C.

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

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

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

For Southern hybridization; genomic DNA was isolated from 100 to 200 mg leaf material of the putative transformants following the protocol of Dellaporta \textit{et al.}. Then 20–22 µg of genomic DNA of putative transformants was either double digested with \textit{EcoRI} and \textit{BamHI} (in case of biolistically obtained plants) or digested with \textit{XhoI} (Agrobacterium-mediated transformation) and fractionated on 1% agarose gel. The DNA was capillary-blotted onto Hybond-N membrane (Amersham International Inc., UK) and incubated in prehybridization solution (50% formamide, 5X Denhardt's reagent, 5% dextran sulphate, 5X SSC, 200 µg/ml Herring sperm DNA) for one day at 37°C with shaking at 40 rpm. The probe was purified PsI fragment of pAHC20 (ref. 3) (carries \textit{Ubi1}-driven \textit{bar} gene) excised from low-melting agarose gel and radio-labelled using Megaprime labelling kit (Amersham International Inc., UK) and (α\textsuperscript{32}P) ATP (BRIT, India), according to manufacturer's specifications. The radiolabelled probe was added to the hybridization solution (same composition as prehybridization solution but with 10% dextran sulphate and 250 µg/ml of Herring sperm DNA) for two days at 37°C and at 40 rpm. The blot was washed and exposed to X-ray film in hypercassettes at – 80°C for the required time. The autoradiogram was developed and fixed using Kodak Affiliate Products, Mumbai, India.

Despite the extensive use of herbicides, productivity losses in wheat due to weeds amount to nearly 12.3%. Weeds commonly found in wheat fields are known for their high degree of resistance to the commonly used herbicides. Therefore, Basta and Glyphosate have been suggested as more suitable herbicides for weed control\textsuperscript{9}. Resistance of wheat towards Basta has the potential to provide substantial relief from yield losses caused by weeds. In the earlier reports, \textit{bar}-resistant plantlets have been obtained by employing either immature embryos or the scutellum tissue\textsuperscript{12}. The present investigation tests the amenability of the basal segment cultures as a recipient explant for herbicide resistance encoded by the \textit{bar} gene and compares transformation efficiency by particle bombardment and Agrobacterium-mediated transformation approaches.

One-month-old basal segment calli were bombarded with pAHHC25 (ref. 3) using the parameters mentioned earlier. The bombarded calli were subjected to two or three rounds of selection, initially at 3 mg l\textsuperscript{-1} PPT followed by 1.5 mg l\textsuperscript{-1} PPT for two weeks and one week respectively (Figure 1 c, d), and finally on a selection-free medium. At maturity (Figure 1), although the number of tillers produced by the transformants was similar to the normal untransformed control plants, they showed reduced height and the number of seeds produced per inflorescence was also less (5–7 seeds). The transformation efficiency (Table 1) of the two genotypes was recorded as the number of regenerating plantlets/total number of explants bom-
barded, according to Takumi and Shimada\(^9\). Although CPAN1676 (\(T. \) aestivum) exhibited maximum transformation efficiency (4.4%), 80% of the transformed plants of PBW343 were fertile. In case of \(T. \) dicoccum, 4.3% transformation efficiency was noted with 33.33% fertility (Table 1).

Presence of the transgene ‘\(\text{bar} \)’ in the primary transformants was confirmed by PCR amplification of genomic DNA samples using \(\text{bar}\)-specific primers. Plasmid pAHC25 was used as a positive control and the genomic DNA of an untransformed plant served as the negative control. Along with the positive control, a 296 bp ampli

For Southern analysis of \(T_0\) transformants of \(T. \) aestivum and \(T. \) dicoccum, genomic DNA was double-digested with \(\text{EcoRI} \) and \(\text{BamHI} \) and probed with \(\text{PstI}\)-fragment of pAH20 (~ 603 bp) spanning the \(\text{bar} \) coding region. Southern analysis revealed the presence of a 860 bp band (\(\text{bar} \) coding region and the \(\text{nos} \) 3’ terminator which is excised upon double digestion with \(\text{EcoRI} \) and \(\text{BamHI} \)) in all the independent transformants (Figure 2 b). The transformation efficiency reported by previous workers generally varied from 0.1 to 5.7% (refs 1, 2 and 10). Uze and co-workers\(^\text{11}\) have, however, reported 14% transformation efficiency of the wheat embryonic tissue via biolistics using a single-stranded linearized DNA. In the present study, a transformation efficiency of approx. 4% has been achieved, which is comparable to that obtained by earlier workers, and is significant since the recipient tissue is of non-embryogenic origin.

Method have been developed for efficient \(Agrobacterium\)-mediated transformation of rice\(^\text{12}\), maize\(^\text{13}\), barley\(^\text{14}\) and sorghum\(^\text{15}\). Cheng and co-workers\(^\text{16}\) described the production of normal, fertile transgenic wheat plants following incubation of immature embryo and embryogenic calli with \(Agrobacterium \) \(tumefaciens\). Though few reports are available on \(Agrobacterium\)-mediated stable wheat transformation, preliminary reports have demonstrated the interaction of the bacteria with wheat tissues, as observed in the scanning electron micrographs\(^\text{17,18}\).

The suitability of various explants for \(Agrobacterium\)-mediated gene delivery and factors influencing \(Agrobacterium\)-mediated introduction of transgene have been extensively investigated\(^\text{19,20}\). Although rapidly dividing tissues are more amenable to \(Agrobacterium\) invasion\(^\text{21}\) paradoxically, meristematic basal segment cultures with actively dividing cells have been rarely exploited for their transformation potential. In the present study, the amenability of the basal segment calli as competent explants for \(Agrobacterium\)-mediated wheat transformation has been investigated by introducing the herbicide resistance gene ‘\(\text{bar} \)’.

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

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

<table>
<thead>
<tr>
<th>Plant material</th>
<th>No. of explants bifornaded</th>
<th>No. of plants regenerared</th>
<th>No. of plants established in soil</th>
<th>No. of fertile plants</th>
<th>Transformation efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Particle bombardment*} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T. ) aestivum var. CPAN1676</td>
<td>181</td>
<td>8</td>
<td>7</td>
<td>3</td>
<td>4.4</td>
</tr>
<tr>
<td>(T. ) aestivum var. PBW343</td>
<td>137</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>3.6</td>
</tr>
<tr>
<td>(T. ) dicoccum var. DDK1001</td>
<td>208</td>
<td>9</td>
<td>6</td>
<td>2</td>
<td>4.3</td>
</tr>
<tr>
<td>(\text{Agrobacterium-mediated**} )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(T. ) aestivum var. CPAN1676</td>
<td>222</td>
<td>15</td>
<td>3</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>(T. ) aestivum var. PBW343</td>
<td>251</td>
<td>21</td>
<td>4</td>
<td>4</td>
<td>8.7</td>
</tr>
<tr>
<td>(T. ) dicoccum var. DDK1001</td>
<td>187</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>6.9</td>
</tr>
</tbody>
</table>

*Calli were bombardcd at 1100 psi and 6 mm barrel distance with pAH25 coated on gold particles (1 micron).

**Calli were co-cultivated with LBA4404 (pCAMBIA3301) for two days followed by washings and transfer to regeneration medium supplemented with 125 mgl\(^{-1}\) cefotaxime. After three days, calli were subjected to selection (1.5–3 mgl\(^{-1}\) phosphinothricin).
employing mature embryos as explants for co-cultivation, and better than the immature embryos and embryogenic calli (4%) \(^16\).

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

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

---

**Figure 2.** \textit{a}, PCR analysis of \textit{T} \textsubscript{0} transformants of \textit{T. aestivum} var. CPAN1676, PBW343 and \textit{T. dicoccum} var. DDK1001 using primers specific to \textit{bar} gene. Transformants were obtained by particle bombardment of the basal segment calli with \textit{pAHC25}. Plasmid and genomic DNA isolated from untransformed plant were used as positive and negative controls respectively. \textit{b}, Southern analysis of \textit{T} \textsubscript{0} transformants of \textit{T. aestivum} var. CPAN1676, PBW343 and \textit{T. dicoccum} var. DDK1001 obtained by particle bombardment with \textit{pAHC25}. Lane 1 (from left), Positive control, \textit{P} [\textit{D}]; lanes 2 and 3, Digested [\textit{D}] and undigested [UD] genomic DNA of a control \textit{T. aestivum} var. CPAN1676 untransformed plant; lanes 4 and 5, Undigested genomic DNA of putative transformants of \textit{T. aestivum} and \textit{T. dicoccum} respectively; lanes 6–11, Double-digested (\textit{EcoRI} + \textit{BamHI}) genomic DNA of transformed lines. Southern blot was probed with 600 bp \textit{bar} fragment obtained by \textit{PstI} digestion of \textit{pAHC20}. Size (kb) represents the fragment size of \textit{\lambda} (\textit{HindIII} digest) marker. A 840 bp band was obtained in all the transformants.

**Figure 3.** Southern analysis of \textit{T} \textsubscript{0} transformants obtained by \textit{Agrobacterium}-mediated transformation of one-month-old basal segment calli co-cultivated with LBA4404 (pcAMBIA3301). Lane 1 (from right), \textit{XhoI} digested [\textit{D}] genomic DNA from a control, untransformed plant; lane 2, Digested positive control; lanes 4–10, \textit{XhoI} digested genomic DNA of \textit{T. dicoccum} and \textit{T. aestivum} with the varieties mentioned at the top; lane 11, Undigested [UD] genomic DNA of control (\textit{T. aestivum} var. CPAN1676), untransformed plant; lanes 12 and 13, Undigested genomic DNA sample of \textit{T. dicoccum} and \textit{T. aestivum} respectively. The Southern blot was probed with 600 bp fragment coding for \textit{bar} gene obtained on \textit{PstI} digestion of \textit{pAHC20}. Autoradiogram shows presence of ~560 bp band coding for \textit{bar} gene in the transformed plants. Size (kb) represents the fragment size of \textit{\lambda} (\textit{HindIII} digest) marker.
ven by CaMV35S promoter during Agrobacterium co-cultivation, whereas for particle bombardment the gene was driven by the monocot promoter Ubi1. The difference in the ploidy level of bread and emmer wheat did not appear to interfere with the transformation efficiency in the ploidy level of bread and emmer wheat did demonstrate to be highly regeneration-dependent. There are few reports describing the transformation efficiency between the two different varieties of wheat for particle bombardment. The present study may be attributed to a well-standardized regenera
tion system for these species and varieties. The present work thus paves the way for introduction of other agriculturally desirable traits in commercially popular Indian wheat types.


ACKNOWLEDGEMENTS. We thank Dr B. S. Mallick, Indian Agricultural Research Institute, New Delhi for providing the seed material, Dr Peter Quail, University of California, Berkeley, USA for the constructs pAHC20 and pAHC25, and Dr R. A. Jefferson, CAMBIA, Canberra, Australia for the CAMBIA vectors. We acknowledge the financial support from Department of Biotechnology, Government of India, New Delhi. A.C. also acknowledges the Senior Research Fellowship awarded by UGC, Government of India, New Delhi.

Received 15 July 2002; revised accepted 19 October 2002.

Proteome maps of flood-tolerant FR 13A and flood-sensitive IR 54 rice types depicting proteins associated with O2-deprivation stress and recovery regimes

Himanshu Dubey‡, Geeta Bhatia§, Santosh Pasha# and Anil Grover‡#*

‡Department of Plant Molecular Biology, University of Delhi South Campus, New Delhi 110 021, India
§Peptide Research Laboratory, CSIR Centre for Biochemical Technology, Mall Road, Delhi 110 007, India
#RIEC-Indian Council of Agricultural Research, New Delhi, India

Rice crop, particularly in lowland ecosystem, is severely affected by flooding stress. To genetically engineer rice with improved flooding tolerance, it is important to gain an understanding of the proteins that constitute its flooding-stress response. A major component of the flooding stress is a decline in availability of O2. Proteomics approach is a powerful tool for analysing global changes in protein profiles. We have constructed proteome maps of flood-tolerant IR 54 and flood-tolerant FR 13A rice types corresponding to several time points during their response to O2 deprivation stress. Several up- and down-regulated proteins have been identified by 1D and 2D protein gel electrophoresis followed by silver staining. Based on peptide sequence analysis, we indicate that sucrose synthase, glyceraldehyde 3-phosphate dehydrogenase, UDP-glucose-6-dehydrogenase and asparagus synthetase are implicated in rice flooding-stress response.

RICE is the most important food crop in the world. Nearly 25% of the world rice (i.e. 38 mha) cultivated in the lowland ecosystem accounts for only 17% of the global rice supply¹. Amongst the South Asian countries, India has the largest area (i.e. 17.2 mha) under rainfed lowland. The

¹For correspondence. (e-mail: grover_anil@hotmail.com)