Agrobacterium-mediated Transformation Efficiency in Blackgram and Rice Enhanced by Multiple Copies of pTiBo542 virB and virG

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Broad host-range Inc W plasmids pBAL3 with 3' end of virB and complete virG of pTiBo542 and pToK47 with complete virB and virG were introduced into Agrobacterium tumefaciens vir helper strains harbouring a binary vector pBAL2 (Inc P). Transformation efficiencies of these strains were evaluated by transforming the primary leaf segments of blackgram [Vigna mungo (Linn.) Hepper cv. CoS] and the scutellum-derived calli of rice [Oryza sativa Linn. cv. Pusa Basmati 1]. Transformation was evaluated on the basis of GUS staining in stably transformed calli. In the octopine vir helper strain LBA4404 harbouring pBAL2, the plasmid pBAL3 with the 3' end of virB and complete virG of pTiBoS42 increased transformation efficiency by 141% to 192%. The plasmid pToK47 that carried pTiBoS42 virB in addition to virG did not promote transformation of blackgram any further. In the L,L-succinamopine vir helper strain EHA105, pBAL3 did not improve blackgram transformation. In rice, transformation of LBA4404(pBAL2) occurred only when multiple copies of pTiBo542 vir genes were present. Transformation efficiencies with pBAL3 and pToK47 were 10 and 18%, respectively. While, multiple copies of 3'end of virB and virG of pTiBo542 harboured on a compatible replicon were effective in blackgram, complete virB was additionally required for efficient transformation of rice.

Keywords: Agrobacterium tumefaciens, virG, GUS expression, blackgram, rice, transformation

Introduction

Agrobacterium tumefaciens Ti plasmid vectors are highly suitable for transforming a wide range of plants (Gelvin, 2000). However, many important crops such as grain legumes and monocots are relatively recalcitrant to A.-mediated transformation (Schroeder et al, 1993; Chan et al, 1993). A number of strategies have been deployed to overcome the limitations in transforming these plants (Hiei et al, 1994; Jaiwal et al, 2001). The type of virG and its copy number in Agrobacterium were found to be

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Abbreviations:
Ap: Ampicillin; BAP: 6-Benzylaminopurine; BCIM: Blackgram callus-induction medium; BRIT: Board of radiation and isotope technology; Cx: Cefotaxirne; 2,4-D: 2,4-Dichlorophenoxyacetic acid; gusA: β-glucuronidase; Hm: Hygromycin; hph: hygromycin phosphotransferase; Inc: Incompatibility group; 2ip: 6-(y, y-dimethylallylamino) purine; Km: Kanamycin; KN: Kinetin; LB: Left border; NAA: α-Naphthaleneacetic acid; nptl/: neomycin phosphotransferase; RB: Right border; RCIM: Rice callus-induction medium; Rm: Rifampcin; RSSIM: Rice shoot-induction medium; Sm: Streptomycin; Sp: Spectinomycin; Te: Tetracycline.

important contributing factors. Rogowsky et al (1987) showed that an increase in the copy number of virG resulted in a proportional, acetosyringone-independent increase in vir gene expression. Jin et al (1987) found that a 2.5 kb DNA fragment harbouring virG and 3' end of the virB operon from pTiBo542 was responsible for the supervirulence phenotype of the L,L-succinamopine type A. tumefaciens strain A281. They also reported that virC, virD and 5' end of the virB operon are not involved in the supervirulence. Komari (1990) showed that A. tumefaciens strains harbouring complete virB, virG and virC of pTiBo542 were able to transform cell suspension cultures of Chenopodium quinoa, which were not transformed by common binary vectors, as pGA437 and pGA472. Multiple copies of virG in A. tumefaciens greatly enhanced the transient transformation efficiency of celery, carrot and rice tissues (Liu et al, 1992).

Multiple copies of pTiBo542 virB, virG and virC have been reported to increase the efficiency of Agrobacterium-mediated transformation of many crops (Hiei et al, 1994; Ishida et al, 1996). Jaiwal et al (2001) generated transgenic Vigna radiata by using the superbinary vector pTOK233 with multiple copies of pTiBo542 virB, virG and virC. Introduction of a
ternary plasmid carrying a constitutive virG mutant into A. tumefaciens strain in combination with a standard binary vector resulted in higher transformation efficiencies in different plant species (van der Fits et al., 2000).

Multiple fold increase in the efficiency of transient transformation of rice and soybean has been demonstrated with constitutive virG mutant carried on the binary vector (Ke et al., 2001).

To date, the generation of transgenic Black gram (Vigna mungo (Linn.) Hepper cv. Co5) plants by A. tumefaciens transformation has not been reported. Karthikeyan et al. (1996) reported the generation of transformed black gram calli through Agrobacterium-mediated transformation at a low frequency. Generation of transgenic rice plants using Agrobacterium has been previously reported (Hiei et al., 1994; Rashid et al., 1996; Mohanty et al., 1999; Khanna & Raina, 1999; Kumria et al., 2001). In a majority of studies on rice transformation, the superbinary vector pTOK233 that carries pTiBo542 virB, virG and virC has been used to improve the efficiency of Agrobacterium-mediated transformation. In the present study, a ternary system, in which a broad host-range plasmid that is compatible with the binary vector and harbours the pTiBo542 vir genes in multiple copies is evaluated, and usefulness of complete virB and virG of pTiBo542 for rice transformation, and 3' end of virB and virG for transformation of blackgram is also reported.

Materials and Methods

Seed Material

Certified seeds of blackgram cv. Co5 were obtained from Tamil Nadu Agricultural University, Coimbatore, India. Seeds were surface-sterilized as described by Karthikeyan et al. (1996). Seeds of indica rice cv. Pusa Basmati 1 (PB1) were obtained from Dr George Thomas, Southern Petrochemicals Industries Corporation (SPIC) Science Foundation, Chennai. Mature rice seeds were dehusked and surface-sterilized as described previously (Vijayachandra et al., 1995).

Bacterial Strains and Growth Conditions

A. tumefaciens and Escherichia coli strains and plasmids used in this study are listed in Table 1. A. tumefaciens was grown at 28°C either in YEP or in AB minimal medium (Chilton et al., 1974), and E. coli was grown in Luria-Bertani medium (Miller, 1972) at 37°C. The concentration of antibiotics (mg l⁻¹) used for culturing A. tumefaciens strains on solid media were as follows: rifampicin (10), tetracycline hydrochloride (5), streptomycin sulphate (300), spectinomycin dihydrochloride (100) and carbenicillin (100 mg l⁻¹ for EHA105 & 5 mg l⁻¹ for LBA4404). Liquid cultures of A. tumefaciens were raised in medium supplemented with half the concentration of the antibiotics specified above. Kanamycin monosulphate (50), ampicillin sodium salt (50) and tetracycline hydrochloride (15) were used for E. coli. These antibiotics were bought from Sigma Chemical Co, St Louis, USA.

Plasmid Constructions

DNA cloning and transformation of E. coli DH5α with plasmid DNA was performed as described by Maniatis et al. (1982). The plasmid pBAL2 is a binary vector derived from pGA472 (An et al., 1985) that carries nptII as a plant selection marker.

The binary plasmid pBAL2 was constructed as follows: a 1.7 kb hph cassette from pTRA151 (Zheng et al., 1991) was cloned as an EcoR I fragment into pBIG (Table 1). Following transformation, the colonies were initially selected on 50 mg l⁻¹ ampicillin and subsequently patched on 50 mg l⁻¹ hygromycin with the appropriate controls. The colony whose growth was comparatively equivalent to the positive control (pTRA151) was chosen, plasmid DNA was extracted and subjected to detailed restriction analysis. The recombinant plasmid containing hph and gusA gene cassettes was named as pBAL1.

The plasmid pBAL1 was digested with Xho I and cloned into pMH1002, a pGA472 derivative (An et al., 1985). Colonies were selected on LB medium containing 50 mg l⁻¹ ampicillin and 15 mg l⁻¹ tetracycline. Detailed restriction analysis of the plasmid DNA from the selected colonies confirmed the clone. The appropriate orientation of the insert in the clone was confirmed by Kpn I digestion. The pBSIINKS+ backbone was removed from plasmid DNA by Kpn I digestion and self-ligation. Following transformation, the colonies were initially selected on 15 mg l⁻¹ tetracycline and patched on 50 mg l⁻¹ ampicillin. Clones with ampicillin sensitivity and tetracycline resistance were chosen. The resultant binary plasmid with nptII and hph as plant selection markers and gusA with catalase intron as a reporter was named as pBAL2 (Fig. 1).

The wide host-range replicon pBAL3 (Fig. 2) with 3' end of virB and complete virG was constructed as follows: a 4.7 kb Sal I fragment 10 representing a
Table I—Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. tumefaciens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LBA4404</td>
<td>A <em>vir</em> helper, harbours disarmed Ti-plasmid pAL4404, a T-DNA deletion derivative of pTiAch5, octopine type (Rm')</td>
<td>Hoekema <em>et al.</em>, 1983</td>
</tr>
<tr>
<td>EHA105</td>
<td>A <em>vir</em> helper, <em>L</em>, L-succinamopine type, harbours T-DNA deletion derivative of pTiBo542, a supervirulent-type Ti-plasmid (Rm')</td>
<td>Hood <em>et al.</em>, 1993</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMH1002</td>
<td>A derivative of the binary vector pGA472 harbouring a multiple cloning site (Tc'), Inc P</td>
<td>Lab collection</td>
</tr>
<tr>
<td>pUCD2</td>
<td>A wide host-range replicon of the incompatibility group, Inc W (Tc', Km', Ap' &amp; Sp'/Sm')</td>
<td>An <em>et al.</em>, 1985</td>
</tr>
<tr>
<td>pSBGA281-G</td>
<td>Harbours the Sal I 10 fragment of pTiBo542 containing the 3' end of <em>virB</em>, complete <em>virG</em>, <em>virC</em> and the 5' end of <em>virD</em> in pUC119 (Ap')</td>
<td>Close <em>et al.</em>, 1984</td>
</tr>
<tr>
<td>pBAL2</td>
<td>A pGA472-based binary vector harbouring <em>nos-nptII</em> into which CaMV 35S-<em>hph</em> from pTRA151 and CaMV 35S-<em>gusA</em> with catalase intron were introduced (Tc'), Inc P</td>
<td>Gelvin, 2000</td>
</tr>
<tr>
<td>pBAL3</td>
<td>The Sal I fragment 10 of pTiBo542 having the 3' end of <em>virB</em>, complete <em>virG</em>, <em>virC</em> and the 5' end of <em>virD</em> in pUCD2 (Ap', Km', Sp'/Sm'), Inc W</td>
<td>This work</td>
</tr>
<tr>
<td>pToK47</td>
<td>A pUCD2 derivative, harbouring the 15.8 kb Kpn I fragment of pTiBo542 containing the entire <em>virB</em>, <em>virG</em> and <em>virC</em> (Ap', Tc' &amp; Sp'/Sm'), Inc W</td>
<td>Zheng <em>et al.</em>, 1991</td>
</tr>
<tr>
<td>pBIG</td>
<td>Harbours the CaMV 35S-<em>gusA</em> gene with catalase intron as Hind III/EcoR I fragment in pBSIIKS+ (Ap')</td>
<td>Ohta <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>pTOK233</td>
<td>A superbinary plasmid harbouring <em>nos-nptII</em>, CaMV 35S-<em>hph</em> and CaMV 35S-<em>gusA</em> with catalase intron within the T-DNA and pTiBo542 <em>virB</em>, <em>virG</em> and <em>virC</em> genes in the region outside the T-DNA (Tc')</td>
<td>S B Gelvin, 2000</td>
</tr>
</tbody>
</table>

portion of the pTiBo542 *vir* region (containing the 3' end of *virB*, *virG* and *virC*) was purified from pSBGA281-G (Table 1) and introduced into pUCD2 at the Sal I site resulting in the insertional inactivation of the tetracycline resistant gene. Clones were screened on the basis of kanamycin resistance and tetracycline sensitivity. The confirmed plasmid was named as pBAL3 (Fig. 2).

**Introduction of Plasmids into A. tumefaciens**

Plasmids from *E. coli* were introduced into *A. tumefaciens* by triparental mating using pRK2013 as mobilization helper (Ditta *et al.*, 1980). The presence of plasmids in the transconjugants was confirmed by Southern analysis (Southern, 1975).

**Plant Transformation**

Transformation of primary leaf segments of black gram was performed as described previously (Karthikeyan *et al.*, 1996). Transformed calli were selected on BCIM supplemented with 50 mg l⁻¹ kanamycin and 250 mg l⁻¹ cefotaxime (Table 2). Since leakiness to kanamycin selection may rarely contribute to kanamycin resistance, transformation efficiencies on the basis of GUS⁺ staining are expressed.

Surface-sterilized PB1 rice seeds were cultured on RCIM in the dark for three weeks (Table 2). Non-embryogenic sectors were removed and the callus was sub-cultured on the same medium and reincubated in the dark for four days. *A. tumefaciens* strains were
grown to 1 OD at 600 nm in liquid AB minimal medium (Chilton et al., 1974) with appropriate antibiotics (Table 1). The bacterial cells were pelleted at 1100 x g at 25°C and resuspended in AA-AS medium (Rashid et al., 1996). Infection of the preincubated calli and cocultivation were performed as described by Rashid et al. (1996). After cocultivation, the infected calli were transferred to selection medium-RCIM (Table 2) supplemented with 50 mg l⁻¹ hygromycin and 250 mg l⁻¹ cefotaxime. Selection of hygromycin-resistant calli was carried out for eleven weeks. First sub-culture of the calli to a fresh selection medium was performed after two weeks. Thereafter, sub-culturing was done at three-week intervals. The proliferated hygromycin-resistant calli obtained were subjected to GUS histochemical staining (Hiei et al., 1994).

Hygromycin-resistant and GUS-positive calli obtained upon transformation with A. tumefaciens strain LBA4404(pTOK233) were transferred to RSIM supplemented with 40 mg l⁻¹ hygromycin and 250 mg l⁻¹ cefotaxime (Table 2). Regenerated rooted plants were acclimatized to soil and maintained in a transgenic greenhouse.

**DNA Isolation and Southern Blot Analysis**

Total DNA was extracted from the leaves of LBA4404 (pTOK233)-transformed and non-transformed control rice plants as described by Rogers and Bendich (1988). DNA concentration was determined by fluorometry (TKO 100, Hoefer Scientific Instruments, San Francisco, USA) using the Hoechst dye 33258. Control and T₀ plant DNA (20 μg) was digested with Hind III for 12 hrs and

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
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<tbody>
<tr>
<td>BCIM</td>
<td>MS salts and vitamins (Murashige &amp; Skoog, 1962), 30 g l⁻¹ sucrose, 45 μM 2,4-D, 0.015 μM each of BAP, 2ip and KN, 8 g l⁻¹ agar, pH 5.7</td>
</tr>
<tr>
<td>RCIM</td>
<td>MS salts, B5 vitamins, 30 g l⁻¹ sucrose, 300 mg l⁻¹ casaminoacids, 500 mg l⁻¹ proline, 11.3 μM 2,4-D, 2.25 g l⁻¹ Phytogel, pH 5.8</td>
</tr>
<tr>
<td>RSIM</td>
<td>MS salts and organic supplements, 30 g l⁻¹ sucrose, 300 mg l⁻¹ casaminoacids, 13.9 μM KN, 8.1 μM NAA, 4 g l⁻¹ Phytogel, pH 5.8</td>
</tr>
</tbody>
</table>
electrophoresed through 1% agarose gels. The DNA was blotted onto the Zeta-Probe membrane (Bio-Rad, Hercules, California) and hybridized with the 0.9 kb nptII coding sequence (RB probe) and 1.1 kb hph coding sequence (LB probe). The probes were labelled with \(^{32}\text{P}dCTP\) (BRIT, Mumbai, India) using a random primer labelling kit (Amersham Pharmacia Biotech, Little Chalfont, England).

**Results and Discussion**

*Multiple Copies of 3' end of virB and virG of pTiBo542 Enhance the Transformation Efficiency in Blackgram*

The effect of multiple copies of 3' end of virB and virG of pTiBo542 by introducing the ternary Inc W plasmid pBAL3 (Table 1; Fig. 2) into the *A. tumefaciens* strain LBA4404 (Hoekema et al., 1983) carrying the Inc P binary vector pBAL2 (Table 1, Fig. 1) was evaluated. The plasmids pBAL2 and pBAL3 are compatible with each other. Stable transformation efficiencies (Km' calli) and percentage of GUS+ calli are presented in Table 3. In a previous report, which included Southern analysis to study T-DNA integration (Karthikeyan et al., 1996), kanamycin-resistant proliferation of calli was established as a reliable index of stable transformation.

*A. tumefaciens* strain LBA4404(pBAL2) without extra copies of vir genes transformed 12% to 17% of the leaf segments in two independent experiments. In the presence of pBAL3 with the 3' end of virB and complete virG of pTiBo542, the transformation efficiency increased from 12% to 35% and 17% to 41% (Table 3). The increase in transformation efficiency contributed by pBAL3 was 192% in the first experiment and 141% in the second experiment. When pToK47 with multiple copies of pTiBo542 virB and virG was used, transformation efficiency did not increase over the levels achieved with pBAL3. Therefore, multiple copies of 3' end of virB and virG of pTiBo542 seem to be adequate to elevate transformation efficiency in blackgram.

Supplementation of a plasmid containing the vir region of pTiBo542 in the octopine strains increased tumourigenesis (supervirulence) on tobacco and tomato plants (Hood et al., 1986b). Increased tumourigenesis was achieved in *Nicotiana glauca* leaf discs by providing 3' end of virB and virG of pTiBo542 in an octopine strain A348 (Jin et al., 1987). Jaiwal et al. (2001) reported the transformation of *V. radiata* using pTOK233 with the pTiBo542 virB, virG and virC. However, they did not compare the transformation efficiencies with and without the pTiBo542 vir genes. The efficiency of Agrobacterium-mediated transformation of blackgram in the presence or absence of pBAL3 (3' end of virB, virG and virC of pTiBo542, Table 1) was evaluated in this study.

van der Fits et al. (2000) reported that expression of a constitutive virG mutant on a ternary plasmid greatly enhanced Agrobacterium-mediated transient transformation efficiencies in a diverse range of plant species. However, in *Dolichos lablab* (a grain legume), no transient transformation was observed with the *A. tumefaciens* strain LBA4404 with and without constitutive virG on a ternary plasmid. The supplementation of pTiBo542 virG on a ternary plasmid pBAL3 in LBA4404(pBAL2) increased the stable transformation of blackgram.

*Multiple Copies of virG and the Type of Ti-plasmid Influence the Transformation Efficiency in Blackgram*

Whether the increase in transformation efficiency of black gram observed in Table 3 is linked to the

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**Table 3—Effect of multiple copies of pTiBo542 virB, virG and virC on the transformation efficiency in primary leaf segments of blackgram**

<table>
<thead>
<tr>
<th><em>A. tumefaciens</em> strain</th>
<th>Number of leaf segments infected</th>
<th>% leaf segments with Km' calli</th>
<th>% leaf segments with GUS+ calli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt1</td>
<td>Expt2</td>
<td>Expt1</td>
</tr>
<tr>
<td>LBA4404(pBAL2)</td>
<td>129</td>
<td>106</td>
<td>33</td>
</tr>
<tr>
<td>LBA4404(pBAL2, pBAL3)</td>
<td>96</td>
<td>112</td>
<td>50</td>
</tr>
<tr>
<td>LBA4404(pBAL2, pToK47)</td>
<td>110</td>
<td>117</td>
<td>43</td>
</tr>
</tbody>
</table>

Scoring of kanamycin resistant (Km') calli and GUS histochemical analysis was performed after 25 days in the selection medium-BCIM (Table 2) supplemented with 50 mg l\(^{-1}\) kanamycin and 250 mg l\(^{-1}\) cefotaxime.
pTiBoS42 vir background or to multiple copies of pBAL3 with the 3' end of virB and virG genes of pTiBoS42 was evaluated. PBAL3 was introduced into A. tumefaciens vir helper strains, LBA4404 (octopine-type) and EHA105 (L,L-succinamopine-type) both harbouring the binary vector pBAL2. Transformation of primary leaf segments of black gram was performed. LBA4404 (pBAL2) without pBAL3 showed 11% transformation, which increased to 31% in the presence of pBAL3 (Table 4). A 182% increase was contributed by pBAL3 in LBA4404. Surprisingly, only a marginal increase of 9% was observed for EHA105 (pBAL2, pBAL3) over EHA105 (pBAL2) (Table 4). Thus, pBAL3 with the 3' end of virB and complete virG of pTiBoS42 improved transformation in LBA4404 but did not improve transformation in EHA105.

Multiple copies of virG and the type of Ti-plasmid harboured by A. tumefaciens strains can influence the transformation efficiency in different plant species. Liu et al (1992) observed that supplementation of multiple copies of octopine virG or pTiBoS42 virG in nopaline Ti-plasmid backgrounds did not bring about a significant increase in transient transformation of celery and rice. However, a five-fold increase in the transient transformation efficiency was obtained by introducing additional copies of octopine virG or pTiBoS42 virG in L,L-succinamopine-type Ti-plasmid background. The effect of additional copies of pTiBoS42 virG in octopine-type Ti-plasmid was not evaluated by Liu et al (1992). It was observed that supplementation of multiple copies of 3' end of virB and virG of pTiBoS42 as a ternary plasmid (pBAL3) in LBA4404 (octopine strain) increased the stable transformation efficiency of blackgram by 182%, but a similar increase was not observed for EHA105.

Multiple Copies of virB and virG Enhance the Stable Transformation Efficiency in Rice

Evaluation of the relative importance of virB, virG and virC (pToK47) and 3' end of virB and complete virG (pBAL3) in a ternary system on stable transformation of rice was done (Table 5). LBA4404 (pBAL2) without the pTiBoS42 vir genes did not transform rice. Supplementation of multiple copies of 3' end of virB and complete virG of pTiBoS42 in the form of pBAL3 in LBA4404 (pBAL2) resulted in stable transformation efficiencies of 8% and 10%. Transformation efficiency increased further from 8% to 18% when pToK47 with virB, virG, and virC of pTiBoS42 was supplemented in LBA4404 (pBAL2). The superbinary vector LBA4404 (pTOK233) transformed rice at a slightly higher efficiency of 22%. Thus, complete virB in addition to virG of pTiBoS42 is required for achieving high transformation efficiency in rice.

Liu et al (1992) reported that additional copies of octopine and L,L-succinamopine virG genes in

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Table 4—Effect of multiple copies of pTiBoS42 virG and the type of Ti-plasmid on the transformation efficiency in primary leaf segments of blackgram

<table>
<thead>
<tr>
<th>A. tumefaciens strain</th>
<th>vir helper type</th>
<th>Presence or absence of extra copies of virG from pTiBoS42 (pBAL3)</th>
<th>Number of leaf segments infected</th>
<th>% leaf segments with Km' calli</th>
<th>% leaf segments with GUS' calli</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA4404(pBAL2)</td>
<td>Octopine</td>
<td>-</td>
<td>209</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>LBA4404(pBAL2, pBAL3)</td>
<td>Octopine</td>
<td>+</td>
<td>207</td>
<td>45</td>
<td>31</td>
</tr>
<tr>
<td>EHA105(pBAL2)</td>
<td>L,L-Succinamopine</td>
<td>-</td>
<td>144</td>
<td>32</td>
<td>22</td>
</tr>
<tr>
<td>EHA105(pBAL2, pBAL3)</td>
<td>L,L-Succinamopine</td>
<td>+</td>
<td>137</td>
<td>31</td>
<td>24</td>
</tr>
</tbody>
</table>

Scoring of kanamycin resistant (Km') calli and GUS histochemical analysis was performed after 25 days in the selection medium-BCIM (Table 2) supplemented with 50 mg l' kanamycin and 250 mg l' cefotaxime.
Table 5—Effect of multiple copies of pTiBo542 virB, virG and virC on stable transformation of scutellum-derived calli of indica rice cv. Pusa Basmati 1 using A. tumefaciens

<table>
<thead>
<tr>
<th>A. tumefaciens strain</th>
<th>% Hm&lt;sup&gt;+&lt;/sup&gt; calli</th>
<th>% GUS&lt;sup&gt;+&lt;/sup&gt; calli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 1</td>
<td>Expt 2</td>
</tr>
<tr>
<td>LBA4404(pBAL2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LBA4404(pBAL2, pBAL3)</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>LBA4404(pBAL2, pToK47)</td>
<td>20</td>
<td>ND</td>
</tr>
<tr>
<td>LBA4404(pTOK233)</td>
<td>24</td>
<td>26</td>
</tr>
</tbody>
</table>

For transformation with each Agrobacterium strain, 50 scutellum-derived calli were taken for cocultivation. NO—Not done.

A. tumefaciens strains containing the L,L-succinamopine-type Ti-plasmid enhanced the efficiency of transient transformation of rice. They did not evaluate the role of complete virB and the effect of additional copies of pTiBo542 virG in A. tumefaciens strain containing an octopine-type Ti-plasmid. From our results, it is clear that in addition to virG, supplementation of complete virB of pTiBo542 is essential for obtaining a high transformation efficiency in rice. Hiei et al. (1994) demonstrated that the pTiBo542 virB, virG and virC contributed to a high transformation efficiency in rice, but did not compare the transformation efficiency in the absence of the complete virB of pTiBo542. The observations presented in Table 5 show that complete virB, in addition to virG of pTiBo542, is required for achieving a high transformation efficiency in rice.

Southern Analysis of T<sub>0</sub> Rice Plants

Southern analysis (Southern, 1975) was done in T<sub>0</sub> rice plants generated from GUS<sup>+</sup> calli to ensure that GUS-staining in rice reflects genuine transformation. T<sub>0</sub> plants obtained from LBA4404 (pTOK233)-infected calli were chosen for Southern analysis to confirm the integration of T-DNA into the rice genome. Genomic DNA (20 μg) from the control plant and eight T<sub>0</sub> plants was digested with Hind III and hybridized with nptII and hph coding sequences as probes to detect right and left border junction sequences, respectively (Fig. 3). The nptII probe is expected to hybridize to the junction sequences longer than 5.65 kb. Hybridization with the nptII probe revealed the presence of two T-DNA copies in plants 8, 10, 12, 17, 21 and 28. Three junction fragments of varying sizes were detected in plants 26 and 35. Surprisingly the T<sub>0</sub> plants 12, 21 and 28 which carried typical junction fragments longer than 5.65 kb also showed shorter junction fragments. These may either be due to truncated T-DNA transfer or due to scrambling of T-DNA prior to integration.

Left border junction sequence analysis with the hph probe revealed that the T-DNA copy numbers were similar to those derived from nptII probe analysis. In a larger proportion of the plants analyzed, junction

![Southern analysis of T<sub>0</sub> rice plants transformed with LBA4404(pTOK233). (A): Analysis of right border junction sequences with nptII probe. (B): Analysis of left border junction sequences with hph probe. Numbers on the top refer to T<sub>0</sub> lines. Twenty microgram aliquots of plant DNA were digested with Hind III and analyzed. Lanes-C: DNA from untransformed control plant digested with Hind III; U: Undigested DNA from T<sub>0</sub> plant 35; L: kb ladder; P: Total DNA from LBA4404(pTOK233) digested with Hind III (100 ng was loaded in panel A and 500 ng was loaded in panel B). Positions and sizes of λ-Hind III fragments (left) and kb ladder (right) are marked.](image-url)
fragments greater than 5.0 kb were observed. However, some plants revealed bands shorter than 5.0 kb. Hybridization patterns were similar in plants 8 and 10 with both probes suggesting that they are siblings that might have arisen from a single transformation event. All the seven independent transgenic plants that regenerated from GUS+ calli carried integrated T-DNA. Thus, GUS+ calli give a reliable score for transformation.

Transformation of different plant species was performed with LBA4404 (pTOK233) which contains virB, virG and virC of pTiBo542 and the T-DNA on the binary vector (Hiei et al, 1994; Mohanty et al, 1999; Jaiwal et al, 2001). In this study, additional copies of vir genes (virB, virG and virC) of pTiBo542 were introduced on a wide host-range ternary plasmids (pBAL3 & pToK47; Table 1). Ternary plasmids can be used in combination with existing A. tumefaciens and standard binary vectors. In this respect, it offers a clear advantage over other improved A. tumefaciens vector systems that provide multiple vir genes on the binary vector itself. The binary vector pTOK233, for example, contains the hypervirulence region of pTiBo542, thereby increasing the size to over 50 kb (Hiei et al, 1994). To introduce genes of interest on the T-DNA of such large binary vectors is often not possible with standard molecular cloning techniques. The Inc P plasmid pSB1 (virB, virG and virC of pTiBo542), cannot be used in A. tumefaciens strains carrying standard Inc P binary vectors for improving the transformation efficiency. Ternary Inc W plasmids (pBAL3 & pToK47) reported in our study enhanced the transformation efficiency of blackgram and rice when contained in A. tumefaciens carrying Inc P binary vectors.

Thus, ternary plasmids expressing multiple copies of virB and virG of pTiBo542 greatly enhance Agrobacterium-mediated stable transformation efficiencies in blackgram and rice. Multiple copies of 3’ end of virB and complete virG of pTiBo542 on a ternary plasmid (pBAL3) were found to be adequate to increase transformation efficiency of blackgram. A. tumefaciens strains harbouring multiple copies of complete virB and virG genes of pTiBo542 transformed rice at the highest efficiency, indicating that complete virB of pTiBo542 in addition to virG is necessary for achieving a higher transformation efficiency in rice. In contrast to improved Agrobacterium strains having hypervirulence regions added on the binary vector backbone, the ternary plasmid pBAL3 reported in this study is more convenient for use in standard A. tumefaciens strains in combination with existing binary vectors.

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