**Transgenic Note**

**Agrobacterium-mediated transformation of indica rice under Acetosyringone-free conditions**

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**Abstract** The possibility of developing transgenic indica rices through Agrobacterium-mediated transformation in the absence of acetosyringone at bacterial preinduction or co-cultivation or both stages was assessed. Four-week-old, scutellum derived calluses of indica rice (Oryza sativa L. cv. 'Pusa Basmati1') were co-cultivated with A. tumefaciens strain LBA4404 (pSB1), harboring the binary vector pCAMBIA 1301 with the β-glucuronidase (GUS) and hygromycin phosphotransferase (HPT) genes in the T-DNA region. Addition of acetosyringone (AS) to both preinduction medium (PIM) and co-cultivation medium (CCM) induced higher levels of transient GUS expression than that obtained with the addition of AS to either of the stages. Addition of only sucrose to both preinduction and co-cultivation media yielded transient expression levels similar to those obtained by the addition of AS. The resultant fertile plants were stable transformants as revealed by GUS histochemical assay and PCR analysis for the GUS and HPT genes. Thus, phenolics like AS may not be essential for induction of vir genes, and development of transgenic indica rice is feasible under AS-free conditions.

**Key words:** Acetosyringone, Agrobacterium, indica rice, transformation, transgenic plant.

Among the currently available plant transformation techniques, Agrobacterium method, a simple natural gene transfer system, is the most widely used one for genetic improvement of several crop species including rice. The processing and transfer of T-DNA from A. tumefaciens to plant cells is mediated by the combined action of both chromosomal (chv) and vir genes (virA, -B, -G, -C, -D and -E). The vir genes are induced by phenolics such AS, and at low AS concentration vir gene induction is augmented by certain wound-induced sugars in plant cells (Stachel et al. 1985; Bolton et al. 1986; Spencer and Towers 1988; Melchers et al. 1989; Huang et al. 1990). To date, all the successful reports on Agrobacterium-mediated transformation of rice have been based on Agrobacterium preinduction and/or co-cultivation in the presence of AS (Hiei et al. 1994; Aldemita et al. 1996; Rashid et al. 1996; Khanna et al. 1999; Datta et al. 2000; Hashizume et al. 2006) or based on co-cultivation in presence of suspension culture of potato cells, a rich source of phenolic compounds (Chan et al. 1993). No transient expression of the GUS gene was observed in the absence of AS even when using a super virulent Agrobacterium strain, and 100 µM of AS was reported to be optimum for transient expression in rice (Azhakanandam et al. 2000).

We examined the influence of AS at different steps of Agrobacterium-mediated transformation of a indica rice and report the feasibility of transformation of recalcitrant indica genotype with simple sugars like sucrose in the absence of AS.

The scutellum-derived embryogenic calluses obtained from a popular long grain aromatic indica rice (Oryza sativa L) cultivar, Pusa Basmati1 was used as a target material for transformation. Media used for culture and transformation are listed in Table 1. Mature seeds washed with sterile water were surface sterilized successively with, 70% ethanol for two min, sodium hypochlorite (contains 4% (v/v) active chlorine) for 15 min and with 0.1% (w/v) aqueous mercuric chloride solution for 5 min with in between repeated washings with sterile water. These seeds were placed on semisolid MS (Murashige and Skoog 1962) callus induction (CI) CI medium contained in culture tubes and incubated at 25±2°C in the dark. After three weeks, the induced calluses were subcultured onto fresh CI medium. After 4–7 days of incubation, the embryogenic callus cultures with somatic embryos were selected for transformation.

The selected embryogenic calluses were transformed with a super virulent A. tumefaciens strain LBA4404 (pSB1) (Komari et al. 1996) containing pCAMBIA 1301 (CAMBIA MGRS Accession number TG148). This binary vector carried intron-containing GUS and HPT genes in the T-DNA region. One loop of bacterial cells was streak-inoculated on yeast extract peptone (YEP).

Abbreviations: AS, acetosyringone; GUS, β-glucuronidase; HPT, hygromycin phosphotransferase; Hyg<sup>R</sup>, hygromycin-resistant

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Table 1. Media used for tissue culture and transformation of rice.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Composition</th>
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<tbody>
<tr>
<td>CI</td>
<td>MS salts and vitamins, 30 g l⁻¹ sucrose, 500 mg l⁻¹ L-proline, 300 mg l⁻¹ casein hydrolysate and 2.0 mg l⁻¹ 2, 4-D, 2.25 g l⁻¹ phytagel, pH 5.8</td>
</tr>
<tr>
<td>CCM</td>
<td>MS salts and vitamins, 30 g l⁻¹ sucrose, 500 mg l⁻¹ L-proline, 300 mg l⁻¹ casein hydrolysate and 2.0 mg l⁻¹ 2, 4-D, 8.5 g l⁻¹ agar, pH 5.8</td>
</tr>
<tr>
<td>CI-SL</td>
<td>MS salts and vitamins, 30 g l⁻¹ sucrose, 500 mg l⁻¹ L-proline, 300 mg l⁻¹ casein hydrolysate and 2.0 mg l⁻¹ 2, 4-D, 30 mg l⁻¹ hygromycin and 125 mg l⁻¹ ceftaxime and 125 mg l⁻¹ carbenicillin, 2.25 g l⁻¹ phytagel, pH 5.8</td>
</tr>
<tr>
<td>PRE-RE</td>
<td>MS salts and vitamins, 30 g l⁻¹ sucrose, 20 g l⁻¹ sorbitol, 1 mg l⁻¹ naphthalenic acid, 2 mg l⁻¹ kinetin, 30 mg l⁻¹ hygromycin, 2.25 g l⁻¹ phytagel, pH 5.8</td>
</tr>
<tr>
<td>RE</td>
<td>MS salts and vitamins, 30 g l⁻¹ sucrose, 0.5 mg l⁻¹ naphthalenic acid, 0.5 mg l⁻¹ kinetin, 1.5 mg l⁻¹ benzylaminopurine, 5 g l⁻¹ phytagel, pH 5.8</td>
</tr>
<tr>
<td>RT</td>
<td>MS salts and vitamins, 50 g l⁻¹ sucrose, 1.0 mg l⁻¹ naphthalenic acid, 0.1 mg l⁻¹ kinetin, 7 g l⁻¹ agar, pH 5.8</td>
</tr>
<tr>
<td>PIM</td>
<td>AB buffer and salts, 5 g l⁻¹ sucrose</td>
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Table 2. Effect of acetosyringone and sucrose on transient transformation frequency.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PIM</th>
<th>CCM</th>
<th>GUS expression zones¹</th>
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<tbody>
<tr>
<td></td>
<td>AS</td>
<td>Sucrose</td>
<td>AS</td>
</tr>
<tr>
<td>A</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>B</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>C</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>D</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
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</table>

¹ Pooled data of two experiments with hundred explants each (SD, standard deviation).

A Preinduction of Agrobacterium in presence of AS and sucrose followed by co-cultivation in the absence of AS.

B Preinduction of Agrobacterium in absence of AS and presence of sucrose followed by co-cultivation in the presence of AS and sucrose.

C Preinduction of Agrobacterium in presence of both AS and sucrose followed by co-cultivation both in the in presence of AS and sucrose.

D Preinduction of Agrobacterium in absence of AS followed by co-cultivation in the absence of AS.

medium (Lichtenstein et al. 1986) dispensed in Petri dishes and the plates were incubated at 28°C for two days. A single colony of the bacterium was picked up and reinoculated on AB minimal medium (Chilton et al. 1974) supplemented with 0.5% (w/v) glucose and incubated for two days at 28°C. From this culture, 2–4 single colonies of the bacterium were transferred to 25 ml of AB minimal medium containing 5 g l⁻¹ sucrose dispensed in 100 ml Erlenmeyer flasks. The flasks were incubated under agitation on an orbital shaker (220 rpm) at 25°C. After 24–48 h, 0.5 ml of the bacterial cell suspension culture was again inoculated to 50 ml of fresh medium of the same composition and incubated under conditions described above for 14–20 h. The bacterial cell suspension, adjusted to 0.6 O.D (A620) with sterile sucrose supplemented AB minimal medium was directly used for infection. Three different antibiotics, kanamycin (100 mg l⁻¹), tetracycline (5 mg l⁻¹), and rifampicin (10 mg l⁻¹) were used for culturing the bacterium on solid medium, while for culturing the bacterium in all liquid media, two antibiotics, kanamycin (50 mg l⁻¹) and tetracycline (2.5 mg l⁻¹) were used. In treatments aimed at evaluating the influence of AS on transformation frequency (Table 2), 50 μM AS was added to the bacterial culture 4 h before co-cultivation.

Inoculation and co-cultivation were performed according to Datta et al. (1997) with minor modifications. Four-week-old embryogenic calluses were inoculated with Agrobacterium by immersing them in the bacterial cell suspension dispensed in Petriplates (90×15 mm) which were gently agitated on an orbital shaker (80–100 rpm) for 15–30 min. The calluses were then blotted on sterile filter paper under aseptic conditions to remove excess bacterial cells and were placed on CCM medium with or without 50 μM AS at 25°C in dark for three days for co-cultivation. At the end of the incubation, the calluses were washed with CI medium without phytagel but supplemented with 125 mg l⁻¹ ceftaxime and 125 mg l⁻¹ carbenicillin, dried on sterile filter paper, and transferred to selection (CI-SL) medium. The calluses were passed through four selection cycles of 15 days each. All growing calluses during the fourth selection cycle (HygR calluses) were transferred to pre regeneration (PRE-RE) medium and incubated for two weeks. The calluses were then transferred to RE medium. Regenerated green shoots thus obtained were transferred to rooting (RT) medium for root induction and development. These putative transgenic plants were planted in earthen pots containing sterile field soil and grown until maturity in an isolated greenhouse.

GUS activity is localized, and generally, transient GUS gene expression analysis is performed with inoculated tissues that are not exposed to antibiotics during cocultivation stage (Rueb et al. 1989). For histochemical
detection, segments (5 mm in length) of rice tissues were incubated in a reaction mixture of 50 mM phosphate buffer (pH 6.8), 1% Triton X-100, 20% methanol and 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-gluc). The reaction was initiated under a mild vacuum for few min and carried out overnight at 37°C. The frequency of transient transformation is expressed as the ratio between the number of calli showing GUS expression and the total number of calli kept for staining. For qualitative assay, the area of GUS expression and intensity of the blue colour at each spot were given due weightage so as to give a holistic picture of the effects of the various treatments tested. Through visual observations, distinction could be made between small spots (<0.5 mm in diameter) representing one or few GUS expressing cells, and large spots (≥1 mm in diameter), representing a complete cell cluster expressing the GUS gene (De Clercq et al. 2002). Based on both quantitative and qualitative assays, the efficacy of acetosyringone was evaluated.

Confirmation for stable integration of GUS and HPT genes in rice genome was obtained by polymerase chain reaction (PCR) analyses. Genomic DNA was extracted from the leaves of putative transgenic plants (T0) and non-transformed (wild type) plants by CTAB method (Murray and Thompson., 1980). Primers (GUS-F-5′-GGT GGG AAA GCG CGT TAC AAG-3′ and GUS-R-5′-GTT TAC GCG TTG CTT CCG CCA-3′) specific for GUS and (HPT-F- 5′-GCC TGA ACT CAC CGC GAC G-3′ and HPT-R-5′-CAC CCA TCG GTC CAG ACG-3′) specific for HPT genes were employed. PCR was performed in a 25 μl reaction volume containing 17.5 μl sterilized nanopure water, 2.5 μl of 10×PCR buffer, 1 μl (concentration 100 ng) of each primer, 1 μl of dNTPs mix (0.2 mM each of dNTP), and 1 μl (0.5 U μl⁻¹) of Taq polymerase and 1 μl (50–100 ng) of template DNA. Amplification was performed in a programmable Thermal cycler (PTC-100) (MJ-Research, US) under the following conditions: GUS gene: 1×(94°C for 5 min), 40×(94°C for 30 s, 55°C for 30 s, 72°C for 1 min), 1×(72°C for 10 min) and HPTgene: 1×(94°C for 5 min), 40×(94°C for 1 min, 58°C for 1 min, 72°C for 1 min), 1×(72°C for 5 min). Fifteen microliters of the PCR product was resolved on ethidium-bromide-stained 0.8% agarose gel for visualization of the bands.

The experiment was aimed to assess the need for

**Table 3. Influence of AS on Agrobacterium-mediated indica rice transformation.**

<table>
<thead>
<tr>
<th>Co-cultivation</th>
<th>Number of scutellum-derived calli</th>
<th>Transformation frequency (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Number of calli co-cultivated (A)</td>
<td>Number of Hyg&lt;sup&gt;+&lt;/sup&gt; calli produced</td>
</tr>
<tr>
<td>With AS</td>
<td>114</td>
<td>22</td>
</tr>
<tr>
<td>Without AS</td>
<td>92</td>
<td>15</td>
</tr>
</tbody>
</table>

Figure 1. Histological GUS assay of calli after co-cultivation with Agrobacterium. (A) Transient GUS gene expression in calli following preinduction in presence of AS and co-cultivation in the absence of AS. Bar=1 mm. (B) Transient GUS gene expression in calli following preinduction in absence of AS and co-cultivation in the presence of AS. Bar=0.6 mm. (C) Transient GUS gene expression in calli following preinduction and co-cultivation both in the in absence of AS. Bar=0.6 mm. (D) Transient GUS gene expression in calli following preinduction and co-cultivation both in the in absence of AS. Bar=1 mm. (E) Stable GUS gene expression in Hyg<sup>+</sup> callus 60 day after co-cultivation. Bar=0.5 mm. (F) Untransformed callus showing no GUS activity. Bar=0.5 mm.
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Figure 2. Polymerase chain reaction analysis, showing integration of the coding region of GUS (1.2 kb) (A) and HPT (0.95 kb) (B) genes in the genome of randomly selected putative transgenic plants. Lane M, λ DNA EcoRI/HindIII double digest as size marker; Lanes 3–8, plants obtained after preinduction and co-cultivation both in the presence of AS; lanes 9–13, plants obtained after preinduction and co-cultivation both in the absence of AS; lane P, pCAMBIA 1301 as a positive control; lane UT, untransformed plant as a negative control.

fragments (~1.2 kb and 0.95 kb respectively) with DNA from Tg generation plants (six plants derived from AS-supplemented calli and five plants derived from sucrose supplemented calli), while amplification was negative with DNA from non transformed control plants (Figure 2A, B). All the transgenic plants were fertile and yielded viable seeds in quantities comparable with those of non-transformed plants. Our results provide strong evidence for the first time that acetosyringone may not be essential for the induction of vir genes of Agrobacterium during co-culture for transformation of recalcitrant genotypes like indica rices.

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