Agrobacterium-mediated transformation of White Ponni, a non-basmati variety of *indica* rice (*Oryza sativa* L.)

G. Sridevi, M. Dhandapani and K. Veluthambi*

Department of Plant Biotechnology, School of Biotechnology, Madurai Kamaraj University, Madurai 625 021, India

We report successful *Agrobacterium*-mediated transformation of a popular rice variety White Ponni, a non-basmati *indica* rice (*Oryza sativa* L.). Scutellum-derived calluses of White Ponni were transformed with *Agrobacterium* strain LBA4404 (pSB1) harbouring the binary vector pMKU-RF2 with rice *chi*11 gene. Five independent transgenic White Ponni plants were generated from hygromycin-resistant calluses. Stable integration of the transgene was confirmed and copy numbers were determined by Southern analysis. Among the five plants, four possessed single-copy T-DNA integration events while one was found to have two integrated copies of T-DNA. Western analysis revealed a higher level of chitinase accumulation in all the five T_0 plants. Progeny analysis of T_0 plants confirmed the inheritance of the transgene to the next generation.

EARLY success in rice transformation was achieved by direct transformation of protoplasts[1]. *Agrobacterium*-mediated transformation was first reported in *japonica* rice[2–4]. Among the three sub-species of rice, *indica* cultivars are recalcitrant for both tissue culture and *Agrobacterium*-mediated transformation[5]. Rashid *et al.*[6] first reported the successful transformation of scutellum-derived calluses of *indica* rice cultivars, Basmati 370 and Basmati 385 using *Agrobacterium*. Subsequently, Aldemita and Hodges[7] showed *Agrobacterium*-mediated transformation of immature embryos of non-basmati (*indica*) varieties (TCS10 and IR72). As Basmati varieties are amenable for transformation, many research groups reported transformation of Pusa Basmati 1 using binary[8,9] and superbinary vectors[9–11]. Many useful genes were introduced into *indica* cultivars. Datta *et al.*[12] introduced rice chitinase (*chi*11) gene into three *indica* cultivars, Basmati 122, Tulsi and Vaidehi. Transgenic Pusa Basmati 1 plants harbouring choline oxidase (*cod*A) gene[13] and heat shock protein gene (*hsp*101)[14] were developed. Khanna and Raina[15] generated transgenic basmati (Pusa Basmati 1 and Karnal Local) and IR74 plants harbouring synthetic *cry*IAc endotoxin gene from *Bacillus thuringiensis*. In a recent report, the snowdrop lectin gene (*gna*) has been introduced into two *indica* cultivars, Chaitanya and Phalguna[16]. The report of the Task Force on Agricultural Biotechnology chaired by M. S. Swaminathan, that addresses both potentials and problems associated with biotechnology applications, makes a strong recommendation that transgenic research should not be undertaken in basmati rice because it is a commodity of international trade[17]. Therefore, there is a pressing need to develop transformation protocols for non-basmati *indica* rice varieties.

Successful production of transgenic plants in *indica* rice variety White Ponni, through *Agrobacterium*-mediated transformation is reported here. White Ponni is a popular non-basmati *indica* variety (obtained from Taichung 65/2× Mayang Ebos-80), widely cultivated in Tamil Nadu. White Ponni is preferred for its grain quality (medium, slender white grain) and palatability. Its average yield is about 5 tonnes per hectare[18].

Agrobacterium tumefaciens strain LBA4404 (pSB1) harbouring the binary plasmid[19], pMKU-RF2 with rice chitinase (*chi*11), *b*-glucuronidase (*gus*) and hygromycin phosphotransferase (*hph*) genes was used for transformation. Integration of the transgene and copy number estimation were done by Southern analysis. Five T_0 plants showed constitutive expression of chitinase at high levels. Inheritance of the transgene was demonstrated by progeny analysis.

Materials and methods

Plant material and Agrobacterium strain

White Ponni (*Oryza sativa* L. *indica* rice variety) seeds were obtained from the Agricultural College and Research Station, Tamil Nadu Agricultural University, Madurai, India. *A. tumefaciens* strain LBA4404 (pSB1)[20] harbouring pMKU-RF2 (binary vector harbouring maize ubiquitin promoter-intron-driven rice chitinase, *chi*11)[19] was used for transformation.

Rice transformation

Mature seeds of White Ponni were surface-sterilized[21] and placed on callus-induction (CI-WP) medium [MS medium[22] supplemented with 2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 0.5 mg/l 6-benzylaminopurine (BAP), 30 g/l sucrose, 200 mg/l casein hydrolysate, 500 mg/l proline and

*For correspondence. (e-mail: kveluthambi@rediffmail.com)

solidified with 2.25 g/l Phytagel (Sigma)] in dark at 25°C for 21 days. Scutellum-derived calluses were then excised and pre-incubated on CI-WP medium for four days.

Infection was performed using 1.0 OD (A_{600}) culture of *A. tumefaciens* strain LBA4404 (pSB1, pMKU-RF2) according to the protocol of Rashid *et al.*[6], with minor modifications. Bacterial suspension was prepared by centrifuging the 1 OD culture at $1100 \times g$ for 10 min and resuspending in AA medium with 50 µM acetosyringone (AS). After 15 min of infection, the calluses were blot-dried and kept on the co-cultivation medium (CI-WP medium supplemented with 10 g/l glucose, 3 g/l Phytagel and 100 µM AS) layered with Whatman No. 1 filter paper.

After three days of co-cultivation in dark, the calluses were washed three times using liquid CI-WP medium (third wash-medium supplemented with 50 mg/l hygromycin and 250 mg/l cefotaxime) and placed on selection medium (CIS-WP, CI-WP medium with 4 g/l Phytagel, 50 mg/l hygromycin and 250 mg/l cefotaxime). The initial selection was carried out for 15 days. Subsequently, two cycles (21 days per cycle) of selection were performed.

Portions of the calluses were analysed for GUS activity[4] at the end of the third cycle. All the hygromycin-resistant calluses (both GUS^+ and GUS^-) were transferred for regeneration to RMS-WP medium [MS medium with 3mg/l kinetin, 2 mg/l *a*-naphthaleneacetic acid (NAA), 6 g/l Phytagel, 20 mg/l hygromycin and 250 mg/l cefotaxime] for 14 days in dark and then transferred to light. In the subsequent subcultures, the Phytagel concentration was reduced to 4 g/l.

Southern analysis

Genomic DNA was extracted[23] from leaves of both control and transformed plants and quantified using Hoechst dye 33258 in a DNA fluorometer. Five-microgram samples of DNA from control and transformed plants were digested with *Hin*dIII, electrophoresed in 0.8% agarose gels and transferred to Zeta-Probe membrane (Bio-Rad, Hercules, CA) for Southern analysis. Three probes, a 2.0-kb *gus* coding sequence with intron, a 1.1-kb *hph* coding sequence and 1.1-kb *chi*11 coding sequence were labelled with [*a*-^{32}P]dCTP (BRIT, Mumbai, India) using the Megaprime™ DNA labelling system (Amersham Pharmacia Biotech, Little Chalfont, England).

Protein extraction and Western analysis

Total protein was extracted from control and T_0 plants and estimated by the method of Bradford[24]. Twenty-microgram protein samples were separated in a 10% SDS–PAGE gel and electrotransferred onto a nitrocellulose membrane using a semi-dry blotting apparatus. The membrane was blocked using 3% gelatin and Tween-Tris buffered saline (TTBS), and probed with chitinase antibody[25] (a polyclonal rabbit antibody raised against barley chitinase used at a dilution of 1:1000 v/v) and goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate (Bangalore Genei Pvt Ltd, Bangalore, India) at a dilution of 1 : 2000. Detection was carried out using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium colour reagent, till the bands appeared.

Segregation analysis

Seeds collected from selfed T_0 plants were screened for hygromycin resistance. The seeds were germinated initially on ½MS basal medium (solidified using 0.8% agar) and placed in dark. The germinated seedlings were then transferred to the same medium supplemented with 50 mg/l hygromycin and placed in light. After ten days, seedlings were scored for hygromycin resistance (Hyg^S and Hyg^R) and the data was validated using c^2 test.

Results and discussion

Production of transgenic White Ponni plants

Scutellum-derived calluses generated from White Ponni seeds were transformed using *A. tumefaciens* strain LBA4404 (pSB1)[20] harbouring pMKU-RF2 (a binary vector with maize ubiquitin promoter-intron driven rice chitinase, *chi*11)[19]. Of a total of 182 co-cultivated calluses transferred to the selection medium (CIS-WP), 102 (56%) proliferated on the selection medium containing 50 mg/l hygromycin. When subjected to regeneration on a medium (RMS-WP) containing 20 mg/l hygromycin, shoots regenerated from five calluses (7, 48, 57, 110 and 130). Among these, only callus no. 110 was GUS-positive. However, the leaves and roots of plants derived from all five calluses were GUS-positive (Figure 1). Transformation efficiency, calculated on the basis of GUS^+ plants, was 3%. The five transgenic (T_0) plants grew normally in the greenhouse. Flowering and seed setting were normal.

Stable integration of transgene

Southern analysis was performed to confirm transgene integration and to determine the copy numbers of the integrated T-DNA. Genomic DNA was extracted from five T_0 plants and one untransformed (control) plant. Five-microgram aliquots of DNA were digested with *Hin*dIII to generate one internal and two junction fragments from the T-DNA region[19]. Three probes, namely a 2.0-kb *gus* coding sequence with intron, a 1.1-kb *hph* coding sequence and 1.1-kb *chi*11 coding sequence were used to determine right border and left border junction fragments and the internal T-DNA fragment respectively.

The *intron–gus* probe is expected to hybridize to a junction fragment comprising the T-DNA (right border) and plant DNA portions. This right-border junction fragment is expected to be longer than 3.1-kb (see Sridevi *et al.*[19]

for restriction map). DNA from all the plants exhibited junction fragments longer than 3.1-kb (Figure 2*a*). The T_0 lines 7, 48 and 110 showed single junction fragments, suggesting single-copy integration events. In the lane corresponding to the plant 57, signal to the *gus* probe was not seen due to DNA degradation. However, a single-junction fragment for plant 57 was observed with the *hph* probe (Figure 2*b*). Line 130 exhibited two junction fragments, indicating two integration events.

The *hph* probe is expected to hybridize to a left-border junction fragment[19] longer than 2.7-kb. In four transgenic lines (7, 48, 57 and 130), the junction fragments were longer than 2.7-kb (Figure 2*b*). Line 110 exhibited a slightly shorter left border junction fragment, possibly due to scrambling during T-DNA integration. With both *gus* and *hph* probes, lines 7, 48, 57 and 110 showed single-copy integrations, while line 130 carried two T-DNA copies.

The *chi*11 probe is expected to hybridize to a 3.1-kb internal T-DNA fragment[19] that is common to all transgenic plants. All five T_0 lines showed the presence of the 3.1-kb fragment (Figure 3). Other *Hin*dIII fragments seen commonly between the control and transgenic plants correspond to the endogenous *chi* genes of rice. Since the transgene *chi*11 is a rice gene, it hybridized to the endogenous rice *chi* genes.

The transgenic lines 7, 48, 57 and 110 showed single junction fragments for both right border and left-border probes and carry the *chi*11 gene in the middle of the T-DNA. Therefore, these four transgenic plants are confirmed to have single integration events with complete T-DNA copies. Transgenic line 130 has two copies of T-DNA.

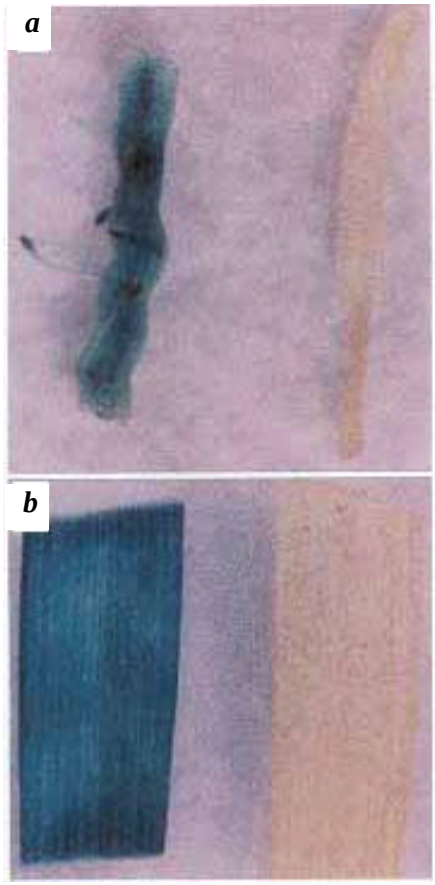

Figure 1. GUS staining of portions of root (***a***) and leaf (***b***) of transformed (left) and control (right) White Ponni rice plants.

Chitinase levels in transgenic plants

Total protein extracted from the leaves of T_0 plants and untransformed (control) plants was subjected to Western analysis to determine the levels of chitinase protein. Twenty microgram aliquots of protein from control and T_0 plants were subjected to Western blot analysis using polyclonal antibody raised against barley chitinase[25]. All the five T_0 lines accumulated 35-kDa chitinase at high levels in comparison to the control plant, which showed a faint signal reflecting very low expression (Figure 4). In addition to the 35-kDa protein, all transgenic plants showed an additional band at 28-kDa, which may have arisen due to proteolysis. The transgenic lines with single copy of T-DNA (7, 48, 57 and 110) and with two copies (130) accumulated comparable levels of chitinase.

Progeny analysis

The seeds of all five selfed T_0 plants were germinated on hygromycin-containing medium to study the segregation pattern (Table 1). Four lines (7, 48, 57 and 110) showed a ratio of 3:1 (Hyg^R and Hyg^S seedlings) expected for a single locus of the transgene. In the case of line 130, the segregation ratio was 15:1, suggesting the presence of two copies of transgene in two unlinked loci.

This is a complete report of *Agrobacterium*-mediated transformation of White Ponni, a popular *indica* rice variety cultivated in South India. The transformation efficiencies of basmati varieties are high[11,19], in the range of about 25 to 35%. However, the non-basmati *indica* rice lines are transformed at a very low efficiency (up to 5%)[7,16]. We report a

Table 1. Segregation analysis of T_0 White Ponni rice plants transformed by LBA4404 (pSB1, pMKU-RF2)

	No. of seedlings				
T_0 plant number	Total	Hyg^R	Hyg^S	c^2 value*	*P*
7	46	35	11	0.028 (3:1)	>0.5
48	26	20	6	0.05 (3:1)	>0.5
57	30	26	4	2.17 (3:1)	>0.1
110	39	32	7	1.02 (3:1)	>0.2
130	37	34	3	0.227 (15:1)	>0.5

*c^2 value is calculated for inheritance of one gene (3:1) or two genes (15:1).

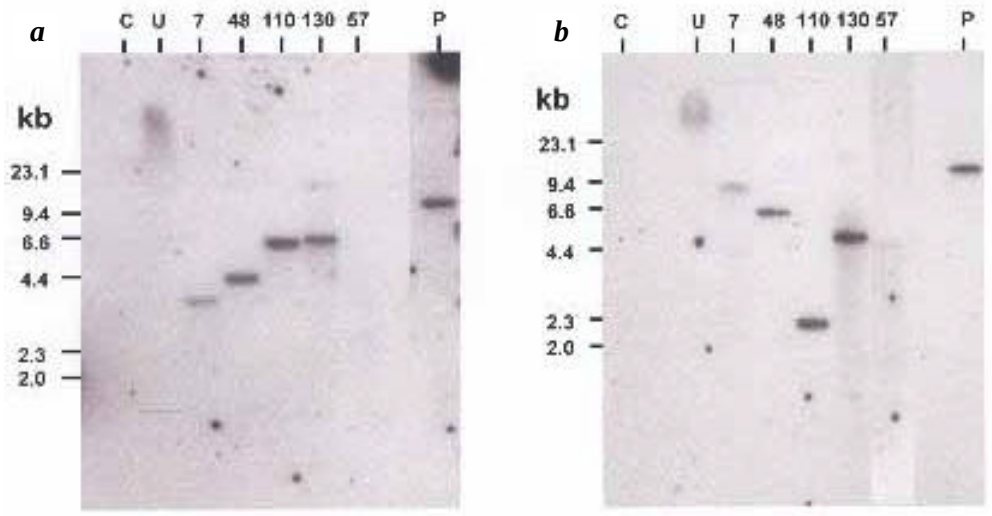

Figure 2. Southern blot hybridization analysis of junction fragments of T_0 White Ponni rice plants using [*a*-^{32}P]dCTP-labelled *gus* gene (***a***) and *hph* gene (***b***). C, DNA from untransformed plant digested with *Hin*dIII; U, Undigested DNA from plant 110. All plant DNA samples (5 µg each) were digested with *Hin*dIII. P, 500 pg of *Hin*dIII-digested plasmid DNA (pMKU-RF2)[19]. Numbers on the top refer to T_0 plant lines. Numerals on the left indicate positions and sizes of *l-Hin*dIII fragments used as size markers. In (***a***), the plant 57 did not show any signal due to DNA degradation. In (***b***), a weak signal for plant 57 is seen due to DNA degradation and the lane for plant 57 is presented from an autoradiogram exposed for a longer period.

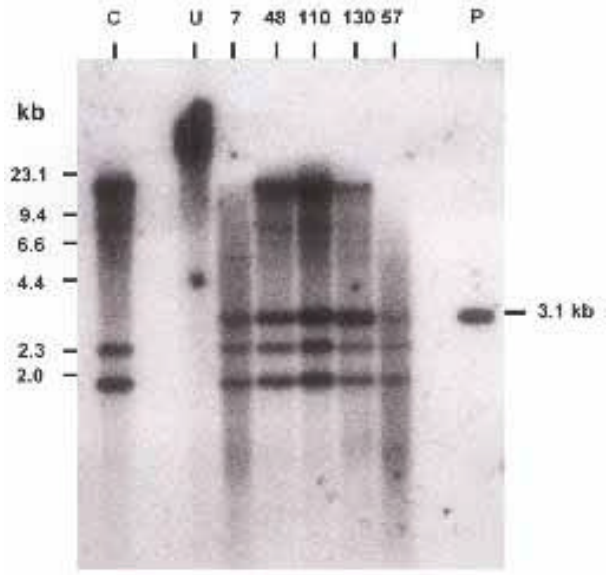

Figure 3. Southern blot hybridization analysis of the *chi*11 transgene in T_0 White Ponni rice plants. Total plant DNA (5 µg) was digested with *Hin*dIII. [*a*-^{32}P]dCTP labelled *chi*11 gene was used as probe. An internal T-DNA fragment of 3.1-kb, which is expected to hybridize with the probe[19], is marked on the right. C, DNA from untransformed plant digested with *Hin*dIII. U, Undigested DNA from plant 110. DNA degradation was noticed in plant 57. Numbers at the top refer to T_0 plant lines. Sizes and positions of *l-Hin*dIII fragments are marked on the left.

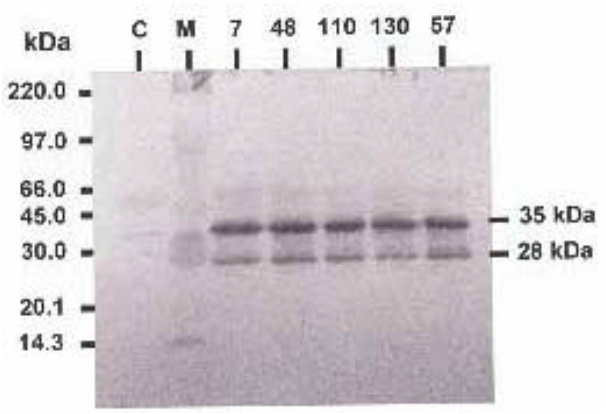

Figure 4. Western blotting analysis of chitinase levels. Protein samples (20 µg) extracted from control (C) and five T_0 White Ponni rice plants (numbered on top) were separated in a 10% SDS–PAGE gel and analysed with barley chitinase antibody. Positions and sizes of protein molecular weight standard (Rainbow marker, M) are marked on the left. Positions of the 35-kDa and 28-kDa proteins are marked on the right.

transformation efficiency of 3% for the *indica* rice variety, White Ponni. Southern analysis with right-border and left-border probes has shown that four T_0 lines carry single-copy insertions of complete T-DNAs. All the transgenic lines accumulated chitinase protein at high levels. The single-copy integration events were further confirmed on the basis of segregation of the transgene in a simple Mendelian ratio of 3 : 1.

In the context of the recommendation of the Task Force on Agricultural Biotechnology that genetic engineering of rice should be confined to non-basmati varieties[17], it has become important to identify elite, non-basmati *indica* varieties that can be taken up for genetic engineering.

Though the transformation frequency for *indica* cultivars remains low, we have shown that White Ponni is amenable for *Agrobacterium*-mediated transformation. White Ponni can be used as a non-basmati variety for routine transformation by improving its transformation frequency. Further increase in the transformation frequency of *indica* variety (White Ponni) could be achieved by adding maltose[9,26] instead of sucrose in the culture medium. Homozygous lines will be established from the single-copy transgenic White Ponni lines. Those lines which express chitinase constitutively, will be evaluated for resistance against *Rhizoctonia solani*, the sheath blight pathogen.

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ACKNOWLEDGEMENTS. We thank Dr Richard A. Jefferson, CAMBIA, Canberra, Australia (pCAMBIA1301), Dr Toshihiko Komari, Japan Tobacco Inc., Japan (pSB1) and Dr S. Muthukrishnan, Kansas State University, Kansas, USA (*chi*11 gene) for providing plasmids. We also thank Dr K. Dharmalingam for providing instrumentation facilities and Dr Vai Ramanathan for help and suggestions. This work was supported by the Department of Biotechnology, New Delhi. G. S. thanks Council of Scientific and Industrial Research, New Delhi for the award of Senior Research Fellowship.

Received 4 June 2004; revised accepted 20 October 2004