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Genetic transformation of pigeonpea with rice chitinase gene

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With 2 figures and 1 table

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Abstract

With a long-term plan to develop transgenic pigeonpea with resistance to fungal disease, the transfer of a rice chitinase gene to pigeonpea [*Cajanus cajan* (L.) Millsp.] is reported here. The rice chitinase gene harboured in the plasmid pCAMBIA 1302:RChit was delivered via the *Agrobacterium*-mediated method to the cotyledonary node explants followed by subsequent regeneration of complete plants on selection media containing hygromycin. Putative transformed pigeonpea plants were recovered with stringent selection pressure and confirmed using molecular techniques. Stable integration and expression of the chitinase gene has been confirmed in the T0 and T1 transgenics through molecular analysis.

Key words: *Agrobacterium tumefaciens* — *Cajanus cajan* — rice chitinase gene — transformation

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is a good source of dietary protein in the tropics and subtropics. It is the second most important food legume of India, valued both as food and fodder. Constraints for enhancing its productivity include the damage caused by fungi, bacteria, virus and insect pests. Among the fungal diseases, the wilt (caused by *Fusarium oxysporum*, *Fusarium udum* (Butler) Snyder and Hansen) drastically limits the crop yield. Genetic engineering technology can be used with conventional methods for the introduction of agronomically useful traits into established cultivars. For successful development of transgenic plants, identification of suitable target tissue and efficient gene transfer protocols are essential (Taylor and Vasil 1991, Devi et al. 2000). Although a number of reports are available on genetic transformation of legumes, there are only two reports on pigeonpea (Geetha et al. 1999, Lawrence and Koundal 2001), with low transformation efficiency.

One of the many natural defence mechanisms plants use to resist pathogen attack is to accumulate proteins (e.g. chitinases) active against disease-causing organisms. In some cases, where this mechanism is too weak or appears too late to fully protect the plant, engineering constitutive expression of a defence protein can boost tolerance to fungal pathogens (Broglie et al. 1991, Grison et al. 1996, Warkentin et al. 1998). With a long-term plan to develop transgenic pigeonpea with resistance to fungal disease, the transfer of a rice chitinase gene (Lin et al. 1995, Krishnaveni et al. 2001) to pigeonpea is reported.

Materials and Methods

Plant materials: Seeds of pigeonpea [*Cajanus cajan* (L.) Millsp.] cultivar 'LRG-30' obtained from the LAM Agricultural Farm, Guntur, India, were surface-sterilized in 70% ethanol for 5 min, washed in sterile distilled water, soaked in 0.1% (w/v) mercuric chloride containing 100 µl Tween-20 for 10 min with intermittent shaking, rinsed three times and soaked in sterile distilled water for 6 h. The seeds (seed-coats were removed aseptically) were germinated on Murashige and Skoog's (MS) basal medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and 0.8% (w/v) Phyta-agar and maintained under a 16-h photoperiod with a light intensity of 60 $\mu E/m^2/s$ at 25 ± 1°C.

Explants and culture conditions: The standardized culture protocol for pigeonpea plant regeneration consisted of cotyledonary nodes (of 7–8 mm in length) excised from 12-day-old aseptically germinated seedlings co-cultivated with *Agrobacterium tumefaciens*. The explants were cultured on shoot initiation medium (SIM) comprising MS medium supplemented with 6-benzyladenine (BA) (2.0 mg/l) for induction of shoot buds. They were transferred after 2 weeks to shoot elongation medium (SEM) comprising MS medium supplemented with 0.5 mg/l gibberellic acid-A (GA_3). The elongated shoots (> 3 cm) were then transferred to Magenta bottles containing the root induction medium (RIM) [comprising the MS medium supplemented with 1.0 mg/l indole butyric acid (IBA)]. The culture conditions employed were the same as described above. Rooted plants were transferred to pots containing a 1 : 1 mixture of sand and soil and incubated for 1 week for acclimatization (by covering with a plastic bag initially and gradually exposing the plant to the open environment) prior to transfer to a glasshouse.

***Agrobacterium* strain and plasmid vector:** The disarmed *Agrobacterium tumefaciens* (strain C-58) harbouring a binary plasmid pCAMBIA 1302:RChit (Fig. 1) was used as vector system for transformation. The rice chitinase (*Rchit*) gene (kindly provided by Dr S. Muthukrishnan, Kansas State University, USA) with the cauliflower mosaic virus (CaMV) 35S promoter and CaMV 35S poly-A terminator inserted at the *Hin*dIII MCS of the pCAMBIA 1302 vector to produce a pCAMBIA 1302:RChit binary plasmid. It also contained the hygromycin phosphotransferase (*hpt*) gene (used as a selectable marker) under the control of CaMV 35S promoter and CaMV 35S poly-A terminator and the green fluorescent protein gene (*gfp*), for possible use as a reporter gene (Fig. 1). Bacteria were maintained on LB (Sambrook et al. 1989) agar plates containing 50 mg/l kanamycin sulphate.

Co-cultivation and transformation: A single bacterial colony was inoculated into 25 ml of liquid LB medium containing 50 mg/l kanamycin sulphate and incubated at 28°C on a shaker at 100 rpm for 16–18 h and used in the late log phase A_{600} at 0.6. The bacterial culture was centrifuged at 5000 × g and half MS liquid medium added to the bacterial pellet to make up a volume of 25 ml. Freshly cut explants were dipped into this suspension, blotted on sterile filter paper and transferred to SIM. Twenty explants were co-cultivated and cultured per petriplate and a total of 200 explants were used with three replicates. The co-cultivated explants were then transferred after 48 h to SIM-Cef medium comprising the SIM, supplemented with 200 mg/l cefotaxime to eliminate the bacteria.

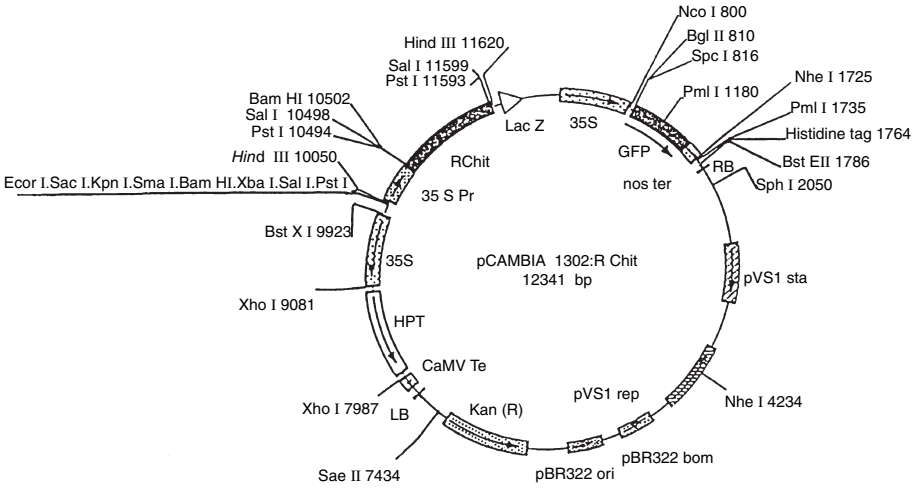

Fig. 1: Restriction map of the plasmid construct pCAMBIA1302: RChit used for *Agrobacterium*-mediated genetic transformation of cotyledonary node explants from *in vitro*-germinated seedlings of pigeonpea

Selection and plant regeneration: To identify the toxic levels of concentration of hygromycin for effective selection of putatively transgenic plants, control explants were cultured on SIM with different concentrations of hygromycin (0.5–10 mg/l). At 4 mg/l and above, the explants turned brown and did not grow further (data not shown). Hence, 5 mg/l was used as selection pressure for the culture of co-cultivated explants. The explants cultured on SIM-Cef for 1 week were transferred to the selection medium, SIM-Sel-1 (comprising the SIM supplemented with 2 mg/l hygromycin and 200 mg/l cefotaxime) and later transferred to SIM-Sel-2 medium (after 2 weeks) comprising SIM supplemented with 5 mg/l hygromycin. The cultures were maintained in stringent selection on SIM-Sel-2 for 3 weeks by which time the regenerated putatively transgenic shoots would have grown considerably. The shoots were then transferred to RIM for rooting and subsequently transferred to pots and moved to a glasshouse after acclimatization (as described previously). Control explants (not co-cultivated) were cultured simultaneously to regenerate untransformed control plants. The T0 plants were grown to maturity and seeds harvested to raise the T1 generation. The T0 and T1 transgenics were subjected to molecular genetic analysis.

Molecular analysis of putative transformants: Molecular analysis was carried out to confirm the integration and expression of the transgene. Genomic DNA isolated from the control and the putatively transgenic plants by a modified method of Rogers and Bendich (1988) was used for the molecular analysis.

PCR analysis: Polymerase chain reaction (PCR) analysis was carried out on the T0 and T1 putative transgenics for amplification of the coding region of the *Rchit* gene. One microgram of RNase-treated DNA was used as template for PCR amplification. Each PCR reaction was performed in 25 µl (final volume) of reaction mixture consisting of 2.5 µl 10X PCR amplification buffer, 2 µl of template DNA, 0.5 µl 10 mM dNTPs, 0.75 µl 50 mM $MgCl_2$, 100 ng (0.5 µl) of each primer, 10.5 µl sterile distilled water, 7.5 µl Enhancer (Invitrogen, Hong Kong Ltd, Tsuen Wan, Hong Kong) and 1 unit (0.25 µl) of Platinum Taq DNA polymerase (Invitrogen). The following primers were used to amplify the 525-bp fragment of the *Rchit* gene: forward primer: 5′-TCT GCC CCA ACT GCC TCT GCT-3′; reverse primer: 5′-CCC CGC GGC CGT AGT TGT AGT-3′. The samples were heated to 94°C for 4 min and then subjected to 34 cycles of 1-min melting at 93°C, 1-min annealing at 63°C and 90-s synthesis at 72°C followed by another 5-min final extension at 72°C. The amplified products were assayed by electrophoresis on 1.2% agarose gels, stained with ethidium bromide (0.5 µg/ml) visualized and photographed under ultraviolet light.

Southern blot hybridization analysis: The T0 transformants were subjected to Southern blot hybridization analysis of the *Rchit* gene. Ten micrograms of genomic DNA from the putatively transformed and untransformed control plants were digested with the enzyme *Xba*I to restrict the genomic DNA which cuts at a single restriction site within the plasmid DNA (pCAMBIA 1302:Rchit), to determine the copy number of the transgene. The digested DNA was separated by electrophoresis through a 0.8% agarose gel and transferred on to a Nylon N+ membrane (Amersham, Uppsala, Sweden) according to the manufacturer's instructions. The blot was probed with a non-radioactively labelled (Alkphos Direct Labelling and Detection system of Amersham Biosciences) 525-bp PCR-amplified *Rchit* gene fragment. The plasmid pCAMBIA1302:RChit was restricted with the restriction enzyme *Hin*dIII to release the 1.57-kb *Rchit* (rice chitinase) gene fragment which was used as the positive control in the Southern hybridization. The blot was exposed to X-Omat film (Eastman Kodak Company, Rochester, NY, USA) for 15 min for autoradiography.

RT-PCR analysis: Reverse transcription followed by the polymerase chain reaction (RT-PCR) leading to the amplification of specific RNA sequences in cDNA form, is a sensitive means for detecting RNA molecules transcribed as a consequence of gene expression for protein synthesis. RT-PCR was carried out on the T1 transformants for the expression of the *Rchit* gene. Total RNA from the putative transformants was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol and the Thermoscript RT-PCR system (Pharmacia) was used for carrying out RT-PCR. To rule out the possibility of having amplified contaminant DNA in the samples, direct PCR amplification of the RNA preparation was carried out without reverse transcription (Satyavathi et al. 2003). One such sample of RNA subjected directly to PCR without reverse transcription served as the negative control and plasmid DNA from pCAMBIA 1302:Rchit served as the positive control. The amplified fragments (using the same components, primers and conditions for *Rchit* gene described earlier for PCR) were separated on 1.2% agarose gels, stained with ethidium bromide (0.5 µg/ml), visualized and photographed under ultraviolet light.

Results

Genetic transformation

The plant regeneration system from cotyledonary node explants used presently for the culture of transformed explants was reliable, reproducible and efficient and capable of producing plantlets independently through organogenesis via development of multiple shoots without any callus phase. Survival rate of the *in vitro* regenerated plantlets was over 70% (data not shown) and a total of 40 healthy putatively transgenic (T0) plants were produced through *Agrobacterium* mediated gene

transfer of the rice chitinase gene and all the plants flowered and set seed normally. Further, some of the T0 (putative) transformants were advanced to the T1 generation to isolate the segregates with the rice chitinase gene with a long-term plan to develop resistance to fungal pathogens.

Molecular characterization of transgenic plants

Molecular analysis of the putative T0 transformants was carried out by PCR and Southern blot hybridization. Out of a total of 40 transformants (T0), 17 plants (P-1, P-2, P-4, P-6, P-8, P-9, P-10, P-11, P-12, P-15, P-16, P-20, P-29 and P-30, P-33, P-35 and P-39) were found to be positive for the amplification of the 525-bp fragment of the *Rchit* gene by PCR (Fig. 2a). There was no amplification observed in case of the untransformed plant DNA. The transformation efficiency out of 600 explants used with respect to the amplification of the expected size of the gene fragment (of *Rchit*) in the T0 transformants was about 2.83%. The transgene integration pattern in the nuclear genome of the putative transformed plants (T0) was confirmed through Southern hybridization analysis of the genomic DNA. The Southern hybridization was carried out in all of the 17 T0 transformants that were positive for PCR (only 10 are visible in Fig. 2b). The hybridization signal for the *Rchit* gene was detected at different locations in only 10 plants (P-1, P-2, P-6, P-9, P-12, P-16, P-20, P-29, P-30 and P-35) (Fig. 2b). Of the 10 plants, four (P-1, P-2, P-29 and P-30) showed distinct single-copy integration, three (P-6, P-9 and P-16) showed double-copy integrations, two (P-12 and P-20) showed three-copy integrations and one (P-35) showed four-copy integrations. No hybridization signal was observed in case of the untransformed plant DNA.

Of the 17 (T0) independently transformed plants, four that showed single copy transgene integration (P-1, P-2, P-29 and P-30) were advanced to the T1 generation. In order to study any Mendelian inheritance pattern in the T1 progeny, 25 seeds from each of the four T0 plants were sown and a total of 83 T1 plants were raised clone-wise in a glasshouse. PCR analysis was carried out on the 83 T1 plants for the amplification of the *Rchit* gene and the progenies of each clone showed a 3 : 1 segregation pattern (Table 1). Figure 2c shows some of these transformants (lanes 4–15). The control plant DNA comprising DNA from untransformed plants did not yield any amplification. The expression of *Rchit* gene in the T1 plants

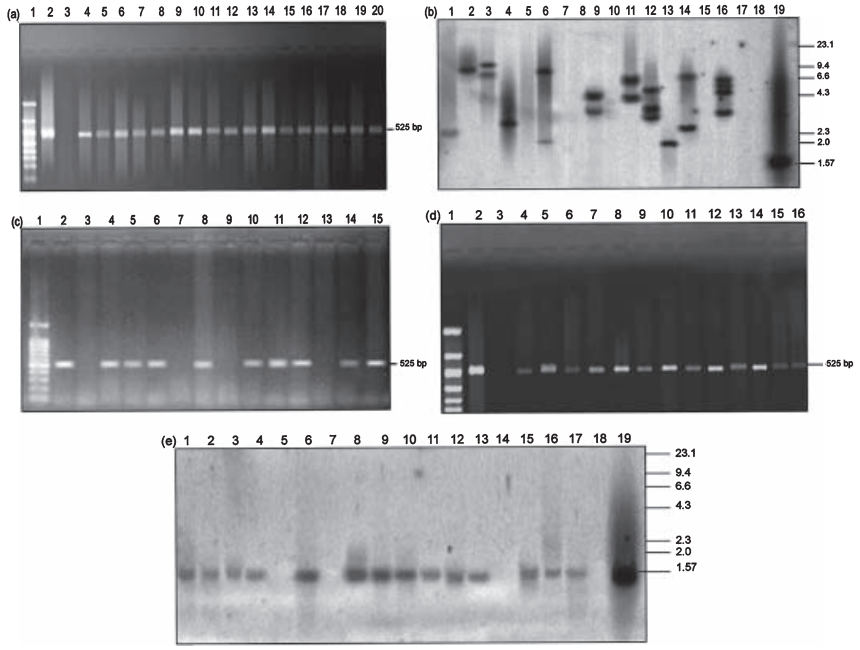

Fig. 2: Molecular analysis of genomic DNA of the T0 and T1 transformants of pigeonpea obtained via *Agrobacterium*-mediated genetic transformation of cotyledonary node explants using the plasmid construct, pCAMBIA 1302:Rchit. (a) PCR amplification of the *Rchit* gene (525 bp) in the T0 (putative) transformants. Lane 1: DNA size marker (100 bp), lane 2: positive control (plasmid pCAMBIA 1302:Rchit), lane 3: negative control (untransformed plant), lanes 4–20: putative transformants (P-1, P-2, P-4, P-6, P-8, P-9, P-10, P-11, P-12, P-15, P-16, P-20, P-29, P-30, P-33, P-35 and P-39, respectively). (b) Southern blot hybridization of the T0 putative transformants. The genomic DNA of the putative transformants was digested with *Xba*I to provide a single cut in the plasmid. Lanes 1–17: putative transformants (P-1, P-2, P-4, P-6, P-8, P-9, P-10, P-11, P-12, P-15, P-16, P-20, P-29, P-30, P-33, P-35 and P-39, respectively), lane 18: negative control (genomic DNA from control untransformed plant), Lane 19: positive control (plasmid pCAMBIA 1302:Rchit digested with *Hin*dIII to release the *Rchit* fragment with CaMV 35S promoter = 1.57 kb). (c) PCR amplification of the *Rchit* gene (525 bp) in the T1 (putative) transformants. Lane 1: DNA size marker (50 bp), lane 2: positive control (plasmid pCAMBIA 1302:Rchit), lane 3: negative control (untransformed plant), lanes 4–15: some of the 83 T1 transformants (P-1-1, P-1-2, P-1-3, P-2-1, P-2-2, P-2-3, P-29-1, P-29-2, P-29-3, P-30-1, P-30-2 and P-30-3, respectively). (d) RT-PCR of the cDNA showing amplification of the 525-bp fragment of *Rchit* gene in the T1 (putative) transformants. Lane 1: DNA size marker (100 bp), lane 2: positive control (plasmid pCAMBIA 1302:Rchit), lane 3: negative control (direct PCR amplified RNA of P-1-1 without reverse transcription), lanes 4–16: a few of the 60 RT-PCR positive T1 transformants (P-1-1, P-1-3, P-1-6, P-1-8, P-2-2, P-2-4, P-2-5, P-29-2, P-29-3, P-29-7, P-30-2, P-30-3 and P-30-7, respectively). (e) Southern blot hybridization of the T1 putative transformants. The genomic DNA of the putative transformants was digested with *Hin*dIII to release the Rice chitinase (*Rchit*) gene fragment. Lanes 1–17: putative transformants (P-1-2, P-1-3, P-1-6, P-1-8, P-2-1, P-2-2, P-2-3, P-2-4, P-2-5, P-29-1, P-29-2, P-29-3, P-29-7, P-30-1, P-30-2, P-30-3 and P-30-7, respectively). Lane 18: negative control (Genomic DNA from control untransformed plant), lane 19: positive control (plasmid pCAMBIA 1302:Rchit digested with *Hin*dIII to release the *Rchit* gene fragment with CaMV 35S promoter = 1.57 kb)

Table 1: Inheritance of the *Rchit* gene in the T1 generation of transgenic pigeonpea

T0 Plants[1]	No of T1 plants[2] characterized by PCR	PCR analysis of *Rchit* gene		3 : 1 Segregation (χ^2 test)	Probability
		Positive	Negative		
P-1	21	15	6	0.142	0.75
P-2	20	14	6	0.26	0.70
P-29	22	17	5	0.06	0.85
P-30	20	14	6	0.26	0.70

[1] P-1, P-2, P-29 and P-30 are independent T0 lines.
[2] T1 transformants were raised from the seeds of the respective T0 transformants.

was analysed by reverse transcriptase (RT)-PCR. Of the 60 PCR-positive T1 plants tested, only 20 plants (P-1-1, P-1-3, P-1-6, P-1-8, P-1-20, P-2-2, P-2-4, P-2-5, P-2-16, P-2-19, P-29-2, P-29-3, P-29-7, P-29-16, P-29-21, P-30-2, P-30-3, P-30-7, P-30-18 and P-30-20) tested positive for RT-PCR. Figure 2d shows some of these transformants (lanes 4–16). No amplified DNA fragments were detectable in the RNA samples of T1 plants subjected to direct PCR amplification without reverse transcription, in order to exclude wrong positive results caused by DNA contamination. Only one such sample is shown in Fig. 2d as a negative control.

Discussion

Although regeneration in pigeonpea was previously reported from cotyledonary node explants (Shiva Prakash et al. 1994) and from different seedling explants (Eapen et al. 1998, Mohan and Krishnamurthy 1998, Geetha et al. 1998) through organogenesis and somatic embryogenesis (Shiva Prakash et al. 1994, Sreenivasu et al. 1998), the protocols were not favourable for genetic transformation, because of low regeneration frequencies and the long time taken for regeneration. However, an efficient and improved protocol for *in vitro* plant regeneration (with a survival rate of $>70\%$) and genetic transformation from cotyledonary node explants of pigeonpea developed in this laboratory (unpublished data) has now been used.

Results show that by fine-tuning the conditions of transformation, even a recalcitrant crop like pigeonpea can be transformed with an optimum frequency. Optimal conditions standardized for efficient transformation of cotyledonary nodes include the use of freshly cut explants, a co-cultivation duration of 48 h, a delay period of 7 days followed by culture on selection medium and application of stringent selection for 3 weeks before rooting. Previous reports on *Agrobacterium*-mediated transformation of pigeonpea utilized shoot apices and cotyledonary nodes to achieve direct regeneration (Geetha et al. 1999, Satyavathi et al. 2003) or embryonic axes to achieve indirect regeneration through callus (Lawrence and Koundal 2001). However, in the former two reports, only three and 13 T0 plants (developed from 898 and 1394 explants, respectively) were characterized through Southern hybridization with an effective transformation frequency of about 1%. The present report with 2.83% efficiency (17 independent T0 plants from 600 explants of which, only 10 are visible in the image; Fig. 2b) is a significant improvement. Some of the PCR-positive T0 plants were negative in Southern blotting analysis. This may be due to the transformation of only a few sectors, giving rise to a chimaeric effect. The *Rchit* gene was randomly integrated at different locations in single and multiple copies. The copy number of the integrated gene in the T0 transformants was one, two, three or four (Fig. 2b). Although the frequency of transformation obtained presently is still low compared to the model species, the protocol is repeatable and can be used to mobilize genes of agronomic importance into elite cultivars.

Of the 83 T1 transformants, 60 plants were positive for PCR and of these, 20 plants were positive for RT-PCR analysis indicating the presence and transcription of the *Rchit* gene and thereby, the inheritance of the gene from T0 to T1 generations. Clear Mendelian gene segregation of the transgene was thus observed in the T1 progeny of plants. The failure to detect the expression of the rice chitinase in some plants may be due to gene silencing. A similar silencing of the rice chitinase gene under the control of the CaMV 35S promoter has been observed in transgenic wheat, rice and sorghum (Lin et al. 1995, Chen et al. 1998, Krishnaveni et al. 2001). The 20 characterized T1 transgenic plants are now being subjected to further genetic analysis and fungal disease assay with *Fusarium* strains on the lines of earlier reports (Lin et al. 1995, Zhu et al. 1998, Nishizawa et al. 1999, Takatsu et al. 1999, Yamamoto et al. 2000, Krishnaveni et al. 2001) to test the effectiveness of the *Rchit* gene against the wilt-causing pathogen.

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