Agrobacterium-mediated transformation of chickpea with α-amylase inhibitor gene for insect resistance

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Chickpea is the world's third most important pulse crop and India produces 75% of the world's supply. Chickpea seeds are attacked by *Callosobruchus maculatus* and *C. chinensis* which cause extensive damage. The α-amylase inhibitor gene isolated from *Phaseolus vulgaris* seeds was introduced into chickpea cultivar K850 through *Agrobacterium*-mediated transformation. A total of 288 kanamycin resistant plants were regenerated. Only 0.3% of these were true transformants. Polymerase chain reaction (PCR) analysis and Southern hybridization confirmed the presence of 4.9 kb α-amylase inhibitor gene in the transformed plants. Western blot confirmed the presence of α-amylase inhibitor protein. The results of bioassay study revealed a significant reduction in the survival rate of bruchid weevil *C. maculatus* reared on transgenic chickpea seeds. All the transgenic plants exhibited a segregation ratio of 3:1.

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1. Introduction

Chickpea is the world's third most important pulse crop and India produces 75% of the world's supply. Chickpea is a good source of carbohydrate (48.2–67.6%), protein (12.4–31.5%), starch (41–50%), fat (6%) and nutritionally important minerals (Geervani and Umadevi 1989). Young leaves, tender shoots and green pods are eaten as a green vegetable. Among the legumes chickpea is the best hypocholesteremic agent, followed by black gram and green gram (Soni *et al* 1982).

Production of chickpea has remained constantly low because of its susceptibility to several pathogens and insect pests. Among the insect pests, bruchids cause substantial loss during storage (Sing *et al* 1994). The cowpea weevil (*Callosobruchus maculatus*) and azuki bean weevil (*C. chinensis*) infest chickpea seeds heavily. Since seeds are used for consumption, use of chemicals to protect the seeds is not recommended. Hence newer approaches such as genetic engineering and molecular breeding have been encouraged (Jaiwal *et al* 2001; Dayal *et al* 2003;Grant *et al* 2003; Saini *et al* 2003).

Many insecticidal proteins and molecules of plant origin such as lectins, α-amylase inhibitor and protease inhibitor can retard insect growth and development when ingested (Boulter 1993; Ussuf *et al* 2001). When α-amylase inhibitor gene from common bean was expressed in transgenic pea, seeds became resistant to the infestation of bruchid weevils (Shade *et al* 1994; Shroeder *et al* 1995). The α-amylase inhibitor markedly suppressed the α-amylase activity in the larval midgut of the weevils (Ryan 1990; Ishimoto and Chrispeels 1996). Since α-amylase inhibitor is easily inactivated by cooking, introducing this gene into host plants can be regarded as a safe strategy.

In this study, α-amylase inhibitor gene (αAI1) isolated from the seeds of *Phaseolus vulgaris* L. (common bean) has been introduced into chickpea through *Agrobacterium*-mediated transformation to analyse the ability of the gene to inhibit growth of bruchid weevil *C. maculatus*, which causes severe damage to chickpea seeds during storage. The gene has been introduced under a strong seed-specific promoter, phytohemagglutinin (PHA), which directs the expression of the gene in chickpea seeds.

Keywords. *Agrobacterium*; α-amylase inhibitor; chickpea; insect resistance.

2. Materials and methods

The plasmid pTA3 (7.5 kb) containing α-amylase inhibitor gene flanked by phytohem-agglutinin promoter and polyA sequences (a gift from T J V Higgins, CSIRO, Australia) was digested with *Hind*III restriction enzyme; the digested fragment (pAPSKαAI1, figure 1) was subcloned into the corresponding site of pCAMBIA 2301 vector (11.5 kb) and the plasmid was mobilized into *Agrobacterium* vir helper strain LBA 4404 by triparental mating (Ditta *et al* 1980) in which LBA 4404 was used as recipient and *Escherichia coli* was used as conjugal helper strain. Southern hybridization of transconjugants using DIG labelling was carried out to confirm the site of integration using *Bam*H1 restriction enzyme.

Seeds of chickpea cultivar K850 were obtained from the National Germplasm Centre, Indian Agricultural Research Institute (IARI), Pusa, New Delhi. This cultivar was chosen because of its desirable characters such as high yield and short duration. The seeds were washed in running tap water several times. Then the seeds were treated with labolene detergent solution for 5 min and rinsed in double distilled water. The seeds were further sterilized with 70% ethanol for 1 min followed by a treatment with 0.1% mercuric chloride for 4 min and washed six times with sterile double distilled water. Sterilized seeds were germinated on seed germination medium [MS salts, MS iron, B_5vitamins, MES (3mM), sucrose 3% and agar 0.8% at 5.6 pH].

Embryogenic axis explants obtained from aseptically germinated seedlings were preincubated for 72 h; they were wounded and infected with LBA 4404 (pAPSKαAI1) *Agrobacterium* strain at 0.8=OD_{600} for 30 min and after removal of excess bacterium by blotting, were cocultivated for 72 h in cocultivation medium with acetosyringone, sodium thiosulphate, L-cysteine and dithiothreitol at 25°C in the dark. Cocultivated embryogenic axis explants were rinsed in liquid MS basal medium fortified with 500 mg/l cefotaxime with a few drops of Tween 20. Then the explants were blotted on sterile paper to remove excess bacteria and moisture and transformed to shoot induction medium (MS salt + B_5 vitamins + BAP 1.0 mg/l) with kanamycin (100 mg/l) for nine weeks. After nine weeks on selection medium the shoots were transferred to regeneration medium [MS salts, MS iron, B_5 vitamins, MES (3 mM), BAP 0.1–5.0 mg/l, sucrose (3%), agar (0.8%), pH 5.6]. Randomly selected transformed shoots derived from embryonic axis were assayed for GUS activity (Jefferson 1987). The shoots of 2–3 cm length were transferred to 0.5 strength MS medium [MS salts, B_5 vitamins, IBA (1.0 mg/l) + Kanamycin (100 mg/l), sucrose (3%), agar (0.8%), pH 5.6] for root induction. The plants were hardened and transferred to controlled green-house conditions and allowed to set seeds which were used for insect bioassay tests. Segregation analysis of the transgenes was also carried out using the R_0 seeds. Total DNA from the transformed plants was extracted using the method of Roger and Bendich (1994). *Hind*III was used as the restriction enzyme. Southern hybridization using DIG labelling was done to confirm the presence of α-amylase inhibitor gene in transformed chickpea plants. Western blot was performed using αAI1 anti-serum raised in rabbit (a gift from T J V Higgins) to confirm the presence of α-amylase inhibitor protein.

The α-amylase inhibitor from the seeds of transformed and untransformed chickepa seeds was quantified following the method of Piergiovanni (1992). In a blind experiment, the first generation (F_1) of transformed and untransformed chickpea seeds were placed in separate glass jars (8 x 6 x 4 cm) and tested for insect resistance. Ten pairs of newly emerged adults, obtained from the stock culture, were introduced into glass jars containing chickpea seeds. After one week dead insects were removed and the percentage of mortality was calculated.

The F_1 adult emergence was noted after 26 days and the weights of the newly emerged adults were recorded using a

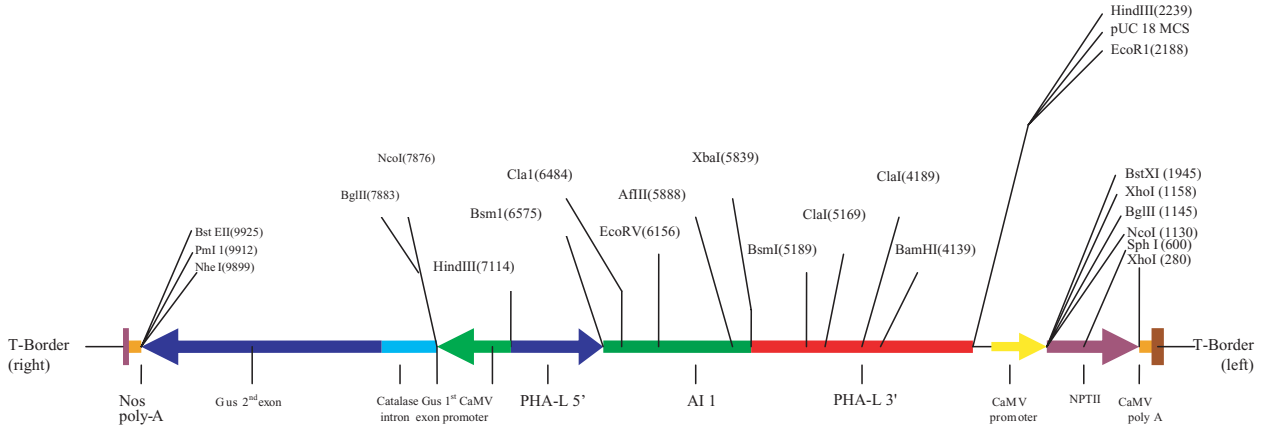

Figure 1. T-DNA map of plasmid pAPSKαAI.

digital balance after anesthetizing them. The longevity of the adults was also recorded. All experiments were replicated three times.

3. Results

Southern hybridization of transconjugants clearly indicated the true integration of *α*-amylase gene and confirmed the mobilization of pAPSKαAI1 into LBA 4404. All the transconjugants showed the expected 1.8 kb and 14.6 kb bands with *Bam*H1 restriction digestion. When the transconjugants were digested with *Hind*III restriction enzyme and subjected to polymerase chain reaction (PCR) analysis, all of them showed the presence of *α*-amylase inhibitor gene (4.9 kb) along with PHA promoter (11.5 kb). Transformed shoots harbouring *α*-amylase gene were green and healthy (figure 2); 982 embryonic axis explants were cocultivated. Eighty-six embryonic axis explants were found to be resistant to kanamycin antibiotic. Nearly 288 plants were produced from these kanamycin resistant embryonic axis explants. Out of these 60% of the shoots showed transient GUS expression. But when the fullygrown plants were tested for *GUS* expression only 0.3% showed blue staining; PCR with *GUS* gene probe confirmed the presence of GUS genes in transformed plants (figure 3).

Southern blot analysis was performed to ascertain the stable integration of *α*-amylase inhibitor gene in transformed plants (figure 4). Western blot analysis confirmed the presence of *α*-amylase inhibitor protein in transformed plants (figure 5). With regard to quantity of *α*-amylase inhibitor protein, the transgenic chickpea plants contained high amounts compared to untransformed plants (table 1).

Segregation of the *α*-amylase inhibitor gene to the next generation was examined through kanamycin resistance and GUS assay experiments. Segregation analysis of transgenic independent R_0 plants revealed that the transgenes were stably inherited to R_1 progeny. All the transgenic plants exhibited a segregation ratio of 3:1 (table 2).

The results of the bioassay tests using *C. maculatus* on different transgenic lines of chickpea are presented in table 3. *C. maculatus* reared on control seeds (untransformed) developed into adults (95%) and only 5% mortality was recorded; the insects reared on transgenic seeds showed a high mortality rate which was found to be significantly higher than in the control (table 3). The number of F_1 individuals that emerged and the weight of the newly emerged adults were also significantly reduced when the insects were reared on transgenic chickpea seeds. The adult longevity of the insects (both male and female) was also recorded and found

Figure 2. Caulogenic response of embryonic axis explants of chickpea. (**A**) Shoot initiation from embryonic axis explant. (**B**) Shoot bud formation from embryonic axis. (**C**) Multiple shoot formation after subculturing on fresh medium. (**D**) Shoot elongation on GA_3 medium. (**E**) Root initiation from elongated shoot. (**F**) Earthen pot with well developed plant.

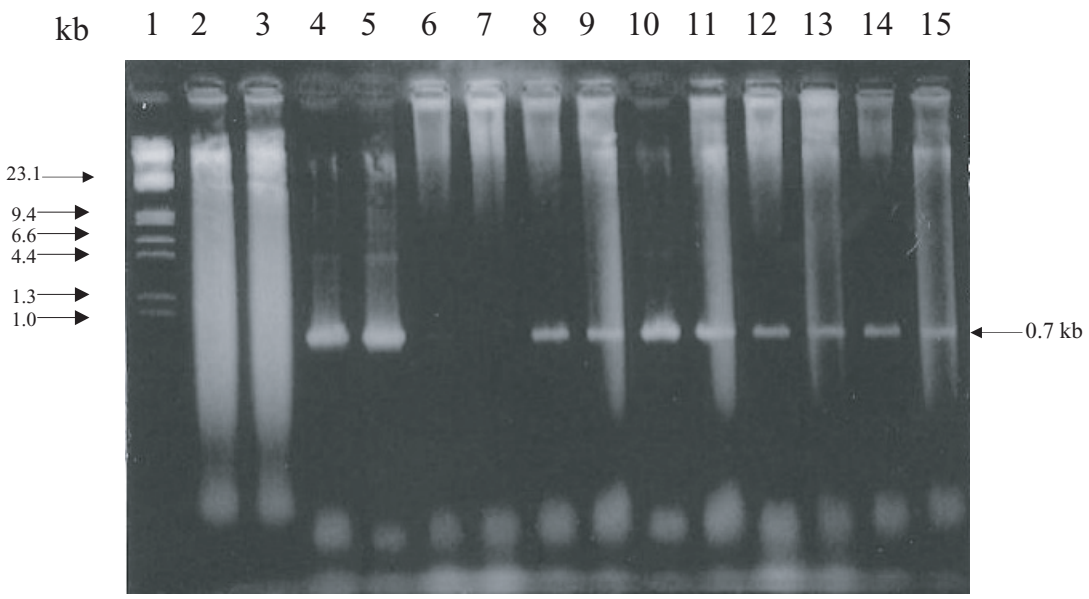

Figure 3. PCR amplification of *GUS* gene from transformed chickpea embryonic axis explants resulting from infection with *Agrobacterium* LBA 4404 strain. Lane 1, Marker lambda DNA/*Hind*III digest; lanes 2-3, negative control (undigested DNA from plants); lanes 4-5, positive control (plasmid DNA); lanes 6-7, negative control (digested DNA from plants); lanes 8-15, transformed chickpea plants.

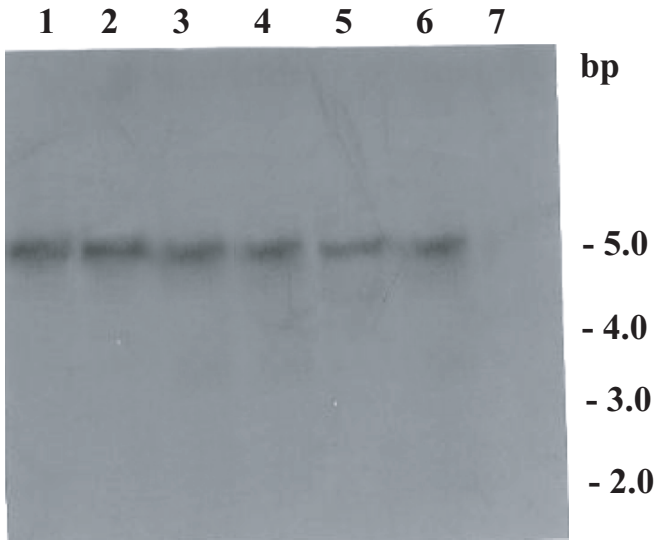

Figure 4. Southern hybridization of *Hind*III digested DNA from transgenic chickpea plants using α-amylase gene probe. Lanes 1-6, Genomic DNA from transformed chickpea plants: CP-1-10, CP-1-12, CP-2-14, CP-2-16, CP-3-18, CP-3-20; lane 7, genomic DNA from untransformed control chickpea plant.

to be significantly reduced in comparison to insects reared on untransformed seeds.

4. Discussion

A major application of gene transfer technology is the introduction of agronomically useful traits into crop plants. Even though there is one report available on the introduction of *Cry1A(c)* gene (Kar *et al* 1997) into chickpea to protect crop from feeding larvae of *Helicoverpa armigera* in field conditions, reports on the resistance to stored product pests are scanty (Sarmah *et al* 2004).

In this study α-amylase inhibitor gene has been introduced into chickepa (K850) through *Agrobacterium*-mediated transfer to provide resistance to bruchid weevil.

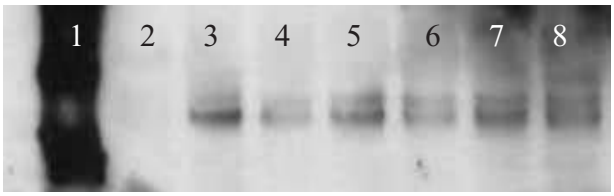

Figure 5. Western blot analysis of α-amylase inhibitor protein from transgenic chickpea plants. Total protein extracts from the transgenic chickpea seeds were fractioned by SDS/PAGE and blotted onto a nitrocellulose membrane and probed with polyclonal antibody to α-amylase inhibitor protein. Lane 1, Marker; lane 2, untransfromed chickpea plant; lanes 3-8, transformed chickpea plants: CP-1-10, CP-1-12, CP-2-14, CP-2-16, CP-3-18, CP-3-20.

Agrobacterium-mediated transformation of chickpea has been attempted earlier (Krishnamurthy *et al* 2000; Sanyal *et al* 2003; Tewari-Singh *et al* 2004) but only one transformation work has been done for stored product insect resistance (Sarmah *et al* 2004). In the present study, a good number of chickpea plants carrying α-amylase inhibitor gene has been produced in K850 cultivar by *Agrobacterium tumefaciens* mediated transformation. This study also proves the efficiency and effectiveness of the virulent *A. tumefaciens* LBA 4404 (pAPSKαAI1) strain in transforming chickpea embryonic axis.

Table 1. Quantitative estimation of α-amylase inhibitor protein content in control and transformed chickpea seeds.

Control and transformed chickpea plants	Percent α-amylase inhibitor protein in the seeds
Control	0.0^{a}
CP-1-10	0.65 ± 0.12^{b}
CP-1-12	0.68 ± 0.24^{b}
CP-2-14	0.71 ± 0.19^{b}
CP-2-16	0.63 ± 0.16^{b}
CP-3-18	0.72 ± 0.31^{bc}
CP-3-20	0.65 ± 0.8^{b}

Numbers with the same alphabet do not differ significantly.

The *GUS* gene expression under the control of a strong seed-specific promoter (CaMV 35S) in transformed chickpea shoots was stable. Similar results have been reported earlier by other workers (Krishnamurthy *et al* 2000; Jaiwal *et al* 2001; Saini *et al* 2003).

Molecular analysis through PCR amplification confirmed the presence of *GUS* gene in transgenic shoots. Genomic analysis of six randomly selected transgenic plants confirmed the integration of α-amylase inhibitor gene. The number of hybridization signals indicated that single copy of the T-DNA had been integrated. Similar results have been obtained by other workers in various plant species using α-amylase inhibitor gene (Schroeder *et al* 1995; Ishimoto and Chrispeels 1996; Ishimoto *et al* 1996; Morton *et al* 2000). Western blot analysis confirmed the presence of α-amylase inhibitor protein. Transgenic plants produced fertile seeds. Segregation of α-amylase inhibitor gene to next generation demonstrated that inheritance occurred in Mendelian ratio of 3:1.

In this study, transformed chickpea plants contained good amount of α-amylase inhibitor protein which confirmed the presence of α-amylase gene. The results of bioassay study

Table 2. Segregation of kanamycin resistant gene and *GUS* gene to R_1 generation of chickpea transformed with *Agrobacterium tumefaciens* LBA 4404 (pAPSKαAI1).

Transformed chickpea plants	No. of seeds tested	Kanamycin esistant				GUS+			
		Resistant	Sensitive	Ratio	χ^2 Value	Positive	Negative	Ratio	χ^2 Value
CP-1-10	56	40	16	3:1	0.39	40	16	3:1	0.38
CP-1-12	42	32	10	3:1	0.10	32	10	3:1	0.10
CP-2-14	60	43	17	3:1	0.35	43	17	3:1	0.35
CP-2-16	37	26	11	3:1	0.44	26	11	3:1	0.44
CP-3-18	45	32	13	3:1	0.36	32	13	3:1	0.36
CP-3-20	64	49	15	3:1	0.08	49	15	3:1	0.08

Table 3. Biological performance of *C. maculatus* reared on control and transformed chickpea seeds.

Control and transformed chickpea plants	Mortality (%)	No. of adult emergence	Mean F_1 adult insect weight (mg)	Adult longevity (days)
Control	7.0 ± 1.5^{a}	324 ± 53.0^{c}	3.4 ± 0.45^{c}	146 ± 23.5^{a}
CP-1-10	75.4 ± 5.4^{b}	25 ± 3.5^{a}	2.4 ± 0.1^{a}	30 ± 5.1^{ab}
CP-1-12	80.7 ± 9.1^{bc}	19 ± 4.0^{a}	2.6 ± 0.21^{ab}	27 ± 4.6^{a}
CP-2-14	78.2 ± 8.6^{b}	28 ± 3.0^{ab}	1.8 ± 0.14^{a}	34 ± 5.2^{b}
CP-2-16	74.6 ± 6.7^{b}	21 ± 5.5^{a}	2.5 ± 0.20^{ab}	19 ± 3.2^{a}
CP-3-18	69.3 ± 7.2^{b}	34 ± 6.0^{ab}	2.4 ± 0.16^{a}	28 ± 4.4^{ab}
CP-3-20	66.5 ± 8.3	26 ± 4.5^{a}	2.0 ± 0.22^{a}	25 ± 4.0^{a}

Numbers with the same alphabet do not differ significantly.

revealed a significant reduction in the survival of bruchid weevil *C. maculatus* reared on transgenic chickpea seeds. Bioassay study proved that the transgenic seeds showed increased resistance to the bruchid weevil than the control seeds. Similar results have been reported earlier (Sarmah *et al* 2004).

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