acid formation. A more or less similar situation was observed with a basidiomycetous yeast *Rhodotorula rubra*[17], where addition of CoA, ATP and NAD in cell-free extract was found essential for *in vitro* conversion of ferulic acid to vanillic acid. Work is being continued with *P. variotii* to characterize the enzymes involved in this biotransformation, the results of which will be presented elsewhere in the near future.

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Agrobacterium-mediated transformation of an elite indica rice for insect resistance

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In India, rice occupies the largest area among all crops and accounts for as much as 21% of the total cropped area. Rice plants are prone to attack from many pests and pathogens. Among them coleopteran pests are well known for causing extensive damage. *Sitophilus oryzae* causes severe damage to rice seeds during storage. Alpha amylase inhibitor gene isolated from *Phaseolus vulgaris* seeds was introduced into Basmati rice (PB1) through *Agrobacterium*-mediated transformation. A total of 174 hygromycin-resistant plants were regenerated. Most of these plants were GUS-positive. PCR analysis and Southern hybridization confirmed the presence of 4.9 kb alpha amylase inhibitor gene in transformed plants. Western blot confirmed presence of alpha amylase inhibitor protein. Results of the bioassay study revealed significant reduction in survival rate of rice weevil, *S. oryzae* reared on transgenic rice seeds.

Keywords: *Agrobacterium*, alpha amylase inhibitor, rice, *Sitophilus oryzae*.

THE Asian cultivated rice (*Oryza sativa* L.) is an economically important cereal crop in the world because more than half of the world's population depends on rice as a primary staple food[1]. In India, rice occupies the largest area among all crops and accounts for as much as 21% of the total cropped area. Rice plants are prone to attack from many pests and pathogens. Among them, coleopteran pests are well known for causing extensive damage[2]. Loss

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of rice yield due to direct insect damage is estimated to cost at least several million dollars per year and further losses are attributed to plant diseases for which insects serve as transmission vectors[3]. Rice seeds are vulnerable to storage insect pests, especially to rice weevil *Sitophilus oryzae*. Damage caused by it results in weight loss of seeds, poor seed germination and quality, which in turn affect nutritive value and hygienic conditions of the processed material.

During the past few years, the success in designing insect-resistant crop plants through gene transfer has been impressive. The first examples of insect-resistant tobacco plants reported in 1987, utilized a native lepidopteran-specific insect toxin gene from *Bacillus thuringiensis*[4]. Recent progress in rice transformation technology has made it possible to produce new rice cultivars with improved resistance to insect pests and diseases by genetic engineering[5,6].

Several insecticidal proteins of plant origin, such as lectins, alpha amylase inhibitor and protease inhibitors have been found to retard insect growth and development[7]. Alpha amylase inhibitor gene is of particular interest because it is part of the plant's natural defence system, and causes moderate mortality, prolonged larval developmental time and reduced fecundity[8]. With the expression of this gene in seeds, harvested seeds can be stored before economic or nutritional losses become intolerable. When alpha amylase inhibitor gene from common bean was expressed in transgenic pea, seeds became resistant to the infestation of bruchid weevils, which usually feed on seeds of pea[9,10] and other pulses. The alpha amylase inhibitor markedly suppressed alpha amylase activity in larval midgut of weevil species[11,12]. Since alpha amylase inhibitor is easily inactivated by cooking, introducing this gene into host plants can be regarded as a safe strategy from the standpoint of food.

In this study, α-amylase inhibitor gene (α-AI) isolated from *Phaseolus vulgaris* L. (common bean) seeds has been introduced into rice through *Agrobacterium*-mediated method, to analyse the ability of the gene to inhibit the growth of rice weevil (*S. oryzae*), which causes severe damage to rice seeds during storage. The gene has been introduced under a strong seed-specific promoter, phytohaemagglutinin (PHA), which directs the expression of the gene in rice seeds[13]. Alpha amylase inhibitor gene has not been used so far for the production of transgenic rice.

Seeds of a basmati rice variety PB1 were obtained from Tamil Nadu Agricultural University, Coimbatore. The plasmid pTA3 (7.5 kb) containing α-amylase inhibitor gene flanked by phytoheme agglutinin promoter and poly A sequences (a gift from T. J. V. Huggins, CSIRO, Australia) was digested with *Hin*dIII restriction enzyme; the digested fragment (pSAMαAI, Figure 1) was subcloned into the corresponding site of pCAMBIA 1301 vector (11.8 kb) and the plasmid was mobilized into *Agrobacterium* vir helper strain LBA 4404 by triparental mating[14], 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.

Dehusked, mature healthy seeds were surface-sterilized with 70% alcohol for 30 s and in 0.1% $HgCl_2$ (w/v) for 5 min, followed by five rinses with sterile double-distilled water. Sterilized seeds were cultured on callus induction medium CIM; MS major and minor salts, B5 vitamins, 300 mg/l cas amino acid, 500 mg/l L-proline (30 g/l sucrose, 2.5 mg/l 2,4-D, 2.25 g/l phytagel or 7 g/l agar, pH 5.8) and incubated in the dark for 21 days at 27 ± 2°C. Pieces of actively proliferating embryogenic calluses (0.2–0.4 mm) were isolated after three weeks and subcultured on fresh callus induction medium to obtain more calluses. These calluses were cocultivated with *Agrobacterium tumefaciens* LBA 4404 (pSAMαAI) strain at 25°C for three days in the dark. Cocultivation medium consisted of CIM + 200 μM acetosyringone at pH 5.8. The calluses were rinsed 4–5 times in 50 ml of sterile water supplemented with 250 mg/l cefotaxime and a drop of Tween 20. Then the calluses were blotted on sterile paper to remove excess bacteria and moisture, and transferred to selection medium (CIM + 250 mg/l cefotaxime and 50 mg/l hygromycin) for inhibition of bacterial growth and preferential growth of the transformed tissues. The cultures were incubated in the dark at 27 ± 2°C.

The hygromycin-resistant calluses obtained after a second round of selection were transferred to fresh selection medium. Once in every three weeks, the fresh and healthy looking hygromycin-resistant calluses were subcultured in fresh selection medium. After three rounds of selection and a total of nine weeks on the selection medium, the calluses were transferred to the regeneration medium (MS major and minor salts, B5 vitamins, 300 mg/l cas amino acid, 500 mg/l L-proline, 30 g/l sucrose, 0.1 mg/l NAA, 2.0 mg/l kinetin, 100 mg/l cefotaxime, 50 mg/l hygromycin, 4.0 g/l phytagel or 8.0 g/l agar, pH 5.8). Small pieces of the hygromycin-resistant calluses were assayed for GUS activity every three weeks during subculture. Similarly, some pieces from resistant calluses that were transferred to the regeneration medium were also stained to check for GUS activity[15]. For regeneration of transformed callus, the cultures were initially incubated in the dark for two weeks and then transferred to light conditions under 16 h photoperiod and incubated for three weeks. Afterwards, shoots of about 2–3 cm length were transferred to half strength MS medium containing MS major and minor salts, MS vitamins, 30 g/l sucrose, 0.5 mg/l NAA, 4.0 g/l phytagel or 7 g/l agar, pH 5.8 for root formation. The plants were hardened and transferred to controlled greenhouse conditions and allowed to set seeds which were used for insect bioassay tests. Total DNA from the transformed plants was extracted using the method of Rogers and Bendich[16]. *Hin*dIII was used as the restriction enzyme. PCR analysis

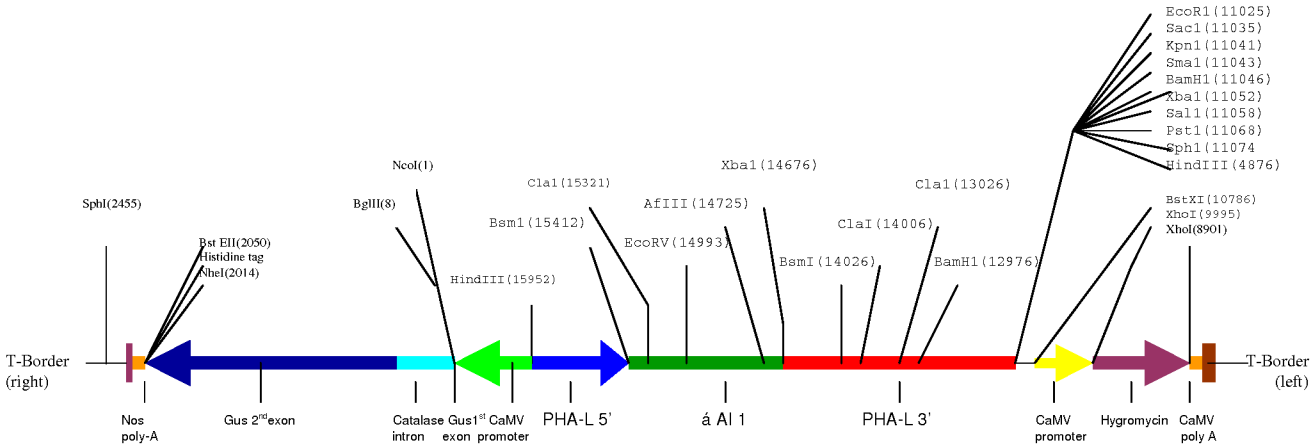

Figure 1. T-DNA map of plasmid pSAMαAI.

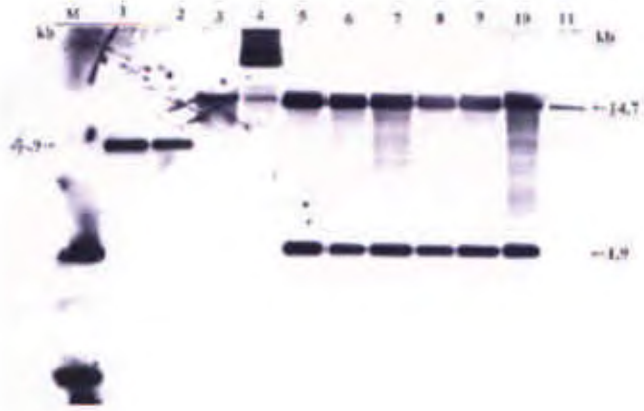

Figure 2. Southern hybridization analysis of transconjugants using DIG-labelling method. Total DNA of six transconjugants (lanes 5–10) were digested with *Bam*HI restriction enzyme, fractionated by electrophoresis, transferred to a nylon membrane and allowed to hybridize with α-amylase inhibitor gene along with its promoter and terminator (4.9 kb, *Hin*dIII fragment of pTA3). M, 1 kb ladder marker DNA; lane 1, Positive control (250 pg of probe DNA, *Hin*dIII fragment of pTA3 plasmid); lane 2, Positive control (50 ng of probe DNA, *Hin*dIII fragment of pTA3 plasmid); lane 3, Total DNA of recipient *Agrobacterium tumefaciens* (LBA 4404 (pSB1); lane 4, Undigested transconjugant; lanes 5–10, Transconjugants digested with *Bam*HI enzyme; lane 11, Binary vector (plasmid isolated from *E. coli*) digested by *Bam*HI enzyme.

and Southern hybridization using DIG-labelling were carried out to confirm the presence of alpha amylase inhibitor gene in the transformed rice plants. DNA from six of the GUS-positive transgenic shoots was used for molecular analysis. Total DNA (20 µg) of putative transformants was digested with *Hin*dIII restriction enzyme, releasing an internal fragment of expected size of 4.9 kb α-amylase inhibitor gene along with the phytohaemagglutinin promoter. The probe for α-amylase inhibitor gene was prepared from pTA3 plasmid by digesting with *Hin*dIII restriction enzyme and DIG-labelled. Western blot was performed to ascertain the presence of alpha amylase inhibitor protein.

Alpha amylase inhibitor from transformed and untransformed rice seeds was quantified according to the method of Piergiovanni[17]. In a blind experiment, the first generation (R_1) of transformed and untransformed dehusked rice seeds (500 seeds) of PB1 cultivar derived from six independent, primary transgenic lines were placed in six separate glass jars (8 × 6 × 4 cm) and tested for insect resistance bioassay. Ten pairs of newly emerged *S. oryzae* adults, obtained from the stock culture were introduced in all these jars. A control jar was also maintained with untransformed seeds. After one week, dead insects were removed and the percentage of mortality calculated. The F_1 adult emergence was noted after 26 days and the weight of the newly emerged adults was recorded using a digital balance. Ten insects were introduced in a small glass container and anesthetized using diethyl ether and weighed individually. The longevity of the adults from the control and transgenic rice was also recorded. All experiments were replicated three times.

Southern hybridization of the transconjugants clearly indicated the true integration of alpha amylase gene and confirmed the mobilization of pSAMαAI into LBA 4404. All the transconjugants showed the expected 1.9 and 14.7 bands with *Bam*H1 restriction digestion (Figure 2).

Alpha amylase inhibitor gene was introduced into embryogenic calluses derived from scutellum explants of the indica variety PB1 by the *Agrobacterium*-mediated gene transfer method. Scutellum calluses cocultivated with LBA 4404 (pSAMαAI) for three days, produced hygromycin-resistant calluses after 42 days in the selection medium with the antibiotic (Figure 3 *a*). Out of 406 calluses cocultivated, 202 hygromycin-resistant calluses were obtained (Figure 3 *b*). After 63 days of selection, the hygromycin-resistant calluses were transferred to the regeneration medium with antibiotics, where small shoot clusters were observed. A total of 174 hygromycin-resistant plants were regenerated from 202 calluses. After six weeks on the regeneration medium, the plantlets were rooted (Figure 3 *c*) on the

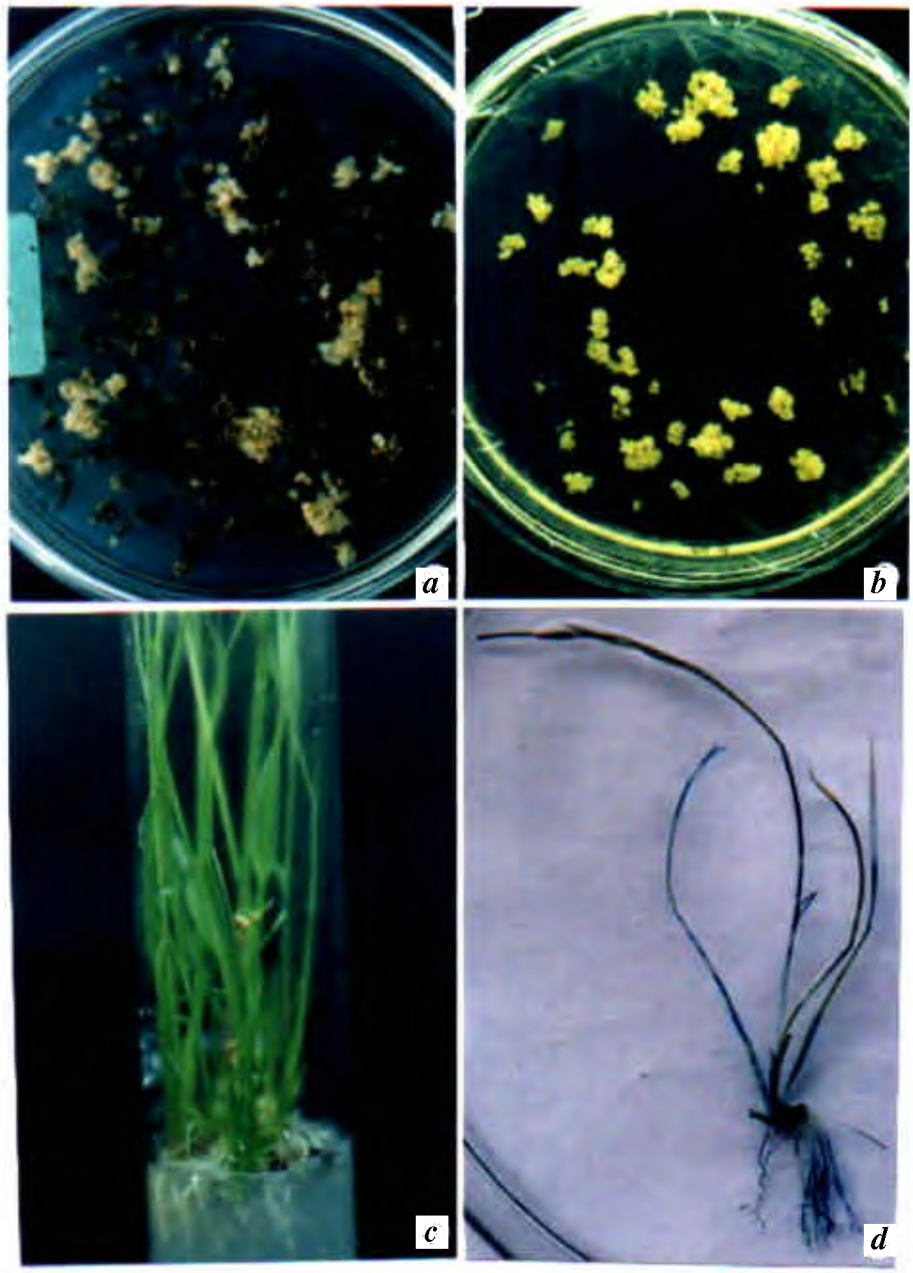

Figure 3. ***a***, Formation of hygromycin-resistant calluses of scutellum explants of *Oryza sativa* L. cv. PB1 after 42 days of culture on selection medium. The calluses were plated on the selection medium after three days of cocultivation with *Agrobacterium tumefaciens* LBA4404 (pSAMαAI). ***b***, Proliferation of hygromycin-resistant calluses of rice (*Oryza sativa* L. cv. PB1) after subculture in selection medium. Photograph was taken after 21 days during third round of selection. ***c***, Plant regeneration and root induction from hygromycin-resistant calluses of *O. sativa* L. cv. PB1. Photograph was taken four weeks after selected calluses had been transferred to root induction medium. ***d***, Expression of GUS in an entire rice (cv. PB1) seedling when stained with X-Gluc after making a wound longitudinally.

rooting medium. Most of the plants subjected to GUS histochemical assay were GUS-positive (Figure 3 *d*). Out of 174 regenerated shoots, 168 showed blue spots.

Total DNA from six PB1 putative transformants were subjected to PCR analysis with α-amylase inhibitor gene primers as well as *nptII* gene primers. Since *nptII* gene is present outside the T-DNA in pSAMαAI, *nptII* gene primers were used to check for the presence of any contaminating *Agrobacterium* cells in the plant tissue, although amplification could also result from T-DNA transfer that extends beyond the T-DNA border[18]. However, none of the plants analysed showed amplification of *nptII* gene sequences, though all the six plants showed amplification of α-amylase inhibitor gene sequences (Figure 4 *a*). This indicates that the tissues were completely free of *Agrobacterium*.

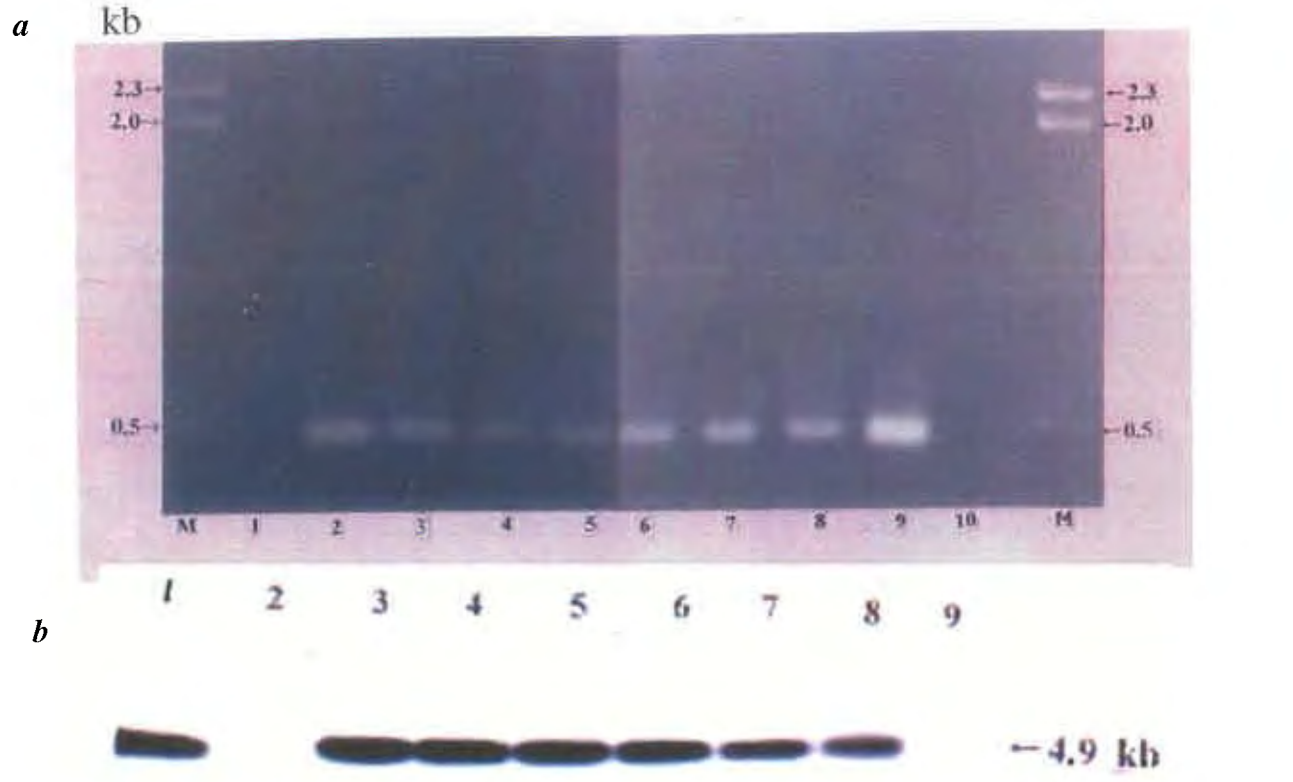

Figure 4. *a*, PCR amplification of α-amylase inhibitor gene from genomic DNA isolated from six putative transgenic rice (*O. sativa* L. cv. PB1) plants resulting from infection with LBA 4404 (pSAMαAI). Each lane represents an individual putative transformant. M, Lambda DNA digested by *Hin*dIII enzyme (marker); lane 1, Untransformed PB1 rice plant; lane 2, Positive control (50 ng of plasmid); lanes 3–8, Putatively transformed rice plants PB1-1-10, PB1-1-12, PB1-2-14, PB1-2-16, PB1-3-18, PB1-3-20 respectively; lane 9, Positive control (50 ng of plasmid); lane 10, Untransformed PB1 rice plant. *b*, Southern blot analysis of six transgenic plants of PB1 cultivar (lanes 3–8) and an untransformed plant using DIG-labelled method. Genomic DNA was digested with *Hin*dIII restriction enzyme, fractionated by electrophoresis, transferred to a nylon membrane and allowed to hybridize with α-amylase inhibitor gene along with the promoter, phytohemagglutinin (*Hin*dIII fragment of plasmid). Lane 1, Positive control (probe DNA); lane 2, Untransformed PB1 rice plant (negative control); lanes 3–8, Putatively transformed rice plants PB1-1-10, PB1-1-12, PB1-2-14, PB1-2-16, PB1-3-18, PB1-3-20 respectively; lane 9, *Hin*dIII enzyme-digested Lambda DNA marker.

Table 1. Quantitative estimation of α-amylase inhibitor content in control and transformed rice (*Oryza sativa* L. cv. PB1) seeds

Transgenic seed	Percentage of α-amylase inhibitor protein
PB1-1-10	0.86 ± 0.001^{ab}
PB1-1-12	0.86 ± 0.02^{ab}
PB1-2-14	1.0 ± 0.02^{b}
PB1-2-16	0.8 ± 0.01^{a}
PB1-3-18	0.73 ± 0.02^{a}
PB1-3-20	0.73 ± 0.04^{a}
Control (PB1)	Not detected

Data represent mean ± SD of three independent assays, expressed as % of α-amylase protein content of transformed rice seeds. Means followed by the same letter do not differ significantly ($P < 0.05$), based on Fisher's LSD test.

Southern blot analysis was performed in order to prove the stable integration of α-amylase inhibitor gene with the plant genome. Alpha amylase inhibitor gene probe only hybridized to DNA from transgenic plants (Figure 4 *b*), but not to DNA from untransformed control plants. These results indicated that the α-amylase inhibitor gene was present in the rice genome. α-amylase inhibitor protein was also present as 19 kDa immunoreactive polypeptide (Figure 5). Results on quantification of α-amylase inhibitor protein indicated that transgenic plants contained high amounts of α-amylase inhibitor protein compared to untransformed rice plants. Alpha amylase inhibitor protein content in the seeds of various independent transformed lines of rice is given in Table 1.

Segregation of the α-amylase inhibitor gene in the next generation was examined through hygromycin resistance and GUS assay experiments. Segregation analysis of these six independent R_0 lines demonstrated that the transgenes were stably inherited to R_1 progeny. All the six transgenic lines exhibited a segregation ratio of 3 : 1. for hygromycin and *GUS* genes (Table 2).

Results of the feeding assays on *S. oryzae* in various lines of transformed rice seeds are presented in Table 3. *S. oryzae* reared on control seeds (untransformed seeds) developed into adults (95%) and only 5% mortality was recorded; 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 individu-

Table 2. Segregation of hygromycin and *GUS* genes in R_1 generation of transformed rice seeds (*O. sativa* L. cv. PB1)

Transgenic line	Number of seeds tested	Hygromycin				*GUS*			
		Resistant	Sensitive	Ratio	χ^2	Positive	Negative	Ratio	χ^2
PB1-1-10	64	49	15	3 : 1	0.08	49	15	3 : 1	0.08
PB1-1-12	42	29	13	3 : 1	0.79	29	13	3 : 1	0.79
PB1-2-14	54	44	10	3 : 1	1.22	44	10	3 : 1	1.22
PB1-2-16	28	22	6	3 : 1	0.19	22	6	3 : 1	0.19
PB1-3-18	12	9	3	3 : 1	0.44	9	3	3 : 1	0.44
PB1-3-20	39	39	9	3 : 1	0.93	39	9	3 : 1	0.93

Table 3. Biological performance of *Sitophilus oryzae* L. reared on control (untransformed) and different lines of transformed rice seeds

Transgenic seed	% Mortality	No. of F_1 adult emergence	F_1 adults weight/mg/insect	Adult longevity/days
PB1-1-10	73.3 ± 8.3^{ad}	26.0 ± 4.0^{a}	1.166 ± 0.001^{a}	28.0 ± 1.0^{ac}
PB1-1-12	76.3 ± 3.0^{ab}	21.0 ± 4.0^{b}	1.123 ± 0.001^{a}	25.3 ± 2.3^{a}
PB1-2-14	81.3 ± 8.3^{b}	16.0 ± 1.0^{c}	0.996 ± 0.002^{b}	20.3 ± 2.3^{b}
PB1-2-16	75.0 ± 25^{a}	26.0 ± 4.0^{a}	1.130 ± 0.001^{a}	28.3 ± 2.3^{ac}
PB1-3-18	66.6 ± 8.3^{c}	33.0 ± 2.3^{d}	1.160 ± 0.001^{a}	31.6 ± 6.3^{c}
PB1-3-20	68.03 ± 8.3^{dc}	27.0 ± 4.0^{a}	1.130 ± 0.001^{a}	29.0 ± 1.0^{c}
Control (PB1)	5.0 ± 25^{e}	367.0 ± 57^{e}	1.29 ± 0.001^{c}	115.6 ± 34.3^{d}

Values expressed as mean ± SD of three replicates.
Mean values followed by the same alphabet do not differ significantly at $P < 0.05$, based on Fisher's LSD test.

Figure 5. Western blot analysis of alpha amylase inhibitor protein in PB1 transgenic rice plants. Total protein extracts from transgenic plants were fractioned by SDS–PAGE and blotted onto a nitrocellulose membrane and probed with polyclonal antibody to alpha amylase inhibitor protein. Lane 1, Marker; lane 2, Untransformed rice plant (PB1); lanes 3–8, Transformed rice plants PB1-1-10, PB1-1-12, PB1-2-14, PB1-2-16, PB1-3-18, PB1-3-20.

als that emerged and the weight of the newly emerged adults were also significantly reduced when the insects were reared on transgenic rice seeds. The adult longevity of the insects (both male and female) was also recorded and found to be significantly reduced in comparison to insects reared on control seeds. Bioassay study proved that the transgenic seeds showed increased resistance to rice weevils than control seeds. Among the seeds of different transgenic lines tested, PB1-2-14 displayed a high level of resistance than other transformed seeds.

In the present investigation, α-amylase inhibitor gene has been introduced into rice (PB1) through *Agrobacterium*-mediated transfer to provide resistance to the rice weevil. Generally *Agrobacterium tumefaciens* has been used previously to produce transgenic rice plants, particularly with the aid of super binary vector, but all these plants carried only marker genes[19–25]. In the present study, a large number of rice plants carrying α-amylase inhibitor gene has been produced in PB1 cultivar by *A. tumefaciens*-mediated transformation method. This study also proves the efficiency and effectiveness of the virulent *A. tumefaciens* (LBA 4404 (pSAMαAI) in transforming indica rice.

The main objective of the present study was to express the α-amylase inhibitor gene in transgenic rice plants specifically in seed to provide resistance to the rice weevil. This gene was introduced under the control of a seed-specific phytohemagglutinin (PHA) promoter, which would direct and ensure the expression of recombinant proteins, specifically in the seed. Such specific expression in the seed would in turn deliver the protein efficiently to the insect, while minimizing the potential undesirable accumulation of the protein in other parts of the plant. This would not affect the non-target organisms, particularly the beneficial insects. This was shown to confer tissue-specific expression in transgenic plants. This study has shown that the dicot gene promoter PHA functions efficiently in monocot plants such as rice. Wu *et al.*[26] evaluated the effectiveness of a dicot gene promoter and wound inducible protease inhibitor II gene (*Pin2*) in monocots. They also reported that the promoter works equally well in transgenic rice plants. This observation suggests that the promoter (PHA) signals and transduction pathway are highly conserved in both dicot and monocot plants. Molecular analysis of transgenic plants clearly revealed the presence of α-amylase inhibitor gene and α-amylase inhibitor protein.

Results of the bioassay study revealed significant reduction of survival rate of rice weevil, *S. oryzae* reared on transgenic rice seeds than on untransformed control rice seeds. The study on quantification of α-amylase inhibitor indicates that transformed plants contained good

amount of inhibitor protein than control rice seeds, which indirectly indicates the presence of the α-amylase inhibitor gene. Biochemical and bioassay studies proved the expression pattern in seeds of six transgenic lines, which revealed the α-amylase activity. Thus there was an indication of the expression of the α-amylase inhibitor gene in rice seeds. This approach would also minimize the risk of insects developing resistance to the inhibitor.

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Validation of markers linked to maximum root length in rice (*Oryza sativa* L.)

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Root characters/traits are difficult to record and require destructive sampling of the plants. DNA-based molecular markers represent a non-destructive method for gathering information regarding the root characteristics. Two root length specific markers, BH14 and RM201 were utilized to determine the maximum root length of 81 diverse genotypes. Polymerase chain reaction

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