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Variability in expression of insecticidal Cry1Ab gene in Indica Basmati rice

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Summary

The expression of an insecticidal gene cry1Ab, under three different promoters was studied in leaves, stem and panicles to determine organ-specificity in Basmati rice. Enhanced resistance against two Lepidopteran insects, stem bore (*Scripophaga incertulas*) and leaf folder (*Cnaphalocrocis medinalis*) was observed. The result of western hybridization and insect bioassays demonstrated that all these promoters express the cry1Ab gene at similar levels in leaves and panicles. The cry1Ab gene was expressed in stems at 0.05% of the total protein under the control of the PEPC promoter alone or in combination with the pollen-specific promoter. On the other hand it was expressed at 0.15% under the control of the ubiquitin promoter. Southern blot hybridization of these plants indicated integration of the complete plant transcriptional unit at multiple insertion sites. These results demonstrated that a specific promoter could be used to limit the expression of cry1Ab gene in the desired parts of Basmati rice plants.

Abbreviations: BCIP – 5-bromo-4-chloro-3-indolyl phosphate; Bt – *Bacillus thuringiensis*; CaMV – cauliflower mosaic virus; DTT – DL-dithiothreitol; NBT – nitroblue terazolium; NOS – nopalin synthase; PBST – Phosphate buffer saline Tween 20; PEPC – phosphoenol pyruvate carboxylase; SDS – sodium dodecyl sulphate

Introduction

Rice is a primary food source for more than three billion people predominantly in developing countries (Toenniessen, 1996). More than 200 tons of rice are lost every year due to biotic and abiotic factors including insects (Wunn et al., 1996). Many devastating diseases in rice, such as tungro and yellow dwarf viruses, are also transmitted by borer insects (Heinrichs et al., 1985). The most destructive insects of rice are Lepidopteran, stem borer (*Scripophaga incertulas* and *S. innotata*) and rice leaf folder (*Cnaphlaococis medinalis)* which cause annual losses in the order of 10 million tons. Occasional outbreaks can destroy between 60 to 95% of the crop (Yambao et al., 1993). Genetic engineering approaches to develop insect resistance in crops has raised the possibility of achieving high levels of resistance to agronomically significant insect pests. Crops expressing the crystal protein genes from *Bacillus thuringiensis* have been shown to result in significant savings in term of cost, time and labor compared to the chemical insecticides conventionally used to control insects (Peferoen, 1997).

Many Lepidopteran insects are sensitive to the Cry1 and Cry2 proteins produced by different strains of *B. thuringiensis* (Hofte & Whiteley, 1989). Cry 1Ab is one protein known to be toxic when fed to stem-borer and leaf folder larvae in artificial diets (Karim et al., 1999). The cry1Ab gene has been transferred into the aromatic rice variety 'Toram Malai' (Ghareyazie et al., 1997), deep water rice (Alam et al., 1998) and into boro season rice (Datta et al., 1998). All these studies used truncated cry1Ab genes from *B. thuringiensis* var. Kurstaki that have been codon-optimized for

plants and placed under the control of either the CaMV 35S promoter or a tissue-specific promoter (PEPC, pith promoter). Here we report the transformation of Basmati rice with the cry1Ab gene under three promoters that express this insecticidal gene differentially in leaves, stem and panicles.

Materials and methods

Seeds and plasmid DNA

Seeds of *Oryza sativa* variety Basmati 370 were obtained from the Rice Research Station, Kala Shah Kaku, Lahore, Pakistan. Plasmid pCIB4421 containing a synthetic *cry1Ab* gene driven by the maize PEPC promoter (Ghareyazie et al., 1997), pIA1 containing *cry1Ab* under the rice ubiquitin promoter and pIA3 containing *cry1Ab* under the pollen specific promoter derived from the Bp10 gene of *Brassica napus* in pGEM 4Z (Sardana et al., 1996) were used for co-transformation experiments. Plasmid pCIB4421 has the 35S terminator and pIA1 and pIA3 have the NOS terminator. Construct maps are shown in Figure 1. All transformation experiments were carried out after mixing of any one of the above plasmid DNAs in combination with pROB5 (Bilang et al., 1991) DNA which contains the *hph* gene driven by CAMV 35S promoter as selectable marker gene in 3:1 ratio or 3:3:1 ratio (when three plasmids were used).

Rice transformation

Scutellum derived embryo-genic callus from mature seeds of indica rice variety Basmati 370 was used as the target tissue for transformation. Two weeks old callus induced on N_6 (Chu et al., 1975) based medium were bombarded on osmoticum medium (MS medium containing 2 Mg/L 2,4-D and 0.2M Mannitol, 2% sorbitol and 3% sucrose) with gold particles coated with plasmids (I) pCIB 4421 and pROB5 (ii) pCIB4421, pIA3 and pROB5 (iii) pIA1 and pROB5. Gold particles were coated with DNA and bombarded on plant tissues exactly as described by Husnain et al. (1997). The coated particles were bombarded using the PDS-1000/He system (Biorad, Hercules, CA). The manufacturer's instructions were followed for bombardment. Plates were incubated at 28 °C for one day in osmtiucum medium and the next day calli were subcultured on N6 based medium containing hygromycin (50 mg/L).

Putatively transformed cell clusters were identified and subcultured after every two weeks. These clusters were subcultured once and regenerated in the presence of hygromycin. Regenerated plantlets were transferred to rooting medium containing hygromycin (50 mg/L). After further 3 or 4 weeks, plants were transferred to soil in the green house. Plants transformed with pIA1(ubiquitin promoter) were numbered Uxxx-x; plants with pCIb4421(PEPC promoter), Pxxx-x and plants with both pIA3 and pCIB4421 constructs (PEPC and pollen promoter), PPxxx-x.

Western blotting of transgenic plants

The expression of the introduced gene in transgenic plants was studied by western blotting. Isolation of protein from transgenic plants and Western blot analysis for *cry1Ab* were performed according to standard procedures (Koziel et al., 1993). Four to five hundred mg tissue from 4 month old plants were homogenized in 100 ul extraction buffer (1XPBS + 1 mg/ml DTT + 10% glycerol + Complete^{TM}; Boehringer Mannheim).

Protein concentration was estimated with the Bio-Rad reagent (Bradford, 1976). Different amounts of total protein (80–100 ug) were separated on 10% polyacrylamide gels. Proteins from SDS polyacrylamide gel were transferred to a nitrocellulose membrane with the help of a semi-dry transblot apparatus (Bio-Rad). After transferring proteins onto nitrocellulose membrane (Towbin et al., 1979), the membrane incubated in 10% skimmed milk at room temperature for one hour or overnight at 4 °C. The membrane was incubated with primary antibodies for one hour. The membrane was washed 3 times with PBST each for 15 minutes, on a platform rocker (Hoeffer Scientific) at room temperature. After the completion of the primary antibody reaction, the membranes were incubated with alkaline phosphatase-conjugated anti-rabbit IgG as the secondary antibody followed by three washes in PBST for 10 minutes each. Color reaction was developed with 66 ul NBT (75 g/L) and 33 ul BCIP (50 g/L) in 10 ml alkaline phosphatase buffer or with sigma fast tablets. Color developed within 10–15 minutes. Protein expression was quantified by scanning densitometry and also by comparison with standards.

Insect feeding bioassays

Leaves from four- to six -month -old transgenic plants were collected for insect bioassay. One leaf was taken from each of three tillers of a plant. Each leaf was further cut into three pieces approximately 6 cm length

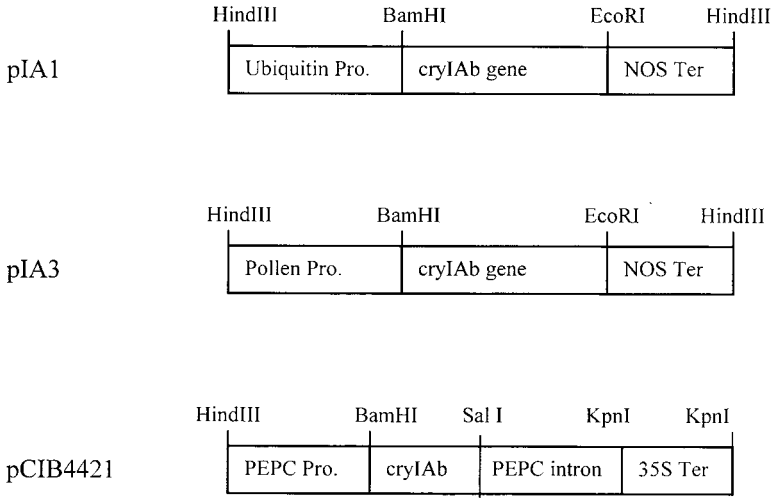

Figure 1. Partial maps of plasmids. A synthetic gene cry1ab was driven by phosphoenol pyruvate carboxylase (PEPC) promoter in a plasmid pUC18 (Yanisch-Perron et al., 1985), pollen specific promoter derived from BP10 gene of *Brassica napus* in plasmid pIA3 plasmid. Plasmids pIA3 and pCIB2221 were based upon pGEM-4z and pUC18 respectively.

and 2 cm in width for feeding. Leaf pieces from control plants were also taken for insect bioassay. Nine Ist and 2nd instar larvae of rice leaf folder (*C. medinalis*) were used for this bioassay. Similarly stem cuttings of 5cm were used for the bioassay of Ist and 2nd instar of yellow stem borer (*S. incertulas*). After 4 days the insect mortality, larval characters and behaviour were recorded.

Dot blot analysis

Dot blot analysis was done to confirm the presence and integration of insect resistant gene in the genome of rice. The plant genomic DNA was isolated from fresh leaves by the procedure described by manufacturer (Nucleon Phytopure Cat No. RPN8511, Amersham). Genomic DNA was denatured at boiling water bath for 10 minutes and then quickly chilled on ice. The DNA was spotted on nylon membrane (Hybond-N Cat No. RPN303N, Amersham) and was fixed on the membrane by incubating at 130 °C for 30 minutes. DIG Labeling Kit (Amersham) was used and the probe prepared according the instruction of manufacturer using PCR. Specific primers: AB1 5' ACAGAAGACCCTTCAATATC; 3' and AB2, 5' GTTACCCTGATTGATTGATTAGGC: 3' were used to amplify a fragment of 550 bp of the cry1Ab gene. Pre-hybridization of the blot was carried out in DIG Easy Hyb solution from the kit. Denatured probe in the same Dig Hyb solution was added and hybridization was carried out for 16 hr. After hybridization, the membrane was washed for 2x15 minutes in 2X, SSC+0.1% SDS solution at room temperature, then, with 0.5X SSC+0.1% SDS at 68 °C for 3x10 minutes. The membrane was washed for 5 minutes in 1X wash buffer IV, incubated in blocking solution for 45 minutes and 60 minutes in digoxigenin antibody as recommended. After incubation the membrane was washed again with wash buffer IV. The membrane was incubated for five minutes in 1X detection buffer and colour was developed using NBT & BCIP.

Southern blotting of transgenic plants

Genomic DNA (10 μg) was separately digested with *Hin*dIII and *Eco*RI+*Bam*HI according to suppliers' instructions. The digested DNA was electrophorosed through 0.8% agarose gels in TBE buffer at 1.5 V/cm and 12 °C for 12–18 h. Gels were stained with ethidium bromide and photographed.

Gels were denatured with NaCl 1.5 M + NaOH 0.5 M for 30 minutes and neutralized with NaCl 1.5 M and Tris-HCl 0.5M, pH 7.5. The DNA was transferred onto a hybond-N^+ nylon membrane (Amersham) with 20X SSC (Na_3 citrate 0.3 M, NaCl 3 M, pH 7.0) by capillary action. The DNA was fixed to the filter by

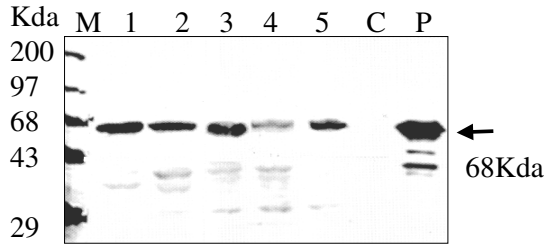

Figure 2. Western blot analysis of primary transgenic rice plants Transformed with *cry1Ab* gene. Lanes 1–5: Transgenic plants of U306-11, P279-2, PP281-1, PP289, and PP304-1, C: Untransformed; P: Positive protein.

baking at 130 °C for 30 minutes. A 550 bp fragment of *cry1Ab* labeled with the Dig-labeling kit was used as the probe for the *cry1Ab* gene. The blots were further processed as described in section 2.5.

Results and discussion

Transformation of Basmati rice

Scutellum-derived calli of Basmati 370 was co-transformed with the selectable marker gene (hygromycin) and the insect resistant *Bt* gene (*cry1Ab*). DNA was coated on gold particles. Transformation efficiency was evaluated on the basis of hygromycin selection. Eleven hundred and sixty scutellum-derived calli were bombarded with plasmid DNA's of pCIB4421 + pIA3 + pROB5 that produced 29 transformed regenerating calli with a transformation efficiency of 2.48%±0.47 and produced 274 plants out of which plants 50 survived. These 50 plants were analyzed for integration and expression of the genes. Two sets of 392 and 96 scutellum-derived calli respectively were bombarded with pIA3 + pROB5 and pCIB4421 + pROB5. These regenerated into 30 plants with a transformation efficiency of 6.8 and 3.1%respectively. Unfortunately none of the plants transformed with pIA3 (pollen specific promoter) alone survived transfer to soil. Three hundred and ninety two calli were bombarded with pIA1+pROB5. These experiments produced 11 regenerating calli with transformation efficiency of 4.5±0.92, giving 80 plants, out of which 45 survived. Plants regenerated from bombarded calli were established in soil.

Western blot analysis of transgenic plants

The first Western blot consisted of U306-11, P279-2, PP281-1, PP289, and PP304-1, with a visible band of cry1Ab protein as a 68 KD (Figure 2). The level of protein expression for *cry1Ab* in leaves of transgenic plants was estimated to be in the range 0.03–0.05% of total soluble protein by comparison with standards (the positive protein of cry1Ab expressed in *E. coli* was used)., The analysis was also carried out on the leaves, panicles and stems of selected plants P279-2, U306-11 and PP289. Although all of these plants showed the presence of a 68 kD Cry1Ab protein band, the concentration of Cry1Ab protein seems to be higher in leaves than in stems or panicles except in plant U306-11 (Figure 3). All promoters showed similar activity in leaves or panicles. However, expression of Cry1Ab in stems under the control of ubiquitin promoter was higher (0.15% the total protein). These and other plants were also used to carry out lab-scale insect bioassay (Table 1).

Insect feeding bioassay

To determine the entomocidal activity of the Cry*1Ab* insecticidal protein in the transgenic rice plants, insect feeding bioassays were performed. Transgenic plants were assayed for resistance to two lepidopteran rice pests (rice leaf folder and yellow stem borer). Leaves and stem cuttings from 4–6 months old plants were fed to the larvae of both insects. Wild type plants were used as controls. Larval survival and growth stage were recorded 4 days after release. In some Petri dishes, not all larvae could be recovered, presumably because of decomposition of larvae that had died earlier in the assay. Plants for which all insects were killed after 96 h were rated as the most promising for enhanced leaf folder and stem borer resistance. Results with rice leaf folder and yellow stem borer are shown in Table 1. Bioassay results showed that plants with the ubiquitin promoter showed toxicity towards both insects, in line with the constitutive nature of expression of this promoter. Rice plants transformed with the *cry1Ab* gene expressed under the PEPC tissue specific promoter had a different activity profile. These plants generally showed higher toxicity towards rice leaf folder than yellow stem borer, which indicated that *cry1Ab* gene expression was higher in rice leaves compared to rice stems (e.g P279-1 and P279-2).

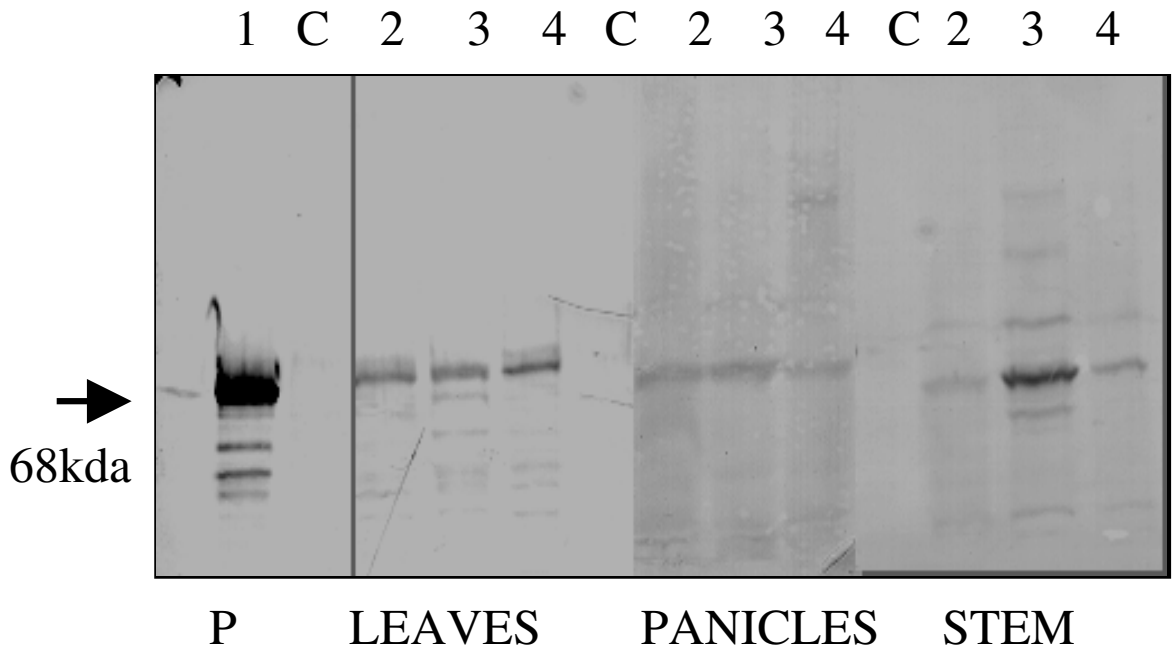

Figure 3. Western blot analysis of different organs of transgenic rice plants transformed with *cry1Ab* gene. Lanes 1 Positive Protein Lane 2–4 Protein isolated from plants # P279-2, U306-11, and PP289 respectively. Lanes C, protein isolated from untransformed plant transgenic.

Integration of cry1Ab gene in Basmati rice

Dot blot analysis provided an initial screen of the transgenic plants for the presence of the transgenes. Total genomic DNA of 41 primary transformed plants was isolated and spotted onto a nylon membrane. DNA isolated from a non- transformed plant was spotted as the negative control. The DNA from plasmid pIA1 and pIA3 containing *cry1Ab* genes was used as the positive controls. Membrane with spotted DNA was hybridized using a labeled PCR amplified probe. Results of Dot blot analysis showed (data not shown) that 32 out of 41 (78%) plants gave a positive indication of presence of the *cry1Ab* gene. No color reaction was observed in the negative control while very dark and distinct colour was developed in the positive controls.

Pattern of integration in the genome of Basmati rice

Plants of a number of positive lines were selected for Southern blot hybridization (Figure 4). The blot indicated that there is stable integration of the synthetic *cry1Ab* gene in the genome of Basmati 370.

A 1.85-kb fragment of the coding region of cry1Ab was released from genomic DNA by double digestion with enzymes EcoR1 and BamH1. The blot consisted of DNA from plants U306-6, PP290-2, PP285-1, PP291-1 and P279-2. In this blot the band pattern of P279-2 was different from the other plants. There was a complex band pattern showing that there were high copy number rearrangements in this line. A 4-kb fragment is expected to be released from genomic DNA when digested with HindIII and probed with cry1Ab (Figure 5). The southern blot consisted of plant lines, PP290-2, PP285-3, PP291-1, PP294-1, P306-6, and U306-8. The band pattern of the blot showed that there was a complete release of the gene cassette in the case of PP290-2, PP285-3, PP291-1 and larger fragment in the case of, U306-6 and U306-8 and no release in case of PP294-1. These complex band patterns confirms (data not shown) that there were high copy rearrangements of the integrated genes in many of the transformants. In addition to the 4 kb internal fragment of the constructs, additional fragments of larger sizes were also observed in all plants, which indicated the presence of rearranged copies of the introduced gene. The type of DNA profile was probably produced through the integration of tandem direct repeats of plasmid DNA resulting from homologous recombination among plasmid molecules before integration

Table 1. Molecular and entomological analysis of transgenic Indica rice Basmati 370 plants

S. No.	Plant #	Promoter	Dot blot	RLF %Mortality±SE	YSB %Mortality±SE
1	U306-4	Ubiquitin	+	62.5±0.23	100±0.00
2	U306-6	Ubiquitin	+	33±.0.35	31±0.00
3	U306-8	Ubiquitin	+	35±0.56	42±o.90
4	U306-10	Ubiquitin	+	100±0.00	100±0.00
5	U306-11	Ubiquitin	+	100±0.00	100±0.00
6	U306-15	Ubiquitin	+	52±0.45	71±0.46
7	U306-17	Ubiquitin	+	43±0.78	10±0.69
8	P279-1	PEPC	+	53±0.69	15±0.23
9	P279-2	PEPC	+	52±0.35	17±0.69
10	PP280	PEPC&Pollen	+	82±0.54	0±0.00
11	PP281-1	PEPC&Pollen	+	100±0.00	84±0.59
12	PP282-1	PEPC&Pollen	+	64±0.69	7±0.69
13	PP282-2	PEPC&Pollen	+	92±0.26	9±0.99
14	PP283-7	PEPC&Pollen	+	71±0.69	35±66
15	PP285-1	PEPC&Pollen	+	33±0.33	29±0.36
16	PP289	PEPC&Pollen	+	100±0.00	31±0.22
17	PP290-2	PEPC&Pollen	+	40±0.68	3±0.36
18	PP294-1	PEPC&Pollen	+	100±0.00	95±0.6
19	PP304-1	PEPC&Pollen	+	100±0.00	0±0.00
20	PP308	PEPC&Pollen	+	55±0.62	33±0.37
21	U306-11-3	Ubiquitin	+	43±0.00	43±0.00
22	U306-11-8	PEPC&Pollen	+	71±0.00	60±0.00
23	PP308-16-1	PEPC&Pollen	+	50±0.56	12.5±0.14
24	PP308-16-6	PEPC&Pollen	+	86±0.96	22±0.24
25	PP309-17-2	PEPC&Pollen	+	75±0.84	20±0.22
26	PP309-17-5	PEPC&Pollen	+	67±0.75	5±0.56
27	Control Plant		–	0.00	0.00
28	Plasmid		+		

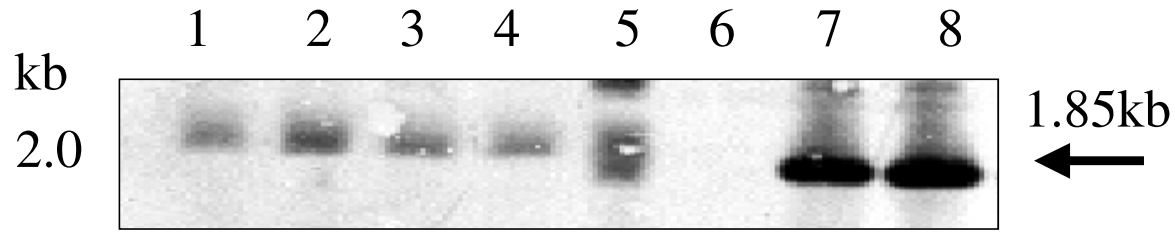

Figure 4. Southern blot analysis of transgenic rice plants Transformed with *cry1Ab* gene. DNA was digested with *Bam*HI and *Eco*R1. Lanes 1–5: Transgenic plants; U306-6, PP290-2, PP285-1, PP291-1, P279-2. Lane 6: Wild type rice plant. Lanes 7–8: Digested plasmid DNA from pIA3 and pIA1 respectively.

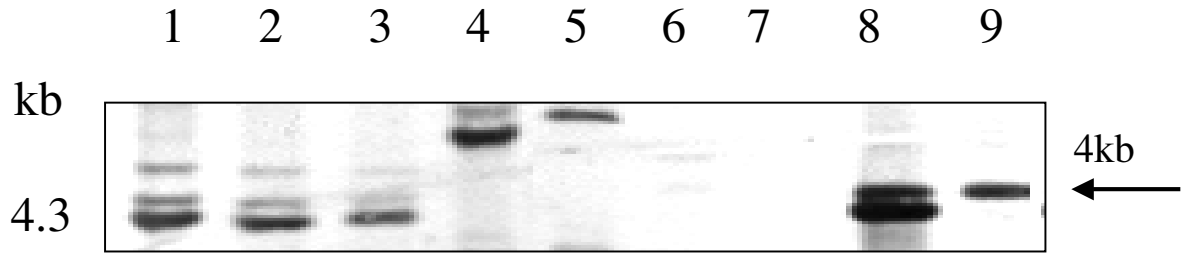

Figure 5. Southern blot analysis of transgenic rice plants transformed with *cry1Ab* gene DNA was digested with *Hin*dIII. Lanes 1–6: Genomic DNA of PP290-2, PP285-3, PP291-1, U306-6, U306-8 and PP294-1. Lane 7: Wild type rice plants. Lanes 8–9: Digested Plasmid DNA from pIA3 and pIA1.

Table 2. Segregation of hygromycin-resistant (*hph*) and insecticidal (*cry1Ab*) genes

Plant line	Transgenes	Total seed cultured	Resistant	Susceptible	Expected ratio	χ^2
PP309-17	*hph*	20	18	2	3:1	2.4
PP301-34	*hph*	12	7	4	3:1	0.44
PP385-1	*hph*	7	4	3	3:1	1.19
PP282-18	*hph*	19	9	10	3:1	7.74
PP385	*cry1Ab*	8	6	2	3:1	0.0
PP294-3	*cry1Ab*	10	5	5	3:1	4.33
PP308-16	*cry1Ab*	10	5	5	3:1	4.33

These blot do not provide precise information for multiple copies because the HindIII release a 4 KB internal fragment in the construct p1A1 and pIA3. Larger fragments and integration was also observed by Peng et al. (1992); Christou et al. (1992) and Tada et al. (1990) in soybean after particle bombardment. It was inferred from the Southern blot band pattern that intact as well as rearranged copies of *cry1Ab* gene were present in the genome of Basmati 370 transformed plants.

The T1 progeny of seven lines were subjected to segregation analysis on the basis of hygromycin gene. One out of four segregated in expected 3:1 ratio on the basis of marker gene while one out of three segregated in expected 3:1 ratio on the basis of cry1Ab gene (Table 2).

Koziel et al. (1993) found in maize that transgenic plants containing the pollen and PEPC promoters expressing cry1Ab genes produced the insecticidal protein in those parts of the plant consumed by both first and second generation of European Corn borer while minimizing expresssion in seeds and other parts of the plants. Matsuoka et al. (1994) showed that the promoter of maize PEPC retains high level of transcriptional activity in the leaf blades and sheaths of rice as judged by expression of the β-glucuronidase reporter gene. Ghareyazie et al. (1997) concluded that the promoter of maize PEPC gene functions effectively in the aromatic vareity 'Toram Malai' and is suitable for the expression of the cry1Ab protein in tissues attacked by early instar larvae of yelllow stem borer but does not allow the protein to accumulate to a detectable levels in tissues used for human consumption. Datta et al. (1998) showed that stem tissues have higher expression of Bt protein irrespective of the promoter used. Cheng et al. (1998) found that the pollen-specific promoter did not direct detectable expression of cry1Ab gene in leaf tissue. Our results showed that in Basmati rice PEPC expresses more in leaves than in stems and panicles as determined by western hybridization (Figure 3) and insect bioassays (Table 1). However the ubiquitin promoter expresses in the all of these tissues including stems as reported earlier (Datta et al., 1998).

This is the first report of differential expression of a cry1Ab gene in Indica Basmati rice. This spatial type of expression is meant to produce the toxic Bt protein

in only those parts where it is required for insect control. This would probably minimize the concerns of regulatory agencies or rice consumers.

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References

Alam, M.F., K. Datta, E. Abrigo, A. Vasquez, D. Senadhira & S.K. Datta, 1998. Production of transgenic deepwater indica rice plants expressing a synthetic *Bacillus thuringiensis* cry1Ab gene with enhanced resistance to yellow stem borer. Plant Sci 135: 25–30.

Bilang, R., S. Linda, I. Peterhans, I. Potrykus & J. Paszkowski, 1991. The 3'-terminal region of the hygromycin-β-resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*. Gene 100: 247–250.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anzl Biochem 72: 248–254.

Cheng, X., R. Sardana, H. Kaplan & I. Altosaar, 1998. *Agrobacterium* transformed rice plants expressing synthetic *cry1Ab* and *cry1Ac* genes are highly toxic to striped stem borer and yellow stem borer. Proc Natl Acad Sci USA 95: 2767–2727.

Christou, P. & W.F. Swain, 1990. Co-transformation frequencies of foreign genes in soybean cell cultures. Theor Appl Genet 79: 337–341.

Christou, P., 1992. Genetic transformation of crop plants using microprojectile bombardment. Plant J 2: 275–281.

Chu, C.C., C. C. Wang, C.S. Sun, K.C. Hsu, K.C. Yin, C.Y. Chu & F.Y. Bi, 1975. Establishment of an efficient medium for anther culture of rice through comparative experiments on nitrogen source. Sci Sinica 18: 659–668.

Datta, K., A. Vasquez, J. Tu, I. Torrizo, M.F. Alam, N. Oliva, E., Abrigo, G.S. Khush & S.K. Datta, 1998. Constitutive and tissue-specific differential expression of the cry1Ab gene in transgenic rice plants conferring resistance to rice insect pest. Theor Appl Genet 97: 20–30.

Ghareyazie, B., F. Alinia, C.A. Menguito, L.G. Rubia, J.M. De Palma, E.A. Liwanag, M.B. Cohen, G.S. Khush & J. Bennett, 1997. Enhanced resistance to two stem borers in an aromatic rice containing a synthetic *cry1Ab* gene. Mol Breed 3: 401–414.

Heinrichs, E.A., F.G. Medramo & H.R. Rapusas, 1985. Genetic evaluation for insect resistance in rice. International Rice Research Institute, Los Banos, Philippines.

Hofte, H. & H.R. Whiteley, 1989. Insecticidal crystal protein of *Bacillus thuringiensis.* Microbiol Rev 53: 242–255.

Husnain, T., T. Malik, S. Riazuddin & M.P. Gordon, 1997. Studies on the expression of marker genes in chickpea. Plant Cell Tiss & Org Cult 49: 7–16.

Karim, S., K. Malik, U. Zafar & S. Riazuddin, 1999. Efficacy of activated toxins from Pakistanian *Bacillus thuringiensis* isolates *Scripophaga incertulas* and *Canaphalocrocis medinalis*. J Asia Pacific Entomol 2(1): 61–67.

Koziel, M.G., G.L. Beland, C. Bowman, N.B. Carozzi, R. Grenshaw, L. Grossland, J. Dawson, N. Desai, M. Hill, S. Kadwell, K. Launis, K. Lewis, D. Maddox, K.. Mc Pherson, M.R. Meghji, E. Merlin, R. Rhodes, G.W. Warren, M. Wright & S.V. Evola, 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Biotech 11: 194–200.

Matsuoka, M., J. Kyozuka, K. Shimamoto & Y. Kano-Murkami, 1994. The promoter of two carboxylases in a C4 plant (maize) direct cell-specific, light regulated expression in a C4 plant (rice). Plant J 6: 331–319.

Peferoen, M., 1997. Progress and prospects for field use of Bt genes in crops. Tib/tech 15: 173–177.

Peng, J., H. Kononowicz & T.K. Hodges, 1992. Transgenic indica rice plant. Theor Appl Genet 83: 855–863.

Sardana, R., S. Dukiandjiev, M. Giband, X. Cheng, K. Cowan, C. Sauder & I. Altosaar, 1996. Construction and rapid testing of synthetic and modified toxin gene sequence cryIA (b & c) by expression in maize endosperm culture. Plant Cell Rep 15: 677–681.

Tada, Y., M. Sakamoto & T. Fujimura, 1990. Efficient gene introduction into rice by electroporation and analysis of transgenic plants: Use of electroporation buffer lacking chloride ions. Theor Appl Genet 80: 475–480.

Toenniessen, G.H., 1996. Progress in rice biotechnology. In: Proceedings of the Fifth Annual Meeting.) National Rice Biotechnology Network IARI, New Delhi, pp. 1–11.

Towbin, H., T. Stachelin & J.I. Gordon, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets: Proc Natl Acad Sci USA 76: 4350–4354.

Wunn, J., A. Kloti, P.K. Burkhardt, C.G. Biswas, K. Launis, V.A. Iglesias & I. Potrykus, 1996. Transgenic indica rice breeding line IR58 expressing a synthetic cry1Ab gene from *Bacillus thuringiensis* provides effective insect pest control. Biotech 14: 171–160.

Yambao, E.B., K.T. Ingrum, E.G. Rubia & B.M. Shepard, 1993. Case study: growth and development of rice in response to artificial stem borer damage. Inter crop Protection. SARP Research Proceedings.

Yanisch-Perron, C., J. Vieira & J. Messing, 1985. Improved cloning vectors and host strains: nucleotide sequences of the new pUC19 vectors. Gene 33: 103–119.