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Constitutive and tissue-specific differential expression of the *cryIA(b)* gene in transgenic rice plants conferring resistance to rice insect pest

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Abstract The truncated chimeric *Bt* gene, *cryIA(b)* of *Bacillus thuringiensis*, driven by two constitutive promoters, 35S from CaMV and Actin-1 from rice, and two tissue-specific promoters, pith tissue and pepcarboxylase (PEPC) for green tissue from maize, was introduced into several varieties of rice (indica and japonica) by microprojectile bombardment and protoplast systems. A total of 1800 putative transgenic *Bt* rice plants could be produced. Southern analysis revealed that more than 100 independently transformed plants could be confirmed for integration of the *cryIA(b)* gene. High levels of CryIA(b*)* proteins were obtained in the green tissue (leaves and stem) of many plants using the PEPC promoter. There was little difference in Bt protein level in leaves and stems from transgenic plants with the 35 S or Actin-1 promoter. Out of 800 Southern-positive plants that were bioassayed, 81 transgenic plants showed 100% mortality of insect larvae of the yellow stem borer (*Scirpophaga incertulas*). The transgene, *cryIA(b)*, driven by different promoters showed a wide range of expression (low to high) of Bt proteins stably inherited in a number of rice varieties with enhanced yellow stem borer resistance. This first report of transgenic indica *Bt* rice plants with the PEPC or pith promoter either alone or in combination should provide a better strategy for providing rice plants with protection against insect pest resistance, minimizing the expression of the CryIA(b) protein in seeds and other tissues.

Key words PEPCP · Pith promoter · *cryIA (b)* · Rice · YSB

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Introduction

Stem borer damage is a serious problem in rice, causing estimated losses of 10–30% of the total yield (Khush and Toenniessen 1991). *Bacillus thuringiensis* (*Bt*) produces characteristic crystalline insecticidal proteins (Bt protein or δ-endotoxin). Bt protein acts by disrupting the midgut cells of the insect pest (Adang 1991). Bt toxins are highly specific (e.g., *cryI* is specifically toxic to the order Lepidoptera) and therefore are not toxic to the beneficial insects, birds, and mammals (including humans) (Vaeck et al. 1987; Stewart et al. 1996). The transgenic approach to obtain insect resistance with the *Bt* gene has been used in cotton (Perlak et al. 1991), maize (Koziel et al. 1993), rice (Fujimoto et al. 1993; Wünn et al. 1996; Nayak et al. 1997; Wu et al. 1997), and other plants (McGaughey and Whalon 1992; Strizhov et al. 1996).

To achieve high expression of the *Cry* genes in crop plants, it is necessary to modify the coding sequence, such as the removal of potential RNA processing and polyadenylation signals and optimization of codon usage (Murray et al. 1991; Perlak et al. 1991). The truncated version of such synthetic *Bt* genes further increases the expression levels, as has been shown in maize (Koziel et al. 1993) and as reported here in rice.

The constitutive promoter, 35SP of CaMV (Odell et al. 1985), is the most commonly used promoter and is active in almost all tissues of dicot species, with variable expression (Benfey and Chua 1990). However, the addition of introns enhances the expression of genes in cereals (Callis et al. 1987). Tissue-specific promoters have been shown to work efficiently in dicots, and a few reports are available describing their expression in monocots (Matsuoka et al. 1994; Yin and Beachy 1995).

The very limited use of PEPCP (pepcarboxylase) and the absence of any report on pith tissue-specific promoter in transgenic plants encouraged us to study the expression and function of such promoters in rice. We report here a large number of independent

transformation events for each construct and provide a comprehensive comparison of the function of the *cryIA(b)* gene driven by four different promoters (35SP of CaMV, Actin-1P, PEPCP, and pith-specific promoter) and its efficacy against rice yellow stem borer (YSB).

Materials and methods

Plant materials

Transformation was carried out with genotypes 'IR72', 'IR64' (extensively grown in several Asian countries), 'CBII' (boro season rice), 'IR51500' (salt-tolerant, anther culture-derived rice), 'IR68899B' (maintainer of a CMS line for hybrid rice), 'MH-63' (restorer line for a commercial Chinese hybrid rice), 'Vaideh-1' (deepwater rice, indica type), IRRI new plant type (derived from Indica tropical japonica crosses), and 'Taipei 309' (japonica type) using both protoplast and biolistic methods (Table 1). Fresh immature embryos for direct bombardment or for callus initiation were isolated from healthy plants grown at the IRRI screenhouse.

Plasmid constructs

Vectors used to transform rice are all derivatives of pUC. pFWW2 contains a synthetic *cryIA(b)* gene driven by the rice Actin-1 promoter (McElroy et al. 1990; Wu et al. 1997). pCIB4418 contains a synthetic gene encoding the amino terminal 648 amino acids of *cryIA(b)* from *Bacillus thuringiensis* var 'kurstaki HD-1' fused with the CaMV 35S promoter (Koziel et al. 1993). pCIB4421 contains a chimeric *cryIA(b)* gene under the control of the maize PEPC promoter (Koziel et al. 1993). pCIB6005 contains the same synthetic *cryIA(b)* gene fused with the pith-specific promoter derived from *Zea mays*. pGL2 or pRob5, which contains the hygromycin phosphotransferase (*hph*) gene driven by 35SP of CaMV, was used as a selectable marker gene (Bilang et al. 1991). Partial diagrams of plasmid DNAs pFWW2, pCIB4418, pCIB4421, and pCIB6005 are shown in Fig. 1a–d.

Improved biolistic transformation, selection, and the regeneration of transgenic plants

Immature embryos and embryogenic calli were used as explants for bombardment. About 80–90 immature embryos, collected at 8–10 days after pollination, were aseptically isolated and plated scutellum side up on solid MS (Murashige and Skoog 1962) medium containing 3% sucrose and 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid). The medium was supplemented with benlate and cefotaxime (approx. 100 mg/l) to reduce contamination. The embryogenic calli were selected from primary, secondary, or tertiary calli. Experiments were conducted with the biolistic PDs-1000 He systems (BioRad). BioRad's instructions on the preparation of gold microcarriers and DNA preparation were as follows. A 50-µl gold aliquot (3 µg/50 µl), 10 µl mixture of plasmid DNA (1 µg/µl; 4 µg *hph* and 8 µg *cryIA(b)* gene), 50 µl 2.5 *M* $CaCl_2$ (autoclaved), and 20 µl spermidine free-base (0.1 *M*) mixture were vortexed for 3 min, followed by centrifugation in a microfuge for 5 s at 10 000 (low) rpm. The supernatant was removed and the pellet was washed in 250 µl cold ($-20^{\circ}C$) 100% ethanol. After washing, the particle-DNA pellet was resuspended in 60 µl 100% ethanol for six bombardments. For each bombardment, 10 µl of particle-DNA suspension was spread onto the surface of the macrocarrier. The petri dish having explants were placed 8 cm beneath the stopping plate of the gun. The He pressure used was either 1100, 1300, or 1500 psi, under partial vacuum (0.3 atm). After bombardment, the explants were first kept in the dark at 25°C for 16–20 h and then subjected to selection on MS/N6 medium containing 2 mg/l 2,4-D, 30 g/l maltose or sucrose, and 50 mg/l hygromycin B. For immature embryos, the medium was supplemented with benlate and cefotaxime. The medium was solidified with 6 g/l agarose or 8 g/l agar. The selection was continued for 42–56 days in the dark at 25°–26°C. For effective selection, the medium was changed every 2 weeks. The embryogenic hygromycin-resistant (Hg^r) calli were placed on preregeneration N6 medium containing 2 mg/l kinetin, 0.1 mg/l NAA (α-naphthalene acetic acid), 30 g/l maltose or sucrose, and 50 mg/l hygromycin B solidified with 6 g or 8 g agar/l. After preregeneration treatment, the putative transgenic calli were placed on MS regeneration medium (with or without hygromycin B) containing 2–5 mg/l kinetin, 1 mg/l NAA, 30 g/l sucrose and 6 g/l or 8 g/l agar. The culture was placed under a 12 : 12-h (day : night) photoperiod at 25–26°C. The regenerated plantlets were transferred to medium containing half-strength MS basal salt, 30 g/l sucrose and 2 g/l gelrite for better

Table 1 *Bt* gene transfer and expression in different rice cultivars

Cultivar	Promoter	Nature of promoter	Method of transformation[a]	Number of plants in greenhouse	Analysis[b]		
					W	S	BA
IR72	Actin-1	Constitutive	B	20	+	+	+
IR64	35SP	Constitutive	B	9	ND	+	+
IR68899B	35SP	Constitutive	B	139	+	+	+
IR68899B	PepC	Tissue Specific	B	6	+	+	+
MH-63	Actin-1	Constitutive	B	64	+	+	+
Vaideh-1	35SP	Constitutive	B	24	+	+	+
New plant type	35SP	Constitutive	B/P	150	+	+	+
T309	35SP, Actin-1	Constitutive	B	568	+	+	+
T309	PepC, Pith	Tissue specific	B	74	+	+	+
IR51500-AC-11	Pith	Tissue specific	B	11	+	+	+
IR51500-AC-11	35S	Constitutive	B	6	ND	+	+
CBII	All constructs	Tissue & constitutive	P	729	+	+	+

[a] B = biolistic, P = protoplast
[b] W = western blot, S = Southern, BA = insect bioassay, ND = not done, + = positive data (based on 90% and above mortality)

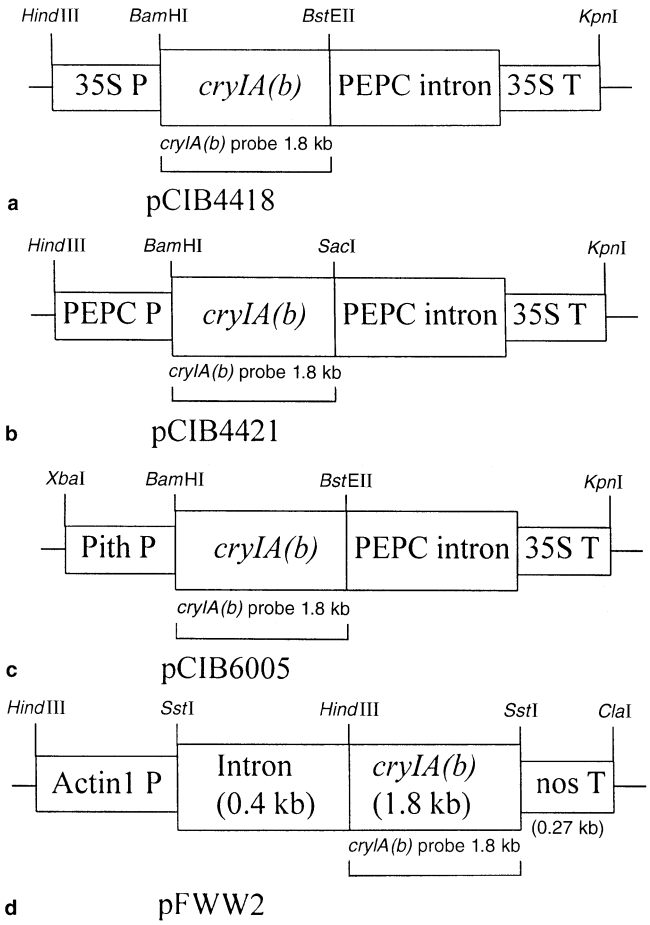

Fig. 1a–d Partial diagrams of plasmid DNAs pCIB4418, pCIB4421, pCIB6005, and pFWW2. **a** Structure of pCIB4418 containing a synthetic *cryIA(b)* gene under control of the expression signals of the 35 S transcript of CaMV, **b** structure of pCIB4421 containing the same *cryIA(b)* gene as in **a** but under the control of pepcarboxylase promoter of maize, **c** structure of pCIB6005 containing the same *cryIA(b)* as in **a** and **b** but under the control of a pith-specific promoter, **d** structure of pFWW2 containing synthetic *cryIA(b)* gene driven by the Actin-1 promoter of rice and NOS terminator. All transformations were carried out along with pGL2 or pRob5 vector containing the *hph* gene driven by 35SP as selectable marker gene

root development. Plantlets with healthy roots were transferred to Yoshida solution (Yoshida et al. 1976). After proper root development and hardening, the plantlets were transferred to soil in pots in the greenhouse. The necessary cultural management and biosafety measures were taken until the seeds were harvested.

Protoplast culture, transformation, and regeneration of transgenic plants

Experiments were carried out with different cultivars ('CBII', 'IR64' and IRRI new plant type). The embryogenic cell suspension, protoplast isolation, and transformation procedures followed were as described by Datta et al. (1990; 1992). Regenerants were transferred to the greenhouse and allowed to mature following the same procedures and precautions taken for plants transformed by the biolistic method.

HPT assay and Southern blot analysis of transgenic rice plants

The HPT assay from fresh or frozen leaf tissue was done following the procedure described earlier (Datta et al. 1990). HPT positive plants were analyzed for integration of *cryIA(b)* gene. For each construct, a minimum of 30 plants were analyzed.

Total DNA was isolated from leaf tissues of primary transgenic (T_0), progenies of T_0 (T_1), and nontransformed control plants. DNA was extracted using a modified procedure (Dellaporta et al. 1983), and 10 µg of DNA was digested with *Bam*HI and *Bst*EII restriction endonucleases. Digested DNA samples were separated by electrophoresis on a 1% (w/v) 1 × TAE agarose gel and transferred to Hybond N^+ nylon membrane (Amersham). The radioactive probe was prepared by the random primer method using α-[^{32}P]dCTP and the Rediprime labeling system (Amersham). The probe consisted of the protein coding region of *cryIA(b)* [1834 bases, *Bam*HI-*Bst*EII fragment of pCIB4418, pCIB4421, and pCIB6005. For pFWW2, a 1.8-kb probe (digested with *Hin*dIII and *Sst*I) was used. Hybridization with the probe was done according to the manufacturer's instructions (Amersham). Following hybridization, the membrane was washed twice in 2 × SSC, 0.1% SDS for 10 min at room temperature, once in 0.2 × SSC, 0.1% SDS for 15 min at 68°C, and finally in 0.1 × SSC, 1% SDS for 15 min at 68°C. The membrane was exposed to X-ray film.

Protein extraction and immunological analysis of transgenic rice plants

Protein extraction from fresh or frozen tissues (about 0.5–0.8 g) of leaves, pith, or seeds from control and transgenic plants was done with 0.05 *M* TRIS-HCl pH 7.0 containing 10% glycerol and 0.1 m*M* phenyl methyl sulphonyl fluoride (PMSF). The extract was clarified by centrifugation at 13 000 *g* twice (10 min followed by 5 min) and the supernatant was collected. The total protein concentration was determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce) with bovine serum albumin as a standard. Absorbance was measured at 550 nm. Western analysis was performed using the horseradish peroxidase procedure. Extracts containing 30 or 50 µg of protein were loaded to each well and separated on a 10% polyacrylamide gel in the presence of sodium dodecylsufate (SDS-PAGE). The separated protein was transferred to a nitrocellulose membrane on the trans-blot to semi-dry transfer cell (BioRad). After washing (100 m*M* TRIS, 1.4 *M* NaCl) and blocking (100 m*M* TRIS, 1.4 *M* NaCl, 0.05% Tween 80, and 5% milk), the membrane was treated with a rabbit anti-*Bt kurstaki* antibody overnight at room temperature. The CryIA(b) protein bound to the membrane was detected by an anti-rabbit horseradish peroxidase conjugate (BIORAD).

Insect bioassay

T_0 and T_1 plants were assayed for resistance to the YSB, *Scripophaga incertulas*. Five tillers were cut from each test plant and the basal 7-cm sections were placed singly in petri dishes lined with moist filter paper. Six neonate YSB larvae were released into each dish, and the dishes were sealed with adhesive tape. Larva survival and growth stage were recorded 96 h after release. In some petri dishes, not all six larvae could be recovered, presumably because of decomposition of larvae that died earlier. Plants for which all insects were killed after 96 h were rated as the most promising for enhanced stem borer resistance. For the whole plant bioassay, 25 larvae were released per plant. The first dissection was made after 4 days, and this was continued for every week until the plants reached maturity.

Results

Transformation and regeneration

The establishment of embryogenic culture (EC) is a prerequisite for the complete process of transformation, particularly in indica rice. Ten genotypes were initially used to develop EC and achieve subsequent plant regeneration (Table 1). Transgenic plants grew well in the CL4 greenhouse when they were transferred at the right stage and time. A large number of transgenic plants obtained by using pCIB4418 (35SP), pFWW2 (Actin-1P), pCIB4421 (PEPC), and pCIB6005 (pith P) constructs (Table 1, Fig. 1 showing a portion of each of plasmid DNA) were grown in the greenhouse (Fig. 2a–c). Detailed data on one indica cultivar 'CBII' are presented here (Table 2). Several other genotypes were transformed with one or more *Bt* constructs as shown in Table 1 (details to be published elsewhere). The co-transformation frequency of the cryIA(b) construct and the *hph* gene was in the 40–70% range in protoplast transformation. Interestingly, in 80% of the cases, both the *hph* and *cryIA(b)* genes were inserted at

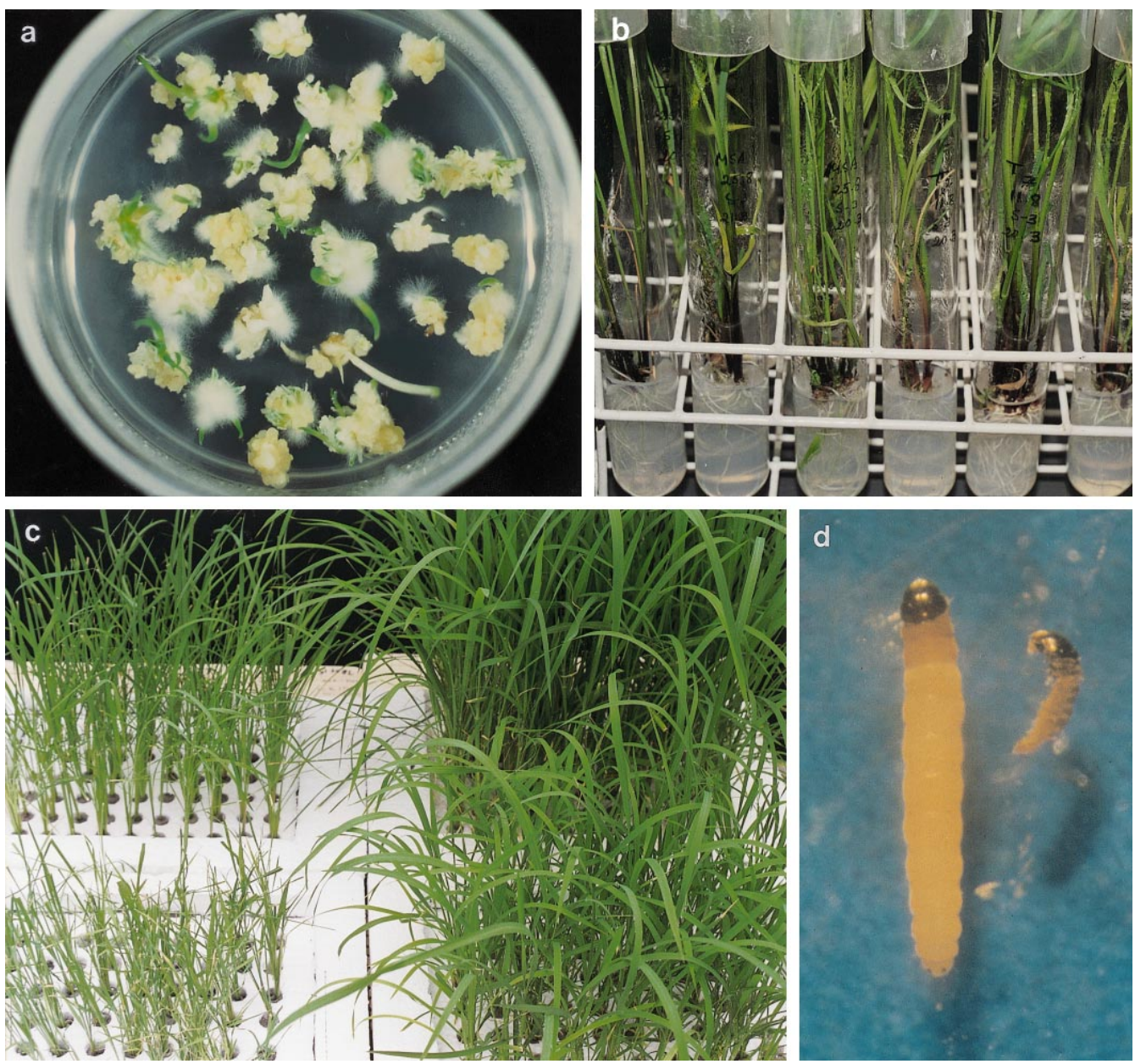

Fig. 2 a Germinating somatic embryos from transformed rice calli, **b** primary transformants growing in regeneration medium, **c** different growing stages of primary transformants in culture solution in transgenic CL4 greenhouse, **d** bioassay of yellow stem borer (*Scirpophaga incertulas*): dead larva (*right*) with black head capsule and brown body at first instar after 96 h on cut stem of a transgenic *Bt* rice plant; live larva (*left*) at second instar after 96 h on cut stem of basal portion of tiller of nontransgenic plant

Table 2 Summary of selected *cryIA(b)* transformation experiments in CBII cultivar with different promoters

Plasmid	Promoter/site of action	Number of protoplasts used	Number of Hg^r plants	Number of plants in greenhouse	Fertility (%)	Southern$^+$ plants\no. of plants analyzed	Number of bioassay$^+$ plants[a]/ no. of plants subjected to assay
pCIB4421	PEPc/green tissue-specific	4.5×10^6	140	119	86.2	30/43	14/18
pCIB6005	Pith-specific	3.0×10^6	136	118	95.4	18/60	8/20
pCIB4418	35S-all tissues	13.5×10^6	520	492	85.0	63/136	4/11

[a] Only a portion of the plants were analyzed; bioassay$^+$: (positive) based on 90% and above mortality

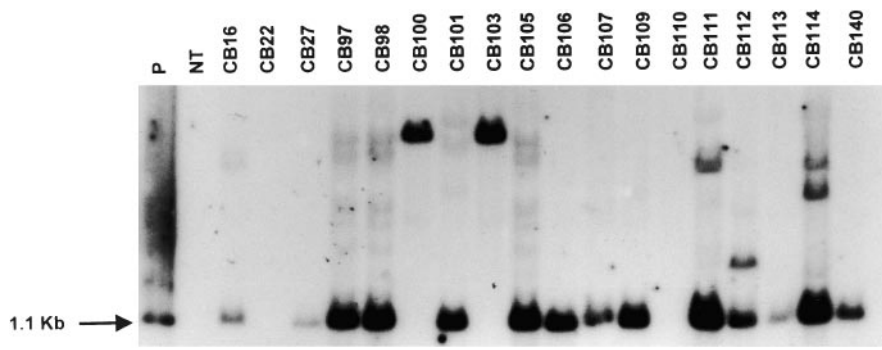

Fig. 3 Southern blot analysis of the *hph* gene in transgenic rice plants. The *left lane* (*P*) refers to plasmid DNA pRob5 used in transformation digested by *Bam*HI, *NT* genomic DNA from a non-transformed plant, *lanes CB22* and *CB110* did not show any hybridization with the probe, whereas all other lanes except for *CB100* and *CB103* showed the expected 1.1-kb fragment indicative of the *hph* gene. Fourteen independent transformants are shown here

the same locus of the rice chromosome of plants generated by biolistic transformation (data not shown).

Hygromycin phosphotransferase assay (HPT)

We used pGL2 or pRob5 containing the *hph* gene as the selectable marker gene, with the *Bt* genes as indicators co-transformation. Initial screening of the transformants was based on the HPT assay. Of 1081 plants analyzed, 70% from transformed protoplasts were positive for HPT (HPT^+), whereas 40% plants from the biolistic experiment were HPT^+. HPT^+ plants were subjected to DNA and protein analysis.

DNA levels

We observed different integration patterns of the *hph* gene with variable copy numbers (1–20) (Fig. 3). Southern blot analysis with the *cryIA(b)* gene showed the transgene with single/multiple copies of the transforming DNA (Figs. 4–8), which was also found consistently in the progeny analysis (Figs. 6–8). Different restriction enzymes and uncut genomic DNA from transgenic plants showed different banding patterns and *cryIA(b)* gene integration, respectively, at high molecular weights (data not shown). Southern blot-positive plants containing fragments consistent with the expected size of the *cryIA(b)* gene were selected for protein analysis and bioassay activity (Tables 1 and 2).

Protein level

Immunological analysis by western blotting showed a wide range of CryIA(b*)* protein levels with the expected size of 65 kDa. Such variation also existed within the transformants with a single promoter, particularly those with a constitutive promoter (data not shown). The results clearly demonstrate that PEPCP in general and 35SP in some lines act as strong promoters in CryIA(b) expression (Fig. 9). Because there was a large amount of variation in expression among transgenic plants containing the same construct (data not shown), we carried out a detailed comparison with 'CBII', for which more than 100 plants were available for each construct. The level of Bt protein was generally high in the leaves for the PEPC promoter (Fig. 10), which was comparable with the levels of a few high Bt protein plants with the 35SP or Actin-1 promoter (Fig. 9). In general, the expression of Bt protein was weak in pith tissue of the stem with the pith promoter (Fig. 11). On the basis of band intensity we estimated that the toxin protein constituted 0.01–0.2% of the total soluble protein of the leaf or stem tissue.

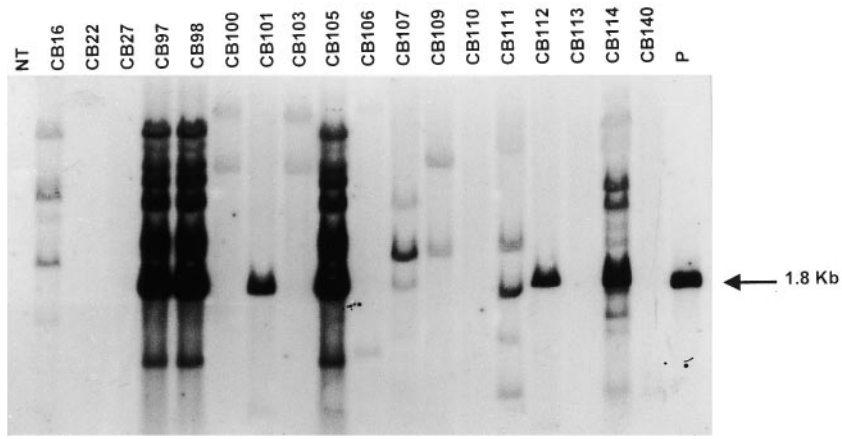

Fig. 4 Southern blot analysis of several independently transformed plants (same blot as shown in **Fig. 3**) of CBII with pCIB4421 containing the *cryIA(b)* gene driven by the PEPC promoter. Most plants, except *CB100*, *CB103*, and *CB106* showed the expected 1.8-kb fragment indicative of the *cryIA(b)* gene digested by *Bam*HI *Bst*EII. *NT* Non-transformed rice DNA, *P* plasmid DNA, pCIB4421 used for transformation

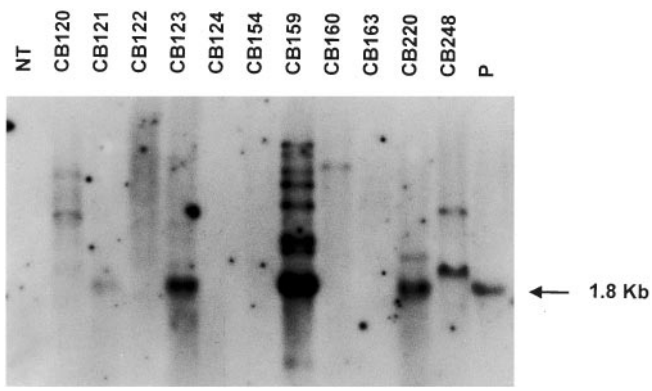

Fig. 5 Southern blot analysis of several independently transformed plants of CBII with pCIB6005 (= *P*)

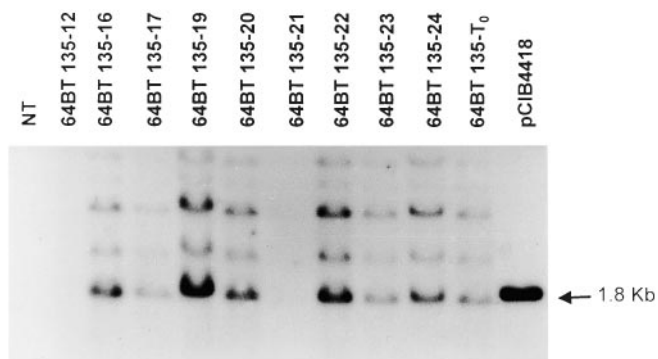

Fig. 6 Southern blot analysis of T_0 and T_1 transgenic rice plants (cv 'IR64') with the same gene shown and described in Fig 1a. *64BT135* $T_0 = T_0$ of one transgenic ('IR64') parental line, and all other lanes are T_1 progeny plants of 64BT135. Aliquots (10 μg) of genomic DNA were digested with *Bam*HI and *Bst*EII and probed with α-[^{32}P]-labeled 1.8-kb *Bam*HI and *Bst*EII fragments of the *cryIA(b)* gene

However, the expression is consistent and correlated with progeny and bioassay data (Tables 3–5). Several Southern-positive plants (CB100, CB103, CB106, CB109, CB16 shown in Fig. 4) not having the correct size of the *cryIA(b)* gene did not show protein expression (data not shown). In some cases, Southern$^+$ *Bt* plants having the right size of *cryIA(b)* gene also showed low protein expression (e.g., CB97, data not shown). However, the stability of transgene and inheritance data were found to be consistent (as in Figs. 10–11). Data are provided for the CB23 (PEPCP) and CB220 (Pith P) lines (Figs. 10–11; Table 4).

Table 1 shows the data of several other genotypes, indicating the availability of transgenic *Bt* rice with one or more *Bt* genes driven by constitutive (Actin-1 and 35SP) or tissue-specific (PEPC and pith) promoters (more details will be published elsewhere). Judging from the protein levels of the selected samples analyzed, it appears that 35SP may be stronger than *Actin-1* in transcribing Bt protein (Fig 9). A direct comparison between the 35S and Actin-1 promoters was not possible as two versions of *cryIA(b)* genes were used under the control of the two promoters. Stem tissues showed a higher expression of Bt protein irrespective of the promoters used.

Insect bioassay

Out of 800 Southern-positive plants that were bioassayed, 81 had Bt protein expression with 100% mortality of YSB larvae (part of results shown in Tables 3 and 4). There were many transgenic plants which showed 70–90% larva mortality. In general, the average mortality (including those missing) in control dishes containing cut stems of nontransgenic plants was 10–40%. Several Southern-positive *Bt* plants showed a low level of larva mortality, comparable with that in the control, that was correlated with protein level (partial data shown in Tables 3 and 4). For selected transgenic lines, (with 100% mortality, Fig. 1d) the experiments showed similar results after several replications, and the mortality was inherited in T_1 and T_2 generations. Significantly, 100% mortality of larvae was found in some plants having low protein expression, particularly *Bt* plants having the pith promoter

(e.g., CB 118, CB 123, CB220). A similar consistent result with high Bt protein expression was found with the PEPC promoter (e.g. CB23, CB98). Most of the data presented in the tables were based on the analyzed plants chosen for either high protein expression or larvae mortality levels up to 100%.

T_1 analysis

A few selected transgenic lines were analyzed by Southern (Figs. 6–8) and western blotting (Figs. 10, 11) and by insect bioassays (Tables 4, 5; Fig. 1d). The data in most cases showed a segregation ratio close to 3 : 1, suggesting the integration of the *Bt* gene at a single locus on one chromosome (Table 4). The results of the insect bioassays using T_1 plants (cut stem and whole plant) corresponded with those of T_0 assays (Tables 3 and 4). Control plants showed live larvae and different stages of pupae development, with the adult stage, whereas there was no live larvae found in many plants of the transgenic lines.

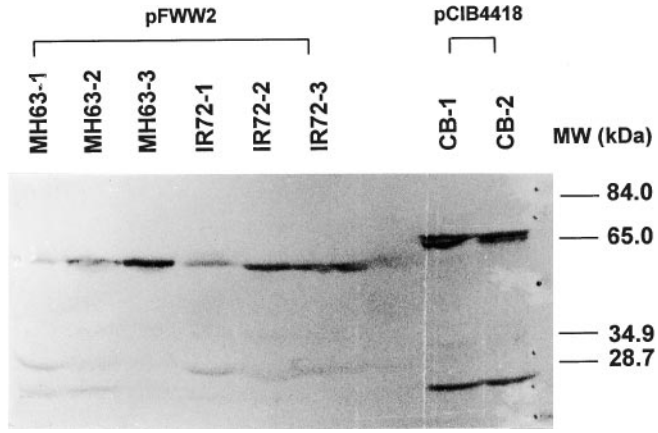

Fig. 9 Western blot analysis showing Bt protein levels in the plants of 3 cultivars, 'MH63', 'IR72', and 'CBII'. Molecular weight of markers given in kilodaltons (kDa) at the extreme *right*. 'MH63' and 'IR72' plants were transformed with the pFWW2 plasmid DNA containing the *cryIA(b)* gene driven by the Actin-1 promoter. The Bt protein of transgenic 'IR72' and 'MH63' plants with the pFWW2 construct showed slightly lower molecular-weight proteins (approximately 60 kDa) than the CBII plants having 65-kDa protein [transformed with *cryIA(b)* driven by 35 S promoter]. Fifty micrograms of leaf extract proteins from freeze-dried samples was analyzed by 10% SDS-PAGE

Discussion

Many different factors are responsible for a successful expression of transgenes in plants; for example, an

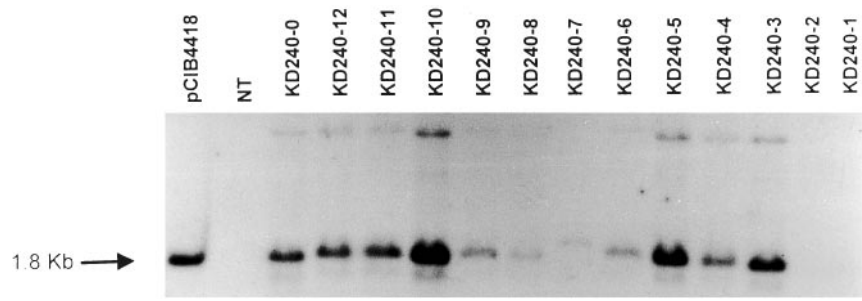

Fig. 7 Southern blot analysis of a transgenic rice plant (KD 240) in T_0 and its progeny (T_1). *KD 240-0* is T_0 and all other lanes are T_1 progeny plants of KD240 showing a segregation pattern. *NT* Lane showing a nontransformed plant, *pCIB4418* plasmid DNA [*cryIA(b)* driven by 35SP] used for transformation

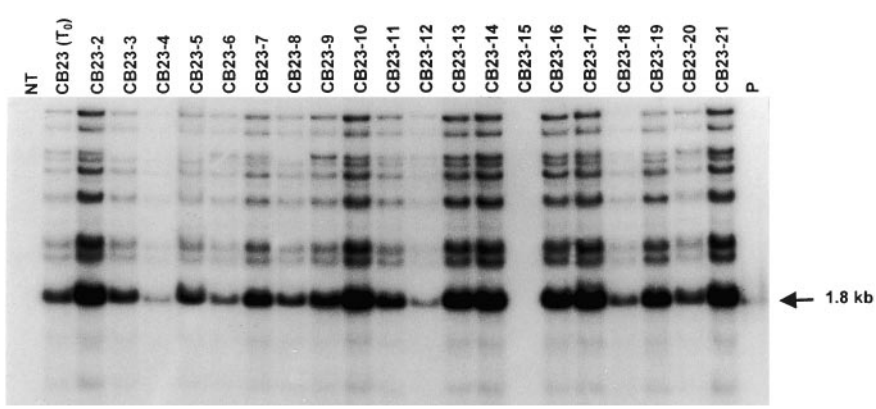

Fig. 8 Southern blot analysis of T_0 and T_1 transgenic rice plants of CBII (CB23) with the same gene as shown in Fig. 7 but with multiple copies of the transforming DNA

Table 3 Bioassay[a] of some selected Southern positive T_0 *Bt* rice plants

Plant code	Plasmid	Status of larvae (Av)			Dead & unrecovered larvae (%)
		Alive ($\bar{X}$) ± SE	Dead ($\bar{X}$) ± SE	Recovered ($\bar{X}$) ± SE	
KD 240	pCIB4418	0.0 ± 0.0	5.25 ± 0.6	0.75 ± 0.6	100
KD 619	pCIB4418	1.2 ± 0.4	2.0 ± 0.6	2.8 ± 0.2	80
KD 808	pCIB4418	0.8 ± 0.3	3.0 ± 0.5	2.2 ± 0.5	86.66
KD 1217	pCIB4418	0.0 ± 0.0	3.4 ± 0.3	2.6 ± 0.3	100
CB 19	pCIB4421	0.2 ± 0.2	3.4 ± 0.6	2.4 ± 0.6	96.7
CB 23	pCIB4421	0.0 ± 0.0	4.8 ± 0.6	1.2 ± 0.6	100
CB 97	pCIB4421	0.0 ± 0.0	5.4 ± 0.2	0.6 ± 0.2	100
CB 98	pCIB4421	0.0 ± 0.0	3.0 ± 0.6	3.0 ± 0.6	100
CB 101	pCIB4421	0.2 ± 0.2	2.6 ± 0.7	3.2 ± 0.5	96.7
CB 106	pCIB4421	0.4 ± 0.4	2.2 ± 0.5	3.4 ± 0.4	93.6
CB 154	pCIB4421	0.4 ± 0.4	5.4 ± 0.4	0.2 ± 0.2	93.3
CB 159	pCIB4421	0.0 ± 0.0	4.4 ± 1.0	1.6 ± 1.0	100
CB 245	pCIB4421	0.0 ± 0.0	2.4 ± 0.9	3.6 ± 0.9	100
CB 251	pCIB4421	0.0 ± 0.0	4.2 ± 0.7	1.8 ± 0.7	100
CB 253	pCIB4421	0.0 ± 0.0	3.8 ± 1.1	2.2 ± 1.1	100
CB 257	pCIB4421	0.0 ± 0.0	4.6 ± 0.6	1.4 ± 0.2	100
CB 713	pCIB4421	0.0 ± 0.0	2.8 ± 0.7	3.2 ± 0.7	100
IR68899B-1	pCIB4421	0.0 ± 0.0	4.2 ± 0.6	1.8 ± 0.8	100
CB 67	pCIB6005	0.2 ± 0.2	4.4 ± 0.7	1.4 ± 0.7	96.7
CB 70	pCIB6005	0.0 ± 0.0	3.0 ± 0.9	3.0 ± 0.9	100
CB 116	pCIB6005	0.0 ± 0.0	5.0 ± 0.6	1.0 ± 0.6	100
CB 118	pCIB6005	0.0 ± 0.0	5.0 ± 0.6	1.0 ± 0.6	100
CB 123	pCIB6005	0.0 ± 0.0	4.4 ± 0.6	1.6 ± 0.6	100
CB 220	pCIB6005	0.0 ± 0.0	4.0 ± 0.6	2.0 ± 0.6	100
CB 313	pCIB6005	0.0 ± 0.0	3.4 ± 0.6	2.6 ± 0.6	96.7
IR 51500-111	pCIB6005	0.0 ± 0.0	5.4 ± 0.3	0.6 ± 0.2	100
Control		3.8 ± 0.2	0.6 ± 0.3	1.6 ± 0.3	37

[a] Mean of five stems analyzed/plant. Each stem was infested with six larvae

efficient vector, suitable promoter, leader sequences, 3′ noncoding sequences, codon frequency, the gene product itself, and unknown factors (Schell 1987; Perlak et al. 1991; Maas et al. 1992). Altogether these result in a large variation in the phenotypes of transgenic lines. The CaMV35S promoter is a strong, constitutive promoter that has been used in many successful transformation studies in both dicot and monocot species (Benfy and Chua 1990; Peterhans et al. 1990; Datta et al. 1990). A few earlier studies, mostly based on transient expression, suggested that 35SP of CaMV is a weak promoter for gene expression in monocots including rice (Wilmink and Dons 1993). Our study clearly confirms that 35SP of CaMV is strongly active in rice (Fig. 9). The Actin-1 promoter from rice also expressed quite well in several elite rice cultivars, 'IR72', 'MH63', and 'IR68899B' (Fig. 9). The green tissue-specific promoter (PEPC) from maize has been used with a *cryIA(b)* gene in maize (Koziel et al. 1993) and the *gus* gene in rice (Matsuoka et al. 1994) (confirmed by our unpublished data). A general conclusion could not be drawn from the earlier work because of the small number of transformation events reported. Our study revealed some simple to complex patterns of transgene expression which would be of interest for multigeneration test (particularly those with a complex pattern) before breeding or field testing. There is so far no published report on the expression of the maize pith-specific promoter in transgenic plants and the efficient expression of the *cryIA(b)* in rice by the PEPC promoter.

The development of EC from immature embryos plays an important role in obtaining transgenic cereals, irrespective of the methods used (Vasil 1994). Three methods of transformation (protoplast, biolistic, *Agrobacterium*) have been reported to be successful in rice (Shimamoto et al. 1989; Datta et al. 1990; 1996; Christou et al. 1991; Hiei et al. 1994). A few reports of transgenic rice with resistance to insects (Fujimoto et al. 1993; Wünn et al. 1996; Duan et al. 1996; Wu et al. 1997) sheath blight, a fungal disease (Lin et al. 1995), and bactereial blight (Song et al. 1995; Tu et al. 1998) have been published. Although gene transfer to indica rice has been demonstrated previously, only one or two genotypes were transformed and, moreover, the transformation efficiency was found to be somehow low. Our present data provides encouraging results, with a good number of indica transgenic plants produced per construct and a considerable variation in gene expression.

Table 4 Bioassay[a] of T_1 *Bt* rice plants

Promoter	Plant code	Status of larvae (Av)			Total dead and unrecovered larvae (%)
		Alive	Dead	Missing	
35SP	KD-240-1	2.4 ± 0.5	2.0 ± 0.8	1.6 ± 0.7	60
	KD-240-2	4.0 ± 0.9	0.4 ± 0.2	1.6 ± 0.7	33.3
	KD-240-3	0.0 ± 0.0	5.4 ± 0.4	0.6 ± 0.6	100
	KD-240-4	0.0 ± 0.0	5.0 ± 0.6	1.0 ± 0.2	100
	KD-240-5	0.0 ± 0.0	4.2 ± 0.4	1.8 ± 0.4	100
	KD-240-6	0.0 ± 0.0	5.4 ± 0.4	0.6 ± 0.4	100
	KD-240-7	4.2 ± 0.4	0.2 ± 0.2	1.6 ± 0.2	29.9
	KD-240-8	0.0 ± 0.0	4.8 ± 0.6	1.2 ± 0.6	100
	KD-240-9	0.0 ± 0.0	4.6 ± 0.7	1.4 ± 0.7	100
	KD-240-10	0.0 ± 0.0	4.8 ± 0.6	1.2 ± 0.6	100
	KD-240-11	0.0 ± 0.0	4.0 ± 0.7	2.0 ± 0.5	100
	KD-240-12	0.0 ± 0.0	3.8 ± 0.5	2.2 ± 0.6	100
Pith specific	CB118-3	0.0 ± 0.0	4.0 ± 0.6	2.0 ± 0.6	100
	CB118-4	0.0 ± 0.0	4.3 ± 0.9	1.7 ± 0.9	100
	CB118-8	3.0 ± 1.5	1.0 ± 0.6	2.0 ± 0.2	50.0
	CB118-10	0.0 ± 0.0	5.3 ± 0.7	0.7 ± 0.7	100
	CB118-14	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
Pith specific	CB220-1	0.3 ± 0.3	5.7 ± 0.3	0.0 ± 0.0	94.4
	CB220-3	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB220-4	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB220-7	0.7 ± 0.3	5.3 ± 0.3	0.0 ± 0.0	88.9
	CB220-8	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB220-11	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
PEPC	CB23-1	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB23-2	0.0 ± 0.0	5.0 ± 0.0	1.0 ± 0.0	100
	CB23-4	0.0 ± 0.0	4.7 ± 0.7	1.3 ± 0.7	100
	CB23-7	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB23-9	0.3 ± 0.3	5.7 ± 0.3	0.0 ± 0.0	94.4
	CB23-10	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
PEPC	CB98-1	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB98-2	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB98-4	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB23-7	0.0 ± 0.0	6.0 ± 0.0	0.0 ± 0.0	100
	CB23-10	0.0 ± 0.0	5.7 ± 0.3	0.3 ± 0.3	100
CBII (C)	Control	4.8 ± 0.7	0.6 ± 0.0	0.6 ± 0.0	19.9

[a] Southern data of some selected plants (T_0 and T_1) shown in Figs. 4–8. Mean of five stems analyzed/plant. Each stem infested with six larvae

The inheritance of transgenes in indica rice has been previously reported (Datta et al. 1990; Christou et al. 1991; Peng et al. 1992). In the present study, we have shown that the inheritance of the *Bt* gene, protein expression, and insect resistance are tightly linked and correlated. Further progeny analyses with several genotypes have yet to be made to accurately confirm these correlation.

Rice is of a divergent nature, grows in different ecosystems (dry upland, irrigated, rainfed lowland, and even in up to 3-m-deep water). However, the same genotype does not grow well in different ecosystems (Khush 1993). It is thus important to have a good number of transgenic lines with wide backgrounds (for suitable adaptation in different ecosystems) in order to be able to select suitable ones for the best performance in a breeding program. Eventually transgenic lines may enhance the germplasm with value-added character. There was a variation of Bt protein expression in transgenic plants with the 35S or Actin-1 promoters, considerably less variation was observed in plants containing PEPC or pith promoters. Total (100%) protection may be achieved in a few plants with low toxin expression as also reported by Strizhov et al. (1996) in alfalfa and tobacco. It may be related to transgene, promoter, or a combination in a certain orientation of expression. A more detailed study would be required to understand the phenomenon. Expression of a transgene, particularly in cereals and recalcitrant indica rice, is often affected by the growth of the plants in tissue culture and conditions of the greenhouse. The development and selection healthy transgenic plants, irrespective of the gene introduced, should be considered with respect to effective gene expression

Table 5 Whole plant bioassay[a] of T_1 and T_2 *BT* rice plants (selected plants)

Background	Progeny status	Inserted gene	Number alive	Number dead	Number missing	Percentage of dead and missing
KD240-4-3	T_2	pCIB4418	0.0	0.0	25.0	100
KD240-4-4	T_2	pCIB4418	0.0	0.0	25.0	100
KD240-4-5	T_2	pCIB4418	0.0	0.0	25.0	100
CB23-4	T_1	pCIB4421	0.0	15.0	10.0	100
CB23-8	T_1	pCIB4421	0.0	9.0	16.0	100
CB23-7	T_1	pCIB4421	0.0	10.0	15.0	100
CB98-3	T_1	pCIB4421	0.0	14.0	11.0	100
CB98-4	T_1	pCIB4421	0.0	6.0	19.0	100
CB98-5	T_1	pCIB4421	0.0	11.0	14.0	100
CB118-3	T_1	pCIB6605	0.0	12.0	13.0	100
CBII-C-11	T_1	Control	18.0	5.0	2.0	28.0

[a] Record taken: 11 days after infestation; 25 larvae infested per plant; 34 days after infestation control plants showed 56% mortality (11/25 alive with 6 at the pupa stage and 5 at the adult stage), whereas the transgenic plants showed 100% mortality

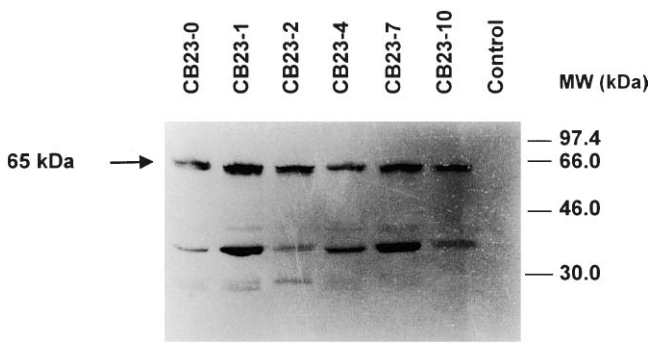

Fig. 10 Western blot analysis of leaves of several CB-23 T_0 and T_1 transgenic lines (*CB23-0* = parental line) transformed with pCIB4421. Leaves were collected from 8-week-old plants. Molecular weight of markers given in kilodaltons (kDa) are indicated on the *right*. The 65-kDa CryIA(b) protein is indicated by the *arrow* on the *left*. Individual lanes show independent transgenic lines. Several low-molecular weight proteins (28–50 kDa), presumably the product of a truncated transcript from the rearranged gene copy, seem to be translated to a shortened peptide

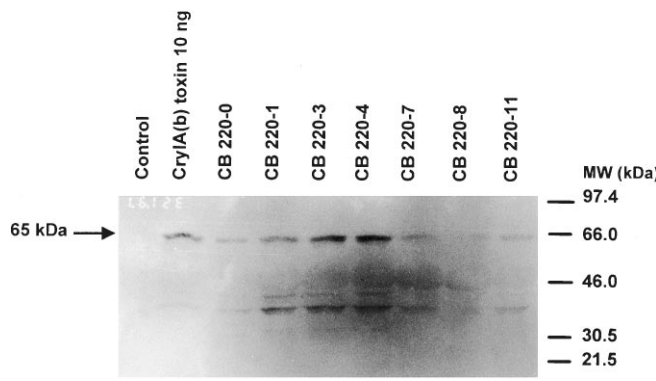

Fig. 11 Western blot showing Bt protein levels in pith tissues of stem in transgenic CB-220 (T_0 and T_1) plants transformed with pCIB6005. Molecular weight of markers are given in kilodaltons (kDa) on the *right*. *Arrow* indicates the expected 65-kDa size of CryIA(b) toxin proteins

to avoid physical and physiological influence (Fig. 2a–c). The data provided here a scenario of different promoter-driven *cryIA(b)* expression in rice which eventually would have a wider application in molecular plant breeding, specifically with tissue-specific expression in rice minimizing the expression of the CryIA(b) protein in seed and other tissue.

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