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Expression and Function of a Hybrid *Bt* Toxin Gene in Transgenic Rice Conferring Resistance to Insect Pest

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Abstract

A hybrid *Bt* toxin gene was made from *cryIA(b)* and *cryIA(c)* after optimization of their codon usage to meet high G+C content in rice and placed under control of the rice Actin I promoter along with its first intron. This hybrid *Bt* toxin gene was introduced into the genome of an elite indica rice cultivar IR72 through biolistic method. Five independent transformants were identified out of 27 transgenic plants obtained. The level of the hybrid *Bt* toxin was estimated to be 0.01%-0.20% of the total soluble protein in leaf or stem tissue of the transgenic plants. This considerable range of expression levels was studied for a strong insecticidal effect. As demonstrated by insect bioassay, neonate larva mortality rates of yellow stem borer (*Scirpophaga incertulas*) (YSB) for selected T_0 transgenic rice plants as well as T_2 homozygous lines were 100%. This is the first report of a hybrid *Bt* toxin gene [*cryIA(b)* and *cryIA(c)*]driven by rice *Actin I* promoter in any cereal crop including rice conferring resistance to insect pest. The present hybrid *Bt* toxin gene deployment strategy will provide sustainable insect pest resistance in rice.

1. Introduction

Rice is one of the most important crops in the world. About 3 billion people, mostly from developing countries, depend on rice as their staple food [1]. It was recognized that production of 70% more rice being required from less land with less labor, less water, and fewer chemicals by 2025 to meet the increase in the number of rice consumers [2]which cannot be achieved without biotechnology [3].

For high efficiency, the application of biotechnology should focus on problems for which solutions are, so far, not available from traditional approaches. In rice, one such priority problem was finding and incorporating resistance to lepidopterous stem borers, which cause high annual yield losses, with occasional outbreaks of up to 60% to 95% [4-6]. Despite the fact that more than 30,000 rice varieties have been screened for stem borer resistance, genes for sufficient levels of resistance have not been found [6]. Furthermore, chemical control is difficult and inefficient because stem borer larvae remain only for a short time on the outer surface of the rice plant before they penetrate the stem [6].

An attractive alternative is the production of protein with insecticidal activity by the rice plant itself. One promising choice is incorporation of the so-called *cry* genes that encode δ-endotoxins from entomocidal, spore-forming soil bacterium *Bacillus thuringiensis*. These endotoxins have been characterized in terms of their safety to mammals, birds, and nontarget insect-predators; their unit insecticidal activity; and their efficiency [7-10]. Because of their favorable features, the *cry* genes have been transferred to higher plants including tobacco [11], tomato [12], cotton [13], maize [14, 15], soybean [16], rape [17] and fusion *cry* toxin in tobaco and tomato [18]. More recently, japonica as well as indica rice have also been transformed with these genes but showed variable protection aganist rice insect pests [19-21]. As there is no report whether hybridized crystal toxin could be effectively used in transgenic rice for efficient plant protection, we used a hybrid *Bt* toxin gene made from *cryIA(b)* and *cryIA(c)* in this study as a possible better *Bt*-rice deployment strategy. A similar study of synergistic effect of PR-protein genes has been reported in tobacco against the fungal disease.

Considering that modern varieties have had a great impact on rice production and that there is a need for new varieties combining higher yield potential with excellent grain quality, moderate resistance to biotic and abiotic stresses, and input use efficiency, we chose the IRRI elite indica rice cultivar IR72 as our target material. This variety not only has high yield potential, suitable growth duration, and favorable grain

quality, but has also been widely used in most rice-producing countries in Asia and elswhere since its release in 1988. Therefore, once successfully transformed with a *Bt* endotoxin gene, it can be directly or indirectly used in traditional rice breeding programs as a new improved line or as a donor parent.

2. Materials and Methods

2. 1 Plasmid Vector

The two plasmid DNA used for rice transformation are shown in Figure 1. Plasmid pFHBT1 contains a hybrid *Bt* toxin gene made from *cryIA(b)* and *cryIA(c)* under cotrol of rice Actin I promoter with its first intron. This plasmid was constructed and available from Biotechnology Research Center in Chinese Academy of Agricultural Science, Beijing. Plasmid pROB5 contains the selectable marker, *hph* coding region, flanked by cauliflower mosaic virus (CaMV) 35S promoter and polyadenylation signals [poly(A)] (**Fig. 1**) [22]. This plasmid provides a selectable marker that confers resistance to hygromycin for cotransformation with pFHBT1 plasmid [22, 23].

2. 2 Modified Bt coding sequence

The sequence of amino acid shown in **Fig. 2** is deduced from a hybrid *Bt* toxin gene made from *cryIA(b)* and *cryIA(c)*. The first 448 amino acids of this hybrid *Bt* toxin gene are identical to the analogous region of the *CryIA(b)* protein exception of C5-R6, D304 and D385 instead of P5-N-I-N-E-C-I11, A309 and Y390 respectively. The remaining sequences of

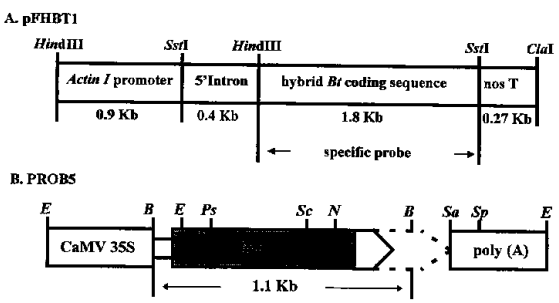

Fig. 1 Diagram of plasmid constructs used in this study.

(A) Structure of pFHBT1, which contains a hybrid *Bt* toxin gene under control of the rice *Actin* I promoter with its first intron and nos terminator. (B) Structure of pROB5, which contains a modified *hph* coding region under control of the CaMV 35S promoter and the CaMV 35S polyadenylation signals [poly (A)]. The arrowed box, shaded part indicate sequence homologous to wild type *hph* information sequence; the hatched box indicate modified *hph* coding region (Bilang *et al.* 1991).

PNINEC

MDNNCRPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQF (40)
LLSEFVPGAGFVLGLVDIIWGIFGPSQWDAFLVQIEQLIN (80)
QRIEEFARNDAISRLEGLSNLYQIYAESFREWEADPTNPA (120)
LREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAA (160)
NLHLSVLRDVSVFGQRWGFDAATINSRYNDLTRLIGNYTD (200)
HAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTVLDIVS (240)
LFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQ (280)
A
GIEGSIRSPHLMDILNSITIYTDDHRGEYYWSGHQIMASP (320)
VGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYRTLSST (360)
Y
LYRRPFNIGINNQQLSVLDGTEFADGTSSNLPSAVYRKSG (400)
TVDSLDEIPPQNNNVPPRQGFSHRLSHVSMFRSGFSNSSV (440)
SIIRAPMFSWIHRSAEFNNIIASDSITQIPAVRGNFLFNG (480)
SVISGPGFTGGDLVRLNSSGNNIQNRGYIEVPINFPSTST (520)
RYRVRVRYASVTPIHLNVNWGNSSIFSNTVPATATSLDNL (560)
QSSDFGYFESANAFTSSLGNIVGVRNFSGTAGVIIDRFEF (600)
IPVTATLEAE (610)

Fig. 2 The sequence of amino acid of a hybrid *Bt* toxin made from *cryIA(b)* and *cryIA(c)*.

The first 448 amino acids of this hybrid *Bt* toxin gene are identical to the analogous region of the *CryIA(b)* protein exception of C5-R6, D304 and D385 replacing with P5-N-I-N-E-C-I11, A309 and Y390 respectively. The remaining sequences of amino acids 449-610 are truncated from *CryIA(c)* without any change. The molecular weight of this hybrid *Bt* toxin checked by western blot analysis is 60 Kda, lower than that of *cryIA(b)* or *cryIA(c)* constructs from NOVARTIS (CIBA-GEIGY) and University of Ottawa respectively. The amino acid sequence of the hybrid *Bt* toxin gene is shown on the numbered line with the corresponding wild type residues directly above.

amino acids 449-610 are truncated from *CryIA(c)* without any change. Before hybridization, the codon usage of this hybrid *Bt* toxin gene was optimized to replace A and T bases at the third position with G or C to meet high G + C content in rice genome. As a result, the overall G + C content in the hybrid gene is 47. 8%, while that of the original DNA sequence from the corresponding part of *cryIA(b)* and *cryIA(c)* toxin gene is 37. 2%.

2. 3 Rice Transformation

Immature grains of IR72 were collected 12 days after pollination from screenhouse-grown plants and the lemma and pelea were removed. Dehulled grains were then immersed in 70% (v/v) ethanol for 1 min and surface-sterilized with 50% (v/v) Clorox for 15-20 min. Immature embryos (IE) were isolated and plated side up onto petri dish of 60 mm in diameter with 10m*l* solid MS medium [24]containing 3% (w/v) maltose, 2 mg l^{-1} 2, 4-D, 0. 8% (w/v) agarose type I

(Sigma) or 0.3 % (w/v) gelrite (MERCK&CO. INC., KELCO, DIV) (MS2). After 16-18 h preculture at 28°C in a dark room, IEs (80-100 pieces per petri dish) were bombarded with PDC-1000/He system (BIO-RAD, Hercules, CA). IE-derived embryogenic primary calli (EC, 4-6 week old) and embryogenic cell suspension cultures (ECS, 2-3 month old) (as described previously by Datta *et al.* [1992] [25]) were also used as target explants following the procedure described by Li *et al.* (1993) [26], and Vasil (1994) [27]. The manufacturer's instructions were followed for coating 1.0 μm gold microcarriers (BIO-RAD, Hercules, CA) with plasmid DNA (*hph: Bt::* 1: 4) prepared using Magic Maxipreps DNA purification system (Promega, Madison, WI). After bombardment, the target explants including ECS cultures were directly transferred to solid MS2 medium supplemented with 50 mg l^{-1} hygromycin B for selection. Developing calli were subcultured every 2 weeks (when IEs are used as explants) or every 3 weeks (when CEs and ECSs are used as explants) on the same medium for 5-7 cycles. Resistant calli were transferred to 20 mL N6 medium [28]supplemented with 2 mg l^{-1} kinetin, 1 mg l^{-1} NAA, 2 mg l^{-1} glycine, 1 g l^{-1} CH (casein hydrolysate), 30 g l^{-1} maltose, 3 g l^{-1} gelrite, and 50 mg l^{-1} hygromycin B (3N6) in the dark and at 28°C for preregeneration. After 7-10 days, the same calli were transferred to 50 ml flasks with 20 ml 3N6 medium without hygromycin B for regeneration. Two to three week-old plantlets were transferred to either Yoshida's culture solution [29]or directly to the soil and placed in the greenhouse under day-time temperature of 29°C followed by night-time temperature of 23°C.

2.4 DNA Extraction and Southern Blot Analysis

Genomic DNA was extracted by an improved CTAB method based on the procedure described by Murray and Thompson (1980) [30]. Five micrograms of DNA of each sample, estimated by agarose gel staining and fluorometry after treatment with RNaseA, was digested with *Hind* III and *Sst*I restriction endonucleases (Gibco-BRL, Geithersburg, MD) in a final volume of 50 μl. The digested DNA was electrophoresed on 1% (w/v) agarose gels. After electrophoresis, DNA fragments were denatured and transferred onto a hybond-N^+ nylon membrane (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. The *Bt* endotoxin coding sequence, from plasmid digested with the same enzyme of the corresponding blotted DNAs, was labeled with (α-32p) dCTP using the Rediprime Labeling Kit (Amersham, Arlington Heights, IL) and used as hybridization probe.

2.5 Protein Extraction and Immunoblot Analysis

For extraction of toxin protein from both transgenic and nontransgenic control plants, 0.5-0.8 g fresh leaf or stem tissue was ground to a slurry in the presence of 1.0-1.5 ml 0.05 M Tris-HCl (pH 7.0) and 10% (v/v) glycerol mixed with 0.1 mM PMSF at 4°C. Supernatants were collected after centrifugation at 13,000 rpm for 10 min followed by a second centrifugation at 13,000 rpm for 5 min. The concentration of total soluble protein was determined by the use of BCA protein assay reagents (using Bicinchoninic acid) standardized using bovin serum albumin according to the manufacturer's instructions (PIERCE Company). Each protein extract was boiled together with sample buffer [12.5 mM Tris (pH 6.8), 20% (v/v) glycerol, 2% (w/v) SDS, 0.001% (w/v) bromphenol blue, 2% (v/v) or 0.3 M 2-Mercaptoethanol]for 5 min, prior to electrophoresis on 10% (w/v) SDS-PAGE gels using a total of 50 μg soluble plant protein per lane. Separated polypeptides were blotted onto nitrocellulose membranes [31]using a semi-dry trans-blot SD transfer cell (BIO-RAD, Hercules, CA). After overnight blocking with 5% (w/v) TBST-milk, Bt toxin proteins were probed with a rabbit anti-Bt toxin serum at room temperature for 20-24 h and detected using a procedure described by Lin et al (1995) [32].

2.6 Bioassay

Insect bioassays were conducted using the petri dish testing method. For each T_0 and T_1 plant, 3 or 5 stems, including sheath, 8 cm in length, were collected at booting stage. Each collected stem was then placed on a moistened filter disc in a 90-mm-diameter petri dish and infested with six neonate larvae of YSB. Petri dishes were incubated at 28°C in the dark. The mortality rate of larvae was determined 4 days later.

3. Results

3.1 Rice transformation and Southern blot analysis

The hybrid *Bt* toxin gene [*cryIA(b)* and *cryIA(c)*] driven by rice Actin I promoter (**Figs. 1** and **2**) was introduced into IE, EC, and ECS of IR72 by co-transformation with the selectable marker *hph* gene. A total of 25 fertile and 2 sterile plants were obtained through selection. Among them, 6 plants (all fertile) were regenerated from IE, 10 plants (9 fertile and 1 sterile) from EC, and 11 plants (10 fertile and 1 sterile) from ECS. Out of 17 plants analyzed, 15 plants showed five independent banding patterns as well as the presence of a 1.8-Kb DNA fragment corresponding to the complete hybrid *Bt* toxin coding sequence as shown by Southern blot analysis (**Fig. 3**).

Table 1. Genetic transformation efficiency of different explants trransformed by particle bombardment with a hybrid *Bt* toxin gene in IR72.

Explants	Start[a]	R-Callus[b]	Plants[c]	*Bt*$^{+d}$	Clones[e]	Fertile
IE	887	200	6	6	4	6
EC	212	31	10	9	1	9
ECS	756	149	11	8[f]	≥1	10

[a] Number of the explants bombarded.
[b] Number of the resistance calli produced during hygromycin selection.
[c] Number of the plants regenerated from each explant-derived calli.
[d] Number of the transgenic plants as shown by Southern blot analysis.
[e] Number of the independent transgenic plants.
[f] Number of the positive plants as detected by insect bioassay or/and western blot analysis.

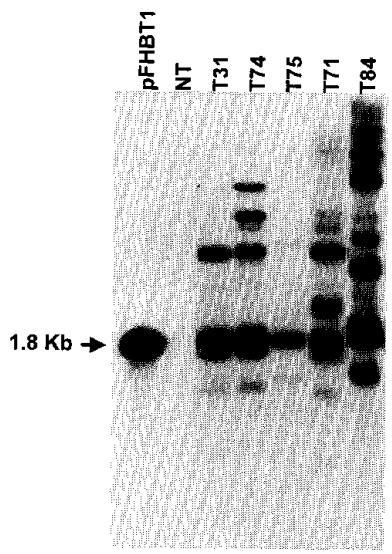

Fig. 3 Southern blot analysis of T_0 transgenic rice plants.

A total of 5 μg plant genomic DNA and 30 pg of plasmid DNA were digested with both *Hind* III and *Sst*I and electophoresed on 1% (w/v) agarose gels. The arrow marks expected 1.8-Kb fragment digested *Bt* plasmid DNA of pFHBT1; NT: nontransformed IR72 control plant; T31, T74, T75, T71, T84; five independent transformants.

Besides the expected band, one to ten additional bands in different transgenic plants were also detected, indicating the presence of rearranged copies of the transgene in rice genome. The transformation efficiency of different explants was summarized in **Table 1**.

3.2 The inheritance of the transgene

The inheritance of the transgene from primary (T_0) generation to next (T_1) generation was examined by Southern blot analysis of 3 selfed progeny lines. The number of the positive and negative plants in the lines T9 and T72, derived from EC and IE respectively, was

Table 2. The inheritance of three selfed T1 progeny lines detected by Southern blot analysis.

Plant code no.	Bt^{+a}	Bt^{-b}	X^2(3:1)	P
T 9	18	7	0.013	0.75-0.90
T 31	15	13	5.753	<0.05
T 72	9	3	0.000	>0.95

[a] Number of the plants with the expected copy corresponding to the complete hybrid *Bt* toxin coding sequence.
[b] Number of the plants without the expected as well as rearranged copy.

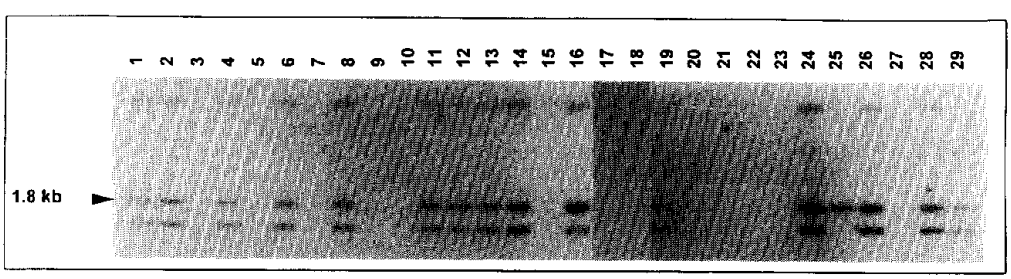

Fig. 4 Southern blot analysis of twenty-eight T31 progeny plants.

A total of 5 μg plant genomic DNA were digested with both *Hind* III and *Sst* I and electrophoresed on 1% (w/v) agarose gel. Lane 1: T31 (To) plant; Lane 2 to 29: DNA from twenty-eight T_1 plants of T31; The arrow marks expected 1.8-Kb fragment digested plasmid DNA of pFHBT1. Note: lane 25 is the segregant T31-23 with only expected fragment after segregation, apparent banding pattern (size difference and distance) appears to be different than that of T31 in Fig. 3 is likely due to different Southern conditions.

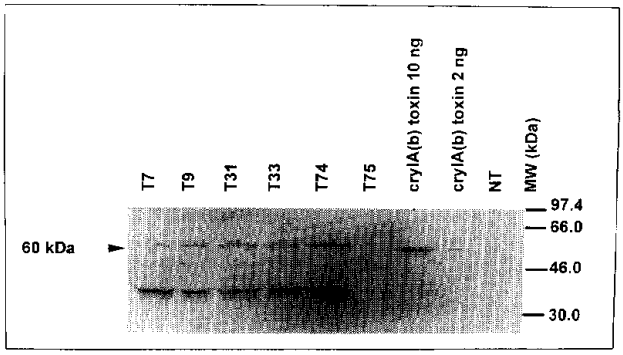

Fig. 5 Western blot analysis of stem protein extracts from T_0 plants with 10% (w/v) SDS-PAGE.
Fifty μg total soluble protein was loaded per lane. The arrow marks the expected 60-kDa size of the hybrid *Bt* toxin. MW: molecular weight marker; NT: protein extract from nontransformed IR72 contol plant. T7, T9, T31, T33 T74, T75: protein extracts from transgenic T_0 plants; *CryIA(b)* toxin: purified *Bt* toxin.

segregated into a 3: 1 ratio, suggesting that the transgenes in these transformants were integrated in a single rice chromosome (**Table 2**). However, that in line T31 fail to fit with this segregating pattern although it derived from the same transformant with the line T9. Out of 28 T_1 plants of this line analyzed, one particular segregant T31-23 inherited only the expected size of the band (**Fig. 4**, lane 25). The other segregants either inherited the same band pattern with T_0 plant or did not give any signal. These results revealed that a recombination event had occurred between the expected and rearranged copies of the transgenes of the detected progeny plants and that event might be responsible for the unfit segregating ratio.

Table 3. Summary of expression level and insect bioassay of T_0 transgenic plant

Transformant		Expression level[c]	Larva mortality(%)[d]
Type[a]	Plant[b]		
I	T31	0.04%	100
II	T71	ND[e]	100
III	T74	0.05%	100
IV	T75	0.01%	100
V	T84	0.20%	100
Control	IR72	0.00%	6

[a] Transformant type was classified based on the Southern band pattern.
[b] Representative plant of each transformant.
[c] Expression levels were approximately estimated by comparing intensities of toxin bands shown on western blot.
[d] Larva mortality was determined 4 days after infestation and scored based on 18-30 larvae per plants (6 larvae per stem and 3 or 5 stem per plant).
[e] ND means no data.

3.3 *Western blot analysis*

The function and expression level of the hybrid *Bt* toxin gene in the transgenic rice plants was further demonstrated by protein immunoblot analysis through T_0 to T_2 generations. As shown in **Fig. 5** and **6**, the hybrid *Bt* toxin with the expected size of 60 kDa as well as unexpected size of 40 kDa was detectable in both positive primary transgenic plants and the tested T_1 progeny plants. The total expression level of these two sizes of toxins in T_0 generation was estimated to be up to 0.01% to 0.20% of the total soluble protein in leaf tissue or stem tissue of the transgenic plants by comparing band intensities with those of

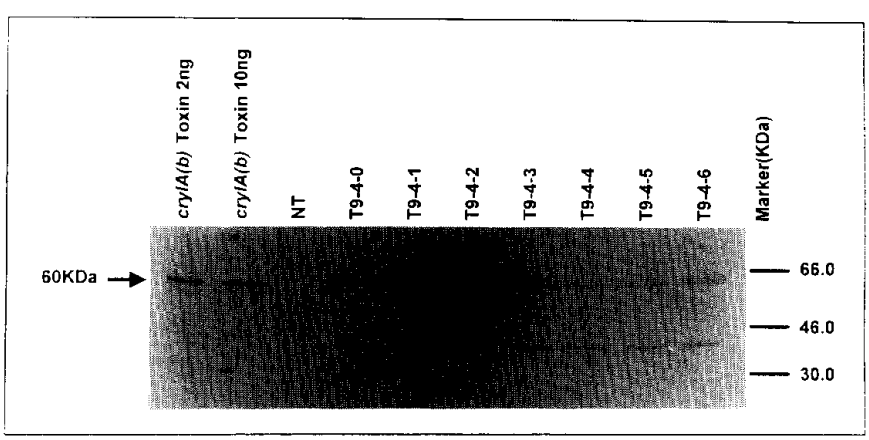

Fig. 6 Western blot analysis of stem protein extracts from T_2 homozygous progenies with 10% (w/v) SDS-PAGE. 50 μg total soluble protein was loaded per lane. The arrow marks the expected 60-kDa size of the hybrid *Bt* toxin. MW: molecular weight marker; NT: protein extracts from nontransformed IR72 control plant; T9-4-0: protein extracts from T_1 positive control plant; T9-4-1 to T9-4-6: protein extracts from six T9-4 homozygous progenies; *CryIA(b)* toxin: purified *Bt* toxin.

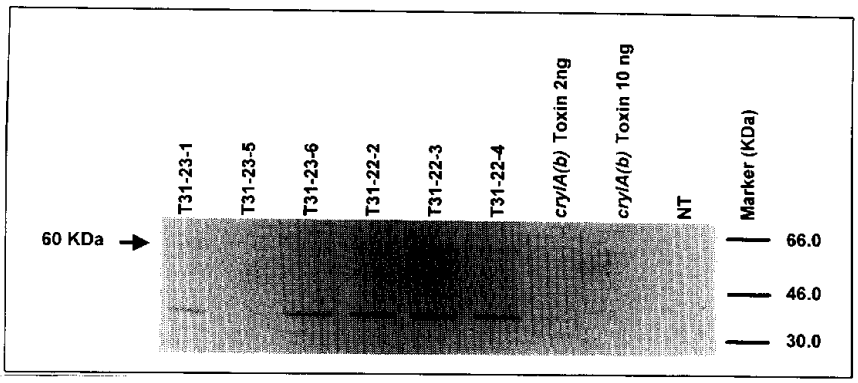

Fig. 7 Western blot analysis of stem protein extracts from two T_2 hemizygous lines.

T31-23: A segregant inherited only one expected fragment of transgene from its parent plant: T31-22: A segregant inherited all fragments of the transgene from its parent plant; *CryIA(b)* toxin: purified *Bt* toxin.

known amounts of purified *Bt* toxin (**Table 3**). The different expression level in the different transformants reflected a differential expression characteristics of the transgenes due to their different copy numbers or/and different insertion sites in rice genome. In addition, we also noticed that the segregant T31-23 with only expected size of DNA band on the Southern blot expressed similar toxin band pattern along with other segregants on the western blot (**Fig. 7**).

3. 4 Insect bioassay

Insecticidal activity of the hybrid *Bt* toxin in the transgenic rice plants was verified by feeding neonate larvae of YSB with stem materials at booting stage in a petri dish assay. The results showed that larva mortality after feeding for 4 days reached 100% in all tested T_0 Southern /western positive plants, irrespective of levels of toxin (0. 01 to 0. 2%) produced by the transgenic plants (**Table 3**). The average mortality in control dishes, which contains stems from untransformed IR72 plants, was 6%, however it varies in different experiments. When the toxicity effect on the insect was examined, the larvae fed with hybrid *Bt* -expressing stems were observed dead, with clinical signs of toxicity, a smaller size, and a deep brownish color of scratching dead body, whereas larvae fed with control stems reached a more advanced develop-

Table 4. Summary of the YSB bioassay and molecular analysis of three selfed T_1 progeny lines selected from two different transformants.

Transformant		Larva mortality[a]		Molecular analysis[b]	
Type	Plant	%	No. of plants	No. of positive	No. of negative
I	T 9	100	15	15	0
		85. 0-99. 9	1	1	0
		70. 0-84. 9	2	2	0
		45. 0-49. 9	1	0	1
		0. 0-44. 9	6	0	6
I	T31	100	7	7	0
		85. 0-99. 9	5	5	0
		70. 0-84. 9	4	3	1
		45. 0-49. 9	1	0	1
		0. 0-44. 9	11	0	11
II	T72	100	8	8	0
		85. 0-99. 9	1	1	0
		70. 0-84. 9	0	0	0
		45. 0-49. 9	3	0	3
		0. 0-44. 9	0	0	0

[a] Larva mortality was determined 4 days after infestation and scored based on 18 larvae per plant (6 larvae per stem and 3 stems per plant).

[b] Molecular analysis includes both Southern blot and/or western blot analysis.

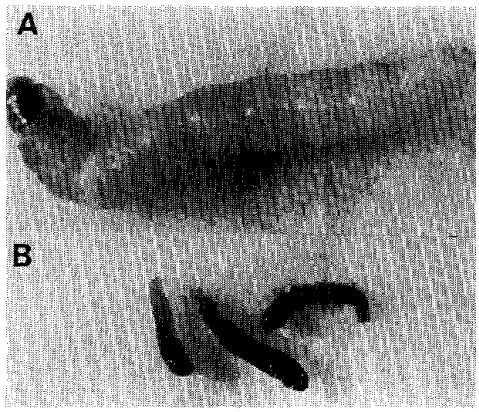

Fig. 8 Live YSB larva after feeding for 4 days on a stem from a nontransformed IR72 control plant (A) and dead larvae on a transgenic stem containing a hybrid *Bt* protein (B).

ment stage (**Fig 8**).

Insect bioassay of the segregating population showed the insecticidal activity of the T_0 generation although the larva mortality varied in some Southern positive T_1 plants (**Table 4**). The range of this variation was observed from 100% down to 71.1%, however, the frequency of the variation was less in the lines in which the transgene showed the normal segregation ratio in the Southern blot analysis. For instance, the number of the plants with the variation of larva mortality in the line T31 having non-Mendelian segregation pattern reached up to nearly 50% of its Southern positive plants. However, such variation accounted only 10-20% in the lines T9 and T72 having Mendelian segregation pattern (3: 1).

Because of this reason, the plants with 100% larva mortality were selected from the lines with normal segregation of the transgene like T9 and T72 and used for generating T_2 population. Through the western blot analysis, two homozygous lines T9-3 and T9-4 in T_2 generation were identified (**Fig. 6**). Insect bioassay of each plant of these homozygous lines showed 100% of larva mortality. However, in hemizygous lines, both the phenotype for resistance to YSB and the number of positive and negative plants in western blot analysis continued to segregate in a 3: 1 ratio (data not shown). A few Southern negative plants carrying rearranged bands other than expected size of *Bt* gene showed considerable high larvae mortality (45-69.9 %) as compared to control (**Table 4**).

4. Discussion

YSB causes considerable yield losses in rice across the ecosystems, where rice is grown throughout the world. Combining more than one insecticidal toxin in transgenic plants is a useful resistance management strategy in agreement with Perlak *et al.* [13]. Hence, the modifications and hybridization of the coding sequences of the two *Bt* toxin genes, *cryIA(b)* and *cryIA(c)*, were used to provide sustainable insect pest resistance in rice. So far, five independent transformants were identified out of 27 transgenic plants derived from three kinds of explants used in this study. The expression level of the hybrid Bt toxin was estimated up to 0.01% to 0.20% of the total soluble protein in leaf or stem tissue of the transgenic plants. This expression level in T_0 generation was comparable to the expression levels of *Bt* toxin detected in transgenic japonica [19]and indica [20, 21] rice as well as in tobacco [11], tomato [12], cotton [13], maize [14, 15], soybean [16], rape [17]tansformed with the modified *cryIA(b)* or *cryIA(c)* gene fused to the 35S or maize ubiquitin promoter. The differential expression levels of the hybrid *Bt* toxin in different transformants provide a good base of rice breeding for resistance to the insect pest YSB. A few Southern negative plants showed relatively high insect mortality compared to control. This is a bit unclear to us but likely to be due to carrying some rearranged *Bt* gene fragment conferring partial resistance. More work is on progress for better understanding the phenomenon.

Our previous data with *cryIA(b)* gene driven by constitutive and tissue-specific promoters (CaMV 35S, PEPC and Pith specific promoters) showed variable protection of plants against feeding larvae of rice YSB in several rice cultivars [33]. The present study dealing with transgenic IR72 plants with the hybrid *Bt* toxin gene under control of Actin I promoter exhibited high levels and less variation of resistance to the feeding larvae of the same insect pest in all T_0 plants as well as homozygous lines. The possible explanation for this observation might be due to that the C-terminal part of *CryIA(b)* protein responsible for the insect host specificity [34]was fused to N-terminal part of *CryIA(c)* protein responsible for insect toxic activity [35]in the hybrid *Bt* toxin gene. It was demonstrated by feeding larvae with artificial diet that the *CryIA(c)* protein is more toxic to most of lepidopteran insects including rice YSB [21, 36]. The LC50 for cabbage looper, tobacco budworm, corn earworm and black cutworm was 2- to 5-fold lower for *CryIA(b)* than for *CryIA(c)* protein [36]. The similar results with a modified *CryIA(c)*-like protein showing more toxic to tobacco budworm than *CryIA(b)* protein was obtained by Perlark *et al* in the transgenic cotton plants in 1990 [13]. However, our (unpublished) data in rice showed *CryIA(b)* is more potentially toxic than *CryIA(c)*.

From the present study, we also noticed that the deletion of P5-N-I-N-E-C-I11 and replacing with C5-R6 made on the 3′ end of *CryIA(b)* half polypeptide have no side effect on insecticidal activity of this

hybrid *Bt* toxin. This was consistent with the earlier results that the first 9 residues of *CryI* toxin were not required for toxic activity [37-41]. However, since lack of proper constructs, we failed to exclude possibility that other two residues of amino acid substitution on this hybrid *Bt* toxin contributed to the observed effects.

To maximize the limited use of insecticides, implementation of integrated pest management programs requires multiple sources of pest mortality such as the use of predators and parasites, continuous monitoring of infestation, and planting of insect and disease-resistant rice varieties. Through insect resistance, the transformed *Bt*-expressing rice will support this strategy. Since it is currently not known whether YSB have any host other than rice, transgenic lines with considerable levels of *Bt* toxin could be potentially effective when grown in conjunction with nontransformed lines to serve as refuges for the pest. The successful expression of the *cryIA(b)* and *cryIA(c)* hybrid *Bt* toxin gene in transgenic rice opens up the possibility to apply the strategy to broaden the genetic source of resistance to rice insect pests.

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