Planta (2016) 244:789–804
DOI 10.1007/s00425-016-2535-1

ORIGINAL ARTICLE

Marker-free transgenic rice expressing the vegetative insecticidal protein (Vip) of *Bacillus thuringiensis* shows broad insecticidal properties

Subrata Pradhan[1] · Anirban Chakraborty[1] · Narattam Sikdar[1] · Saikat Chakraborty[1] · Jagannath Bhattacharyya[1] · Joy Mitra[1] · Anulina Manna[1] · Snehasish Dutta Gupta[2] · Soumitra Kumar Sen[1]

Received: 19 February 2016 / Accepted: 15 April 2016 / Published online: 10 May 2016

Abstract

Main conclusion **Genetically engineered rice lines with broad insecticidal properties against major lepidopteran pests were generated using a synthetic, truncated form of vegetative insecticidal protein (Syn vip3BR) from *Bacillus thuringiensis*. The selectable marker gene and the redundant transgene(s) were eliminated through Cre/*lox* mediated recombination and genetic segregation to make consumer friendly *Bt*-rice.**

For sustainable resistance against lepidopteran insect pests, chloroplast targeted synthetic version of bioactive core component of a vegetative insecticidal protein (Syn - vip3BR) of *Bacillus thuringiensis* was expressed in rice under the control of green-tissue specific ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit gene promoter. The transgenic plants (in *Oryza sativa indica* Swarna cultivar) showed high insect mortality rate in vitro against major rice pests, yellow stem borer (*Scirpophaga incertulas*), rice leaf folder (*Cnaphalocrocis medinalis*) and rice horn caterpillar (*Melanitis leda ismene*) in T_1 generation, indicating insecticidal potency of Syn vip3BR. Under field conditions, the T_1 plants showed considerable resistance against leaf folders and stem borers. The expression cassette (*vip-lox-hpt-lox*) as well as another vector with chimeric *cre recombinase* gene under constitutive rice *ubiquitin1* gene promoter was designed for the elimination of selectable marker *hygromycin phosphotransferase* (*hpt*II) gene. Crossing experiments were performed between T_1 plants with single insertion site of *vip-lox-hpt-lox* T-DNA and one T_1 plant with moderate expression of *cre recombinase* with linked bialaphos resistance (*syn bar*) gene. Marker gene excision was achieved in hybrids with up to 41.18 % recombination efficiency. Insect resistant transgenic lines, devoid of selectable marker and redundant transgene(s) (*hpt*II + *cre-syn bar*), were established in subsequent generation through genetic segregation.

Keywords Vegetative insecticidal protein (Vip) · Green-tissue specific expression · Field assay · Cre/*lox* recombination · Marker free transgenic rice

Introduction

Rice is staple food for a large population of the world, particularly Asia, Africa, Central America, and the Middle East (Zhang et al. 2011; Durand-Morat et al. 2015). Reports predict gradual increase in the global demand for rice between 0.9 and 1.2 percent every year over the next decade (Durand-Morat et al. 2015). However, yield loss (5–25 % every year) due to lepidopteran insect inflicted damage (Pathak and Khan 1994) remains a major challenge to meet this demand. Intense use of chemical pesticides may lead to the risk of environmental pollutions and health hazards to producers and consumers (Chelliah and Gunathilagaraj, http://www.rkmp.co.in). *Bacillus thuringiensis* (*Bt*)-technology, the most economic and environmentally viable method for pest management, has

Electronic supplementary material The online version of this article (doi:10.1007/s00425-016-2535-1) contains supplementary material, which is available to authorized users.

✉ Subrata Pradhan
subratapradhan.bio@gmail.com

[1] Advanced Laboratory for Plant Genetic Engineering, Indian Institute of Technology Kharagpur, Kharagpur 721302, India

[2] Department of Agricultural and Food Engineering, Indian Institute of Technology Kharagpur, Kharagpur 721302, India

been intensively developed for last two decades (Fujimoto et al. 1993; Nayak et al. 1997; Sreevathsa et al. 2015) to overcome this difficulty. Concurrently, the global participation towards genetically engineered (GE) crop cultivation has gained momentum in past years (James 2014). Given the progress of the technology, rice can be no exception and various *Bt* rice lines have been developed, harboring Cry-toxins, with moderate to high resistance to target pests in laboratory and in field conditions (Nayak et al. 1997; Shu et al. 2000; Khanna and Raina 2002; Dandapat et al. 2013). These GE rice varieties with agronomic benefits have been in the pipeline for over a decade (Demont et al. 2013) and large scale field assessment of the Huahui 1 and *Bt* Shanyou 63 *Bt* rice lines in Hubei Province have already been issued biosafety certification by China's Ministry of Agriculture in 2009. The biosafety certificates were renewed in 2014; however, the *Bt* rice lines have not been commercially planted till date (Li et al. 2015).

Persistent exposure to *Bt* toxin can lead to resistant insect population and this has become the most serious threat to the continued efficacy of *Bt* crops (Zhao et al. 2003; Tabashnik et al. 2013). Thus, there is urgent need to diversify the pool of insecticidal proteins, apart from Cry-toxins, to get maximum benefit from the technology. Vegetative insecticidal proteins (Vips) from *Bacillus thuringiensis*, having wide spectrum of activities against lepidopteran pests (Estruch et al. 1996; Selvapandiyan et al. 2001; Gayen et al. 2012, 2015) can serve as potential alternative to Cry proteins and are currently being employed in commercial insect-resistant transgenic crops, like cotton (Fang et al. 2007). The mode of action of Vip is significantly different from Cry proteins in terms of receptor recognition and ion channel formation in the midgut of target insect pests (Lee et al. 2003). *Bt* genes have been engineered via deletion of redundant sequences for enhancement of the insecticidal activity (Mandal et al. 2007). Furthermore, the synthetic versions of the full length or truncated toxin genes with plant preferred codon usage have been employed for high level expression in plants (Dandapat et al. 2013). In one of our recent studies, it was revealed that the deletion of 200 amino acids at N-terminus of *vip*3BR gene (identified from local *Bt* isolate) enhanced insecticidal potency against cotton boll worm (CBW), cotton leaf worm (CLW), black cut worm (BCW) and major rice pest, Yellow Stem borer (YSB) compared to the native toxin as well as Cry1Ac and CryIIA in transgenic tobacco plants (Gayen et al. 2012, 2015). However, the efficacy of the same has not yet been tested in major edible crop, rice. In addition to this, enhancement of *Bt* expression level (high-dose strategy) by tissue-specific expression is highly recommended as an alternative approach of resistance management (Gould 1998). Localization of *Bt*-protein in sub-cellular organelle can provide substantial resistance against the action of cytoplasmic proteases resulting in the higher level of accumulation of *Bt*-protein in tissues (Kim et al. 2009; Pillay et al. 2014). Also, there is no utility of constitutive *Bt* expression as the lepidopteran insect pests prefer only green parts of plant body as food material. Chloroplast-targeted *Bt* expression is an option in this regard. The green-tissue specific expression also lowers *Bt* expression in endosperm or embryos (Ye et al. 2009), thereby allowing better consumer acceptance in view of ongoing debate about *Bt*-technology to be based on environmentally sound science.

Selectable marker genes (SMGs) are essential components for transgenic plant development but their retention in transgenic plants can cause possible toxicity or allergenicity in humans and hazards to the environment (Miki et al. 2009; Yau and Stewart 2013). Therefore, the production of marker-free transgenic crops is dire need of the hour to promote their commercial deployment. The site specific Cre/*lox* recombination system has been extensively used for this purpose in the recent years (reviewed by Tuteja et al. 2012). The 38 kDa Cre recombinase, a member of the tyrosine recombinase family (Gilbertson 2003), recognizes two directly repeated asymmetric 34 bp *lox*P sites for the accurate excision (Wang et al. 2005) of the target gene from the host genome.

To execute precise elimination of marker gene, the expression of *cre* gene can be spatio-temporally regulated in different manner, viz., constitutive, and transient. The constitutively expressed *cre* gene is delivered to targeted *lox*P sites by cross-pollination (Hoa et al. 2002). Auto excision of marker genes by Cre/*lox*P system, using heat-shock inducible promoter (Khattri et al. 2011), chemically inducible promoter (Zuo et al. 2001), pollen/seed-specific promoters, like *PAB5* (Luo et al. 2007) and embryo-globulin *REG-2* (Chong-Pérez et al. 2013) are examples of temporal expression. There are reports of successful marker elimination using both constitutive and inducible promoters (Kopertekh and Schiemann 2012). However, subsequent removal of the *cre* gene through genetic segregation can eliminate potential undesired effect of the Cre protein (Coppoolse et al. 2003).

With this background, the present study illustrates the ectopic expression of a codon optimized, synthetic, chloroplast targeted core component of *vip*3BR gene (*syn vip*3BR) in a rice variety of agronomic importance (*Oryza sativa indica* cv. Swarna). The insecticidal potency of *syn vip*3BR, under the control of a green tissue specific ribulose-1,5- bisphosphate carboxylase/oxygenase small subunit gene promoter (*rbcS*) and chloroplast targeting signal (Almeida et al. 1989) was tested against major lepidopteran pests of rice under laboratory as well as field conditions. The Cre/*lox*P system was utilized for elimination of *hpt*II

marker gene from the insect-resistant rice lines. In addition to this, genetic segregation of the redundant *cre-syn bar* linked gene was also accomplished in subsequent generation. The present study, thus for the first time, documents a comprehensive strategy to develop consumer friendly, second generation, marker free broad spectrum insect resistant rice plants.

Materials and methods

Plant material

Oryza sativa indica Swarna cultivar (MTU 7029) was used for plant transformation experiments.

Bacterial strains and plasmid vectors

The super virulent *Agrobacterium tumefaciens* strain EHA105 (Cheng et al. 1998) and binary vector pCAMBIA1300 were used for the rice transformation experiments. *Escherichia coli* strain DH10B (BRL), and cloning bacterial vector pUC18 were applied for standard gene cloning procedure (Sambrook et al. 1989).

Reconstruction of *vip*3BR gene using plant preferred codons and generation of plant transformation vectors pLHRV and pBUC

The N-terminal 200 amino acids deleted *vip*3BR gene [(Ndv200) (Gayen et al. 2012, 2015); GenBank: AY739647] fused with chloroplast targeting signal sequence (obtained from Impact Vector version 1.4, Plant Research International, Wageningen, Netherlands) at 5′ end, was synthesized at GenScript Corporation (USA) using rice nuclear gene preferred codons (http://www.kazusa.or.jp/) for optimal expression in rice following Murray et al. (1989) and Grantham et al. (1986). Essentially, the AT rich (68.88 %) bacterial gene was converted to GC rich (58.77 %) without altering the amino acid sequence. Also, the splicing signals, eukaryotic ribosome binding sites, direct and indirect repeats (up to 8 mer), CpG gene silencing signals and TATA/CAAT box like sequences, that generated during the process of codon optimization, were subsequently removed to achieve effective gene expression. The synthetic gene (Suppl. Fig. S1) was referred to as *syn vip*3BR, henceforth.

The plant selection marker, *hpt*II gene was amplified from pCAMBIA1300 vector (Cambia, Canberra; GenBank: AF234296) by Polymerase Chain Reaction (PCR) using the primers LHFP/LHRP. The primers were designed to contain *lox*P sequence in direct orientation. Thus, after amplification, the Coding DNA Sequence (CDS) of *hpt*II gene (under the regulatory control of CaMV35S promoter and terminator) was flanked by two *lox*P sites in direct orientation. The *lox*P containing PCR product was cloned into pCAMBIA1300 at *Xho*I sites incorporated in the primers (Suppl. Fig. S2a). The *syn vip*3BR gene, described above, was cloned into pCAMBIA1300 at *Xba*I and *Kpn*I to form pLHV. The *rbcS* promoter was PCR amplified from Impact vector 1.4 using the primers RBFP/RBRP and subsequently cloned into pLHV at *Hin*dIII/*Xba*I to obtain pLHRV (Suppl. Fig. S2a). The transcription termination signal from *Nopaline synthase* (*Nos*) gene was used at 3′ end of *syn vip*3BR gene in pLHRV cassette.

For the preparation of recombination cassette, the CDS of *cre* recombinase gene (1202 bp) was PCR amplified from pX6-GFP plant DNA excision vector (GenBank: AF330636.1) using the primers CRFP/CRRP. The amplified product was cloned into pCAMBIA1300 vector at *Kpn*I and *Sac*I sites to obtain pC. The synthetically designed herbicide resistant gene, *syn bar* was PCR amplified from pBAR (Bhattacharyya et al. 2015) with *Xho*I sites in the oligos. Then *syn bar* gene was cloned in pCAMBIA1300 by replacement of *hpt*II gene (also between *Xho*I sites) to obtain pCB. The *OsUbi*1 promoter (GenBank: AY785814, Bhattacharyya et al. 2012) was cloned at 5′ end at *Hin*dIII and *Kpn*I sites and *Nos* terminator, mentioned above, was incorporated at 3′ end of *cre* gene at *Sac*I and *Eco*RI sites to obtain pBUC (Suppl. Fig. S2b).

Agrobacterium-mediated rice transformation and generation of T_1 parental lines for in vitro and field bioassay, crossing and marker elimination

Standard *Agrobacterium tumefaciens* mediated rice transformation protocol was followed as described previously in Bhattacharyya et al. (2012). Individual transformation cassettes (pLHRV or pBUC) were used separately in transformation events to generate two types of GE plant lines, viz., harboring *syn vip3BR* and *cre* gene cassette(s). Putative transformed calli masses were selected on MS medium (Murashige and Skoog 1962) containing appropriate doses of selection markers (hygromycin B at 50 mg/L for pLHRV and bialaphos at 2.5 mg/L for pBUC) and regenerated plants, after root development, were grown to maturity in greenhouse after proper acclimatization. To obtain the T_1 progeny plants, the individual transgenic rice lines, harboring pLHRV and pBUC expression cassettes, were self-pollinated under containment conditions (to avoid cross-pollination) and T_1 seeds were harvested. The T_1 seeds were germinated in half strength MS media with hygromycin B (50 mg/L) and bialaphos (2.5 mg/L),

respectively, and scored for surviving (green) vs etiolated seedlings in each case. The segregation pattern of the *hpt*II and *syn bar* transgene(s) through the T_1 progenies was analyzed by χ^2 test to determine the number of functional gene loci, which directly reflect the presence of linked *syn vip*3BR and *cre* transgene(s) in rice genome, respectively.

Southern blot analysis

Genomic DNA was isolated from the fresh leaves (tillering stage) of T_0 and T_1 plants following the protocol of Doyle and Doyle (1990). Southern hybridization was performed according to Sambrook et al. (1989). Briefly, 10 µg genomic DNA was digested with restriction enzyme (*Hin*dIII) and electrophoresed on 0.8 % agarose gel. The DNA from the gel was then transferred to Hybond–N^+ nylon membrane (Amershan Pharmacia Biotech) using a vacuum blot transfer unit (model 785, Bio-Rad Laboratories, Inc.). Hybridization was carried out separately at 65 °C in CHURCH buffer [0.25 M Phosphate buffer (pH 7.2), 1 mM EDTA and 7 % SDS. 1 % BSA; Sambrook et al. 1989] for 16 h, with α-[^{32}P]-dCTP-labeled *syn vip*3BR, *cre* and *hpt*II gene as probes. The membrane was stripped with boiling distilled water and 1 % SDS and re-probed with a different probe in each case. The autoradiograms were developed by exposing the membrane to Super Resolution screen (PerkinElmer) and scanned in Storage Phosphor system (Cyclone Plus, PerkinElmer) at 300 dpi resolution.

Real time qPCR analysis of T_1 transgenic plants

Total RNA was isolated from fresh rice leaves (sampled at the same stage as in Southern blot experiment) and the grain tissue (dehusked mature seeds; 125 days old from the day of sowing) using RNeasy Plant Mini Kit (Qiagen), followed by on-spin column DNase treatment to remove any DNA contamination, as per manufacturer's protocol. First strand cDNA was synthesized using respective reverse primers (VIP RTRP for *syn vip*3BR and CR RTRP for *cre*) using transcriptor 1st strand cDNA synthesis kit (Roche). 2 µl cDNA was used for subsequent qPCR reactions. The thermal cycling conditions for qPCR comprised of 2 min at 94 °C followed by 40 cycles of 94 °C for 30 s, 58 °C for 15 s, and 68 °C for 30 s, for respective samples in different setup. For each reading, triplicate C_t values were averaged. Melting curve analysis in each case confirmed the amplification of specific product.

The relative expression levels of *syn vip*3BR and *cre* recombinase gene in different T_1 transgenic plant lines were measured by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). The gene expression was normalized by *β-actin* gene of *Oryza sativa* (GenBank: X15865.1) as internal control.

Western blot analysis

Total soluble protein was extracted from leaf (tillering stage) and grain tissue of respective T_1 transgenic lines using extraction buffer [50 mM Tris (pH 8.0), 2 mM EDTA, 100 mM NaCl, 0.02 % Tween-20, 1 % beta mercaptoethanol, and 10 mM PMSF], and the quantity was estimated by the standard method (Bradford 1976). Equal amounts (30 µg) of total protein were loaded onto 12 % SDS-PAGE, electrophoresed, and blotted onto Hybond C nylon membrane (Amersham Pharmacia Biotech) applying a wet transfer method. The rabbit polyclonal antibody against bacterially expressed Vip3BR (developed in–house) and rabbit polyclonal anti Cre recombinase antibody (Product no. ab41104, Abcam, UK) were used as primary antibody (1:1000 dilution), anti-isotype IgG-POD (Horseradish peroxidase) (1:2000 dilution) was used as the secondary antibody and mouse monoclonal plant Actin antibody (Sigma, mabGPa) was used as loading control (1:500 dilution) for the Western blot. Western blot analysis was carried out using the Western Chromogenic Kit (Roche Molecular Biochemical) according to the manufacturer's instructions.

ELISA

Expression of *syn vip*3BR and *cre* gene(s) in transgenic lines was quantitated by enzyme linked immunosorbent assay (ELISA) using Express ELISA Kit (GenScript Corporation USA, Cat. no. L00251). Total soluble protein from untransformed control plant was used as negative control. Total soluble proteins were extracted and measured as in Western blot analysis. ELISA was performed according to vendor's instructions and quantification of Syn vip3BR and Cre recombinase in absolute terms was done by comparing the absorbance values of the samples with the standard curve generated with purified Ndv200 and Cre proteins. The result was expressed as nanogram of Syn vip3BR and Cre protein per microgram of total soluble protein.

In vitro insect feeding assay of detached leaf and stem tissue from T_1 *syn vip*3BR transgenic plants

In vitro insect feeding assays were performed according to established protocols (Nayak et al. 1997; Ye et al. 2000; Gayen et al. 2015). The assay was carried out from one representative T_1 pLHRV plant of each category (with single insertion site of *syn vip*3BR; the same plant line was subjected to Southern blot to confirm presence of transgene and real time qPCR, Western blot and ELISA to check for level of expression of the transgene) against second instar

larvae of rice leaf folder (RLF) (*Cnaphalocrocis medinalis*), rice horn caterpillar (RHC) (*Melanitis leda ismene*) and first-instar (<24 h) larvae of yellow stem borer (YSB) (*Scirpophaga incertulas*). The insects were maintained in an artificial diet [chickpea grain flour 150.00 g, methyl parabenzoate 2.4 g, sorbic acid 1.28 g, ascorbic acid 3.98 g, streptomycin sulfate 0.19 g, multivitamin tablet 0.25 g, agar 12.00 g, baking yeast 10.00 g, formaldehyde 10 % (v/v) in 800 ml of distilled water] under laboratory conditions. In case of YSB, 50 g of young (green), soft rice stem tissue homogenate was used to supplement 100 g of chickpea grain flour. Freshly cut (4–5 cm) terminal leaves of main stem of transgenic rice plants (tillering stage) were placed on moist tissue paper in Petri dishes. The leaves of untransformed plant (designated as UC) were used as negative control. Each experimental set comprised of 15 larvae with 5 insect larvae per Petri plate with 3 replicates of each experiment. The plates were incubated at 28 °C in 70 % relative humidity, photo periodic regime of 16/8 h of light (3000 lux) and dark. Feeding area in each treatment were recorded after 24, 48 and 72 h (following a diet change after 48 h, assay continued with same set of insects), respectively and the insect survival rate [in terms of % mortality (Gayen et al. 2012)] was recorded after 72 h. Young, freshly cut (4–5 cm) stems of transgenic rice plants were used for bioassay of first-instar YSB larvae following same protocol as above.

Field bioassay of T_1 *syn vip*3BR transgenic plants

Small-scale evaluation for insect resistance at field level was performed at our laboratory field in 2014. Transgenic trials were carried out according to the guidelines (1998) by Department of Biotechnology (DBT), Govt. of India (http://dbtbiosafety.nic.in/). The materials tested were four *syn vip*3BR T_1 transgenic lines (containing single insertion site of *syn vip3BR* expression cassette), and an untransformed control (UC). The seedlings developing from hygromycin screened seeds were subsequently transplanted to a specially designed net-house (2 m × 1 m × 2 m) (Dandapat et al. 2013) on mid-June. The field layout followed a randomized complete block design with three replications. Each plot consisted of 20 UC plants and 20 transgenic plants of each type in rows (10 plants in each row), with distances of 12.0 cm between plants within a row and 16.0 cm between rows. Evaluation of insect resistance of transgenic plants in the field was conducted by artificial infestation using YSB combined with occasional natural infestation of stem borers. The same bioassay was conducted separately for the RLF. No chemical insecticide was applied throughout the growth period. Each rice plant was artificially infested with 15–20 first-instar larvae of YSB and RLF at the tillering stage, separately. The extent of damage caused by RLF was recorded within 9 days after visible scrapes or folds appeared in leaves. Dead hearts caused by YSB were counted at the late maximum tillering stage, and white heads were counted at the flowering stage.

Crossing of T_1 transgenic lines for Cre/*lox* recombination mediated marker elimination

Four independent T_1 progeny plants from different category, harboring pLHRV (as female parent) were crossed with a T_1 progeny plant, harboring pBUC (as male parent) with moderate level of Cre recombinase expression. T_1F_1 (hybrid progeny derived from crosses between T_1 pBUC and pLHRV plants; subsequent generations of this population were designated as T_1F_2 and so on) seeds were harvested from all four independent crossing experiments. The seeds were surface sterilized and germinated on half strength MS basal medium with 2.5 mg/L bialaphos at 25 °C. Germinated seedlings were transferred to greenhouse and grown to maturity for further analysis.

Screening of T_1F_1 and T_1F_2 plants by PCR

PCR were performed in various rice lines generated after crossing using *syn vip*3BR, *hpt*II, *cre* and *syn bar* specific primers in separate reaction tubes for each gene. Each 25 µl PCR reaction mixture contained 1× PCR master mix including *Taq* DNA polymerase. Primers were used in 0.2 µM concentration. The thermal cycling conditions were 4 min at 94 °C followed by 35 cycles of 30 s at 94 °C, 45 s at 58 °C, 60 s at 72 °C and final extension with 10 min at 72 °C, for *syn vip*3BR. The extension time for *hptII* and *cre* genes were 90 s and for *syn bar* was 30 s at 72 °C and all other conditions were similar to *syn vip*3BR amplification. pLHRV plasmid was taken as positive control for *syn vip*3BR, *hpt*II genes and pBUC plasmid was taken as positive control for *cre*, *syn bar* genes. The amplification products of *syn vip*3BR, *cre*, *hpt*II and *syn bar* genes were checked in ethidium bromide stained-agarose gel (1 %).

Statistical analyses

The time course of detached leaf/stem bioassay and field bioassay were statistically evaluated using Student's *t* test.

Oligonucleotides used in the study

Oligonucleotides used in the study were listed in Suppl. Table 1.

Results

Generation of independent transgenic rice lines harboring *syn vip*3BR and progeny analysis

The numbers of independent primary rice transformants generated following the optimized *Agrobacterium*-mediated rice transformation protocol and stringent screening were seven (SA, SB, SC, SD, SE, SF, SG) in case of pLHRV (harboring *syn vip*3BR). Southern blot analysis confirmed the presence of *syn vip*3BR (Fig. 1a, left panel) in respective transformants in T_0 generation. The Chi square analysis of *hpt*II from a population of 40 T_1 seeds indicated a 3:1 segregation ratio, implying transgene integration in a single chromosome (Suppl. Table 2). Randomly chosen T_1 progenies ($n = 2$) from each category were subjected to Southern hybridization. The autoradiogram showed similar pattern of *syn vip*3BR integration (Fig. 1a, right panel) in all T_1 transgenic plants as in T_0 lines (Fig. 1a, left panel). This provided evidence for stable integration of the transgene as well as its transfer to next generation through seeds.

Expression analysis of stable T_1 transgenic rice lines harboring *syn vip*3BR

Transgenic rice lines with single insertion site of *syn vip*3BR (SA, SD, SE, SG) were subjected to expression analysis. The expression level varied among the four independent T_1 transgenic lines (Fig. 1b), with significant higher expression in the line SD compared to other lines. Transgenic lines with relatively higher expression level of *syn vip*3BR mRNA also showed higher expression of Syn vip3BR protein as determined by Western blotting (Fig. 1c). No band was detected in untransformed control line in the specific size range of the transgenic protein.

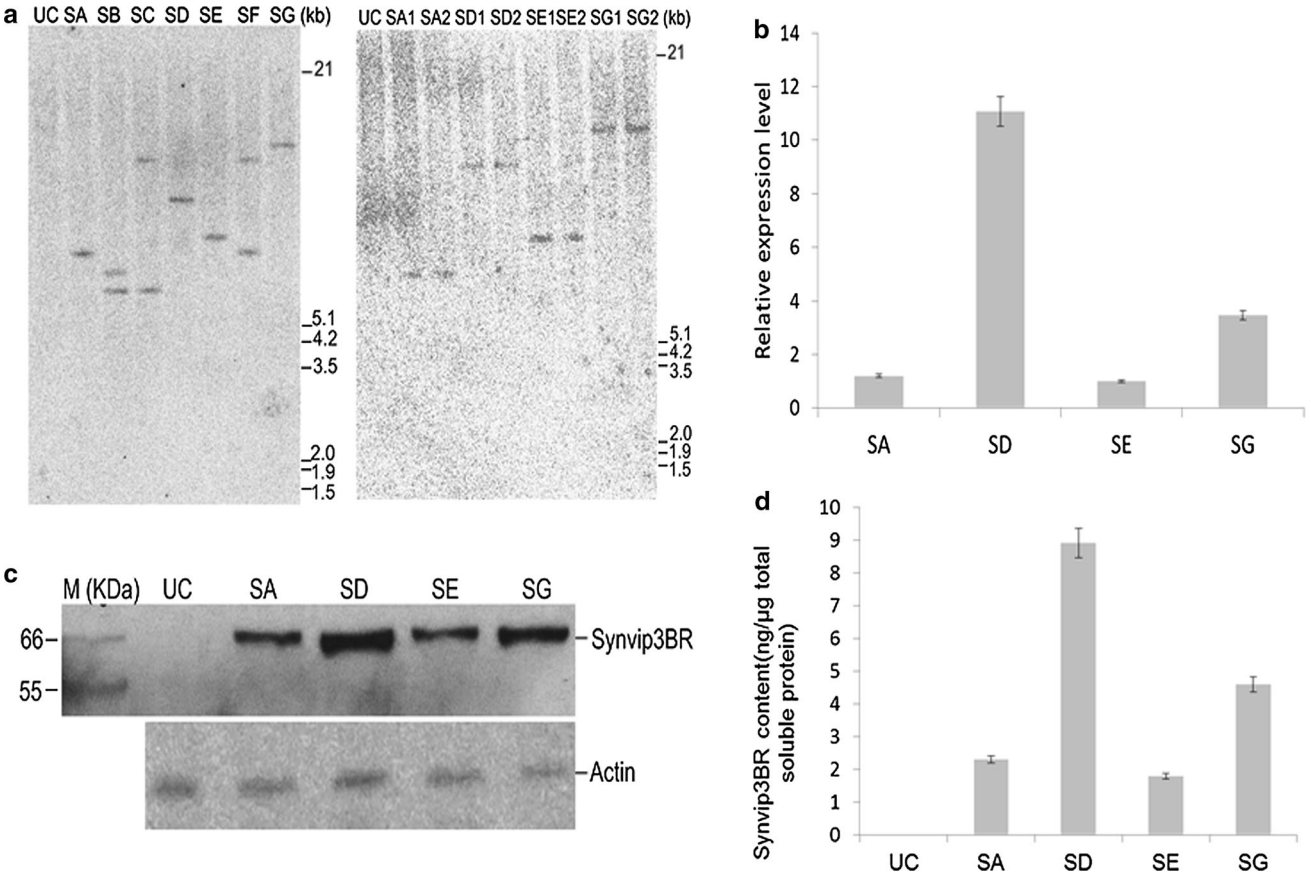

Fig. 1 Molecular analysis of *syn vip*3BR gene integration and expression in T_1 rice lines. **a** Southern blot analysis of *Hin*dIII digested genomic DNA from leaves of T_0 (*left panel*) and representative ($n = 2$) T_1 (*right panel*) pLHRV rice lines probed with (α-32P) dCTP labeled *syn vip*3BR gene. **b** Normalized, relative expression level of *syn vip*3BR gene in T_1 transgenic rice lines by quantitative RT-PCR analysis. Results are expressed as mean ± SD, for three biological replicates (three individual T_1 plants from same category were sampled). **c** Immunoblot analysis of Syn vip3BR protein in T_1 progeny plants (*Upper panel*). Actin is used as loading control (*Lower panel*). **d** Quantitative expression analysis of Syn vip3BR protein in total soluble protein from leaves of T_1 plants by ELISA. Results are expressed as mean ± SD, for three biological replicates (sampled similar to qRT-PCR). *UC* untransformed control, *M* molecular weight marker

Finally, ELISA analysis showed expression of Syn vip3BR in the range of 0.18–0.89 % of the total soluble protein (Fig. 1d) with T_1 progeny of SD showing the highest expression level consistent with the qRT-PCR results. In a further attempt to ascertain spatial expression pattern of the *syn vip*3BR, qRT-PCR and Western blot were carried out from leaf and grain tissue of two T_1 rice lines (SD and SG) with relative higher expression level of *syn vip*3BR (Suppl. Fig. S3). The results revealed low to negligible expression (both in mRNA and protein level) of *syn vip*3BR in rice grains as compared to their high level expression in leaf tissues. This underscores the biosafety of the *Bt*-line as grain is used as primary food material.

In vitro insect feeding assay of T_1 transgenic plants harboring *syn vip*3BR

The young, fresh leaf and stem tissues from four representative T_1 rice lines were used for the insect feeding bioassay to test the efficacy and the sensitivity of the expressed Syn vip3BR toxin against the second instar larvae of RLF, RHC and first-instar larvae of YSB. The leaf tissues and stems of untransformed plant were used as negative control (Fig. 2a, d, g). The larvae fed on untransformed control plant grew well whereas the larvae fed on transgenic rice lines showed high percentage of mortality scored after 72 h (Fig. 2b, e, h; representative figure for plant line SD). The cumulative feeding area in all insect bioassays did not show significant differences between control (UC) and transgenic lines after 24 h and 48 h of infestation ($P > 0.05$). However, significant differences were observed 72 h post infestation ($P < 0.05$) (Table 1). This indicated that there was no feed avoidance by the insects, at least for first 48 h post infestation. Thus, the insects exhibit mortality due to toxicity from the expressed Syn vip3BR. The % mortality ranged between 46.6–80 % in case of RLF (Fig. 2c), 40–73.2 % for RHC (Fig. 2f) and 40–86.6 % for YSB (Fig. 2i) after 72 h. All three types of larvae showed highest percentage of mortality against the transgenic line SD, in which the highest expression of toxin was detected.

Field bioassay of T_1 *syn vip*3BR transgenic rice lines

The above four T_1 transgenic plant lines were further tested under field conditions, in small scale, for evaluating resistance against major rice pests, leaf folders (RLF) and stem borers (YSB). The T_1 seeds which survived hygromycin screening during progeny analysis were used for the purpose. It was found that 92.22 % of tillers of the untransformed line (UC) were damaged to various extents (Fig. 3a, right panel) by RLF (Fig. 3a, left panel), with an average of 2.76 leaves affected per tiller, while no damage (0.00–0.35 leaves per tiller) was observed in all four transgenic lines (Table 2). Similarly, untransformed line (UC) was also severely damaged by YSB (Fig. 3b, right panel), with 86.66 % of the plant tillers having dead hearts or white heads (Fig. 3b, left panel and inset), while only 18.33 % of dead hearts were observed in SD (Fig. 3b, right panel and Table 2). The damage was relatively high, however, in SA (55.0 %) and SG (48.33 %) transgenic plant lines. The transgenic line SE showed no significant insect resistance (Table 2). On the basis of above results, it can be concluded that these specific transgenic plant lines, SA, SG and SD, exhibited significant resistance against two major lepidopteran pests under field conditions. The SE showed significant resistance against RLF, however, resistance against YSB was not so prominent (Table 2) and thus, this line may not be suitable enough for broad spectrum insect resistance.

Expression analysis of stable T_1 transgenic rice lines with *cre* transgene

The number of independent primary rice transformants were five (CA, CB, CC, CD, CE) in case of pBUC (harboring *cre recombinase*). Southern blot analysis confirmed the presence of *cre* (Fig. 4a, left panel) transgene in respective transformants in T_0 generation. The Chi square analysis of syn *bar* from a population of 40 T_1 seeds indicated a 3:1 segregation ratio, implying transgene integration in a single chromosome (Suppl. Table 3). Randomly chosen T_1 progenies ($n = 2$) from each category were subjected to Southern hybridization. The autoradiogram showed similar pattern of *cre* integration (Fig. 4a, right panel) in all T_1 transgenic plants as in T_0 lines (Fig. 4a, left panel). This provided evidence for stable transgene integration and its transfer to next generation through seeds. Transgenic rice lines with single insertion site of *cre* (CB, CD) showed variation in expression level (Fig. 4b, c). The ELISA analysis revealed the expression level of 0.52 and 0.78 % of total soluble protein in T_1 progeny of CB and CD, respectively (Fig. 4d). This relatively high expression of the Cre recombinase could be attributed to its expression driven by constitutive *Os Ubi*1 promoter. The plants, however, showed no phenotypic abnormality. Based on some earlier observations about adverse effect of high level expression of Cre recombinase on plant growth (Coppoolse et al. 2003), the plant line CB with moderate expression of Cre recombinase was chosen as the candidate T_1 progeny for subsequent crossing experiments.

Molecular analysis of selectable marker gene excision following Cre/*lox*P mediated recombination

From the four independent crossing events (Table 3), T_1F_1 seeds were collected separately and germinated under bialaphos (2.5 mg/L) selection. All the progeny plants,

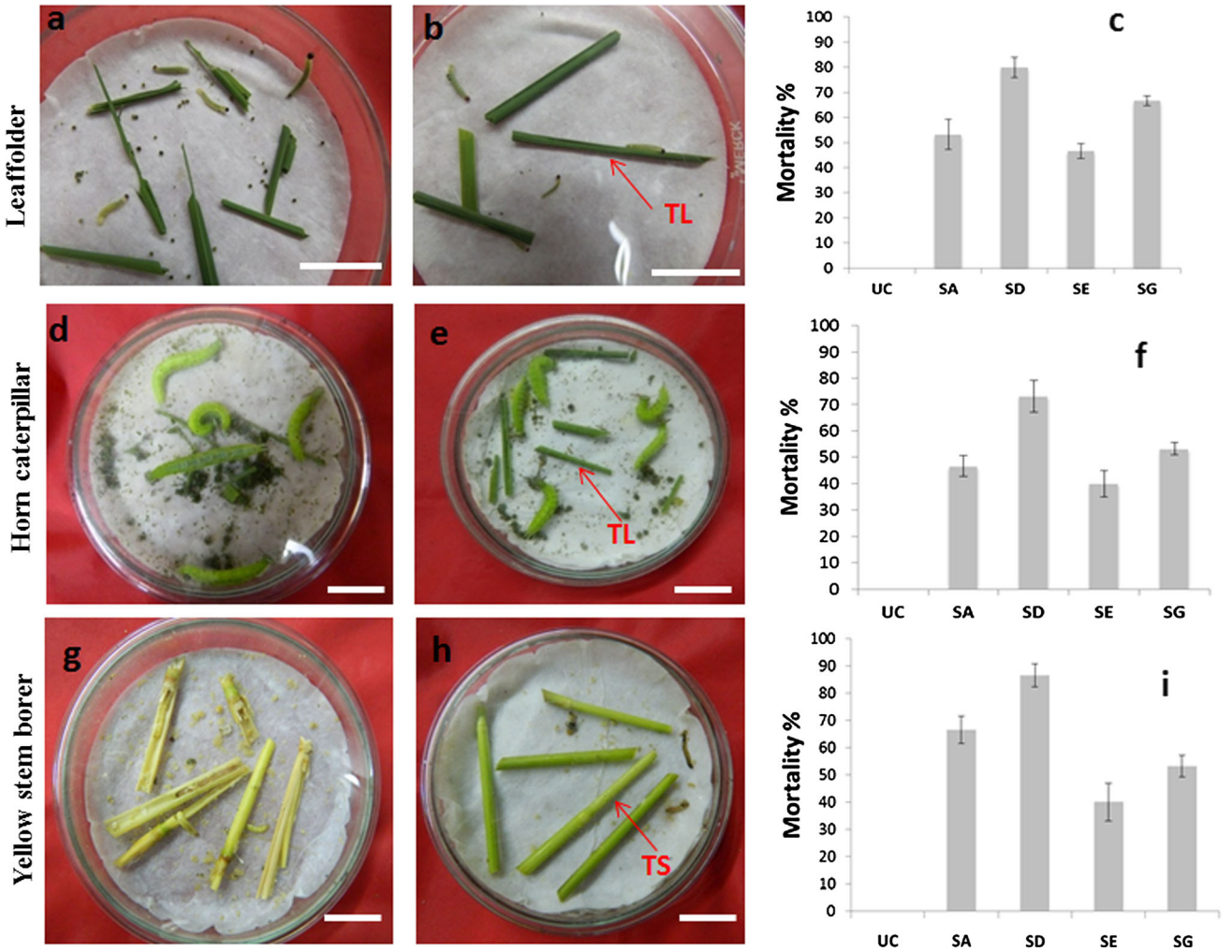

Fig. 2 In vitro insect feeding assay of T_1 transgenic plants harboring *syn vip*3BR transgene (results recorded after 72 h following a diet change after 48 h). Rice leaf folder **a** against untransformed plant leaves (*bar* 2 cm) **b** against leaves of T_1 progeny plant expressing Syn vip3BR protein (*bar* 2 cm) **c** mortality % against T_1 rice lines. Rice horn caterpillar against **d** untransformed control plant leaves (*bar* 1.6 cm) **e** T_1 progeny plant leaves expressing Syn vip3BR protein (*bar* 1.6 cm) **f** mortality % against same rice lines. Yellow stem borer against **g** untransformed control plant stems (*bar* 1.6 cm) **h** T_1 progeny plant stems expressing Syn vip3BR protein (*bar* 1.6 cm) **i** mortality % against same rice lines. *TL* transformed leaves. *TS* transformed stems. Results are expressed as mean ± SD, for three replicates in each case. The figures (**a**, **b**, **d**, **e**, **g**, **h**) represent insect feeding profile after 72 h of initial diet supply (time = 0 h) with diet change after 48 h

azygous for the *syn bar* and linked *cre* transgene, were eliminated as a result of this stringent screening. PCR were performed with *cre*, *syn vip*3BR and *hpt*II specific primers for initial screening of the hybrid plants. The representative data from hybrid line SD9CB is shown (Fig. 5a, c). Forty seeds were chosen from hybrid line SD9CB (Table 3). PCR were performed with *cre* and *syn vip*3BR gene specific primers. Among the 25 progeny plants, obtained after stringent bialaphos screening, 17 plants were found to be positive for both *cre* and *syn vip*3BR genes; 8 plants were positive for only *cre* gene (Fig. 5a, b). No plant line could be recovered harboring only *syn vip3BR* cassette, as these plants did not contain bialaphos resistance gene and thus, were eliminated in the stringent screening process. The marker gene excision among the 17 *syn vip*3BR and *cre* positive hybrid plants of SD9CB were further verified through PCR using *hpt*II gene specific primers (Fig. 5c). This amplification identified seven *hpt*II negative plants where *cre* did work efficiently and as a result, excision of *hpt*II took place through recombination event. Based on the results, the Cre/*lox*P mediated recombination frequency was determined to be 41.18 % in case of hybrid line SD9CB. The seven hybrid plants were further screened through Southern hybridization using three different probes for *syn vip*3BR, *cre* and *hpt*II (Suppl. Fig. S4a-S4c). The results clearly showed the absence of *hpt*II gene (Suppl.

Table 1 In vitro insect feeding assay of T_1 transgenic plants harboring *syn vip*3BR transgene

Rice line	Cumulative feeding area of leaf/stem (mm^2/larva) (mean ± SD, for triplicate readings)								
	Rice leaf folder			Rice horn caterpillar			Yellow stem borer		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Control (UC)	3.80 ± 0.40	9.26 ± 0.51	16.96 ± 0.37	6.10 ± 0.36	13.06 ± 0.65	27.83 ± 0.55	2.73 ± 0.35	6.30 ± 0.45	11.60 ± 0.60
SA	3.66 ± 0.15	8.60 ± 1.05	10.80 ± 1.40*	6.06 ± 0.70	12.40 ± 1.10	20.86 ± 3.32*	2.50 ± 0.78	5.50 ± 0.55	6.93 ± 1.30*
SD	3.93 ± 0.25	7.83 ± 1.02	8.03 ± 0.98***	6.26 ± 0.25	12.30 ± 0.79	16.03 ± 3.79***	2.60 ± 0.26	5.36 ± 0.15	5.63 ± 0.15**
SE	4.2 ± 0.40	8.93 ± 0.49	11.40 ± 2.06*	6.06 ± 0.45	12.03 ± 0.70	21.36 ± 4.90*	2.73 ± 0.66	6.23 ± 0.61	9.03 ± 1.42
SG	3.96 ± 0.55	8.96 ± 0.25	10.03 ± 0.40*	5.96 ± 0.45	11.40 ± 1.15	20.36 ± 3.09*	2.63 ± 0.61	6.03 ± 0.60	6.86 ± 0.83*

Means significantly different from the control (* $P < 0.05$), (** $P < 0.01$), (*** $P < 0.001$)

Fig. S4c) but the presence of *syn vip*3BR (Suppl. Fig. 4a) and *cre* gene (Suppl. Fig. S4b). Recombination frequency in three other hybrid lines varied between 33.33–35.71 % (Table 3).

Establishment of T_1F_2 transgenic plants with complete elimination of selectable marker

Since the T_1F_1 *hpt*II negative plants were *cre* positive, it is very likely that they still bear the *syn bar* gene. The PCR assay with *syn bar* gene specific oligos (data not shown) confirmed this. Therefore, some of the T_1F_1 progeny plants were allowed to self-fertilize to obtain T_1F_2 plants and were analyzed for complete elimination of any selection marker through genetic segregation. Twenty T_1F_2 progeny plants of the *hpt*II negative line SD9CB (5) were analyzed for the presence of *syn vip*3BR, *cre* and *syn bar* genes. Twelve plants contained *syn vip*3BR, *cre* and *syn bar* genes [T_1F_2 plant SD9CB (5)—1, 2, 4, 5, 8, 9, 10, 12, 13, 15, 17, 19], three plants [SD9CB (5)—3, 14, 20] showed the presence of *cre* and *syn bar,* and five plants [SD9CB (5)—6, 7, 11, 16, 18] were found to contain only *syn vip*3BR without presence of *cre* or *syn bar* gene (Fig. 6a, b, c). Thus the five plants were considered as complete marker free *Bt*-rice lines.

Discussion

Over the last two decades, *Bt*-toxins, particularly the Cry-toxin has come up as a major tool for protection against insect pests, resulting in enhanced crop yield and lesser pesticide use (Naranjo 2009; Qaim 2009). Rice (*Oryza sativa* L.) is a staple food providing 75 % of the daily calorie intake (Khush 2005). Despite recent progress of the *Bt*-technology, a new threat emerged as insect pests have become resistant to *Bt* toxin resulting in loss of the economic, health and environmental benefits of the technology (Raybould and Vlachos 2011). The major reason for development of resistance against *Bt* toxin is probably change in binding affinity of receptors with the toxin peptide (Van Rie et al. 1990; Deist et al. 2014). In this context, the discovery of secretory vegetative insecticidal proteins (Vips) as a new class of insecticidal toxin, with a different mode of action than Cry protein (Estruch et al. 1996), opened up possibilities for development of second generation insect resistant *Bt* crops. Since then, various laboratories are engaged in search for Vips (Selvapandiyan et al. 2001; Bhalla et al. 2005) with variation of sequence and protein engineering approaches to develop diversity to reduce chances of emergence of resistance. In our laboratory, Vip3A-like toxin molecule, Vip3BR was recently isolated and characterized (Gayen et al. 2012, 2015). The

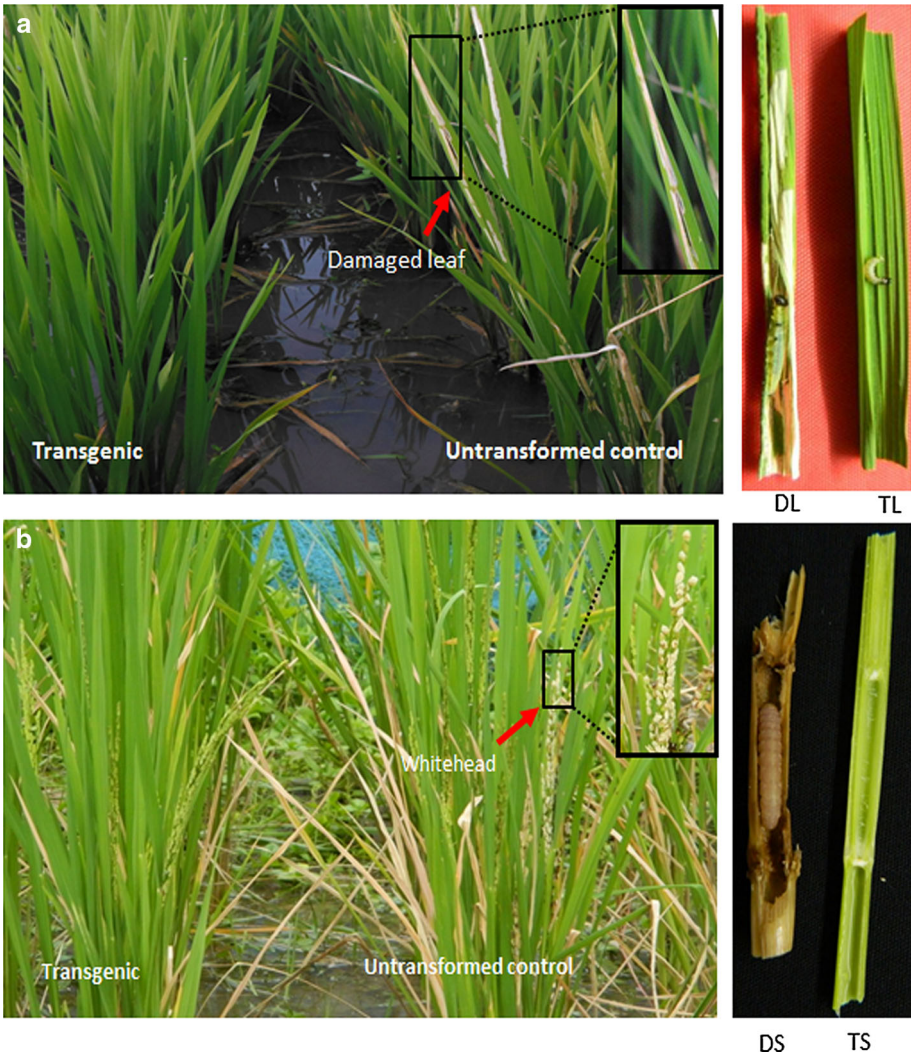

Fig. 3 Field bioassay of T_1 *syn vip*3BR transgenic rice lines **a** estimation of RLF mediated damage (*left panel*); untransformed control line (*right*), transgenic line (*left*) and damaged white leaf in untransformed control line (*inset*) **b** estimation of YSB mediated damage (*left panel*); untransformed control line (*right*), transgenic line (*left*) and whiteheads seen in untransformed control line (*inset*). Detached damaged leaf/stem from untransformed control line vs intact leaf/stem from transgenic lines is shown in respective *right panels*. *DL* damaged leaf, *TL* leaf from transgenic plant, *DS* damaged stem, *TS* stem from transgenic plant

Table 2 Field bioassay of T_1 *syn vip*3BR transgenic plant lines and untransformed Swarna (ALPGE, IIT Kharagpur, 2014)

Line name	Damaged by stem borers (%)[a]	Damaged by leaf folders[b]	
		Number of tillers affected (%)	Number of damaged leaves per tiller
Control (UC)	86.66 ± 0.57	92.22 ± 4.50	2.76 ± 0.11
SA	55.00 ± 1.73*	9.72 ± 2.51***	0.29 ± 0.06*
SD	18.33 ± 0.57***	0.00 ± 0.00***	0.00 ± 0.00*
SE	78.33 ± 0.57	11.94 ± 2.08***	0.35 ± 0.05*
SG	48.33 ± 1.15**	1.94 ± 0.57***	0.05 ± 0.01*

[a] , [b]Values are presented as mean ± SD, for triplicate readings of 20 sample plants in each case. Means are significantly different from the control (* $P < 0.05$), (* * $P < 0.01$), (* * * $P < 0.001$)

N-terminal 200 amino acid deletion mutant (Ndv200) of *vip3BR* gene was put to test as a prospective candidate for broad spectrum insect resistance in rice. Codon optimization was carried out as earlier reports show that the modification of the coding sequence with plant preferred codon usage enhanced the expression of *Bt* gene in plant (Perlak et al. 1991). Constitutive expression of toxin proteins in transgenic plants may cause several adverse effects, such as adding metabolic burden on cell, potential risk of resistance of the target insects towards *Bt* toxins (Corrado and Karali 2009; Ye et al. 2009) and this could also raise potential bio-safety concerns about GE rice (Conner et al. 2003; Ye et al. 2012). The common consumers are unlikely to accept unnecessary expression of the foreign toxin

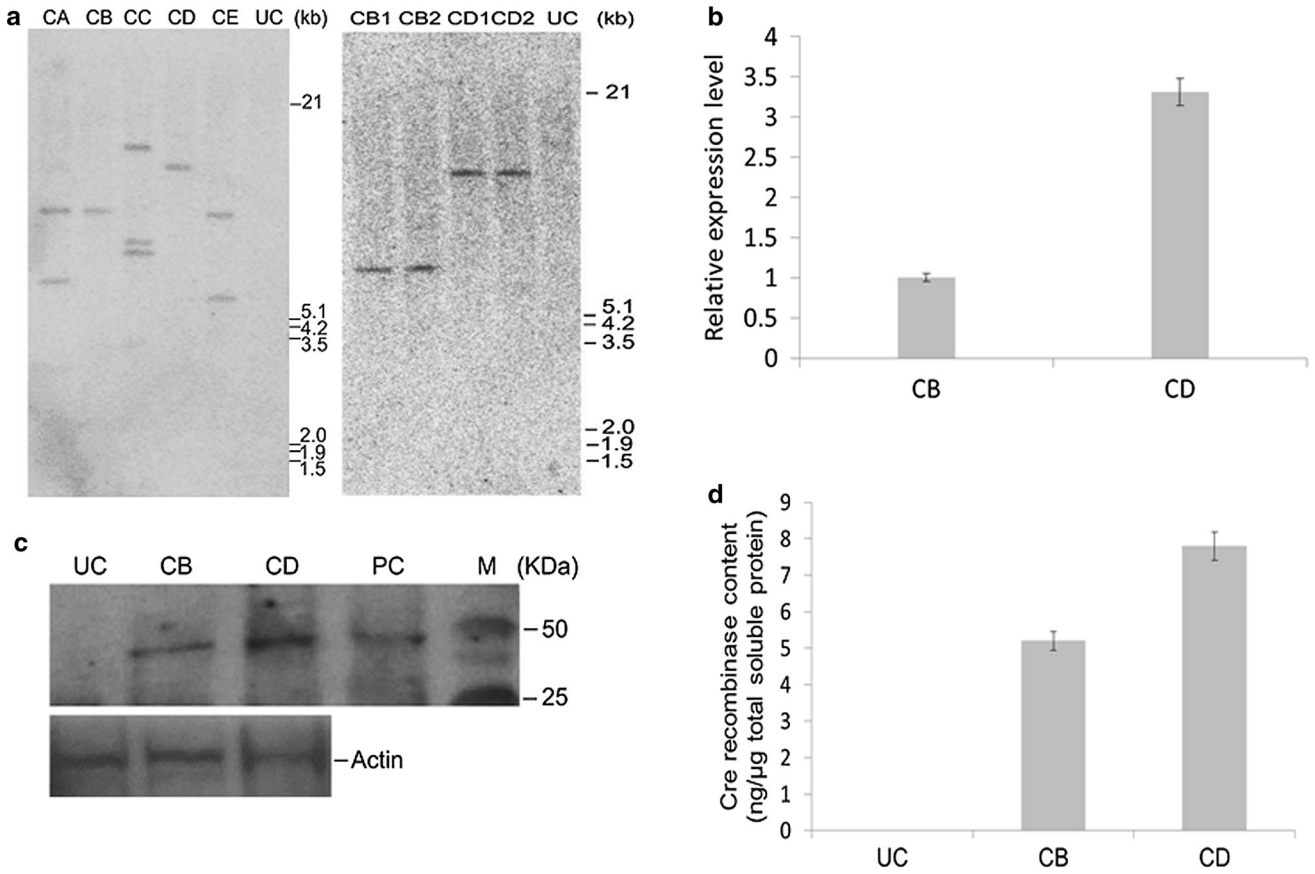

Fig. 4 Molecular analysis of *cre* recombinase gene integration and expression in T_1 rice lines **a** Southern blot analysis of *Hin*dIII digested genomic DNA from leaves of T_0 (*left panel*) and representative ($n = 2$) T_1 (*right panel*) pBUC rice lines probed with (α-32P) dCTP labeled *cre* recombinase gene. **b** Normalized, relative expression level of *cre* recombinase gene in T_1 transgenic rice lines by quantitative RT-PCR analysis. Results are expressed as mean ± SD, for three biological replicates (three individual T_1 plants from same category were sampled). **c** Immunoblot analysis of Cre recombinase protein in T_1 progeny plants (*Upper panel*). Actin is used as loading control (*Lower panel*). **d** Quantitative expression analysis of Cre recombinase protein in total soluble protein of T_1 plant leaves by ELISA. Results are expressed as mean ± SD, for three biological replicates (three individual T_1 plants from same category were sampled). *UC* untransformed control, *PC* purified Cre, *M* molecular mass marker

Table 3 Crosses between T_1 *syn vip*3BR and *cre* lines and estimation of the frequency of Cre recombinase mediated excision

Cross no.	T_1 parent (female)	T_1 parent (male)	Hybrid line ID	Total plants	*cre* positive plants	*syn vip*3BR and *cre* positive plants	*hpt*II negative plants	Recombination frequency (%)
1	SD9	CB	SD9CB	40	25	17	7	41.18
2	SA2	CB	SA2CB	37	25	14	5	35.71
3	SG8	CB	SG8CB	41	27	17	6	35.29
4	SE3	CB	SE3CB	32	18	12	4	33.33
Total				150	95	60	22	36.66

throughout plant system (Bakhsh et al. 2012), particularly the edible parts. Chloroplast targeting of toxin proteins, driven by green tissue specific promoter (*rbcS*) can serve as an alternative strategy in this regard (Kiani et al. 2013), as majority of the newly hatched lepidopteran larvae initially feed by scraping chlorophyll in the tender leaves (Gore et al. 2002). Following similar strategy, Wong et al. (1992) and Kim et al. (2009) reported a 10 to 20-fold increase in both the mRNA and protein levels, as compared to the expression driven by the CaMV35S promoter, when *cry1Ac* gene is fused with *Arabidopsis rbcS* promoter and chloroplast-targeting transit peptide. In the present study, all the above listed modifications reflected in high level expression of transgenic toxin Syn vip3BR (ranging from

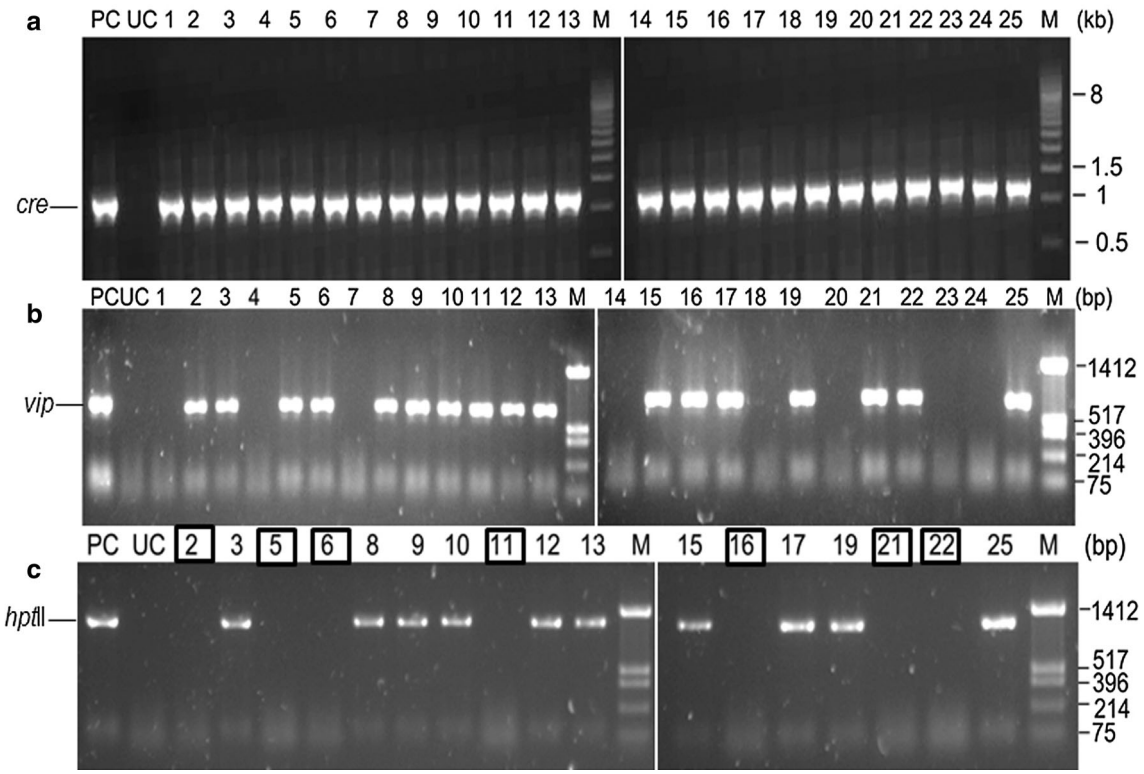

Fig. 5 Molecular analysis of marker gene excision **a** PCR analysis of bialaphos selected T_1F_1 hybrid line SD9CB with *cre* specific primers. Lane PC: PCR from pBUC plasmid as positive control; Lanes 1–25: 25 T_1F_1 hybrid progenies of line SD9CB. The 1214 bp amplicon represented the *cre* recombinase. **b** PCR with *syn vip*3BR specific primers of 25 *cre* positive plants of T_1F_1 hybrid line SD9CB. 17 plants showed amplification of representative 721 bp region of *syn vip*3BR gene. Lane PC: pLHRV as template; **c** PCR with *hpt*II specific primers of 17 *syn vip*3BR and *cre* positive plants of SD9CB line. Lanes 1–17: T_1F_1 hybrid plants, where plant no. 2, 5, 6, 11, 16, 21 and 22 showed absence of *hpt*II. Lane PC: PCR from pLHRV; the 1060 bp amplicon represented the *hpt*II gene. Plant lines boxed in *black* showed successful elimination of *hpt*II gene. Lane M in (**a**), SimplyLoad^{TM} 500 bp DNA ladder, Lonza. Lane M in (**b**) and (**c**), *Hin*fI digested pUC18 DNA as molecular weight marker. Lane UC: represents PCR from genomic DNA of untransformed plant in each case. Independent gel figures are separated by *white border* in each case

0.18 to 0.89 % of total soluble protein in the fresh leaves). This probably resulted from chloroplast targeted expression under the control of green-tissue specific promoter, which probably helped the transgenic protein to evade the cytoplasmic proteases. This indicated the usefulness of the present study in optimizing the expression level of *syn vip*3BR gene. This should also be helpful in addressing the food safety concerns in a section of common consumers as this promoter activity is mostly limited to green tissue parts of rice plants with low to negligible activity in edible parts like endosperms or whole grain, as tested in the present study.

The truncated, codon optimized, chloroplast targeted Syn vip3BR toxin clearly demonstrates the insecticidal potency against YSB, RLF and RHC, as shown by in vitro detached leaf/stem bioassay experiments. The highly expressive *syn vip*3BR T_1 transgenic plant, SD (0.89 % Syn vip3BR of total soluble leaf protein) showed significant protection against major rice lepidopteran pests, resulting in highest mortality of YSB (86.6 %), RLF (80 %) as well as RHC (73.2 %) populations. This expression of Syn vip3BR was higher than the expression levels of earlier reported Cry1Ab and Cry1Ac in the range of 0.02–0.05 % of soluble proteins, causing a 10–50 and 76–92 % mortality for the striped stem borer (Fujimoto et al. 1993) and yellow stem borer (Nayak et al. 1997), respectively. As in in vitro bioassay, the transgenic rice lines with stable, high level expression (mostly confined to green tissue parts) of Syn vip3BR toxin also offered significant protection against RLF and YSB under small scale field conditions. All four *syn vip*3BR T_1 transgenic rice lines showed significant resistance against RLF in laboratory as well as field conditions. However, the line SE could not efficiently protect from YSB, unlike other three lines in field conditions. These happened probably due to low expression level of the Syn vip3BR toxin combined with the fact that stem tissues are not very rich in chlorophyll and thus, plant lines with higher level of Syn vip3BR expression only is capable of protecting the plants from YSB infestation. Thus, *syn vip*3BR serves as a prospective

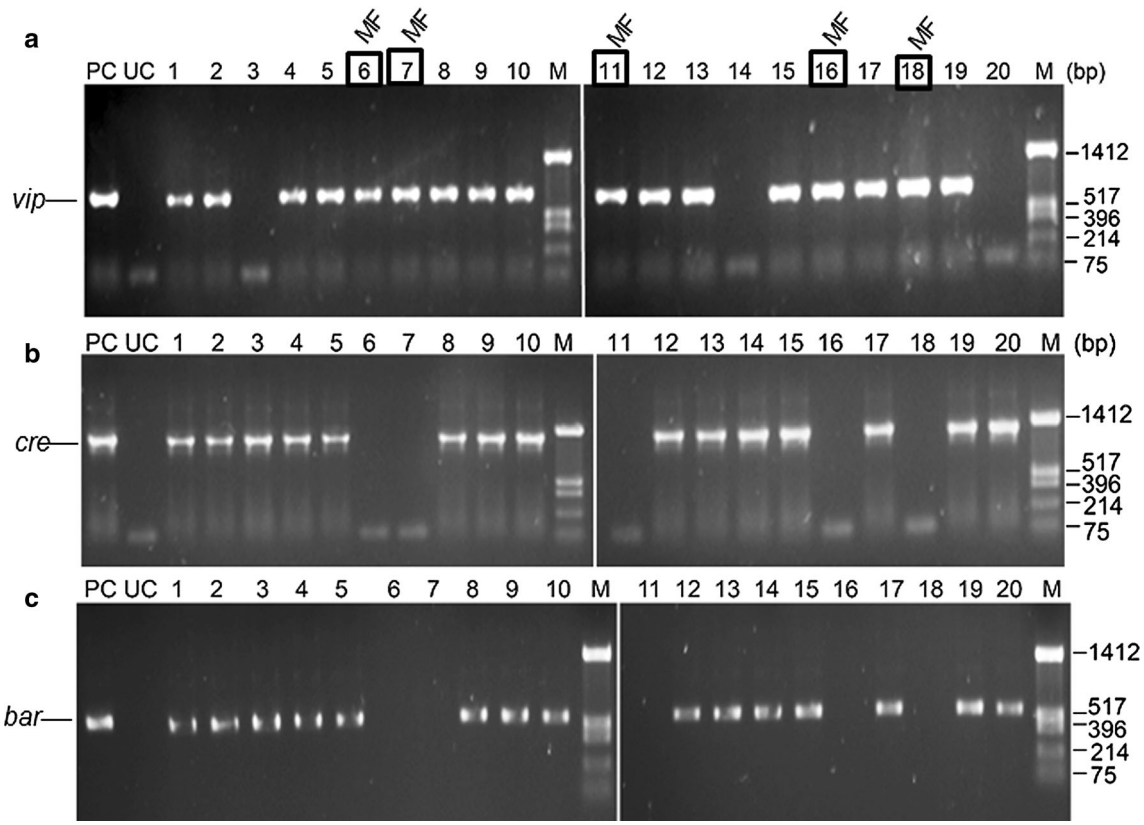

Fig. 6 PCR analysis of 20 randomly selected *hpt*II negative SD9CB (5) T_1F_2 plants to establish complete elimination of *cre-syn bar* gene. **a** PCR with *syn vip*3BR specific primers, **b** PCR with *cre* specific primers, **c** PCR with *syn bar* specific primers. *PC* positive control (amplification from *syn vip3BR* or *cre* gene containing expression constructs) and *UC* showing no amplification from untransformed control line. MF (Marker Free): represents T_1F_2 plants with complete elimination of all the selection markers (*hpt*II) + redundant transgenes (*cre-syn bar* linked gene) with retention of toxin gene. M *Hin*fI digested pUC18 as molecular weight marker. Independent gel figures are separated by *white border* in each case

candidate for developing second generation GE rice, with potential of broad spectrum insect resistance and also it is a suitable candidate for gene pyramiding with established Cry-toxins to further evaluate their potency in integrated pest management.

Retention of SMGs poses a major barrier towards development of consumer friendly GE rice because of perceived risk of horizontal gene transfer from plant to bacteria or from plant products consumed as food to intestinal microorganisms, with possibility of emergence of antibiotic resistance in them (Wogerbauer 2007; Ramessar et al. 2007; EFSA 2009). Together with this, use of lower numbers of transgene(s) with high efficacy towards broad spectrum insect pest mortality remained key issue for biosafety evaluation of transgenic rice. In the present study, an attempt was undertaken to extend the well-established Cre/*lox* technology (Hoa et al. 2002; Sreekala et al. 2005; Meszaros et al. 2014) for obtaining marker free transgenic rice lines with potential broad spectrum insecticidal activity. Some of the earlier reports in rice showed variable Cre/*lox*P mediated recombination frequency when different promoters were used to drive *cre* gene expression: 26.02 % by constitutive *ubiquitin* promoter (Hoa et al. 2002), 29.1 % by beta-estradiol inducible XVE promoter (Sreekala et al. 2005) and 11.1–54.5 % by constitutive CaMV35S promoter (Sengupta et al. 2010). The reason for variation of the recombination efficiency might be attributed to chromosomal location of *cre* transgene affecting expression level ("position effect" Matzke and Matzke 1998) or variations in the Cre recombinase activity in different events (Russell et al. 1992). In the present study, a consistent recombination efficiency or marker excision frequency was achieved in the range of 33.33–41.18 % among different crosses. The excision efficiency obtained in the present study is comparable or in some cases, higher compared to other systems where constitutive or transient *cre* expression was used such as 29–66 % in *Arabidopsis thaliana* using a beta-estradiol inducible XVE promoter (Zuo et al. 2001), 30–40 or 70–80 % in tobacco using a heat-shock inducible promoter in seeds or leaves, respectively (Wang et al. 2005), 40–59.7 % using heat shock inducible promoter in banana (Chong-Perez et al. 2012),

22.2–56.25 % in Brassica with constitutive CaMV35S promoter (Bala et al. 2013), 44–51 % in wheat using cold inducible promoter (Meszaros et al. 2014). Also, the hybrid plants in T_1F_1 generation showed no phenotypic abnormality after introgression of *cre* transgene.

Elimination of the antibiotic resistance marker from the *syn vip*3BR expression cassette could not generate complete marker free plants as the *cre* and *syn bar* genes remained in T_1F_1 hybrid plants. Further elimination of the transgenes (redundant with regard to our ultimate goal of broad spectrum insect resistance) was achieved in the subsequent generation (T_1F_2) as a result of genetic segregation. The successful event of complete marker elimination (*hpt*II and *cre-syn bar* linked gene) was confirmed by PCR analysis. Five plants were generated that harbored only the *syn vip*3BR expression cassette and *syn vip*3BR homozygous lines can be developed from them in future course of research.

In summary, our study, for the first time, demonstrated the transgenic expression of a codon optimized, chloroplast targeted, bioactive core toxic component of *vip*3BR gene under the control of green tissue specific promoter in an agronomically important rice variety, Swarna, with substantial insecticidal property against major lepidopteran insect pests both in vitro and under field assay conditions and elimination of all the selectable marker gene(s) from its progeny plants. Such an integrated approach itself advances the present state of art in the field of transgenic *Bt*-rice and the novelty of the present study is underscored in successful implementation of this all-inclusive strategy. The study will potentially be of importance in the future integrated pest management and should allay concerns in the minds of a section of common consumer. This strategy should contribute towards acceptability of engineered crop in consumer forum in accordance with the biosafety regulations and thus, facilitate future commercial deployment of transgenic *Bt*-rice, which in turn should help small-land holder farmers to effectively boost yield.

Author contribution statement SKS conceived and designed the research. Experiments were conducted by SP, AC, NS, SC, JB, JM and AM. SDG provided valuable scientific inputs during the course of study. SP and AC analyzed the data and wrote the manuscript in consultation with SKS and SDG. All the authors read and approved the MS.

Acknowledgments The authors extend their sincere appreciation for Partha Das and Sona Dogra for their assistance in generating the transgenic rice lines related to this study, Sudarshan Maity for maintenance and rearing of the genetic lines under outdoor cultural conditions. pX6-GFP plasmid DNA was a generous gift from Dr. N-H Chua, Rockefeller University, USA. Financial assistance from National Agricultural Innovation Project, Indian Council of Agricultural Research (NAIP/ICAR) in terms of grant support to Advanced Laboratory for Plant Genetic Engineering and fellowship to SP is thankfully acknowledged. Finally, the authors extend their sincere gratitude to the distinguished editor and the anonymous reviewers, whose incisive comments have helped immensely to improve the clarity of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no financial or commercial conflict of interest.

References

Almeida E, Gossele V, Muller CG, Dockx J, Reynaerts A, Botterman J, Krebbers E, Timko MP (1989) Transgenic expression of two marker genes under the control of an *Arabidopsis rbcS* promoter: sequences encoding the Rubisco transit peptide increase expression levels. Mol Gen Genet 218:78–86

Bakhsh A, Siddique S, Husnain T (2012) A molecular approach to combat spatio-temporal variation in insecticidal gene (Cry1Ac) expression in cotton. Euphytica 183:65–74

Bala A, Roy A, Das A, Chakraborti D, Das S (2013) Development of selectable marker free, insect resistant, transgenic mustard (*Brassica juncea*) plants using Cre/lox mediated recombination. BMC Biotechnol 13:88. doi:10.1186/1472-6750-13-88

Bhalla R, Dalal M, Panguluri SK, Jagadish B, Mandaokar AD, Singh AK, Kumar PA (2005) Isolation, characterization and expression of a novel vegetative insecticidal protein gene of *Bacillus thuringiensis*. FEMS Microbiol Lett 243:467–472

Bhattacharyya J, Chowdhury AH, Ray S, Jha JK, Das S, Gayen S, Chakraborty A, Mitra J, Maiti MK, Basu A, Sen SK (2012) Native polyubiquitin promoter of rice provides increased constitutive expression in stable transgenic rice plants. Plant Cell Rep 31:271–279

Bhattacharyya J, Chakraborty A, Roy S, Pradhan S, Mitra J, Chakraborty M, Manna A, Sikdar N, Chakraborty S, Sen SK (2015) Genetic transformation of cultivated jute (*Corchorus capsularis* L.) by particle bombardment using apical meristem tissue and development of stable transgenic plant. Plant Cell Tiss. Organ. doi:10.1007/s11240-014-0702-2

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

Chelliah S, Gunathilagaraj K (2009) Pest management in rice-current status and future prospects. http://www.rkmp.co.in. Accessed 16 Mar 2015

Cheng X, Sardana R, Kaplan H, Altosaar I (1998) *Agrobacterium* transformed rice plants expression synthetic cryIA(b) and cryIA(c) genes are highly toxic to striped stem borer and yellow stem borer. Proc Natl Acad Sci 95:2767–2772

Chong-Perez B, Kosky RG, Reyes M, Rojas L, Ocana B, Tejeda M, Perez B, Angenon G (2012) Heat shock induced excision of selectable marker genes in transgenic banana by the Cre-lox site-specific recombination system. J Biotechnol 159:265–273

Chong-Pérez B, Reyes M, Rojas L, Ocaña B, Ramos A, Kosky RG, Angenon G (2013) Excision of a selectable marker gene in transgenic banana using a Cre/lox system controlled by an embryo specific promoter. Plant Mol Biol 83:143–152

Conner AJ, Glare TR, Nap JP (2003) The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. Plant J 33:19–46

Coppoolse ER, De Vroomen MJ, Roelofs D, Smit J, Van Gennip F, Hersmus BJM, Nijkamp HJJ, Van Haaren MJJ (2003) Cre

recombinase expression can result in phenotypic aberrations in plants. Plant Mol Biol 51:263–279
Corrado G, Karali M (2009) Inducible gene expression systems and plant biotechnology. Biotechnol Adv 27:733–743
Dandapat A, Bhattacharyya J, Gayen S, Chakraborty A, Banga A, Mukherjee R, Mandal CC, Hossain MA, Roy S, Basu A, Sen SK (2013) Variant *cry1Ab* entomocidal *Bacillus thuringiensis* toxin gene facilitates the recovery of an increased number of lepidopteran insect resistant independent rice transformants against yellow stem borer (*Scirpophaga incertulus*) inflicted damage. J Plant Biochem Biotechnol 23(1):81–92
Deist BR, Rausch MA, Fernandez-Luna MT, Adang MJ, Bonning BC (2014) Bt toxin modification for enhanced efficacy. Toxins 6:3005–3027
Demont M, Chen M, Ye G, Stein AJ (2013) Rice. In: Smyth SJ, Castle D, Phillips PWB (eds) Handbook on agriculture, biotechnology and development. Edward Elgar Publishing, Northampton **(Chapter 42)**
Doyle JJ, Doyle JJ (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15
Durand-Morat A, Chavez EC, Wailes EJ (2015) GM rice commercialization and its impact on the global rice economy. SAEA Annual Meeting, Atlanta, Georgia, February 2–3
EFSA (2009) Consolidated presentation of the joint Scientific Opinion of the GMO and BIOHAZ panels on the "Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants" and the scientific opinion of the GMO panel on "Consequences of the Opinion on the Use of Antibiotic Resistance Genes as Marker Genes in Genetically Modified Plants on Previous EFSA Assessments of Individual GM Plants". EFSA J 1108:1–8
Estruch JJ, Warren GW, Mullins MA, Nye GJ, Craig JA, Koziel MG (1996) Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc Natl Acad Sci 93:5389–5394
Fang J, Xu XL, Wang P, Zhao JZ, Shelton AM, Cheng J, Feng MG, Shen ZC (2007) Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Appl Environ Microbiol 73:956–961
Fujimoto H, Itoh K, Yamamoto M, Kyozuka J, Shimamoto K (1993) Insect resistant rice generated by introduction of a modified delta-endotoxin gene of *Bacillus thuringiensis*. Nat Biotechnol 11:1151–1155
Gayen S, Hossain MA, Sen SK (2012) Identification of the bioactive core component of the insecticidal Vip3A toxin peptide of *Bacillus thuringiensis*. J Plant Biochem Biotechnol 21(Suppl 1):S128–S135. doi:10.1007/s13562-012-0148-8
Gayen S, Samanta MK, Hossain MA, Mandal CC, Sen SK (2015) A deletion mutant ndv200 of the *Bacillus thuringiensis vip3BR* insecticidal toxin gene is a prospective candidate for the next generation of genetically modified crop plants resistant to lepidopteran insect damage. Planta. doi:10.1007/s00425-015-2309-1
Gilbertson L (2003) Cre-lox recombination: cre-active tools for plant biotechnology. Trends Biotechnol 21:550–555
Gore J, Leonard BR, Church CE, Cook DR (2002) Behavior of bollworm (Lepidoptera: Noctuidae) larvae on genetically engineered cotton. J Econ Entomol 95:763–769
Gould F (1998) Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu Rev Entomol 43:701–726
Grantham R, Perrin P, Mouchiroud D (1986) Pattern of codon usage of different kinds of species. Oxf Surv Evol Biol 3:48–81
Hoa TTC, Bong BB, Huq E, Hodge TK (2002) Cre/lox site-specific recombination controls the excision of a transgene from the rice genome. Theor Appl Genet 104:518–525
James C (2014) Global status of commercialised biotech/GM crops. ISAAA Briefs ISAAA, New York **(37)**
Khanna HK, Raina SK (2002) Elite indica transgenic rice plants expressing modified Cry1Ac endotoxin of *Bacillus thuringiensis* show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). Transgenic Res 11:411–423
Khattri A, Nandy S, Srivastava V (2011) Heat-inducible Cre-lox system for marker excision in transgenic rice. J Biosci 36:37–42
Khush GS (2005) What it will take to Feed 5.0 Billion Rice consumers in 2030. Plant Mol Bio 59:1–6
Kiani S, Mohamed BB, Shehzad K, Jamal A, Shahid MN, Shahid AA, Husnain T (2013) Chloroplast-targeted expression of recombinant crystal-protein gene in cotton: an unconventional combat with resistant pests. J Biotechnol 166:88–96
Kim EH, Suh SC, Park BS, Shin KS, Kweon SJ, Han JE, Park SH, Kim YS, Kim JK (2009) Chloroplast-targeted expression of synthetic cry1Ac in transgenic rice as an alternative strategy for increased pest protection. Planta 230:397–405
Kopertekh L, Schiemann J (2012) Elimination of transgenic sequences in plants by *cre* Gene expression, transgenic plants—Advances and Limitations, PhD. Yelda Ozden Çiftçi (ed), ISBN: 978-953-51-0181-9, InTech, Available from: http://www.intechopen.com/books/transgenic-plants-advances-andlimitations/elimination-of-transgenic-sequences-in-plants-by-cre-gene-expression
Lee MK, Walters FS, Hart H, Palekar N, Chen JS (2003) The mode of action of the *Bacillus thuringiensis* vegetative insecticidal Protein Vip3A differs from that of Cry1Ab δ endotoxin. Appl Environ Microbiol 69:4648–4657
Li Y, Hallerman EM, Liu Q, Wu K, Peng Y (2015) The development and status of *Bt* rice in China. Plant Biotechnol J. doi:10.1111/pbi.12464
Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408
Luo K, Duan H, Zhao D, Zheng X, Deng W, Chen Y, Stewart CN, McAvoy R, Jiang X, Wu Y, He A, Pei Y, Li Y (2007) "GM-gene-deletor": fused loxP-FRT recognition sequences dramatically improve the efficiency of FLP or Cre recombinase on transgene excision from pollen and seed of tobacco plants. Plant Biotechnol J 5(2):263–274
Mandal CC, Gayen S, Basu A, Ghosh KS, Dasgupta S, Maiti MK, Sen SK (2007) Prediction-based protein engineering of domain I of Cry2A entomocidal toxin of *Bacillus thuringiensis* for the enhancement of toxicity against lepidopteran insects. Protein Eng Des Sel 20(12):599–606
Matzke AJM, Matzke MA (1998) Position effects and epigenetic silencing of plant transgenes. Curr Opin Plant Biol 1:142–148
Meszaros K, Eva C, Kiss T, Banyai J, Kiss E, Teglas F, Lang L, Karsai I, Tamas L (2014) Generating marker-free transgenic wheat using minimal gene cassette and cold-inducible Cre/Lox system. Plant Mol Biol Rep. doi:10.1007/s11105-014-0830-1
Miki B, Abdeen A, Manabe Y, MacDonald P (2009) Selectable marker genes and unintended changes to the plant transcriptome. Plant Biotechnol J 7:211–218
Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:437–497
Murray EE, Lotzer J, Eberle M (1989) Codon use in plant genes. Nucl Acids Res 17:477–498
Naranjo S (2009) Impacts of Bt crops on non-target invertebrates and insecticide use patterns. CAB Rev Perspect Agric Vet Sci Nutrit Nat Resour 4:1–23
Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, Sen SK (1997) Transgenic elite indica rice plants expressing CryIAc δ-endotoxin of *Bacillus thuringiensis* are

resistant against yellow stem borer (*Scirpophaga incertulas*). Proc Natl Acad Sci 94:2111–2116

Pathak MD, Khan Z (1994) Insect pests of rice. International Rice Research Institute, Los Banos. ISBN 971-22-0028-0

Perlak FJ, Fuchs RL, Dean DA, Mc Pherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci 88:3324–3328

Pillay P, Schluter U, van Wyk S, Kunert KJ, Vorster BJ (2014) Proteolysis of recombinant proteins in bioengineered plant cells. Bioengineered 5(1):15–20

Qaim M (2009) The economics of genetically modified crops. Annu Rev Resour Econ 1:665–693

Ramessar K, Peremarti A, Gomez-Galera S, Naqvi S, Moralejo M, Munoz P, Capell T, Christou P (2007) Biosafety and risk assessment framework for selectable marker genes in transgenic crop plants: a case of the science not supporting the politics. Transgenic Res 16:261–280

Raybould A, Vlachos D (2011) Non-target organism effects tests on Vip3A and their application to the ecological risk assessment for cultivation of MIR162 maize. Transgenic Res 20:599–611

Russell SH, Hoopes JL, Odell JT (1992) Directed excision of a transgene from the plant genome. Mol Gen Genet 234:49–59

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Selvapandiyan A, Arora N, Rajagopal R, Jalali SK, Venkatesan T, Singh SP, Bhatnagar RK (2001) Toxicity analysis of N- and C-terminus-deleted vegetative insecticidal protein from *Bacillus thuringiensis*. Appl Environ Microbiol 67(12):5855–5858

Sengupta S, Chakraborti D, Mondal HA, Das S (2010) Selectable antibiotic resistance marker gene-free transgenic rice harbouring the garlic leaf lectin gene exhibits resistance to sap-sucking planthoppers. Plant Cell Rep 29:261–271

Shu Q, Ye G, Cui H, Cheng X, Xiang Y, Wu D, Gao M, Xia Y, Hu C, Sardana R, Altosaar I (2000) Transgenic rice plants with a synthetic *cry*1Ab gene from *Bacillus thuringiensis* were highly resistant to eight lepidopteran rice pest species. Mol Breeding 6:433–439

Sreekala C, Wu L, Gu K, Wang D, Tian D, Yin Z (2005) Excision of a selectable marker in transgenic rice (*Oryza sativa* L.) using a chemically regulated Cre/*lox*P system. Plant Cell Rep 24:86–94

Sreevathsa R, Solanke AU, Kumar PA (2015) Biotechnology for insect pest management in vegetable crops. Recent advancements in gene expression and enabling technologies in crop plants, Chapter 10. Springer, New York, pp 313–340

Tabashnik BE, Brévault T, Carrière Y (2013) Insect resistance to Bt crops: lessons from the first billion acres. Nat Biotechnol 31:510–521

Tuteja N, Verma S, Sahoo RK, Raveendar S, Reddy IBL (2012) Recent advances in development of marker free transgenic plants: regulation and biosafety concern. J Biosci 37:162–197

Van Rie J, McGaughey WH, Johnson DE, Barnett BD, Van Mellaert H (1990) Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science 247:72–74

Wang Y, Chen B, Hu Y, Li J, Lin Z (2005) Inducible excision of selectable marker gene from transgenic plants by the Cre/*lox* site-specific recombination system. Transgenic Res 14:605–614

Wogerbauer MM (2007) Risk assessment of antibiotic resistance marker genes in genetically modified organisms. Forschungsberichte der Sektion IV, Band 5/2007:1–135

Wong EY, Hironaka CM, Fischhoff DA (1992) *Arabidopsis thaliana* small subunit leader and transit peptide enhance the expression of *Bacillus thuringiensis* proteins in transgenic plants. Plant Mol Biol 20:81–93

Yau Y-Y, Stewart CN Jr (2013) Less is more: strategies to remove marker genes from transgenic plants. BMC Biotechnol 13:36

Ye GY, Shu QY, Cui HR, Hu C, Gao MW, Xia YW, Cheng X, Altosaar I (2000) A leaf-section bioassay for evaluating rice stem borer resistance in transgenic rice containing a synthetic *cry*1Ab gene from *Bacillus thuringiensis* Berliner. Bull Entomol Res 90:179–182

Ye RJ, Huang HQ, Yang Z, Chen TY, Liu L, Li XH, Chen H, Lin YJ (2009) Development of insect resistant transgenic rice with Cry1C*-free endosperm. Pest Manag Sci 65:1015–1020

Ye R, Zhou F, Lin Y (2012) Two novel positive cis-regulatory elements involved in green tissue-specific promoter activity in rice (*Oryza sativa* L ssp.). Plant Cell Rep 31:1159–1172

Zhang YJ, Li YH, Zhang Y, Chen Y, Wu KM, Peng YF, Guo YY (2011) Seasonal expression of Cry1Ab and Cry1Ac proteins in transgenic rice lines and their resistance against striped rice borer *Chilo suppressalis* (Walker). Transgenic Plants Insects 40(5):1323–1330

Zhao J-Z, Cao J, Li Y, Collins HL, Roush RT, Earle ED, Shelton AM (2003) Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat Biotechnol 21:1493–1497

Zuo J, Niu QW, Moller SG, Chua NH (2001) Chemical-regulated, site-specific DNA excision in transgenic plants. Nat Biotechnol 19:157–161