GENERATION AND EVALUATION OF TWO-GENE *Bt* TRANSGENICS OF *INDICA* RICE (*Oryza sativa* L.)

Suresh Kumar

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Dedicated to :

my Beloved Parents and Niece Dolly

GENERATION AND EVALUATION OF TWO-GENE *Bt* TRANSGENICS OF *INDICA* RICE (*Oryza sativa* L.)

A Thesis

by

Suresh Kumar

submitted to the faculty of Post-Graduate School,
Indian Agricultural Research Institute, New Delhi,
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Molecular Biology and Biotechnology

2004

Approved by:

Advisory Committee

Chairman:

Dr. S. K. Raina

Members:

Dr. N. K. Singh

Dr. K. V. Prabhu

Dr. A. Sachdev

Certificate

This is to certify that the thesis entitled "Generation and Evaluation of Two-gene Bt Transgenics of Indica Rice (Oryza sativa L.)" submitted in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology, to the Faculty of Post Graduate School, Indian Agricultural Research Institute New Delhi, embodies the results of *bonafide* research work carried out by Mr. Suresh Kumar, under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma. The assistance and help received during the course of investigation has been duly acknowledged by him.

Place: *New Delhi*

Date: *24th December, 2004.*

(S.K.Raina)
Chairman
Advisory Committee

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Suresh Kumar

(Suresh Kumar)

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INTRODUCTION

Rice (*Oryza sativa* L.) is the world's most important crop as it is the major source of food and income for about 40% of the global population. In Asia, rice is the lifeline. More than 90% of the world's rice production, currently about 520 million tonnes, is produced in Asia, providing livelihood for 3100 million people in the region. With the growing population of the continent, by about 56 million every year, the domestic demand of rice is expected to exceed 770 million tonnes by the year 2025. If the present trends continue, within 20 years, most of the Asian countries will no longer be self-sufficient in rice. In the latest session of the International Rice Commission, held in Cairo, rice experts were describing the challenges ahead as "mind boggling". It was estimated that over the next 30 years, the yield ceiling of irrigated rice in Asia will need to be doubled, and this will have to be achieved using less land, water, labour and fewer chemical inputs, particularly pesticides.

Out of the 22 species of the genus *Oryza*, only two, *O. sativa* and *O. glaberrima*, are cultivated. The three subspecies of *O. sativa* (*indica*, *japonica* and *javanica*) are grown in different regions of the world. *Indica* rice is the most widely grown subspecies, occupying 80% of the cultivated area. Maintaining adequate supply of rice, with diminishing natural resources and increasing biotic and abiotic stresses, for the alarming annual increase in rice consuming population of Asia, is a formidable challenge to the rice growing and consuming countries.

Rice improvement through conventional breeding methods has met with considerable success. Green Revolution technologies, which spurred increases in annual rice production by more than 3%, are now considered "almost exhausted". Recent advances in modern biology, especially molecular biology and genetic engineering, offer numerous opportunities for genetic enhancement and meeting the challenges of declining productivity of rice. Genetic transformation methods have made it possible to introduce novel genes into rice, across barriers of genomic and sexual incompatibilities (Cao *et al.*, 1992; Lucca *et al.*, 2001). Transgenic rice plants carrying desirable traits such as enhanced resistance to insect pest, disease or herbicide tolerance, are becoming available (Christou *et al.*, 1991; Fujimoto *et al.*, 1993; Rathore *et al.*, 1993; Lin *et al.*, 1995; Wunn *et al.*, 1996; Alam *et al.*, 1998; Tu *et al.*, 1998; Maqbool *et al.*, 2001; Khanna and Raina, 2002). Commercialization of transgenic Bt crops has been approved in several countries including some Asian countries such as India and China. In the year 2003, more than 18% of the global area under transgenic crops was planted with Bt transgenic crops (James 2003). Philippines is the first Asian country to have approved a transgenic Bt-food crop (corn) for commercial cultivation, in 2003.

Out of approximately 67000 pest species that damage agricultural crops, about 9000 species are insects and mites (Ross and Lembi, 1985). Pest associated losses in major crops vary from 52% in wheat to about 84% in cotton (Oerke *et al.*, 1994). Insects not only inflict direct losses to the agricultural production, but also inflict damage indirectly due to their role as vectors of various plant pathogens. In addition to these losses in production, they also increase the cost of production by way of pesticides used, currently valued at US $10 billion annually. Extensive use

of pesticides impacts insect ecology, soil health, human health and ground water resources.

About 100 insect species are known to visit rice. Of these, 20 are known to cause significant economic damage. The lepidopteran stem borers are generally considered to be the most destructive pests, causing estimated losses of 5-30% of the total yield of rice. Recovery of 5% of this yield loss (equivalent to 25 million tons of rice) could provide food for one year for the approximately 140 million people. Yellow stem borer (*Scirpophaga incertulas*) and striped stem borer (*Chilo suppressalis*) are widely distributed in Asia.

Enhancing insect resistance in crop plants has been one of the major objectives in crop improvement. However, only a little is understood about the mechanisms of insect resistance. Despite screening of more than 30,000 accessions of rice at IRRI, Philippines, none of the rice varieties developed so far have more than a moderate level of resistance (Khan *et al.*, 1991). Hence recombinant-DNA technologies are being considered to enhance resistance.

In this period of transition, from "Green Revolution" to "Gene Revolution" technology, we are witnessing remarkable changes in biological sciences made possible by advances in biotechnology and bioinformatics. The power of genetic engineering has created unprecedented opportunities, not only for the manipulation of biological systems, but also for undertaking studies to understand the fundamental processes of life. It provides unusual scope for tailoring plants to suit different growing conditions and consumer needs. For example, the use of

transgenes to enhance insect-resistance in crop plants provides an effective alternative to the massive use of insecticides for control of insect pests in agricultural fields.

Chemical control of stem borers is difficult and often ineffective because the larvae hide inside the stem, protected from sprayed insecticides. Genetic transformation of crop plants with *Bt* gene(s) is an important technological advancement, as it is highly toxic, yet highly specific. However, as is the case with other genes for enhancing resistance, insect pests will eventually develop resistance to Bt toxins. Therefore, concerted efforts must be made to delay breakdown of resistance against the powerful tool. Many strategies to manage resistance to Bt have been proposed (Cohen *et al.*, 2000; Shelton *et al.*, 2000, 2002). There is widespread agreement that the "high-dose/refuge" strategy is the most promising and practical approach to prolong the effectiveness of Bt toxin. Another important recommendation has been the deployment of two or more insecticidal genes with different binding sites and/or different mode of action.

The present research work was taken up for genetic transformation of an elite *indica* rice breeding line, Pusa Basmati-1 (a semi-dwarf, high yielding and fine grain aromatic rice variety, but highly susceptible to many insect pests and diseases). The specific objective was to develop Bt transgenic lines having more than one Bt *cry* genes driven by specific promoters. It was also proposed that only 'clean DNA' transformation approach will be used to develop such transgenics, in view of concerns voiced by several stakeholders against incorporation of plasmid vector backbone sequences into the plant genome.

REVIEW OF LITERATURE

Rice is the major staple food crop in India, grown on 25% of the total cropped area and accounts for about 42% of the country's food production. While the current rice production is about 90 million tons, the projected requirement (year 2025) has been estimated to be about 140 million tons. Since independence, the population has grown from about 350 million to more than 1 billion and is projected to reach 1.6 billion by the year 2050. Though Indian agriculture has traveled a long way, however the growing population is a grim reminder of the need to still produce more food if the nation were not to relapse into deficit. The challenges in crop improvement are appearing at a much faster pace than ever before, with diminishing natural resources and increasing biotic and abiotic stresses. Biotic stresses take a heavy toll of crop productivity. In India, insects (Table 1), nematodes, weeds and fungal, bacterial and viral pathogens, collectively result an average of about 45% yield loss (Agnihotri 1999). World crop losses without pesticide use and other non-chemical control strategies have been estimated to be about 70% of the production. Despite all efforts to prevent pre-harvest losses, pests continue to destroy over half of the global crop production (Pimentil and Raven 2000). Only 5% recovery of the damage caused by the rice stem borers can provide food for 140 million people for one year.

Stem borers belong to the order Lepidoptera. Adult moths (Fig. 1a) lay eggs mostly on the leaf blades (Fig. 1b). The larvae (Fig. 1c) bore into the plant stem

Table 1. Losses caused by some important pests

Pest		Productivity Loss (%)
Common Name	**Scientific Name**	
Brown Plant Hopper	*Nilaparvata lugens*	10-70
Rice gall midge	*Orseolia oryzae*	15-60
Rice stem borer	***Scirpophaga incertulas***	**25-30**
Sorghum shoot fly	*Antherigona soccata*	30-60
Sorghum midge	*Contarinia sorghicola*	30-50
Maize pink borer	*Sesamia inferens*	25-75
Pulse stem fly	*Ophiomyia sp.*	50-72
Mustard aphid	*Lipaphis erysimi*	30-70
Cotton boll worm	*Spodoptera sp. & others*	50-60
Cotton white fly	*Bemisia tabaci*	53-80
Groundnut leafminer	*Aproaerema modicella*	24-92
Safflower aphid	*Dactynotus carthami*	9-67
Caster semilooper	*Achea janata*	40
Linseed budfly	*Dasineura lini*	30
Brinjal fruit & stem borer	*Lucinodes orbonalis*	48
Chilli midge	*Aspordylia capsici*	60-80

Source: Agnihotri, 1999.

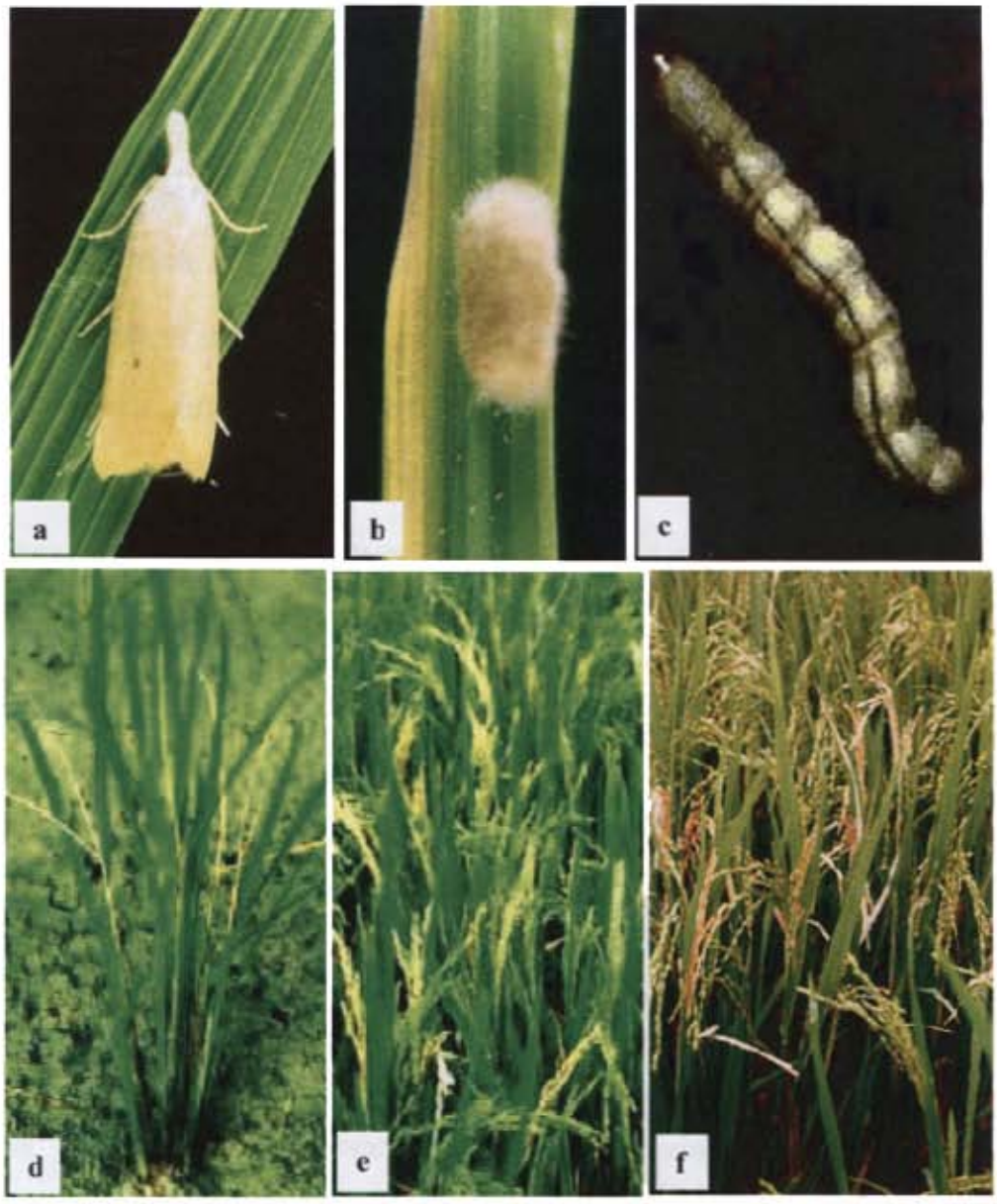


Fig. 1. Important insect pest of rice and the damages caused: **(a)** adult moth of yellow stem borer (YSB), **(b)** eggs of YSB, **(c)** larva of YSB, **(d)** dead-hearts: the damage caused by YSB at vegetative stage, **(e)** white-heads: the damage caused by YSB at reproductive stage, and **(f)** the protected crop with normally filled grains.

and damage it while feeding inside the stem. Attacks during the vegetative growth stage of the plant (seedling to stem elongation) result into the death of the central shoot, the symptom typically known as "dead-heart" (Fig. 1d). The damaged shoots do not produce panicle, and thus, no grain. Attacks of stem borers during the reproductive stage (panicle initiation to milk grain) cause severing of the developing panicle at its base. As a result, the panicle becomes chaffy and white in color. Such empty panicles are called "white-heads" (Fig. 1e). Chemical control of stem borers is difficult and often ineffective, moreover, the extensive use of pesticides results in adverse effects on the beneficial organisms, leaves pesticide residues in the food and results in the environmental pollution. Except for some moderate levels, genetic sources of resistance have not been identified despite extensive screening of rice accessions (Khan *et al.*, 1991). It is in this context transgenic technology becomes an important and relevant part of the equation.

2.1 Genetic transformation of rice

Several methods of introducing foreign DNA into protoplasts and intact cells are available. Following methods have been repeatedly successful: (i) polyethylene-glycol (PEG) and electroporation methods for introduction of DNA into protoplasts, (ii) Biolistic, and (iii) *Agrobacterium*-mediated methods of gene transfer for intact cells. In the absence of *Agrobacterium*-mediated T-DNA delivery system, protoplast transformation method was the only system of choice for transformation in rice. However, development of fertile transgenic plants from protoplasts is a laborious, time-consuming and genotype dependent process. In the past few years, methods for transformation of intact cells have been developed, and these include Biolistic and *Agrobacterium*-mediated methods of transformation.

Biolistic method of gene transfer has emerged as a simple and promising alternative for cereal transformation and has been successfully used in maize, rice, sugarcane and wheat transformation.

Since monocots, including rice, are not a natural host to *Agrobacterium tumefaciens* infection, the transformation methods employed were largely restricted to direct gene transfer into isolated protoplasts. In early 1990s, attempts were made to introduce marker genes into some cereal plants using the T-DNA delivery system of *Agrobacterium.* (Raineri *et al.*, 1990; Mooney *et al.*, 1991; Chan *et al.*, 1992; Li *et al.*, 1992). Chan *et al.* (1993) were among the pioneers to demonstrate inheritance of transferred DNA in the progenies of transgenic rice plants generated using *Agrobacterium*. The most convincing demonstration of *Agrobacterium*-mediated rice transformation was shown by Hiei *et al.* (1994). *Agrobacterium*-mediated transformation method is preferred in many cases, as it does not involve protoplasts, and in general, results in higher transformation efficiency and a more predictable pattern of foreign DNA integration than any other transformation method (Hiei *et al.*, 1994; Sivamani *et al.*, 1996; Rashid *et al.*, 1996; Toki 1997). Although, *Agrobacterium* relies on a host-parasite interaction in order to be successful, the limitations have been overcome with our increased knowledge of the biological vector (Hansen *et al.*, 1994; Hiei *et al.*, 1994; Ishida *et al.*, 1996).

The first transgenic rice was reported for japonica varieties by three independent groups (Toriyama *et al.*, 1988; Zhang *et al.*, 1988; Zhang and Wu, 1988). The first fertile transgenic *indica* rice was obtained by Datta *et al.* (1990). In the recent past,

reports on transformation of *indica* rice have been accumulating (Christou *et al.*, 1991; Datta *et al.*, 1992; Hayakawa *et al.*, 1992; Peng *et al.*, 1992; Cao *et al.*, 1992; Fujimoto *et al.*, 1993; Rathore *et al.*, 1993). Currently, several protocols for transformation of *indica* rice by *Agrobacterium*-mediated (Hiei *et al.*, 1994; Sivamani *et al.*, 1996; Rashid *et al.*, 1996; Toki 1997; Cheng *et al.*, 1998; Khanna and Raina, 1999) and biolistic (Christou and Ford, 1995; Wunn *et al.*, 1996; Ghareyazie *et al.*, 1997; Khanna and Raina, 2002) methods are available.

2.2 Biolistic transformation

Biolistic method was invented by John Sanford at Cornell University (Sanford *et al.*, 1987). The particle acceleration system as a method of gene delivery in intact cells and tissues has been also employed in cereals, including rice. Christou *et al.* (1991) were the first to obtain transgenic rice plants by DNA delivery into immature embryos of several commercially important *indica* and *japonica* rice cultivars using Acell technology. Later, Cao *et al.* (1992) reported the production of transgenic rice plants from embryogenic cell suspensions using the helium driven (PDS-1000/He) biolistic gun. Since then, this method has been widely used for the generation of transgenic rice plants (Li *et al.*, 1993; Duan *et al.*, 1996; Wunn *et al.*, 1996; Ghareyazie *et al.*, 1997; Khanna *et al.*, 1997; Datta *et al.*, 1998; Tu *et al.*, 1998; Khanna and Raina, 2002).

A major advantage of biolistic transformation is that a wide variety of explant tissues can be used as targets for gene delivery. Therefore, this technique can be tested for transformation of species and cultivars that are not readily amenable to plantlet regeneration from protoplasts. Moreover, this method can be used to study

developmentally regulated gene expression in specialized cells or tissues by introducing different gene constructs into spatially distinct plant parts at particular stages in their development (Khush and Toenniessen, 1991). Multiple genes can be introduced by co-transformation (Hadi *et al.*, 1996; Chen *et al.*, 1998) and larger fragments of DNA, including yeast artificial chromosome, can be introduced (VanEck *et al.*, 1995). When combined with the 'clean DNA' transformation method, it produces transgenic plants with very simple integration patterns and low transgene copy numbers, resulting in stable expression of the transgene over many generations and almost no gene silencing (Fu *et al.*, 2000; Loc *et al.*, 2002).

2.2.1 Clean DNA transformation

The two commonly used rice transformation techniques, viz. Direct DNA transfer (Christou *et al.*, 1991), and *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994), generally leads to the integration of vector backbone sequences into the host genome (Knonov *et al.*, 1997). The integration of vector backbone may exert undesirable effects in *cis* (Artelt *et al.*, 1991) as well as promotes transgene rearrangements. Although, it has been a common practice for all the vector backbone sequences to be removed before microinjecting the foreign DNA into animal eggs or embryos (Palmiter and Brinster, 1986), whole plasmid bombardment is a common practice for plant transformation. In case of microprojectile-mediated direct DNA transfer, the vector backbone sequences of the linear or super-coiled plasmid, get integrated into the host plant genome along with the transgene(s). Even in the case of *Agrobacterium*-mediated transformation, initially thought to generate only 'clean' transgenic events, however, may show as

high as 33% transformants having T-DNA exceeding the left border repeat (Wenck *et al.*, 1997). Integrated vector backbone sequences may serve as recombination hotspots leading to illegitimate recombination, transgene rearrangement and plasmid multimerization, made possible by the presence of any AT rich recombinogenic sequences in the plasmid backbone (Muller *et al.*, 1999). Besides, a large transgenic locus may be meiotically unstable and, thereby, more prone to excision and loss of transgene expression (Stoger *et al.*, 1998). There is also possibility of new replicons, comprising of plasmid *ori* and host plant genomic DNA, may escape into the environment (Fu *et al.*, 2000).

Unlike the *Agrobacterium*-mediated transformation, in which the use of whole plasmid is obligatory, vector backbone serves no specific purpose in biolistic transformation. Therefore, Fu *et al.*, (2000) investigated transgene integration and expression patterns in rice, following microprojectile-mediated direct DNA transformation using minimal transgene cassettes (promoter-open reading frame-terminator). Results revealed that such transformants had predominantly 'simple' integration events, produced transgenic plants with low-copy number and a low frequency of transgene re-arrangements. In a subsequent study, Loc *et al.* (2002) confirmed the utility of 'clean DNA' transformation technique for rice. Higher levels of transgene product accumulation were found in transgenics derived from minimal gene cassette transformations, in comparison to those derived from whole plasmids. Besides, they did not find any instances of transgene silencing after transformation with minimal gene cassettes, and co-integration of three genes was not affected by co-transformation with minimal gene-cassettes fragments. Co-

bombardment with two 'clean DNA' gene cassette fragments, one carrying the gene of interest and plant selectable marker gene on the other, allows integration of transgenes at different loci of the host genome. Such integrations facilitate separation of any unwanted marker gene from the gene of interest during segregation of the genes in the progenies.

A large number of Bt-transgenic lines of elite *indica* rice breeding line has been generated in our laboratory, mainly through the process of biolistic transformation (Khanna and Raina, 2002; Talwar and Raina, unpublished). 'Clean DNA' gene-cassette fragments, generated by restriction digestion and gel extraction, have been used for rice transformation. However, isolating the gene-cassette fragment DNA, in sufficient quantities, is not only cumbersome and time consuming but also laborious and expensive process. Low yield of the desired fragment DNA, following restriction digestion and gel extraction, has been the main limitation, while availability of suitable restriction sites flanking the gene expression cassette was the other. Certain amounts of flanking vector backbone sequences are still retained, depending upon the availability of restriction sites for appropriate restriction enzyme(s).

2.3 *Bacillus thuringiensis*

Bacillus thuringiensis Berliner, commonly known as Bt, is a naturally occurring, ubiquitous, facultative anaerobic, gram-positive, rod shaped, spore forming soil bacterium (Fig. 2a). Bt research began when a Japanese bacteriologist, Shingetane Ishawata, isolated a bacillus from diseased silkworm larvae (Ishawata 1901).

About a decade later, a similar organism was isolated and named as *Bacillus thuringiensis* by Ernst Berliner (Berliner 1911). Berliner reported the existence of crystal within *B. thuringiensis*, and in 1916, Aoki and Chigasaki found that the insecticidal activity was due to the toxin present in the sporulated cultures (Beegle and Yamamoto, 1992). Bt produces crystalline inclusions during sporulation (Fig. 2b) which contain insecticidal proteins called δ-endotoxins. More than 67 subspecies of *B. thuringiensis* have been identified, the most commonly known and useful in agriculture is the subspecies *kurstaki*, which is active against a wide range of lepidopteran insects (Bajwa and Kogan, 2001).

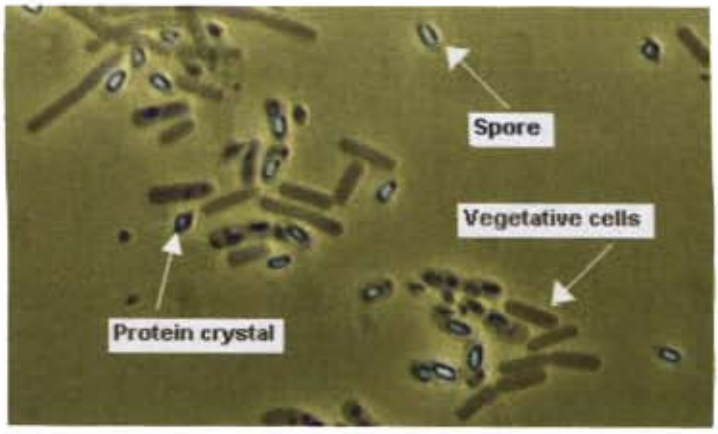
One of the main classification characteristics of *B. thuringiensis* is its ability to form insecticidal crystal protein (ICP) during sporulation (Fig. 2c). It is usually located outside the spore, often in the same cell. Bt isolates can be classified into about 30 serotypes based on biochemical properties and flagellar H-antigens (de Barjac and Frachon, 1990). However, this classification does not reflect the bacterial pathotype, which is essentially defined by the δ-endotoxins (Lereclus *et al.*, 1993). This insecticidal protein is synthesized after stage II of sporulation (*t2-t3*) and accumulates in the mother cell as crystal, which can account for up to 25% of the dry weight of the sporulated cells (Ribier and Lecadet, 1973). Höfte and Whiteley (1989) proposed nomenclature and classification of δ-endotoxins according to their insecticidal properties and molecular relationships. Initially, Bt toxins were classified into 14 distinct groups and 4 classes (Höfte and Whiteley, 1989) based on their insect specificity (host spectrum designated in roman numerals). These are:

- CryI (contain proteins of 130 kDa, and are specifically active against Lepidopteran larvae)
- CryII (contain proteins of 70 kDa that are specifically active against lepidopteran and dipteran larvae)
- CryIII (contain proteins of 70 kDa specifically active against coleopteran larvae)
- CryIV (contain proteins that are active against the dipteran larvae).

Nomenclature of the proteins considers differences in the amino acid sequences with the already known ICP and chronological order of discovery of the proteins (Fig. 3). The system was further extended to include type V genes that encode for proteins effective against lepidopteran and coleopteran larvae (Tailor *et al.*, 1992). Later many more classes have been added (Feitelson *et al.*, 1992; Crickmore *et al.*, 1996). New isolates effective against nematodes and other pests have also been described (Edwards *et al.*, 1988; Schwab and Halloran, 1989). More than 50 Cry proteins are known now, of which 28 genes have been isolated from 14 different Bt subspecies that have been shown to be effective against insects (Krattiger 1997). Classifications of new *Bt* toxin according to the old system (Höfte and Whiteley classification) became difficult, therefore a new system was designed (OSU, 1997; Krattiger 1997).

2.3.1 Bt as biopesticide

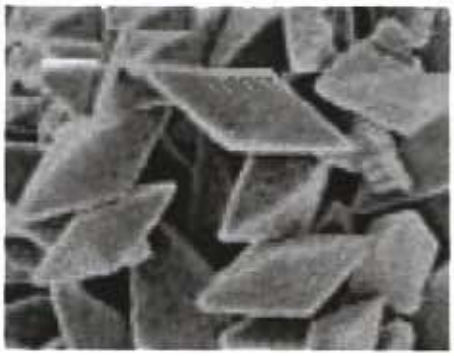
The attempts to use Bt for insect control started in late 1920s against European corn borer (*Ostrinia nubilalis*) in southeastern Europe (Van Frankenhuyzen 1993).



(a)



(b)



(c)

Fig. 2. *Bacillus thuringiensis*. **(a)** rod shaped vegetative cells of *B. thuringiensis*. **(b)** a *B. thuringiensis* cell in the stationary phase of its growth, containing endospore and insecticidal protein crystal and **(c)** electron micrograph of purified crystalline inclusion bodies (ICP).

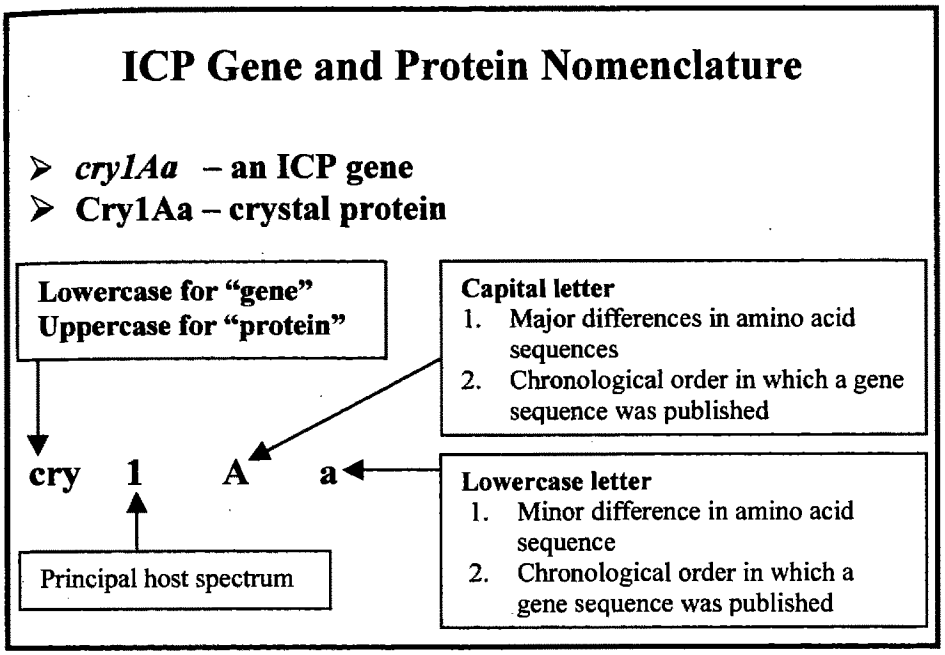


Fig. 3. Nomenclature system for Insecticidal Crystaline Proteins (ICPs) and genes.

The first Bt product, as biopesticide, was commercialized in 1938 in France for the control of flour moth (Jacos 1951). The first Bt formulations were available for field testing in USA in 1958 (Faust 1974). Many wild type Bt isolates produce multiple δ-endotoxins, each having a characteristic insecticidal activity. In the past decades, many Bt strains with different insect host spectra have been identified and used in biopesticide formulations. Identification of the subspecies *kurstaki* provided a boost for commercialization of Bt. The HD-1 strain, identified by Dulmage (1981), is the most important Bt product in the market. Moreover, the problems associated with specificity, shelflife, potency and presence of viable spores have been overcome by using modern tools in microbiology and genetic engineering.

Bt has been used for a long time as an effective biopesticide in agriculture, yet it represents less than 1% of the insecticides used on global basis. Compared to chemical pesticides, microbial preparations of Bt have certain advantages: they are highly toxic and yet highly specific. Bt toxins are totally harmless to human, animals and a wide array of non-target pests. Therefore, it is ideally suited for integration in Integrated Pest Management (IPM) programmes (Nester *et al.*, 2002). In spite of the advantages, Bt based biopesticides have several disadvantages also (McGaughey and Whalon, 1992). Production of the biopesticide is relatively expensive and its application requires the use of agricultural machinery. Most applications need to be repeated several times since sunlight (UV) breaks down the active ingredient and water (rain or dew) washes off the protein from plants, thus limiting the time when insects are exposed to it. The biopesticides, therefore, need to be applied repeatedly and are only effective against immature stages of lepidoptera, diptera, and coleoptera feeding on exposed plant surfaces. This limits its usefulness against insect pests that tunnel into the plants or plant parts. Examples include codling moth and corn earworm, which are susceptible to Bt but rarely have an opportunity to eat it in field use. These limitations of microbial preparations can be overcome by expression of *Bt* genes in transgenic plants (Krattiger 1997). There are two noteworthy differences between the form of Bt toxins that occurs in transgenic plants and that present in Bt sprays. First, the Bt toxins in spray formulations are in the form of crystals and need to be dissolved in digestive tract of the insect before becoming toxic, while the toxins expressed in plants are already in a dissolved state. Second, the toxins expressed in plants are different from that of bacteria. Generally, *Bt* genes are modified (codon optimized and/or attached with eukaryotic promoter) to enable plants to produce

sufficient amount of toxins. Due to these modifications, additional biosafety tests of the toxins producing plants are needed. The tests conducted in United States have indicated that plant-produced toxins are not toxic or allergenic to test animals such as mice, rats, and quail, or to beneficial insects such as honeybees and ladybird beetles.

2.3.2 Molecular biology of Bt toxin

The Bt δ–endotoxins constitute a family of related proteins for which more than 140 genes have been identified (Crickmore *et al.* 1998). Crystalline proteins are inactive until they get solubilised by the midgut proteases (Tojo and Aijawa, 1983; Milne and Kaplan, 1993) in the insect's midgut at high pH (>9.5), releasing proteins called δ-endotoxins. The toxin portion is derived from the N-terminal half of the protoxin, while the C-terminal half is involved in the formation of parasporal inclusion bodies and is usually hydrolyzed into small peptides (Choma *et al.* 1990). The region within the N-terminal toxin domain (amino acids 1 to 279) is composed of α–helices, which are considered important in penetrating the peritrophic membrane of midgut epithelial cells. Cry protein has three domains: Domain I consists of a bundle of seven antiparallel α-helices in which 5th helix is encircled by the remaining helices (Fig. 4b). Domain II consists of three antiparallel β-sheets joined in a typical "Greek key" topology and arranged in a so-called β-prism fold (Sankaranarayanan *et al.*, 1996; Shimizu and Morikawa, 1996). Domain III consists of two twisted, antiparallel β-sheets forming a β-sandwich with a "jelly roll" topology (Fig. 4a,b). The toxicity of Bt protein lies in the organization of α–helices derived from domain I. The C-terminal region (amino acids 461–695)

and the highly variable region (amino acids 280–460) are considered to be important in toxin specificity by coding for open β–sheets that bind to glycoprotein receptors on brush border membrane vesicles (BBMV) in the midgut (Lee *et al.*, 1992).

There may be many toxin binding protein (12 to 180 kDa glycoprotein) receptors (Garczynski *et al.*, 1991; Knowles *et al.*, 1991). The toxin gets inserted irreversibly into the brush border membrane leading to lesion formation. Positive correlation between toxin activity and its ability to bind BBMV (the number of receptors) has been observed (Gill *et al.*, 1992; Van Rie *et al.*, 1990).

2.3.3 Mechanism of action of Bt toxin

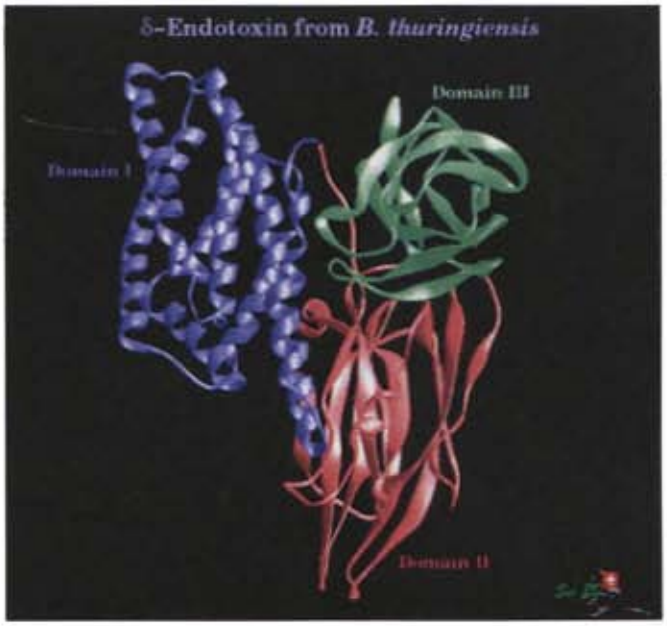
When insect ingest the bacterium and/or the protein, the functions of its digestive systems get disrupted, resulting into insect death. The crystalline inclusions are solubilized under alkaline conditions of insect midgut (Hofmann *et al.*, 1988a), releasing the δ-endotoxins which, upon proteolytic activation (Lecadet and Dedonder, 1967; Tojo and Aizawa, 1983) exhibit a highly specific insecticidal activity (Fig. 5). Activated toxins have two known functions, receptor binding and ion channel activity. The activated toxin binds readily to specific receptors on the apical brush border of the midgut microvillae of susceptible insects (Hofmann and Luthy, 1986; Hofmann *et al.*, 1988a, 1988b). Binding is a two-stage process involving reversible and irreversible steps, the latter step involves a tight binding between the toxin and receptor and insertion of domain I of the toxin into the apical brush border membrane (Flores *et al.*, 1997). After binding of active toxin to the specific receptors, the α–helices penetrate the membrane to form an ion

channel into the apical brush border membrane (Fig. 6) (Knowels and Dow, 1993). The formation of toxin induced pores in the columnar cell apical membrane allows rapid fluxes of ions. The pores are K^+ selective (Sacchi *et al.*, 1986), permeable to cations (Wolfersberger 1989), anions (Hendrickx *et al.*, 1989) and solutes such as sucrose, irrespective of the charge (Schwartz *et al.*, 1991). The disruption of gut integrity leads to death of the insect through starvation or septicaemia.

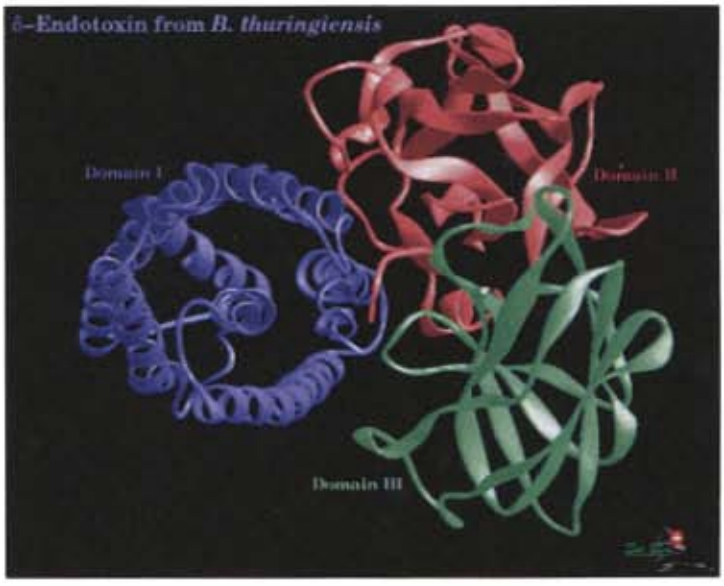
The Bt toxins are highly insoluble in normal digestive system conditions, therefore not harmful to mammals, birds, fish or to most of the beneficial insects. Mammals, including humans, do not have δ-endotoxin receptors in their guts and all the Bt toxins tested so far (except Cry9) are degraded within 20 seconds by the mammalian digestive enzymes (EPA 1998). Recent developments in cloning of δ-endotoxin genes and their expression in transgenic crop plants have provided an efficient strategy for insect resistance.

2.3.4 *Bt* gene for insect control

Each Bt strain produces its own unique insecticidal crystal protein, which is encoded by a single gene on plasmid in the bacterium (Whalon and McGuaghey, 1998). The genes encoding δ–endotoxins have been cloned since 1980s (Schnepf and Whitley, 1981). Expression of the gene in tobacco and tomato provided the first example of genetically engineered plants for resistance to insects (Barton *et al.*, 1987; Vaeck *et al.*, 1987). Since then, several *cry* genes have been introduced into crop plants (Estruch *et al.*, 1997; Peferoen 1997).



(a)



(b)

Fig. 4. Three-dimensional structures of Bt Cry3A protein. **(a)** side view **(b)** top view.

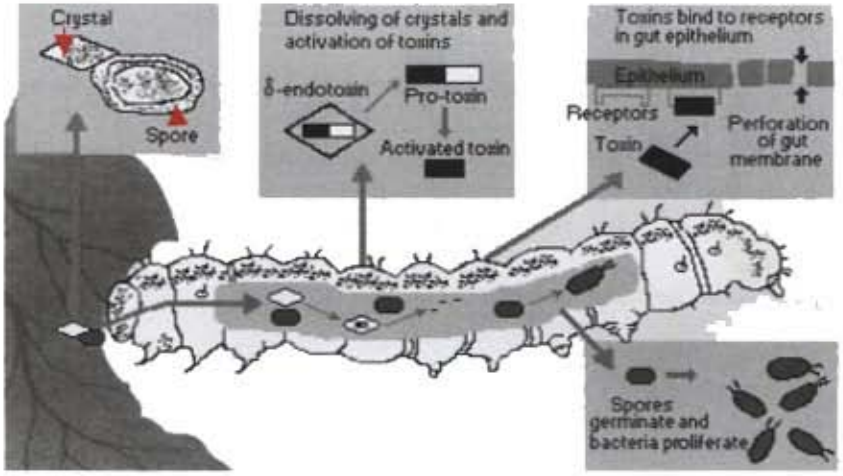


Fig. 5. Proposed mechanism of toxicity of *Bacillus thuringiensis*.

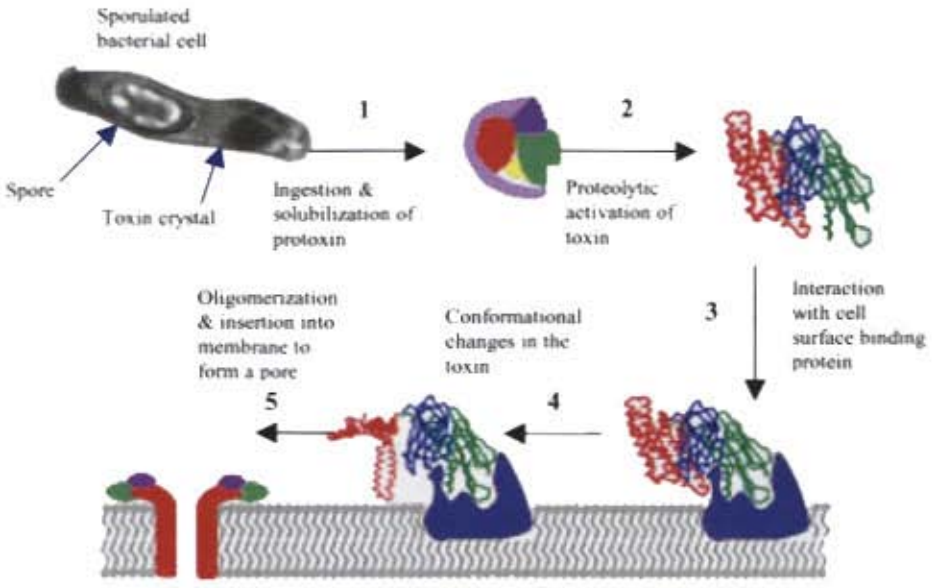


Fig. 6. Proposed mode of action of the Bt toxin.

2.3.5 Bt transgenic crops

First reports of Bt transgenic plants encoding δ-endotoxin gene were those of tobacco (Vaeck *et al.*, 1987; Barton *et al.*, 1987; Adang *et al.*, 1987) and tomato (Fischhoff *et al.*, 1987). None of the regenerated transgenic plants for native full-length *cry1Ab* gene produced detectable level of insecticidal protein. Adang *et al.*, (1987) used *cry1Ac* (*Bt* var. *kurstaki* HD73) for *Agrobacterium*-mediated transformation of tobacco. One of the transformants showed some degree of toxicity to tobacco hornworm.

Fischhoff *et al.* (1987) used two truncated versions of *cry1Ab*: one encoding a protein with 646 amino acid and the other with 725 amino acid. Their findings demonstrated that expression of N-terminal fragment of δ-endotoxin produced plants with a high level of insecticidal activity while plants containing full-length δ-endotoxin gene did not. Subsequently, there were several reports of δ-endotoxin expressing transgenic plants (Table 2), including potato, cotton, tobacco, eggplants and tomato.

The first report on field performance of transgenic tomato plants, expressing δ-endotoxin gene, was that of Delannay *et al.* (1989). Though *cry*1Ab gene, encoding 725 amino acids derived from Bt var. *kurstaki* HD1, was insecticidal against tobacco hornworm, however, higher level of the gene expression was needed for the control of agronomically important pests such as tomato fruit worm (*Helicoverpa sp.*).

Table 2. Reports of field-tested Bt transgenic crops.

Crop	*Bt* gene used	Against the pest	References
Corn	*cry*1Ab	*Heliothis zea*	Koziel *et al.*, 1993; Armstrong *et al.*, 1995
	*cry*1Ac	*Pectinophora gossippiella*	Buschman *et al.*, 1998.
Potato	*Cry*3A	*Leptinotarsa decemlineata*	Perlak *et al.*, 1993
	*cry*1Ab	Potato tuber worm	Duck and Evola, 1997
	*cry*1Ac	Potato tuber worm	Ebora *et al.*, 1994.
Cotton	*cry*1Ac	Cotton ball worm	Perlak *et al.*, 1990; Jenkins *et al.*, 1997
	*cry*1Ab	Pink ball worm	Wilson *et al.*, 1992; Benedict *et al.*, 1996.
Tomato	*cry*1Ac	Tobacco ball worm	Peferoen *et al.*, 1987
	*cry*1Ab	Tobacco hornworm	Delannay *et al.*, 1989.
Egg plant	*Cry1*Ab	Fruit borer	Kumar *et al.*, 1998
	*cry*3B	Fruit borer	Iannacone *et al.*, 1997.
Chickpea	*cry*1Ac	Pod borer	Kar *et al.*, 1997.
Sugarcane	*cry*1Ab	Shoot borer	Arencibia *et al.*, 1995, 1997.
Rice	*cry*1Ab	Yellow stem borer, Striped stem borer	Wunn *et al.*, 1996; Datta *et al.*, 1998; Shu *et al.*, 2000
	*cry*1Ac	Yellow stem borer	Nayak *et al.*, 1997
	*cry*3A	Rice weevil, Rice hispa	Johanson *et al.*, 1996
	*cry*2A-1Ac-*gna*	Rice weevil, Rice hispa	Bano *et al.*, 1999
	*cry*1B - 1Aa	Yellow Stem Borer	Raina *et al.*, 2002.

Field trial results of Bt transgenic tobacco (Carozzi *et al.*, 1992; Hofmann *et al.*, 1992) and cotton (Wilson *et al.*, 1992) using truncated δ-endotoxin genes were encouraging, but expression level of the gene was found to be lower due to the production of truncated transcripts. Modifications in the gene and/or regulatory sequences were considered necessary for enhancing gene expression. The year 1996 marked a milestone in agricultural biotechnology: for the first time Bt transgenic varieties of potato, cotton and corn containing modified *cry* genes were released for commercial cultivation.

2.3.5.1 *Enhanced gene expression*

Murray *et al.* (1991) examined expression of *cry1Ab*, *cry1Ac* and *cry3A* genes in electroporated protoplasts to study mRNA stability. Most of the transcripts were truncated and this was attributed to inefficient post-transcriptional processing or rapid turnover of mRNA. The instability of mRNA could be the result of endonucleolytic or exonucleolytic degradation at specific sequences. Codon usage in the native δ-endotoxin genes is considerably different from that found in typical plant genes, which have a higher GC content. The production of truncated transcripts in the transgenic plants can be attributed to the high AT content of the bacterial native gene, which may leads to premature transcription termination or polyadenylation in the AT rich region or inappropriate splicing of mRNA. Later it was found that the truncated transcripts were the result of mRNA instability rather than truncation due to premature transcriptional termination. Considerable differences in the ORFs led to the anticipation that increasing GC content of the Bt genes and/or by using plant preferred codons could improve the expression of insecticidal proteins (Frutos *et al.*, 1999).

Various strategies have been adopted to enhance Bt δ-endotoxin gene expression in transgenic plants. These include truncation and sequence modification of the gene, use of regulatory elements, including tissue and temporal specific promoters. The problem of low level of *Bt* gene expression, due to AT rich sequences, was overcome by increasing GC content of the gene by partial or complete modification of the gene which resulted into significant increase in the gene expression (Perlak *et al.*, 1990; Koziel *et al.*, 1993). Perlak *et al.* (1990) achieved 100-fold increase in expression level, as compared to the truncated wild type gene

of *cry1Ab* and *cry1Ac* genes, in cotton by modifying the DNA sequence without changing the amino acid sequence. Koziel *et al.* (1993) synthesized a truncated *cry1Ab* gene encoding 1-648 amino acids of the protein wherein bacterial codons were replaced with maize preferred codons. The synthetic *cry1Ab* gene had 65% homology with the wild type gene and had 65% GC content instead of 37% GC content in the native gene. The synthetic gene was cloned under control of maize phophoenolpyruvate carboxylase (*pepc*) promoter, which gives green tissue specific expression of the gene, and under the control of maize pollen specific promoter. Later, synthetic genes were extensively used to enhance δ-endotoxin expression in corn, potato, and rapeseed (Adang *et al.*, 1993; Hoy and Head, 1995; Stewart *et al.*, 1997). Only four insect-resistant transgenic crops, viz. cotton, maize, tomato and potato producing Bt toxin have been commercialized, and only four Bt toxins, viz. Cry1Aa, Cry1Ab, Cry1Ac and Cry3Aa, are being produced in the commercialized Bt transgenic crop plants.

2.3.5.2 *Bt transgenic rice*

Fujimoto *et al.* (1993) were the first to generate Bt transgenic rice expressing codon-optimized truncated *cry1Ab* gene driven by constitutive CaMV35S promoter. The transgenic plants conferred mortality up to 50% for striped stem borer (SSB). Significant levels of protection against yellow stem borer (YSB), SSB and rice leaf folder (RLF) were observed in an *indica* rice cultivar IR-58 expressing a synthetic *cry1Ab* gene under the control of CaMV35S promoter (Wunn *et al.*, (1996). High level of expression of synthetic *cry1Ac* gene, driven by constitutive maize ubiquitin-1 promoter, was observed in another *indica* rice cultivar IR-64 (Nayak *et al.*, 1997). Insect bioassay results of the transgenic plants

showed effective protection of the transgenic plants against YSB larvae. Nayak *et al.* (1997) proposed, as suggested earlier by McGaughey and Whalon (1992), insect resistance management using their Bt transgenic lines grown alongside untransformed lines to serve as refuges. Enhanced resistance against YSB was observed in a deepwater *indica* rice variety Vaidehi expressing synthetic *cry1Ab* gene under the control of CaMV35S promoter (Alam *et al.*, 1998). Another synthetic gene, *cry2Aa*, encoding a Bt toxin with different receptor binding sites and known to be active against lepidopteran and dipteran insects, was mobilized into *indica* rice (Basmati-370 and M-7) varieties (Maqbool *et al.*, 1998). The selected transgenic lines showed 100% mortality of YSB as well as leaf folders. Brietler *et al.* (2000) tested a codon-optimized *cry1B* gene, driven by ubiquitin promoter, for enhanced resistance against SSB. Transgenic plants exhibited 85-100% mortality of SSB larvae.

Tissue specific expression of the *Bt* genes is considered to be a better strategy to manage insect resistance and also addresses certain biosafety concerns. Enhanced stem borer resistance was observed by Ghareyazie *et al.* (1997) in *indica* rice cv. Tarom Molaii transformed with *cry1Ab* under the regulatory control of *pepc* promoter. The gene expression was detected in leaf blades but not in dehulled mature grains and provided effective resistance to first-instar larvae of SSB and YSB. No dead-heart or whiteheads were observed on transgenic plants. Datta *et al.* (1998) transformed *indica* and *japonica* rice varieties by microprojectile bombardment using *cry1Ab* gene driven by green tissue specific and/or pith specific promoters. Cut-stem insect bioassays showed up to 100% mortality of YSB larvae. High level Cry1Ab protein expression in green tissues (leaves and

stem) of the plants resulted in enhanced YSB resistance, and provided a better strategy for protection against the target insect pests, as also minimized the expression of the toxin in seeds and other tissues. Several other groups have succeeded in mobilizing Bt insecticidal genes into rice (Table 3). At the Center for International Co-operation in Agricultural Research for Development (CIRAD), France, the group working on Engineering Rice for Resistance to Insects used *cry1Aa* or *cry1B* gene under the control of either the constitutive ubiquitin promoter or a wound-inducible promoter of maize proteinase inhibitor gene (*mpi*) for transformation of two rice cultivars. Insect bioassays of the transgenic plants, transformed with either *ubi-cry1B* or *mpi-cry1B* construct, provided full protection against SSB larvae in T_0 to T_4 generations. Accumulation of Cry1B toxin in leaf and pith tissues upon wounding, and its absence in pollen grains and endosperms was demonstrated in *mpi-cry1B* transgenic plants.

With the objective of prolonging effectiveness of the Bt toxin genes, and in keeping with the strategy of resistance management, a fusion toxin gene was used for rice transformation (Tu *et al.*, 1998). The fusion gene comprised of 1344 bp (encoding the N-terminus) of *cry1Ab* and 486 bp (encoding the C-terminus) of *cry1Ac*. Cut-stem insect bioassays of a homozygous transgenic line showed 100% YSB larval mortality. Maqbool and Christou (1999) co-transformed rice with two *Bt* genes (*cry1Ac* and *cry2A*) and a snowdrop lectin gene (*gna*). All the insecticidal genes were found to co-express, although at different levels. Based on a detailed molecular and expression profiling of 29 independent transgenic lines, the authors suggested that it would be appropriate to select transgenic lines with moderate levels of protein expression to minimize problems of developmental abnormalities

in transgenic plants. Co-transformants for all the three genes showed 100% protection against RLF and YSB, and 25% reduction in the survival of brown plant-hopper (BPH). Loc *et al.* (2002) used the same three genes for rice transformation with minimal gene-expression-cassette bombardment. A simpler transgene integration pattern was observed, along with higher level accumulation of the insecticidal gene products and enhanced resistance against BPH and SSB, in comparison to the transformants generated by whole-plasmid bombardments. Raina *et al.* (2002) tested translational fusion of *cy1B-1Aa* genes for transformation of *indica* rice cultivars IR-64 and Pusa Basmati-1. Insect bioassays showed high level mortality of YSB neonate larvae.

2.4 Status of transgenic crops

From a rather modest beginning (in 1992) of about 8,000 ha. in the Central Hunan Province of China, the global area of transgenic crops has grown to 67.7 million hectares in 2003 (James, 2003). In the year 2003, 99% of the global transgenic crop area was grown in six major countries with ten countries now growing 50,000 hectares or more of GM crops in all by 7 million farmers in 18 countries world over (see Tables 4, 5, 6 and Fig. 7).

2.5 Future of transgenic crops

There is increasing number of candidate genes that can be deployed for the development of insect-resistant crop plants. Recombinant-DNA technology and Bioinformatics, on the other hand, offer the possibility of developing entirely new biological insecticides (smart proteins) that have the advantages of classical biological control agents but have fewer of their drawbacks.

Table 3. Transgenic rice for Bt δ-endotoxin genes

Genes	Target pests	Cultivars (japonica/indica)	References
cry1Ab	SSB, RLF	Nipponbare	Fujimoto *et al.*, 1993.
	YSB, SSB, RLF	IR-58	Wunn *et al.*, 1996.
	YSB, SSB	Tarom Molaii	Ghareyazie *et al.*, 1997.
	YSB, SSB	9 Cultivars	Cheng *et al.*, 1998.
	YSB	Vaidehi	Alam *et al.*, 1998.
	YSB	IR-68899B (maintainer line)	Alam *et al.*, 1999.
cry1Ac	YSB	IR-64	Nayak *et al.*, 1997.
	YSB, SSB	9 Cultivars	Cheng *et al.*, 1998.
	YSB	10 Cultivars	Datta *et al.*, 1998.
	YSB, RLF, BPH	Basmati-370, M7	Maqbool and Christou, 1999.
	YSB, SSB, etc.	Xiushui-11	Ye *et al.*, 2001.
	YSB	PB-1, IR-64, Karnal Local	Khanna and Raina, 2002.
	SSB	Minghui 81, Minghui 86, Shuhui 162 (restorer line)	Zeng *et al.*, 2002.
cry2A	YSB, RLF, BPH	Basmati 370, M7	Maqbool *et al.*, 1998.
(Multiple genes)	YSB, RLF, BPH	Basmati 370, M7	Maqbool and Christou, 1999.
(Multiple genes)	YSB, RLF, BPH	Basmati 370, M7	Maqbool *et al.*, 2001.
(Multiple genes)	YSB, RLF, BPH	Eyi-105, Bengal	Loc *et al.*, 2002.
cry1B	SSB	Ariete, Senia	Brietler *et al.*, 2000.
	SSB	Ariete	Brietler *et al.*, 2001.
Fusion genes			
cry1Ab - 1Ac	YSB, RLF	Minghui 63	Tu *et al.*, 1998.
	YSB, RLF	Minghui 63	Tu *et al.*, 2000.
cry1B - 1Aa	YSB	PB-1, IR-64	Raina *et al.*, 2002.

YSB = Yellow Stem Borer; SSB = Striped Stem Borer; RLF = Rice Leaf Folder; BHP = Brown Plant Hopper, PB-1 = Pusa Basmati-1

Table 4. Global distribution of transgenic crops.

S. No.	Country	2003		
		Area (m hac.)	%	
1	USA	42.8	63	
2	Argentine	13.9	21	
3	Canada	4.4	6	
4	Brazil	3.0	4	
5	China	2.8	4	
6	South Africa	0.4	1	
7	Australia	0.1	-	< 1%
8	India	0.1	-	
9	Romania	0.05	-	
10	Uruguay	0.05	-	

Source: James, 2003.

Table 5. Global area of different transgenic crops.

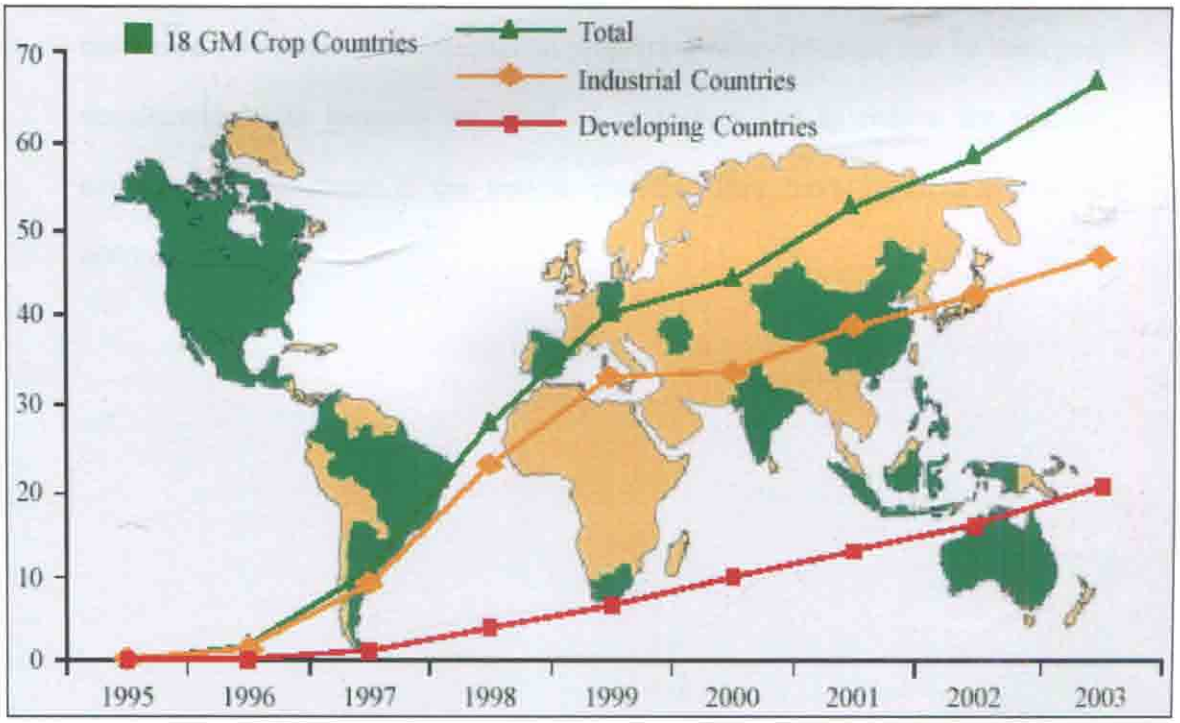
Transgenic crop	2002		2003	
	Area (m hac.)	%	Area (m hac.)	%
Soybean	36.5	62	41.4	61
Corn	12.4	21	15.5	23
Cotton	6.8	12	7.2	11
Canola	3.0	5	3.6	5

Source: James, 2003.

Table 6. Global area of transgenic crops with different agronomic traits.

Transgenic trait	2002		2003	
	Area (m hac.)	%	Area (m hac.)	%
Herbicide tolerance	44.2	75	49.7	73.5
Insect resistance	10.1	17	12.2	18.0
Herbicide tolerance and insect resistance	4.4	8	5.8	8.5

Source: James, 2003.



Source: James, 2003.

Fig. 7. Global acceptance of commercialized transgenic crops.

Commercial and ethical considerations have placed this technology beyond the reach of poorer section of society and have generated considerable public debate about its usefulness, effects on consumers and the environment, thus preventing its use as an additional tool for increasing and improving productivity of crops (Sharma *et al.*, 2000).

Biotechnological tools and techniques, if properly integrated into production systems, offer new opportunities to increase production and productivity by using a more sustainable and ecologically friendly agricultural system. Such a near-term biotechnological application is the insect resistant Bt transgenic crops. Review of the known insecticidal genes and possible insect resistance technologies reveal that Bt is beginning of new and safer technologies to augment productivity, to bring about sustainable agriculture, and to protect the environment. Bt alone is having more than 100 genes with insecticidal properties. Many of these can be deployed simultaneously to increase the level of protection and to reduce the risk of resistance development in the insects, provided they have different modes of action.

MATERIALS AND METHODS

3.1 Plant Material

Pusa Basmati-1 (PB-1), a semi-dwarf, high yielding and fine grain aromatic *indica* rice breeding line, highly susceptible to many insect pests and diseases, was mainly used for transformation experiments. Mature seeds from pure line PB-1 were used for the study. Panicles, free from fungal infection, were collected from pure line seed maintenance plot at IARI. After drying at 37° C for a week, the seeds were stored in cloth bags at room temperature. Embryogenic calli induced from scutellar region of mature seeds (Khanna and Raina, 1997) were used as explants for microprojectile bombardments.

3.2 *Bt* genes and Promoters

The synthetic and codon-optimized δ-endotoxin genes of *Bacillus thuringiensis*, *cry1Aa*, *cry1B* and *cry1B-1Aa* translational fusion gene, obtained from Dr. R. Frutos, CIRAD, France, and tissue/temporal specific promoters were used for the construction of suitable gene-expression-cassettes. *pepc* promoter (driving expression of phosphoenolpyruvate carboxylase gene, specifically in the green tissues of maize, obtained from Novartis Agribusiness Biotechnology Research Inc. North Carolina, USA) and *mpi* promoter (driving expression of maize proteinase inhibitor gene in response to wounding to the plant) obtained from Dr. R. Frutos, CIRAD, were used.

To ascertain the exact base sequences and availability of restriction sites of the genes and promoters used for the construction of gene-expression-cassette, the promoters and genes were sequenced by single strand non-assembled primer walking reactions (Microsynth GmbH, Switzerland). Some of the sequencing results obtained for non-assembled primer walking reactions are given below.

A few sequencing reaction results received for translational fusion gene *cry1B-Aa*. The highlighted base sequences show overlapping sequences of the two consecutive reactions.

318708 80207 FGBA v.Hand Ubi-1rev.1
TCTCATAAATAACGTCATGCATTACATGTTAATTATTACATGCTTAACGTAATTCAACAGAA
ATTATATGATAATCATCGCAAGACCGGCAACAGGATTCAATCTTAAGAAACTTTATTGCCA
AATGTTTGAACGATCTGCAGGTCGGGGTACCGGGCCCCCCCTCGAGGTCGACGGTATCGAT
AAGCTTGATGGGAATTCATTACCTCTCCAAGTCGTACTCGGCCTCGAACGTGACCTCGGCTG
GGACGAACTCGATGCGGTCGATGTACACCTCGTTGCCGCTGTTGAAGACGTGAGCGCTGAG
GGTGAAGACGCTGGAGCCGTTGGAGAAGTTGAATGGGGTGGTGAAGCCCACGGTCCTGAA
GGAGCCGGATTGGAGGTTGCTGCCGGAGGACATGGTGGCGGAGAAGTTGCCTTGGTTGATT
GGCCTGCCGTCGATGGAGGTGTGGAACTGCAGGTTCGTGGTGGAGGCGTAGCGGATGCGGA
CGCGGTAGCGTTGGCTGAGTGGCGCGGTGATGTTCACGCGGAGGGTGGAGATTTGGCCT
GGGGAGGTGCGGCGGAGGATGTCGCCCCCGGTGAAGCCTGGCCCCTTGACGACGGAG
GTGCCGGAGCCGAGGTTGGTGGACTTGGTGAGTGGGATTTGGGTGATTTGGGAGGATGG
GATGATGT

328623 82669 FGBA v.Hand Ubi-1rev.2
AGGTGCGGCGGAGGATGTCGCCCCCGGTGAAGCCTGGCCCCTTGACGACGGAGGTGCCGG
AGCCGAGGTTGGTGGACTTGGTGAGTGGGATTTGGGTGATTTGGGAGGATGGGATGATGTT
GTTGAACTCGGCGGAGCGGTGTTGCCAGGAGAAGGTTGGAGCTCTGAGGGTGTACACAGCG
CCAGCGGCTTGAGAGAGCATGGTCACGTGGGACAGGCGGTGGGAGAAGCCAGCGCGTGGT
GGGACGGAGTTGTCTTGTGGTGGGATCACGTCGAGGGAGTCCACGGTGCCCCTTTGCCTGT
AGATGGTGGATGGGAGGTTGGTGGTGAGGGAGGCGAAGGAGAACTCGGTGCCGTCCAGGA
CGAACAGCTCTTGGTTGTTTGGGCCGGAGCCGAGGATGATGCGCCTGTACAGTGGGCTGGA
GAGGGTGCGGAAGATGCCGAGGCCAGTGAGGGAGACGAGGACTGGTGGAGCGGCGTTACC
GGCGTTGCCGAAGAGTGGGAAGGCGAACTCTGGGCCGGAGAAGCCGACTGGGGAGGCGGT
GATTTGGTGGCCGGACCAGTAGTTGAAGCCCCTGTGGACGTCGGTGTAGATGGTGATGGAG
TTGAGGATATCCATGAGGTGTGGTTGCCTGATGTTTTGCTCGATGCGTTGAGCCATACCCCT
GAAGCTACCGTCGAAGTTCTCAAGGACTGGGTTAGTATAGATTTCTCTGGTA

329213 82869 FGBA v.Hand Ubi-1rev.3
GTCGGTGTAGATGGTGATGGAGTTGAGGATATCCATGAGGTGTGGTTGCCTGATGTTTTGC
TCGATGCGTTGAGCCATACCCCTGAAGCTACCGTCGAAGTTCTCAAGGACTGGGTTAGTAT
AGATTTCTCTGGTAAGTTGGGACACTGTACGGATAGGGTAGCGTCTGGAGTCATAGTTGGA
GAAGAGAGCCACGATGTCGAGAACTGTGAGGGTCAATTCTCTCCTGAACTGGTTGTATCTC
ACCCAATCTCTAGAGTCTGGCCCCCAGACGCGCTCCAGGCCGGTGTTGTACCACCTGACGG
CGTAGTCGGTGTAGTTGCCGATGAGCCTGGTGAGGTCGTTGTAGCGGGAGTTGATGGTAGC
GGCGTCGAAGCCCCAGCGTTGGCCGAAGACGGACACGTCGCGGAGGACGGAGAGGTGGA
GGTTGGCGGCTTGGACGTACACGGAGAGGAGTGGGACTTGGTAGTTTTGGACAGCGAGGA
GTGGGATCGCTGTGGTGAGGGCGGAGTTCATGTCGTTGAATTGAATCCTCATCTCCTCGCG
GAGGGCTGGGTTGGTTGGGTCGGCCTCCCACTCCCTGAAGGACTCGGCGTAGATTTGGTAG
AGGTTGGAGAGACCCTCCAGGCGGGAGATGGCTTGGTTGCGGGCGAACTCCTCGATGCGT
TGGTTGATGAGTTGCTCGATTTGGACTAGAAAGGCGTCCCATTG

The primer walking reaction results were assembled based on the overlapping sequences of two consecutive reactions. The assembled base sequences for the genes and promoters (Appendix A-6) were verified with the information received along with the genes and promoters as well as by comparing with the gene sequence information available in the NCBI (National Center for Biotechnology Information) GenBank using the Internet based program, Nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

3.3 Plasmid constructs

The *Bt* genes and promoters were initially cloned in pCAMBIA-1305.1 (Accession No. AF354045; Fig. 8a) obtained from CAMBIA, Australia.

3.3.1 Cloning of *pepc* promoter

The synthetic *pepc* promoter was received in pCIB8759 (Fig. 8b). The plasmid was digested with *Hin*dIII - *Bam*HI to take out 2.3 kb fragment of the promoter. The promoter fragment was then cloned into pCAMBIA-1305.1 plasmid digested with *Hin*d III - *Bam*HI which led to the construction of pSKPPC-05 (Fig. 8c).

3.3.2 Cloning of fusion genes

Translational fusion of *cry1B-1Aa* along with the *nos* terminator was excised using *Bam*HI restriction digestion of the construct (pC1300-Ubi-1B-1Aa) received from CIRAD. The gene + *nos* terminator fragment was then cloned into the pSKPPC-05 at *Bam*HI site, which led to the construction of pSKPBA-05 (Fig. 9).

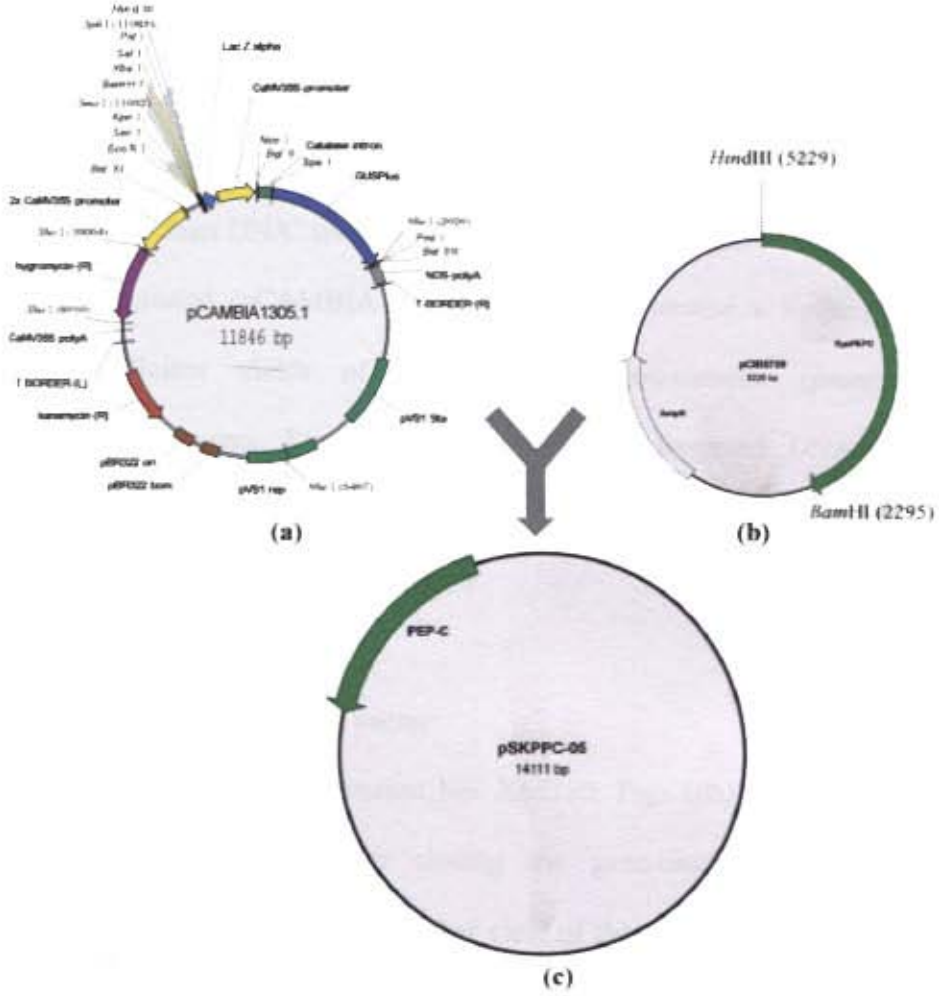


Fig. 8. Cloning of synthetic *pepc* promoter in pCAMBIA-1305.1 between *Hin*dIII - *Bam*HI sites of MCS led to the construction of pSKPPC-05.

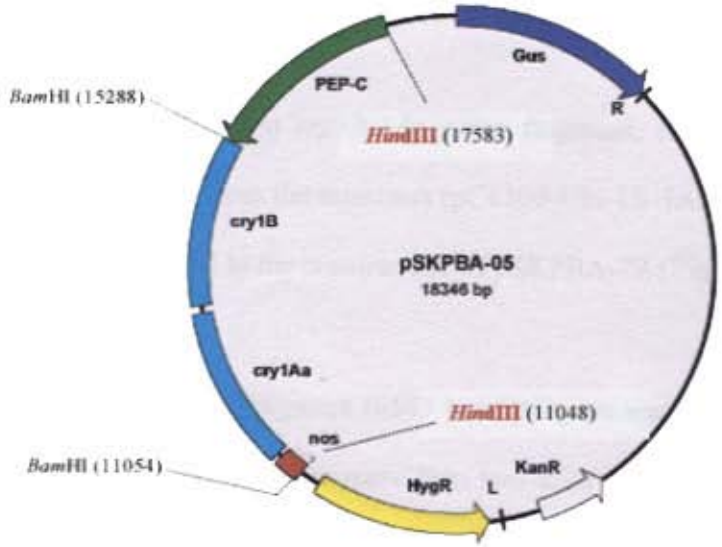


Fig. 9. Cloning of translational fusion genes *cry1B-1Aa* under the control of *pepc* promoter in pSKPPC-05 at *Bam*HI site led to the construction of pSKPBA-05.

Cloning was carried out in a high copy number plasmid (pCAMBIA-1305.1). Such plasmid was especially useful when using restriction digestion and gel extraction approach for 'clean DNA' transformation experiments. However, larger size of the vector background (pCAMBIA-1305.1; 11846 bp) became a limiting factor in getting sufficient yields of the gene-expression-cassette (promoter-ORF-terminator) fragments. Low yield of the desired fragment DNA, following restriction digestion and gel extraction, made it necessary to clone the specific gene-cassette in a smaller plasmid vector.

3.3.3 Cloning in pGEM-7Z vector

Plasmid pGEM-7Zf (+) (Accession No. X65310; Fig. 10b) was identified as a suitable small size vector for cloning the gene-cassette of interest and, subsequently, obtaining relatively higher yield of the desired fragment DNA. The *Hin*dIII - *Bam*HI digested 2.3 kb fragment of the synthetic *pepc* promoter was cloned into pGEM-7Z plasmid, which led to the construction of pSKPPC-7Z (Fig. 10c).

Subsequently, translational fusion *cry1B-1Aa* genes fragment, excised out using *Bam*HI restriction digestion from the construct (pC1300-Ubi-1B-1Aa), was cloned in the pSKPPC-7Z, which led to the construction of pSKPBA-7Z (Fig. 11).

The yield of the gene-cassette fragment (6563 bp) DNA, on restriction digestion with *Sma*I – *Mlu*I, was expected to be more than two-third of the quantity of the plasmid DNA (9526 bp) used for restriction digestion.

3.3.4 Addition of restriction sites to the termini by PCR amplification of *mpi* promoter

To add suitable restriction sites at both the ends of *mpi* promoter (Fig. 12), PCR amplification strategy was adopted (Sambrook and Russell, 2001). *Hin*dIII site at the 5' end and *Bam*HI site at 3' end were added suitably designed PCR primers. A high fidelity proofreading enzyme, DyNAzyme EXT was used for the PCR amplification.

Forward primer:

*Hin*dIII site
5' - CGC AAG CTT GTC GAG AGA CGA TAT TTA G - 3'

Reverse primer:

*Bam*HI site
5' - CAC GGA TCC CGA ACT GCC GGA CCA GTT GA - 3'

3.3.4 Addition of restriction sites to the PCR amplified *cry 1B* gene

Suitable restriction site at the 5' end of *cry1B* was added using PCR strategy. *Bam*HI site was already present at the 3' end of the gene. DyNAzyme EXT was used for the PCR amplification.

Forward primer:

5' - CAT GGT TAC CTC CAA CCG - 3'

Reverse primer:

*Bam*HI site
5' - GGA GCT CTC CGG ATC CAA G - 3'

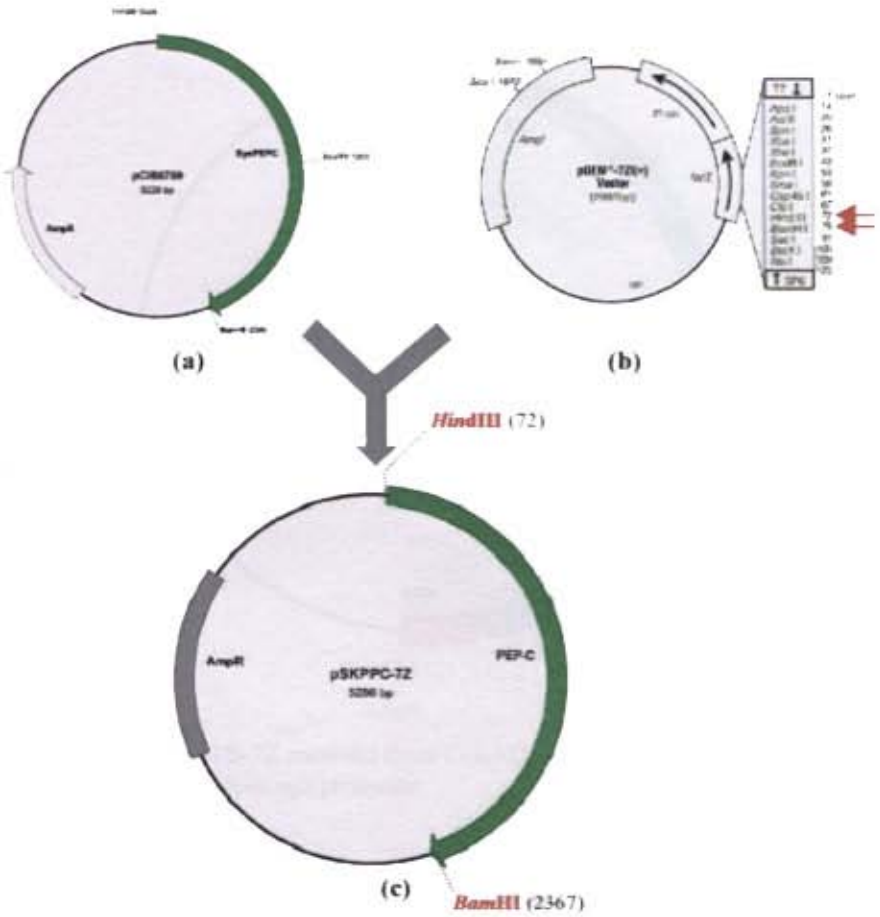


Fig. 10. Cloning of synthetic *pepc* promoter in pGEM-7Z vector in the MCS at *Hin*dIII - *Bam*HI sites led to the construction of pSKPPC-7Z.

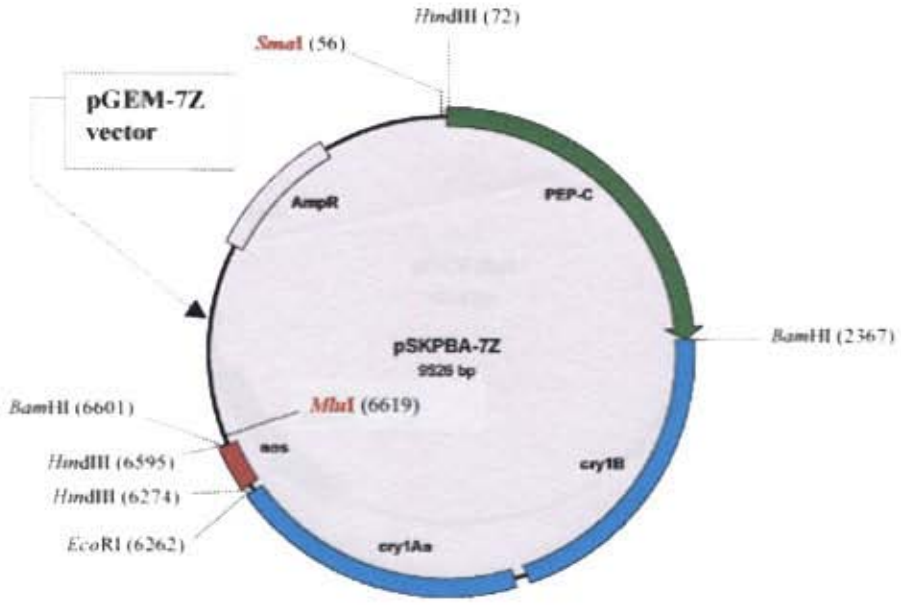


Fig. 11. Cloning of translational fusion gene *cry1B-1Aa* in pSKPPC-7Z plasmid at *Bam*HI site led to the construction of pSKPBA-7Z.

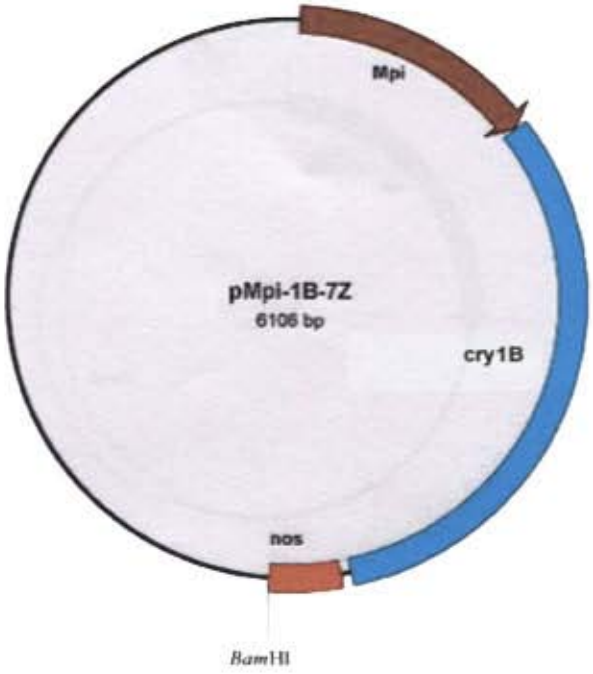


Fig. 12. pMpi-1B-7Z received from CIRAD, carrying *cry1B* driven by the wound inducible *mpi* promoter.

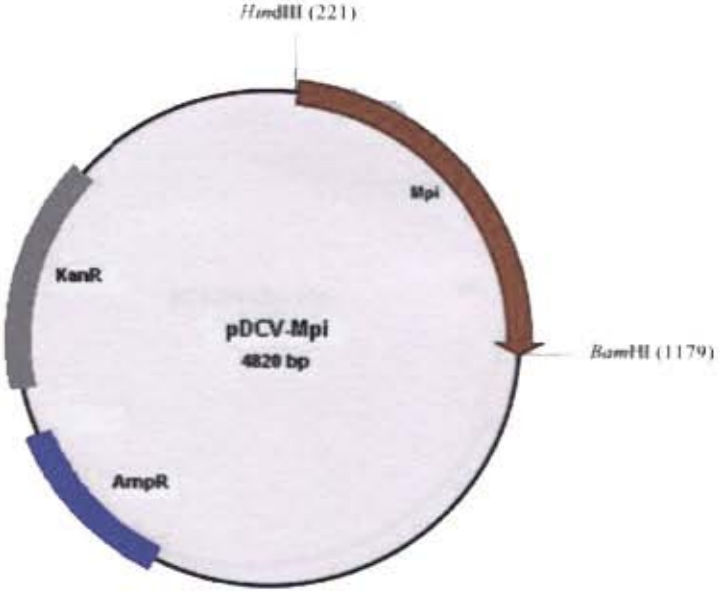


Fig. 13. PCR amplified *mpi* promoter cloned in pDrive Cloning Vector (pDCV-Mpi).

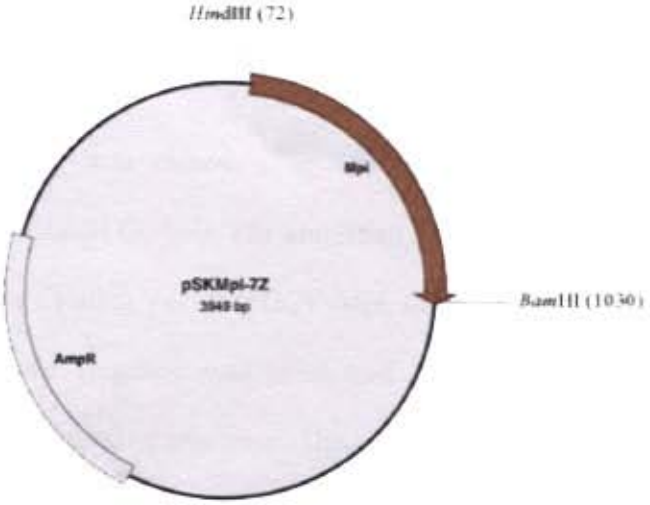


Fig. 14. Cloning of *mpi* promoter in pGEM-7Z in the MCS at *Hin*dIII-*Bam*II sites led to the construction of pSKMpi-7Z.

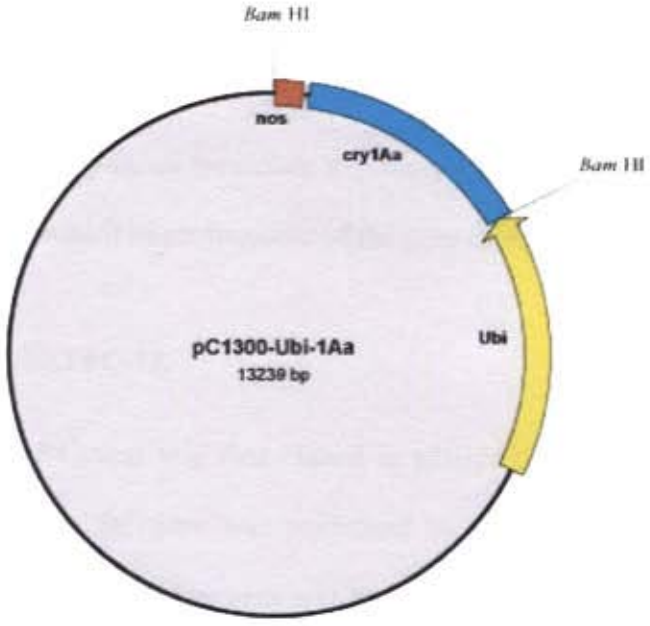


Fig. 15. pC1300-Ubi-1Aa received from CIRAD, carrying *cry1Aa* driven by the Ubiquitin promoter.

3.3.5 Cloning *mpi* promoter in pGEM-7Z

PCR amplified *mpi* promoter was cloned using UA hybridization technique (pDrive Cloning Vector; QIAGEN GmbH). The amplified *mpi* promoter fragment was first cloned into pDrive Cloning Vector (pDCV-Mpi, Fig. 13). Base sequence of the PCR cloned promoter fragment was confirmed by single strand non-assembled primer walking sequencing reactions. The promoter was then excised from pDCV-Mpi using *Hin*dIII- *Bam*HI restriction enzymes. The *mpi* promoter fragment, thus generated, was then cloned into pGEM-7Z at the multiple cloning site. The plasmid thus constructed was named pSKMpi-7Z (Fig. 14).

3.3.6 Cloning *cry1Aa* gene in pSKMpi-7Z

The *cry1Aa* fragment was taken out from the pC1300-Ubi-1Aa (Fig. 15) using *Bam*HI digestion. The gene was then cloned into pSKMpi-7Z. The plasmid thus constructed (pSKMAa-7Z; Fig. 16), on restriction digestion with *Sma*I and *Mlu*I, gave 3194 bp (52% of the plasmid) linear fragment of the gene expression cassette.

3.3.7 Cloning *cry1B* in pSKPPC-7Z

The PCR amplified *cry1B* fragment was first cloned in pDrive Cloning Vector (pDCV-1B). Base sequence of the gene was confirmed by single strand non-assembled primer walking sequencing. The gene was then excised from pDCV-1B using *Bam*HI restriction enzyme and cloned into pSKPPC-7Z. The plasmid thus constructed was named pSKP1B-7Z (Fig. 17).

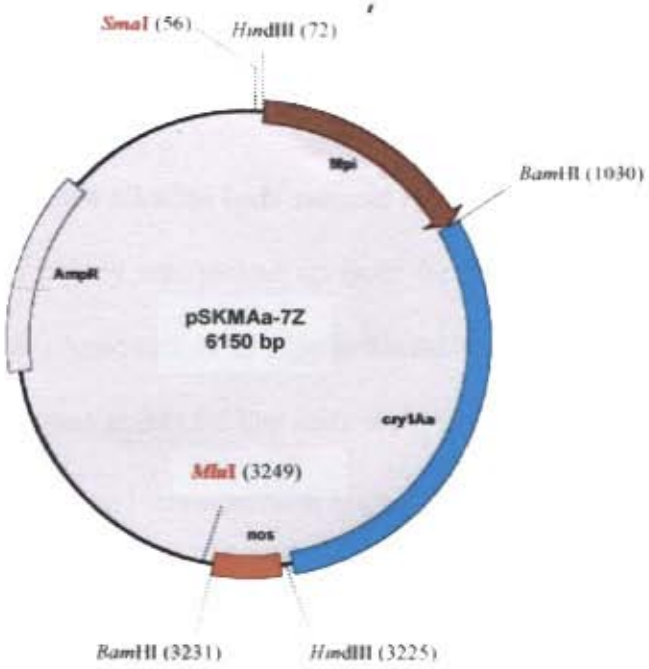


Fig. 16. Cloning of *cry1Aa* in pSKMpi-7Z at *Bam*HI site led to the construction of pSKMAa-7Z.

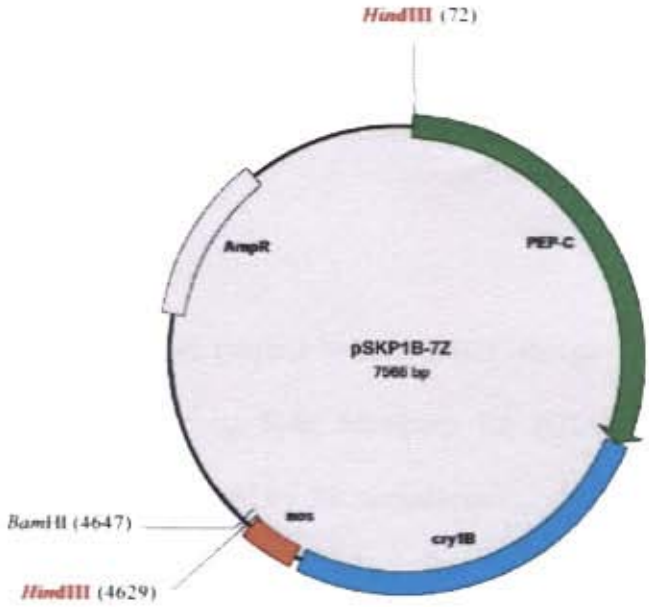


Fig. 17. Cloning of *cry1B* in pSKPPC-7Z at *Bam*HI site led to the construction of pSKP1B-7Z.

3.4 Plasmid isolation

3.4.1 Alkaline lysis method

For plasmid miniprep, modified alkaline lysis method (Brinboim and Doly, 1979) was used. Single bacterial colony was picked up from freshly streaked LA plate, inoculated into 5 ml of LB (Appendix A-1) supplemented with suitable antibiotic and grown overnight (200 rpm) at 37° C. The cells were harvested and pellet was resuspended in 250 µl of Solution I (resuspension buffer, Appendix A-2). 250 µl of Solution II (lysis buffer, Appendix A-2) was added, mixed by inverting the tube 4-5 times and incubated at room temperature for not more than 5 min. 350 µl of Solution III (neutralization solution, Appendix A-2) was added. Centrifuged the content (10 min, 13000 rpm) and supernatant was transferred to a fresh microcentrifuge tube. Isopropanol (0.6 vol.) precipitated DNA pellet was washed with 70% ethanol, air dried and dissolved in 25 µl of 10 mM Tris buffer. The plasmid DNA was used for downstream processes.

3.4.2 Plasmid isolation kit

For cloning, PCR amplification, particle bombardment, etc. purified plasmid DNA was isolated using the QIAprep Spin Miniprep kit (QIAGEN GmbH). The isolation procedure was as described by the manufacturer.

3.5 Restriction digestion

Restriction digestion of DNA was carried out by adding the components, in a given order, to an autoclaved microcentrifuge tube and incubated at the

recommended temperature (generally 37° C) for 3-4 hrs or longer as per the requirements.

Components		Volume
Sterile double distilled water	calculated volume	23 µl
10 X buffer	$^1/_{10}$ volume	3 µl
DNA	1 µg	3 µl
Restriction Enzyme	10 units/µg DNA	1 µl (10 u/µl)
Total reaction volume	**30 µl/µg DNA**	**30 µl**

3.6 DNA ligation

DNA ligation was performed by incubating the DNA fragment with appropriately linearized cloning vector in the presence of buffer and T_4 DNA ligase. The ligation reaction was set up by adding the components in a given order and incubated overnight at 7° C. A control reaction was set up that contained all the reagents listed below except the DNA fragment to be cloned. The control reaction helped in determining competence of the bacterial cells and/or working of ligation process, particularly when insertion of the fragment failed. 5 µl of the ligation product was used for transformation of competent bacterial cells.

Components		Volume
Sterile double distilled water	calculated volume	5 µl
10 X buffer	$^1/_{10}$ volume	1 µl
DNA fragment	100-500 µg	2 µl
Linearized cloning vector	50-100 µg	1 µl
T_4 DNA ligase enzyme	10 units/µl	1 µl
Total reaction volume		**10 µl**

3.7. Bacterial transformation

3.7.1 Preparation of competent cells

Competent cells were prepared as per the standard $CaCl_2$ method with minor modifications (Sambrook *et al.*, 1989). A single colony (*E. coli*, DH-5α) from freshly grown LA plate was used as inoculum. The bacterial culture of 0.6 OD_{600} and ice cold 0.1 M $CaCl_2$ was used to prepare fresh competent cells.

3.7.2 Transformation of competent cells :

Transformation of competent cells was performed by heat-shock method (Sambrook *et al.*, 1989). 200 µl of the competent cells and 50 ng of the plasmid DNA was mixed and incubated on ice for 30 min. Heat-shock was given for 90 sec at 42° C. Colonies from the transformed cells were seen after overnight incubation at 37° C.

3.8 Rice transformation

3.8.1 Generation of transgenic plants

The whole process of generation of transgenic rice was carried out in the following steps:

3.8.1.1 *Callus induction*

Matured seeds of rice cultivar PB-1 (Fig. 18a) were dehusked manually and used as explants to induce embryo-derived calli. The dehusked seeds (Fig. 18b) were surface sterilized (70% ethanol) for one minute. The seeds were washed thrice before treatment with 0.1% $HgCl_2$ for ten minutes with constant shaking. After the

treatment, seeds were thoroughly washed with sterile distilled water, 4-5 times, to remove any traces of mercuric chloride. The seeds were then spread over sterile filter paper pads to remove excess of water. Sterilized seeds (25-30) were plated on callus induction medium (Appendix, A-4) in 90 mm petri-plates. The plates were sealed with parafilm and incubated in dark at 28° C for five days. The tissue culture protocol of Khanna *et al.* (1997) was adopted with minor modifications.



Fig. 18. Rice (PB-1) seeds used as explants: mature seeds **(a)** and dehusked seeds **(b)**. Callus induction from germinated seeds **(c)**, and calli arranged on MS callusing medium in the center of petri-plate for bombardment **(d)**.

3.8.1.2 *Particle bombardment*

Five day old scutellar calli (Fig. 18c) were excised from the germinated seeds and 25-30 such calli were closely arranged within 2 cm (diameter) circular area in the center of a pre-sterilized plate containing fresh MS callus induction medium (Fig. 18d).

Gold particles (0.6 μm diameter) were prepared (Appendix A-11) and coated with DNA (Appendix A-12) using procedure described by Klein *et al.* (1988) with minor modifications. The gene-cassette fragment DNA for the genes of interest and selectable marker gene (2.5 μg, in a molar ratio of 4:1) were used for precipitation on the gold particles. Occasionally, GUS gene-cassette fragment DNA was used for bombardment to monitor the gene delivery.

The Biolistic apparatus (He-driven Particle Delivery System; PDS-1000/BioRad), placed on the working bench of a laminar air flow hood, was thoroughly cleaned with 70% ethanol. The accessories for bombardment, such as rupture discs, stopping screens, macrocarriers and macrocarrier holders were sterilized by dipping in absolute ethanol for 10 min and air dried under the clean air laminar flow. Embryogenic calli, arranged in the centre of a petri plate were placed at the fixed distance (9 cm) from the microcarrier launch assembly (Fig. 19). The calli were bombarded once under vacuum (25mm Hg) using 900 psi rupture discs. The bombardment process was carried out under aseptic conditions and the protocol of Khanna *et al.* (1997) was adopted with minor modifications. After bombardment, the calli were incubated on the same medium at 28° C in dark for 24 hrs.

3.8.1.3 *Clean DNA transformation*

Minimal gene-expression-cassettes (promoter-open reading frame-terminator) were used for clean DNA transformation. Co-bombardments involved two or more 'clean DNA' gene-cassette fragments: one carried the selectable marker gene and the other carried gene(s) of interest. For most of the bombardments, minimal gene-cassette fragments were isolated using restriction digestion of the plasmid DNA followed by agarose gel electrophoresis and gel extraction of the fragment of interest. Restriction enzymes, flanking the gene-cassette (Fig. 11, 20), were used and the gene-cassette fragment DNA was separated from remaining vector backbone by agarose (1.0%) gel electrophoresis. The DNA was eluted from gel using QIAEX II gel extraction kit (QIAGEN GmbH).

Restriction digestion and gel extraction of gene-cassette was not only cumbersome and time consuming process but also low yielding. Considering the complexities involved in generating sufficient quantities of gene-cassette fragment DNA, an alternative approach of generating gene-cassette DNA was investigated. A pilot experiment was designed to test the utility of PCR amplified gene-expression-cassette.

The experiment involved PCR amplification of GUS reporter gene-cassette using DNA polymerases with 3'-5' proof reading activity. Amplification products of three such high fidelity DNA polymerases, along with *Taq* DNA polymerase, were tested. The quantity and quality of amplification products were compared with that of the gene-cassette generated by restriction digestion and gel extraction.

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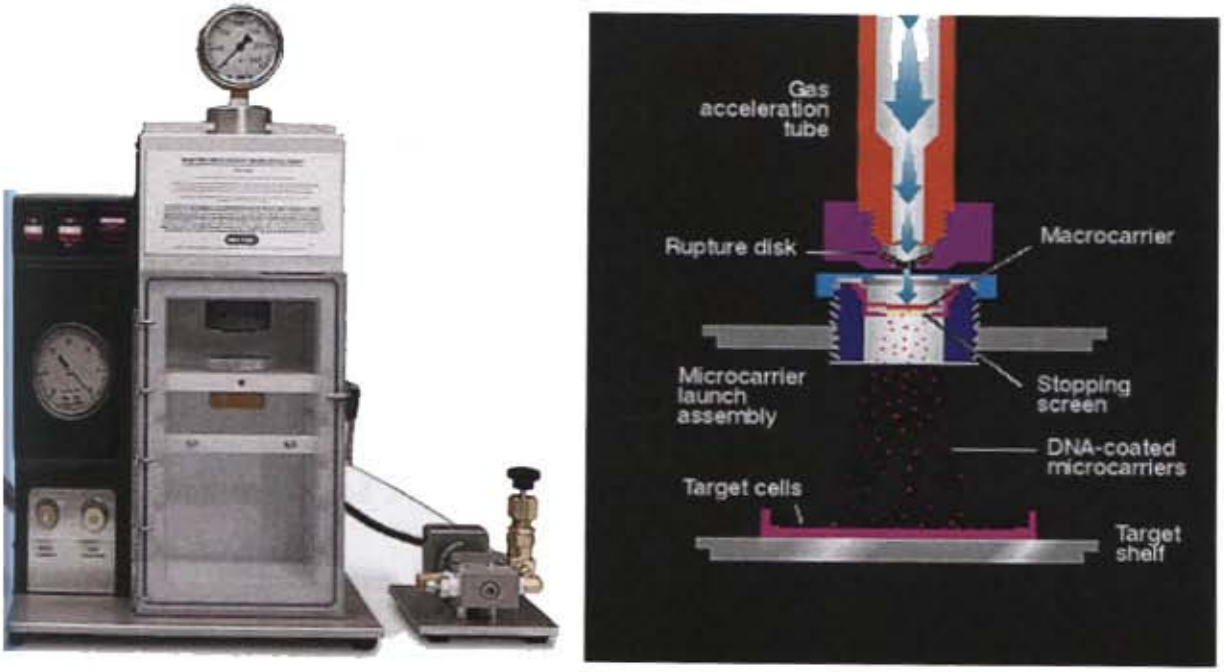


Fig. 19. He-driven Particle Delivery System. **(a)** PDS-1000/BioRad, **(b)** Sectional view of microcarrier launch assembly.

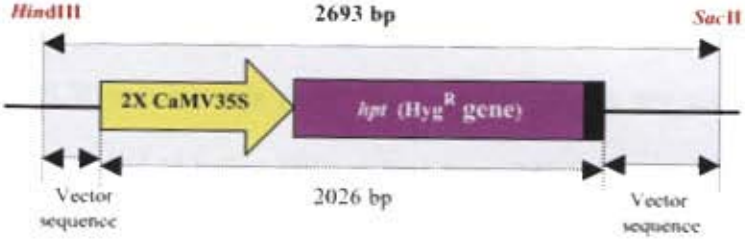


Fig. 20. Minimal gene-expression-cassette for the hygromycin resistance gene (*hpt*) was excised from pCAMBIA-1305.1 using restriction digestion with *Hin*dIII - *Sac*II. However, in restriction digestion with *Hin*dIII - *Sac*II, inclusion of some vector backbone sequences (268 bp upstream and 399 bp downstream) was inevitable. The 2X CaMV35S promoter is having duplicated enhancer region.

3.8.1.3.1 *PCR amplification* of *GUS gene-expression-cassette* :

Minimal gene-expression-cassette for the GUS reporter gene (Jefferson *et al.*, 1987) was generated from the source plasmid pCAMBIA-1305.1 (CAMBIA, Australia) by PCR amplification as well as by restriction digestion with *Sph*I. pCAMBIA-1305.1 (11,846 bp) has GUS*Plus* under the control of CaMV35S promoter and polyA signal from *nos* (nopaline synthase gene). Due to limitations of restriction sites, 220 bp upstream of the promoter and 129 bp downstream of the terminator (Fig. 21) were retained along with the isolated gene cassette (total 3227 bp). On the other hand, the PCR amplified gene expression cassette (2878 bp), amplified using 21 and 24 bases from the ends of the gene cassette as primers (Appendix A-13), was devoid of any vector backbone sequence. PCR amplifications were performed in 50 µl reaction volume, using standard 10x buffer supplied with the enzyme, 1.5 mM $MgCl_2$, 50 µM each of the four dNTPs, 20 ρM each of forward and reverse primer, 50 ng pCAMBIA-1305.1 as template DNA and 1 unit of DNA polymerase.

Three different DNA polymerases with proofreading activity were tested for comparative evaluation of their performance: i) DyNAzyme EXT (Finnzymes); ii) High Fidelity PCR enzyme mix (MBI Fermentas); and iii) XT-5 PCR system (Bangalore Genei Pvt. Ltd.). *Taq* DNA polymerase (MBI Fermentas), having no proofreading activity, was also included to serve as control. The reaction conditions were as follows: denaturation at 94° C for 3 min, then 32 cycles (94° C, 1 min; 54° C, 1 min; 72° C, 4 min.) followed by 7 min final extension at 72° C. PCR amplified gene-cassette DNA and gel extracted gene-cassette fragment were

quantified by running (electrophoresis) standard DNA marker (λ DNA/*Hin*d III digest) along with the test DNAs, as well as by spectrophotometry.

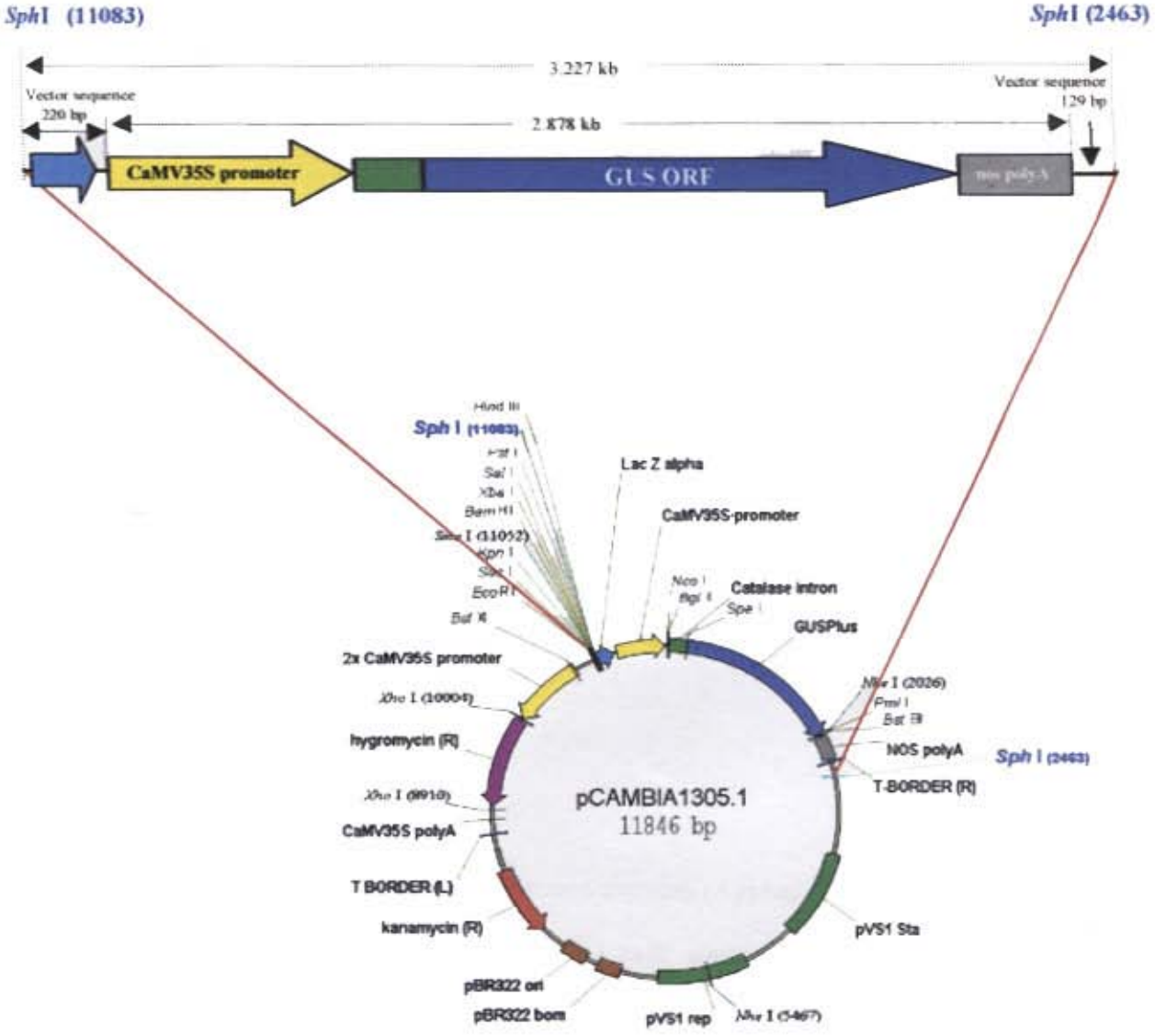


Fig. 21. Map of pCAMBIA-1305.1. The minimal gene expression cassette for the GUS reporter gene was excised by restriction digestion with *Sph*I. However, with *Sph*I, inclusion of some vector backbone sequences (220 bp upstream and 129 bp downstream) was inevitable.

3.8.1.3.2 ***Comparative evaluation of PCR amplified 'clean DNA' fragment*** **:**

Mature embryo-derived scutellar calli of *indica* rice breeding line IR-64 were used as explants for microprojectile bombardment. However, instead of using 2.5μg DNA for precipitation on 3mg gold particles for a set of six bombardments, only 0.5μg DNA was precipitated on 1mg gold particles (0.6 μm) for a set of two bombardments.

3.8.1.4 ***Transient GUS expression***

Transient *GUS* expression was observed in the calli 24 hrs after bombardment, as described by Jefferson (1987). The calli were submersed in GUS assay solution (Appendix A-6) and given a mild vacuum treatment for 15 min. The tubes were incubated at 37° C overnight. The calli were examined under microscope for the blue spots (GUS expression units). The average numbers of blue spots for each treatment (two plates of bombarded calli), over three replications, were statistically analyzed (Duncan's Multiple Range Test) to find significant differences among the treatment means.

3.8.1.5 ***Selection of transformed calli***

Bombarded calli were transferred to the selection medium (Appendix A-4) 24 hrs after bombardment. One-third of the individual calli was pressed inside the selection medium. The plates were incubated at 28° C in dark for 21 days. Small secondary calli observed to be appearing from the selected calli (Fig. 22a). These secondary calli were transferred to the freshly prepared selection medium and subjected to second and third selection rounds of selection, each of 21 days.

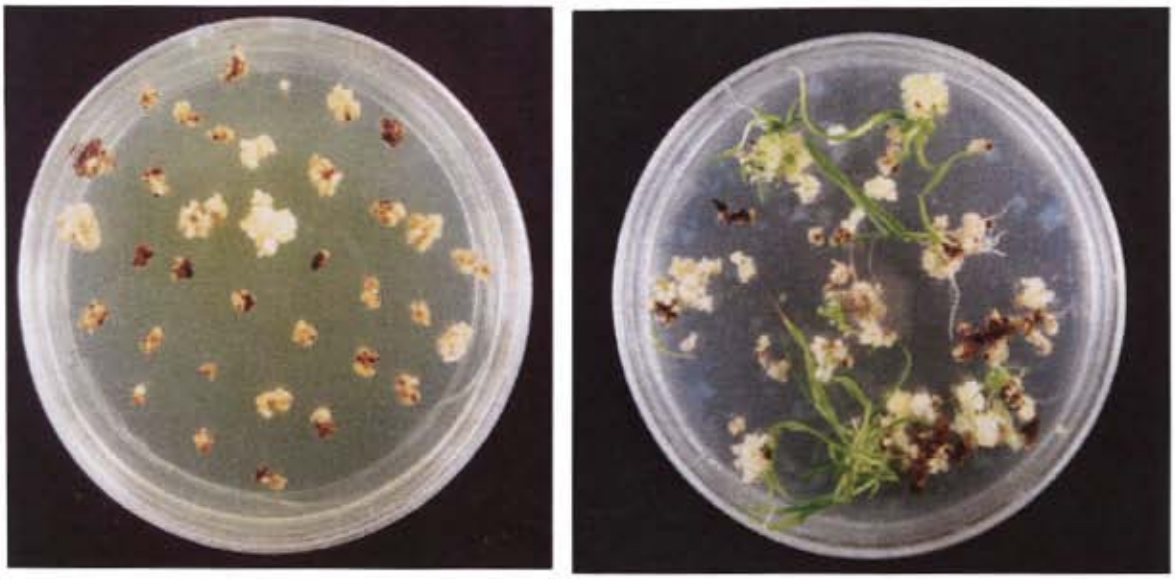
Untransformed calli (those not bombarded) were also plated on the selection medium, as control, and treated similar to that for the bombarded calli.

3.8.1.6 *Regeneration of transgenic plants*

The hygromycin selected calli were transferred to pre-regeneration medium in petri plates (Appendix A-4) and incubated at 28° C in dark for 14 days. The calli were then transferred to regeneration medium (Appendix A-4) and incubated at 26° C under 16 hrs photoperiod with light intensity of 110-130 $mM/m^2/s$ PAR using Philips F40/CW fluorescent tubes. Greening of the calli was observed, which emerged into shootlets (Fig. 22b). As the shoots reached 4-5 cm in length, they were transferred to the rooting medium (Appendix A-5).

3.8.1.7 *Rooting and hardening of putative transformants*

The regenerated shootlets were transferred to rooting medium (Appendix A-4) in culture tubes for root organogenesis (Fig. 23a). The tubes were incubated at 26° C under 16 hrs photoperiod. The plants with well developed roots and shoots were removed from culture tubes and adhering medium was removed by wash under running tap water. The plants were hardened by growing in hardening medium (Appendix A-4) in culture tubes for 14 days (Fig. 23b). The well developed hardened plants were then transferred to phytotron glasshouse and transplanted into pots filled with Agropeat (Fig. 23c). Plants were irrigated regularly with Hoagland's solution (Appendix A-15). The putative transgenic plants were grown in the phytotron till maturity (Fig. 24).



(a) (b)

Fig. 22. Selection of bombarded calli and plantlet regeneration. **(a)** secondary calli from the selected callus on hygromycin containing medium, **(b)** shoot organogenesis from the hygromycin selected calli.

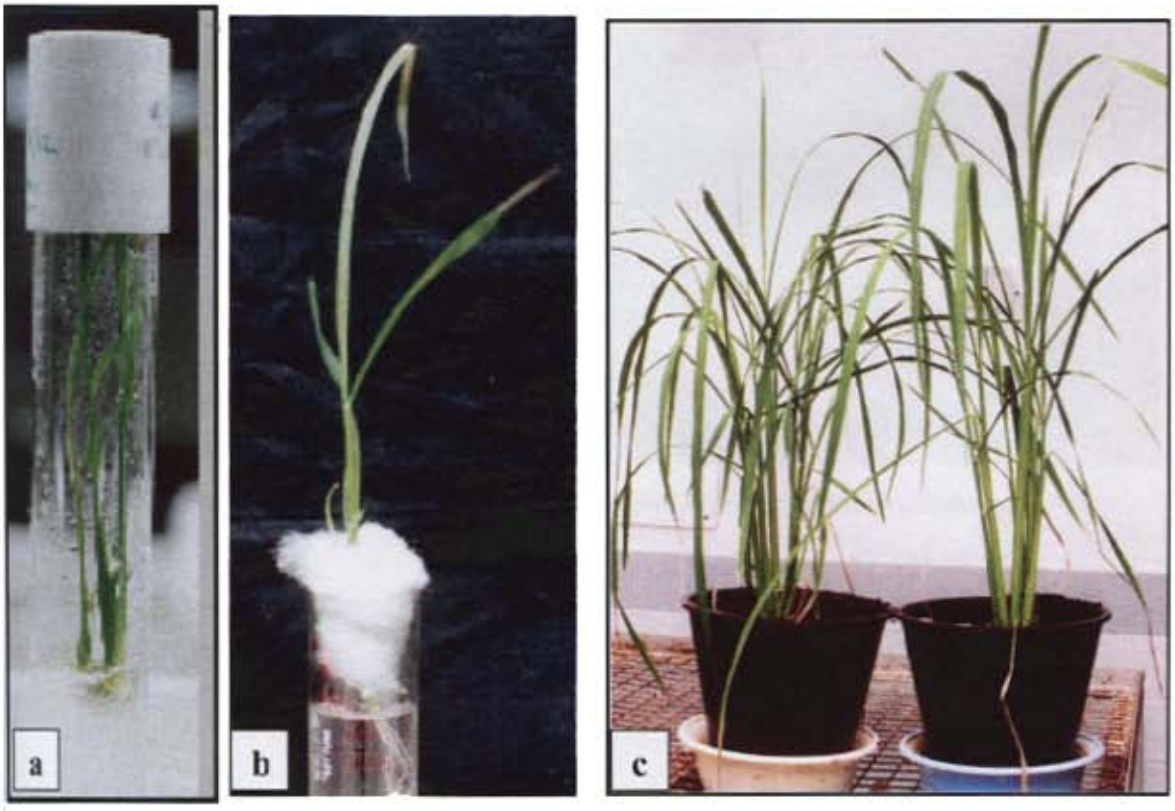


Fig. 23. Root-organogenesis and hardening of regenerated plants. **(a)** regenerated shootlets on rooting medium, **(b)** regenerated plant in liquid hardening medium, **(c)** hardened plants transplanted into pots filled with Agropeat being grown in phytotron.

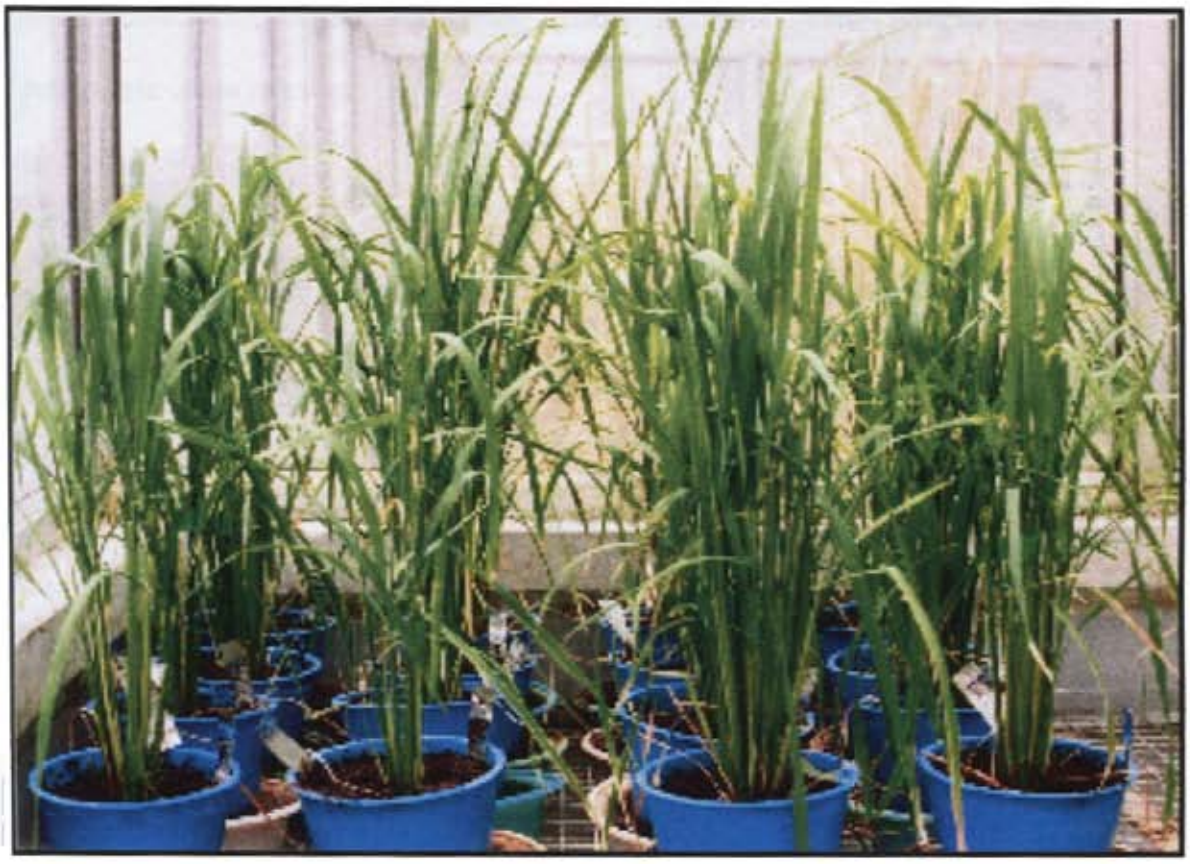


Fig. 24. Putative transgenic (PB-1) plants being grown in pots in phytotron glasshouse.

3.8.2 Molecular characterization of putative transgenic plants

The regenerated putative transgenic plants were screened for the presence of the gene(s) of interest using PCR. Southern analysis confirmed integration of the gene(s). Finally, RT-PCR was used to confirm transcription of the integrated gene.

3.8.2.1 *PCR screening of putative transformants*

The regenerated putative transgenic plants of PB-1 were screened using polymerase chain reaction.

3.8.2.1.1 *Genomic DNA isolation* :

Genomic DNA from putative transgenic plants was isolated using a simple protocol standardized in our laboratory. A small piece (1 cm) of young leaf tissues was ground in 200 µl of AP1 buffer (QIAGEN DNeasy Plant Mini Kit) with a disposable plastic pestle, specially designed for grinding soft tissues in microcentrifuge tube. A few grains of acid washed, sterilized sand was added to help grinding of the leaf tissues. The ground sample was incubated at 65° C for 15 minutes. To this, 65 µl of AP2 buffer (DNeasy Plant Mini Kit) was added and incubated on ice for 5 min. The content was centrifuged at 13,000 rpm for 10 min. Isopropanol (0.6 volumes) was mixed with supernatant and centrifuged at 13,000 rpm for 10 min. The pellet was washed with 70% ethanol, air dried and dissolved in 30 µl of sterilized double distilled water.

3.8.2.1.2 *Polymerase Chain Reaction (PCR)* :

Gene specific primers (Appendix A-13) were used for PCR amplification of a part of transgene(s) to screen the putative transgenic plants.

Master mix of the components of PCR reaction was prepared on ice, and dispensed into 0.2 ml thin walled PCR tubes. The required amount of template DNA was added in the individual tubes, a quick spin was given to collect the content in the bottom of the tube.

PCR cocktail prepared by mixing the components

Component	Volume
Sterilized distilled water	38.5 µl
10X buffer	5 µl
dNTPs mix (10 mM)	1 µl
Primer-F	1 µl
Primer-R	1 µl
Taq Polymerase (3 u/µl)	0.5 µl
Template DNA	3 µl
Total	**50 µl**

Biometra thermocycler was used for PCR amplification of the targeted template DNA using the following programme.

	Step	Temperature (°C)	Duration (min)
1.	Initial denaturation	94	3
2.	Denaturation	94	1
3.	Primer annealing	Variable (according to the primer pair)	1
4.	Primer extension	72	1:30 (repeated steps 2 - 4 for 32 cycles)
5.	Final extension	72	7
6.	Pause	4	∞

The PCR amplification products were subjected to agarose gel (1.2%) electrophoresis and visualized under UV light. Gels were photographed using gel documentation system.

3.8.2.2 *Southern hybridization*

The PCR positive T_0 plants were further characterized by Southern hybridization (Southern 1975).

3.8.2.2.1 *Genomic DNA isolation* :

Genomic DNA was isolated from young leaf tissues of the PCR positive T_0 plants and untransformed PB-1 plant (negative control) using DNeasy Plant Mini Kit (QIAGEN GmbH) following the procedure recommended by the manufacturer. The concentration of isolated genomic DNAs was gel estimated by running standard DNA marker (λ DNA/*Hind* III digest) along with the test DNA samples.

3.8.2.2.2 *Restriction digestion of genomic DNA* :

About 15 µg of genomic DNA (equivalent to 100 copies of rice genome) of every PCR positive T_0 plant as well as that of untransformed control (PB-1) plants was digested with *Bam*HI (which takes out the Bt gene, see Fig. 25) or with *Sac*II (which cuts the insert once, see Fig. 26). The plasmid construct used for generating 'clean DNA' fragments for transformation was also restricted with the restriction enzyme and used as positive control. Reaction mix was prepared as follows:

Linear Sequence: *pepc - 1B-1Aa - nos*

NEW ENGLAND BioLabs Inc.

Cleavage code	Enzyme name code
▼ | blunt end cut	Available from NEB
▼ | 5′ extension	Has other supplier
▲ | 3′ extension	Not commercially available
▼ | cuts 1 strand	*: cleavage affected by CpG meth.
	▮: cleavage affected by other meth.
	(enz.name): ambiguous site

Display: - NEB double cutter restriction enzymes
- Main non-overlapping, min. 100 aa ORFs

GC = 52%, AT = 48%

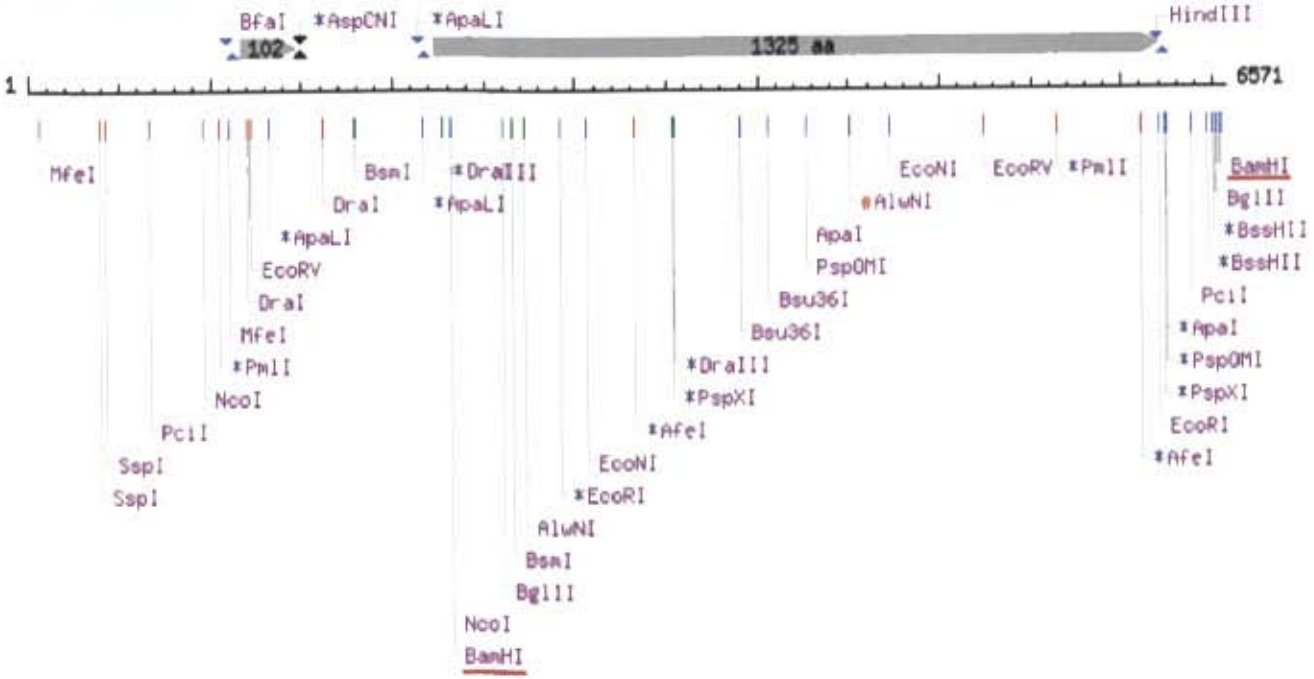


Fig. 25. Double cutter restriction enzymes of the linear fragment used for 'Clean DNA' transformation. *Bam*HI was used for restriction digestion of genomic DNA from transgenic plants to take out the gene (*1B-1Aa)* for detection of the transgene.

Linear Sequence: ***pepc - 1B-1Aa - nos***

NEW ENGLAND BioLabs Inc.

Cleavage code	Enzyme name code
▼▲ | blunt end cut	Available from NEB
▼▲ | 5′ extension	Has other supplier
▲▼ | 3′ extension	Not commercially available
▼ | cuts 1 strand	*: cleavage affected by CpG meth.
	#: cleavage affected by other meth.
	(enz.name): ambiguous site

Display:
- **NEB single cutter restriction enzymes**
- **Main non-overlapping, min. 100 aa ORFs**

GC = 52%, AT = 48%

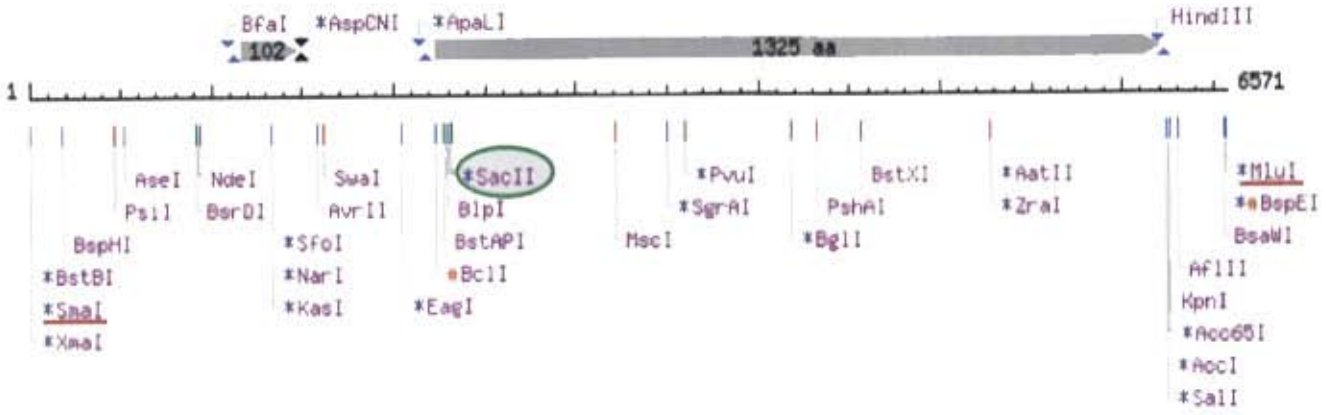
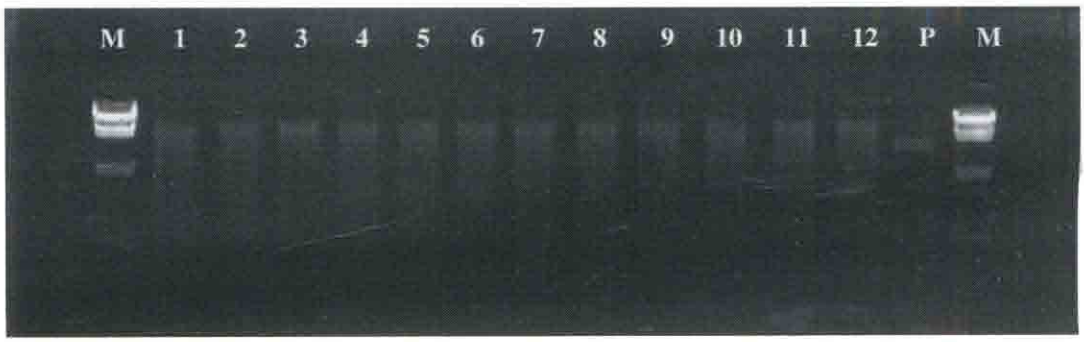


Fig. 26. Single cutter restriction enzymes of the linear fragment used for 'Clean DNA' transformation. *Sma* I and *Mlu* I was used to generate 'Clean DNA' fragments, while *Sac* I was used for restriction digestion of genomic DNA from transgenic plants to analyze the copy number of the insert.

Components		Volume
Sterile double distilled water	calculated volume	560 µl
10 X buffer	$^1/_{10}$ volume	75 µl
Genomic DNA	15 µg	100 µl
Restriction Enzyme	10 units/µg DNA	15 µl (10 u/µl)
Total reaction volume	**50 µl/µg DNA**	**750 µl**

- The restriction digestion was carried out at 37° C for 16 to 20 hrs. Complete restriction of the genomic DNA was confirmed by loading 200 ng aliquots on 0.8% agarose gel (Fig. 27). The restricted DNA was concentrated by isopropanol precipitation and dissolving in 30 µl sterile distilled water.



Lane No.	Description
1 & 15	Marker (λ/*Hin*dIII digest)
2 - 13	Genomic DNA *Bam*HI digestion
14	Plasmid DNA *Bam*HI digestion

20 µl of reaction mix was loaded on 0.8% agarose gel, run at 60 volt for 60 minutes.

Fig. 27. Agarose gel electrophoresis to check complete digestion of plant genomic DNA with *Bam*HI.

3.8.2.2.3 *Agarose gel electrophoresis* :

The restricted genomic and plasmid DNA was run on 0.8% agarose gel prepared in 1X TAE. DNA size marker (1kb ladder) was also loaded in one of the wells. Electrophoresis was carried out at 30 volt overnight. The gel was observed under

UV and position of marker bands was traced on a polythene sheet. The marker lane was cut removed from the gel before processing of the gel for transfer.

3.8.2.2.4 *Blotting of DNA from agarose gel* :

To facilitate transfer of restricted DNA to the positively charged nylon membrane (Hybond N^+) the gel was immersed in 500 ml depurination solution (Appendix A-14), followed by slow shaking till the blue dye turned yellow. The gel was rinsed with sterile distilled water and immersed in 500 ml of denaturation solution (Appendix A-14) for 30 min on shaker. The gel was rinsed and submerged in 500 ml of neutralization solution (Appendix A-14) for 30 min.

An appropriate volume of 20X SSC (transfer buffer; Appendix A-14) was poured into a plastic tray. The upward capillary transfer assembly was prepared on a platform made by keeping a glass plate over the plastic tray. The different components of the assembly were arranged in order, following the procedure described by Sambrook *et al.* (1989) (Fig. 28). The gel was placed upside down on the wick. The trapped air bubbles were removed carefully.

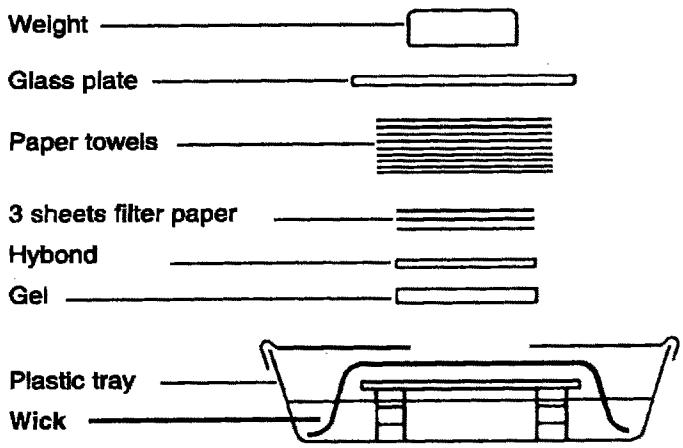


Fig. 28. Upward capillary transfer assembly.

The capillary transfer of DNA was continued for 16-18 hrs. Once the transfer was over, the assembly was dismantled. Downward processing of the membrane was carried out as described by Sambrook *et al.* (1989). The membrane was used for pre-hybridization treatment.

3.8.2.2.5 *Probe preparation* :

Radio labeled probe was prepared by using HexaLabel Plus DNA labeling kit (MBI Fermentas). PCR amplified internal regions of the Bt genes was used as template for generation of gene-specific radiolabeled (with α-^{32}P dCTP) DNA probe. The protocol used was as recommended by the manufacturer.

3.8.2.2.6 *Prehybridization and hybridization* :

The membrane was incubated in 20 ml of pre-hybridization buffer (Appendix A-14) for 4 hours at 65° C in hybridization oven under continuous rotation. Then pre-hybridization buffer was replaced with 10 ml of hybridization buffer (Appendix A-14). The radiolabelled probe was added and hybridization was carried out at 60° C for 12-16 hrs under continuous rotation.

3.8.2.2.7 *Washing* :

After hybridization, the membrane was washed for 20 min at 60° C with each of the low, medium and high stringency wash solution (Appendix A-14).

3.8.2.2.8 *Autoradiography* :

The membrane was wrapped in saran wrap and placed inside a lead cassette. An X-ray film (Kodak) was placed over the membrane while working inside a dark

room. The cassette was incubated at -70° C for 3-7 days. The film was processed by submerging in developer and fixer (Kodak) for 5 min. The film was washed thoroughly under running tap water and air-dried.

3.8.2.3 *Reverse Transcription-PCR*

Normal growing Southern positive transgenic plants for *cry1B-1Aa* translational fusion genes were used for RT-PCR analysis. Four PCR positive but Southern negative plants (Nos. 26, 41, 48 and 61) were excluded from RT-PCR analysis.

3.8.2.3.1 *Isolation of total RNA* :

Total RNA was isolated from 100 mg young leaf tissues of transgenic plants as well as from untransformed PB-1 (negative control) plant using RNeasy Plant Mini Kit (QIAGEN) following procedure recommended by the manufacturer. Glassware, plasticware, pestles, mortars, etc. used for RNA isolation were treated with 0.1% DEPC (an RNase inhibitor) for 24 hrs and autoclaved before use. Appropriate precautions were taken while handling DEPC, since it is carcinogenic. The quality of RNA preparation was checked by loading 3 µl of isolated RNA on a denaturing agarose gel electrophoresis (Appendix A-15). The concentration of RNA was estimated spectrophotometrically.

3.8.2.3.2 *DNase treatment* :

The isolated RNA was treated with DNase I to make sure that the RNA preparation was free from any DNA contamination. The DNase treatment reaction was set as below :

Component	Volume
Total RNA	1 µg (4 - 5 µl)
10X reaction buffer	1 µl
DNase I (1 u/µl)	1 µl
RNase free water	to make up the final volume to 10 µl
Total volume	**10 µl**

The reaction mix was incubated at 37° C for 1 hr. Reaction was stopped by adding 1µl of 0.5 M EDTA, incubated at 65° C for 10 min and stored at - 20° C until used.

3.8.2.3.3 *RT-PCR* :

OneStep RT-PCR Kit (QIAGEN) was used for RT-PCR. It involved, reverse transcription at 50° C for 30 min, followed by simultaneous inactivation of reverse transcriptases and activation of HotStar *Taq* DNA polymerase by heating at 95° C for 15 min. The protocol recommended by the manufacturer was used. The reaction mix was prepared as follows:

Component	Volume
5X OneStep RT-PCR buffer	10 µl
dNTP mix (10 mM)	2 µl
Forward primer (1BF-2)	1.5 µl
Reverse primer (1BR-2)	1.5 µl
OneStep RT-PCR enzyme mix	2.0 µl
Template RNA	2 µl (100-200 ng)
RNase free water	to make up the final volume to 50 µl
Total volume	**50 µl**

RNA template was added into the reaction tubes and loaded on thermocycler (Biometra T-Personal). A negative control, without template RNA, was included in the experiment. Following programme was used for the RT-PCR.

	Step	Temp (°C)	Duration (Sec)
1.	Lid temperature	105	
2.	Reverse transcription	50	30
3.	Initial PCR activation	95	15
4.	Cycle denaturation	94	60
5.	Annealing	59	45
6.	Extension	72	60 (repeated steps 4 - 6 for 32 cycles)
7.	Final extension	72	600

The RT-PCR products were subjected to agarose gel (1.2%) electrophoresis and visualized under UV light. Gel was photographed using gel documentation system.

3.9 Evaluation of Transgenic plants

The PCR and Southern positive transgenic plants were evaluated for resistance against yellow stem borer (YSB). Cut-stem bioassay as well as whole plant bioassays of potted plants were carried out to evaluate the transgenic plants.

3.9.1 Cut-stem bioassay

Seventeen PCR positive plants, showing normal growth, were evaluated for YSB resistance. Two of the non-transgenic plants (Plant Nos. 66 and 67) as well as untransformed control plants were also included in the cut-stem bioassay.

Individual tillers of the selected T_0 plants were tested in a three-replication cut-stem assay.

3.9.1.1 *Collection of YSB moths*

Yellow Stem Borer moths were collected from unprotected paddy fields of the research farms at Rice Research Station, Kapurthala (Punjab), a hot-spot area of rice stem borers. The moths were collected in round bottom flasks, either early in the morning or late in the evening, transferred to cloth-bag caged potted rice plants (Fig. 29a). The caged plants were carried to IARI, New Delhi. Generally, the moths laid eggs masses on potted plants within 24 hrs. The plants were transferred to lighted growth chamber maintained at 28° C for 7 days. Leaf blades carrying one or two egg masses were collected in conical flask and incubated at 37° C to facilitate egg hatching. The freshly released neonate larvae were used for infestation within 3 hrs of hatching.

3.9.1.2 *Infestation of YSB larvae*

Tillers of transgenic and untransformed control PB-1 plants were collected from potted plants. Stems were cut into 3 pieces of 5-7 cm length. The stem pieces were plugged with wet cotton at both the ends (Fig. 29b) to prevent entry of the larvae from the cut ends. The prepared cut-stem was placed on a moist filter paper in polystyrene petri plate and infested with 5 freshly hatched neonate larvae (Fig. 29c). The plates were properly sealed and incubated under 16 hrs photoperiod at 28° C (Fig. 30). After 4 days of incubation, the stem pieces were dissected using scalpel and forceps (Fig. 31), and the larval growth and mortality was recorded (Appendix A-17).



Fig. 29. Cut-stem bio-assay of Bt transgenic PB-1 plants. **(a)** Caged potted rice plant for the collection of YSB moths, **(b)** cut-stem of transgenic plant plugged at both the ends with cotton **(c)** neonate larva of yellow stem borer released on cut-stem.

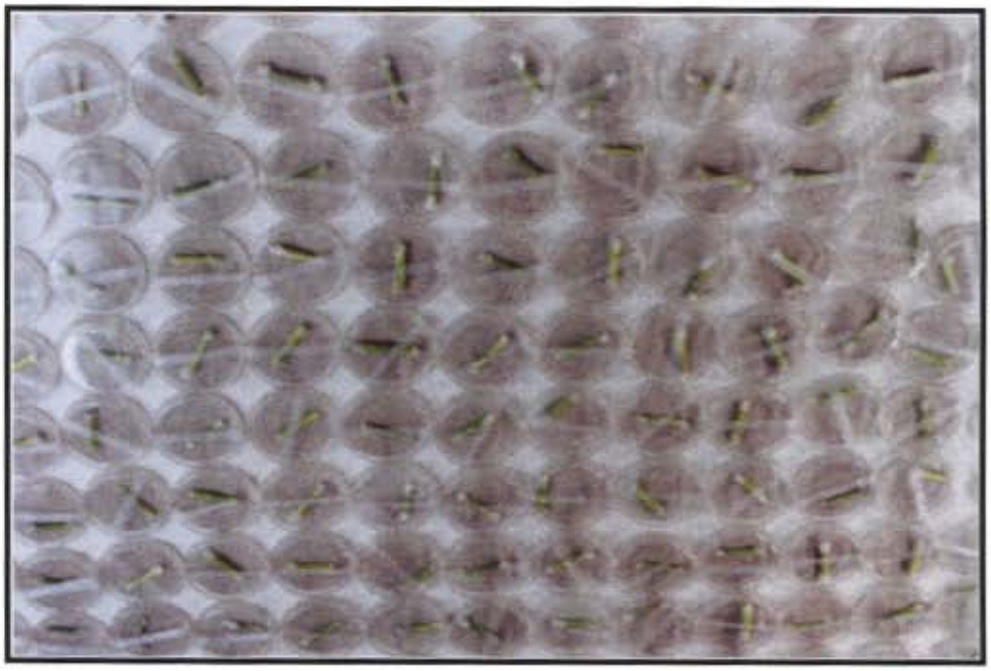


Fig. 30. Cut-stem bioassay of PB-1 Bt transgenic plants.

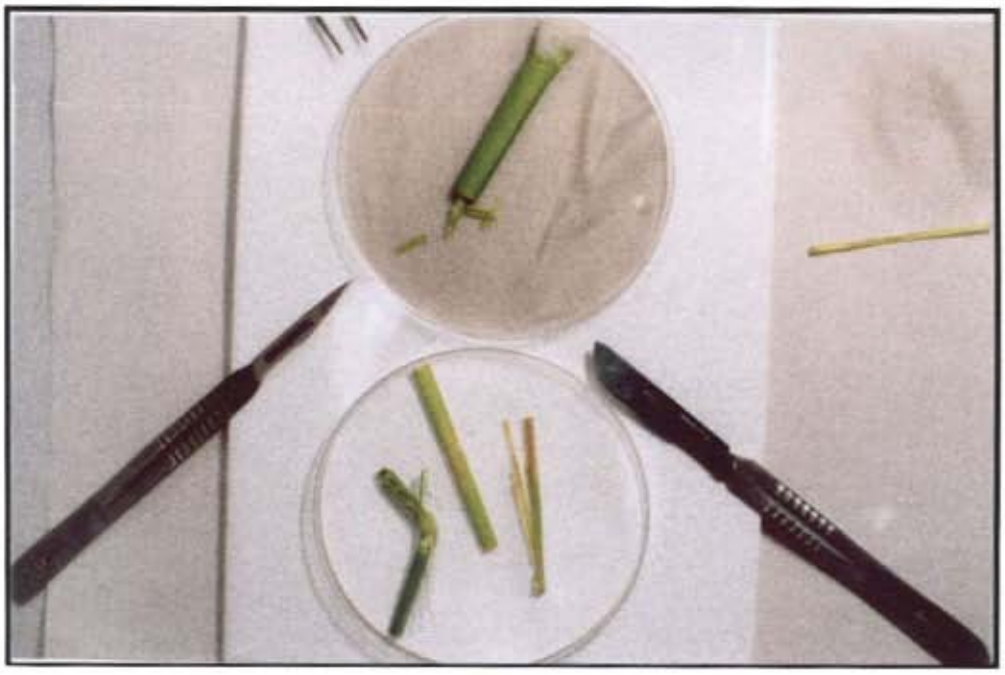


Fig. 31. Dissection of bio-assayed cut-stems to record mortality and growth of yellow stem borer larvae.

3.9.2 Whole plant bioassay

Only selected transformants were tested by whole plant bioassay. Four of the selected plants (T_0 plant Nos. 7, 11, 38 and 64) were Southern and RT-PCR positive while two of the plants (T_0 plant Nos. 47 and 59) were Southern positive but RT-PCR negative. One of the non-transgenic plants (T_0 plant Nos. 67) was also included in the whole plant bioassay. Freshly hatched neonate larvae of YSB were used for whole plant bioassays. The potted transgenic plants along with the untransformed control were placed in isolation in polyhouse. Each potted plant was infested with 25 larvae. The plants were maintained under conditions suitable for growing rice in polyhouse. After 15 days of infestation, observations were made on the plants for the symptoms of YSB infestation. Affected tillers were dissected and critically analyzed for larval growth and mortality.

RESULTS

Gene-expression-cassettes were successfully constructed using specific promoters and *Bt* genes for 'clean DNA' transformation of *indica* rice. A large number of Pusa Basmati-1 putative transgenic rice plants were generated using microprojectile-mediated gene delivery method. Putative transformants were analyzed for the integration of transgene(s) using PCR, Southern hybridization and RT-PCR. Southern positive transformants were tested for yellow stem borer (YSB) resistance by cut-stem and whole plant bioassays under controlled conditions. Detailed results of generation and evaluation of Bt transgenic PB-1 plants are described.

4.1 Sequencing of genes and promoters

Sequencing results obtained for single strand non-assembled primer walking reactions (from Microsynth GmbH, Switzerland) for the genes and promoters were validated by BLAST with NCBI GenBank nucleotide sequences. Although, portion of the query sequence was found to have alignment with many different database sequences, complete alignment was seen only with the subject of interest. For example, when BLAST was performed for the assembled base sequence of *mpi* promoter, complete homology was observed with the base sequence for *mpi* gene from *Zea mays* available in the GenBank database (Fig. 32; Appendix A-7). The sequencing results for PCR amplified gene (*cry1B*) and promoter (*mpi*) showed complete conformity to the respective known sequences. No errors (substitutions/deletions/insertions) were detected.

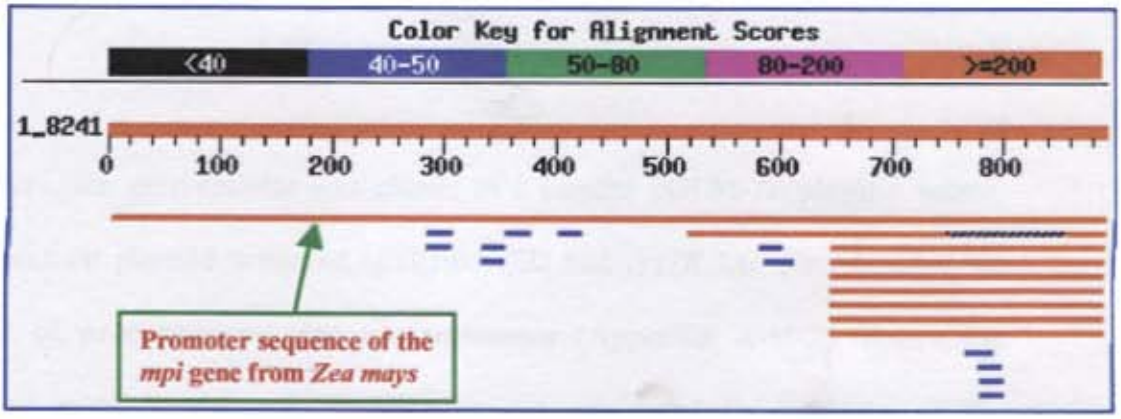


Fig. 32. Distribution of BLAST hits on the query (*mpi* promoter) sequence showing complete alignment with the subject of interest (i.e. promoter of the *mpi* gene from *Zea mays*).

4.2 Cloning genes and promoters

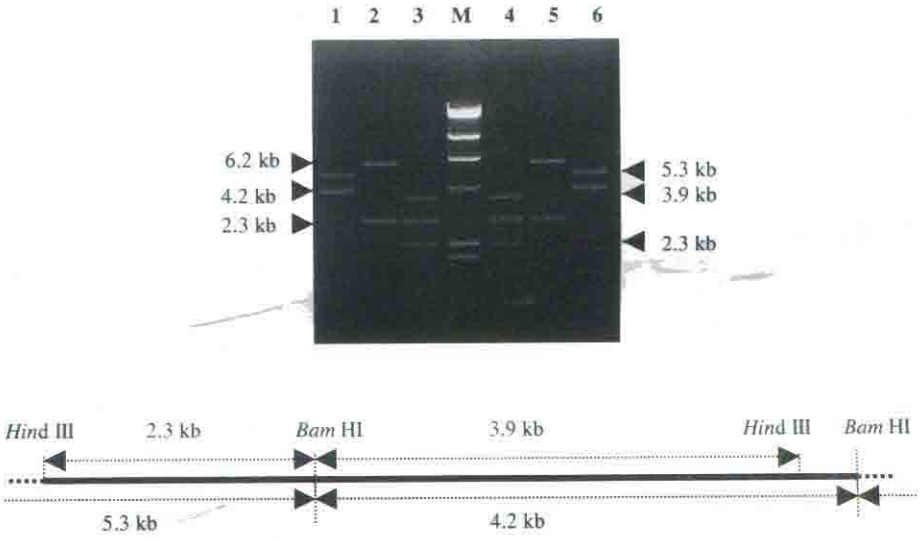
4.2.1 Cloning *pepc* promoter

The synthetic *pepc* promoter was initially cloned in pCAMBIA-1305.1 and later in pGEM-7Z at MCS between *Hin*dIII-*Bam*HI sites, which resulted into the constructs pSKPPC-05 (14,111 bp) and pSKPPC-7Z (5,286 bp), respectively.

4.2.2 Cloning of translational fusion genes

Translational fusion of *cry1B* and *cry1Aa* genes was cloned into the pSKPPC-05 at *Bam*HI site, which resulted into the construct pSKPBA-05 (Appendix A-10.1). The larger size of recombinant plasmid pSKPBA-05 (18,346 bp) was limiting factor in getting sufficient yield of the gene-expression-cassette (Promoter-ORF-Terminator) fragment DNA. The amount of gene-cassette fragment DNA was expected to be one-third (3.8 µg) of the amount of plasmid used (10 µg) for restriction digestion and gel extraction, however in real terms the amount obtained was only one-fifth (2.2 µg) of the plasmid used. The low yield of the desired fragment DNA became a major limitation in using this construct for 'clean DNA' transformation.

Therefore, the gene-cassette was cloned in a smaller pGEM-7Z plasmid vector. The resultant plasmid construct (pSKPBA-7Z) had *cry1B-1Aa* placed under the control of *pepc* promoter and *nos* terminator (Appendix A-10.2). Restriction mapping with *Bam*HI and *Hin*dIII enzymes confirmed the integrity of the recombinant plasmid (Fig. 33). The expected yield of gene-cassette fragment DNA (6,563 bp) in this case was more than two-third of the quantity of the plasmid (9,526 bp) DNA used for restriction digestion.



Lane No.	Description
1 & 6.	*Bam* HI digestion
2 & 5	*Hin*d III digestion
3 & 4	*Bam* HI - *Hin*d III digestion
M	Marker (λ/*Hin*d III digest)

Fig. 33. Confirmation of the different elements in the recombinant plasmid pSKPBA-7Z by restriction mapping.

4.2.3 Addition of restriction sites to the termini of *mpi* promoter by PCR amplification

The desired restriction sites were added to the ends of the promoter using suitably designed primers and high fidelity DNA polymerase for PCR amplification. The amplification product (970 bp; Fig. 34) had *Hin*dIII site at 5' end and *Bam*HI site at 3' end of the promoter.

4.2.4 Cloning *mpi* promoter in pGEM-7Z

The PCR amplified *mpi* promoter fragment was first cloned in pDrive Cloning Vector (pDCV-Mpi, Fig. 13). After sequence confirmation of the promoter, the fragment was digested out (*Hin*dIII - *Bam*HI) and cloned in pGEM-7Z at the multiple cloning site which resulted into the plasmid construct pSKMpi-7Z (Fig. 14).

4.2.5 Cloning *cry1Aa* in pSKMpi-7Z

The *cry1Aa* fragment, digested with *Bam*HI enzyme from the pC1300-Ubi-*1Aa*, was cloned into pSKMpi-7Z. The derived plasmid construct (pSKMAa-7Z) had *cry1Aa* under the control of *mpi* promoter and *nos* terminator (Appendix A-10.3). Restriction mapping with *Bam*HI and *Hin*dIII confirmed the structural details of the plasmid.

4.2.6 Addition of a restriction site to the PCR amplified *cry1B* gene

The *cry1B-nos* fragment was PCR amplified using suitable primers and high fidelity DNA polymerase. To the amplification product (2277 bp; Fig. 35) *Bam*HI site at 5' end was added, while it had one already at 3' end (Appendix A-8.3).

4.2.7 Cloning *cry1B* in pSKPPC-7Z

The PCR amplified *cry1B-nos* fragment was first cloned into pDrive Cloning Vector (pDCV-1B). Base sequence verified gene fragment was then cloned into pSKPPC-7Z at *Bam*HI site. The derived plasmid construct (pSKP1B-7Z) had *cry1B* placed under the control of *pepc* promoter and *nos* terminator (Appendix A-10.4). Structural organization of the plasmid was verified with *Bam*HI and *Hin*dIII restriction mapping.

4.3 Generation of Bt transgenic plants

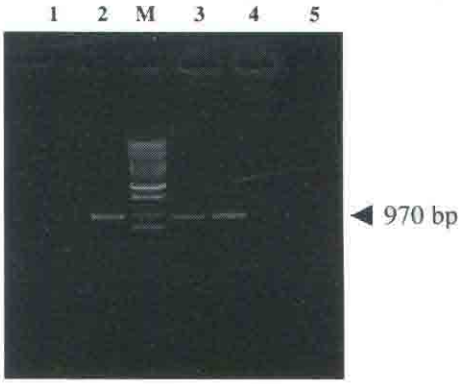
In all, 98 putative transformants were generated following microprojectile delivery of minimal gene-expression-cassette DNA encoding different Bt proteins and a selectable marker (Table 7).

Table 7. Bombardment of PB-1 calli with minimal gene-expression-cassette DNA for generation of Bt transgenics of *indica* rice.

Gene-expression-cassettes used for co-bombardment	Calli bombarded #	Putative transformants #	Transformation frequency φ (%)
pepc-1B-1Aa + hpt *	1440	67	0.90
pepc-1B + mpi-1Aa + hpt *	720	31	0.27
Total	**2160**	**98**	**0.69**

* Gene-expression-cassette for hygromycin resistance (selectable marker) gene comprised of 2x CaMV35S promoter-ORF-CaMV35S polyA.

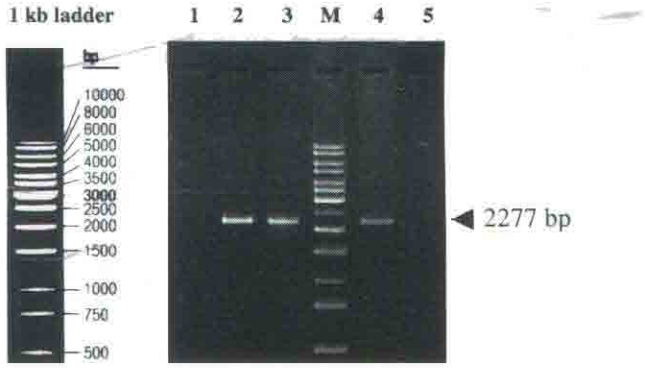
φ Transformation frequency = No. of embryonic-calli bombarded / No. of independent transformants for both the *cry* genes X 100.



Lane No.	Description
1.	- ve control (no template)
2.	Amplification product
M	Marker (1 kb ladder)
3.	Amplification product
4.	Amplification product
5.	- ve control (a different plasmid DNA)

2 µl of amplification product was loaded on 1.2% agarose gel, run at 75 volt for 25 minutes.

Fig. 34. PCR amplified *mpi* promoter fragment (970 bp)



Lane No.	Description
1.	- ve control (no template)
2.	Amplification product
3.	Amplification product
M	Marker (1 kb ladder)
4.	Amplification product
5.	- ve control (a different plasmid DNA)

2 µl of amplification product was loaded on 1.0% agarose gel, run at 75 volt for 45 minutes.

Fig. 35. PCR amplified *cry1B* gene fragment (2277 bp)

4.3.1 'Clean DNA' transformation

To avoid any undesirable consequences due to integration of vector backbone sequences, 'clean DNA' transformation method was used. Minimal gene-expression-cassettes were generated either by restriction digestion and gel extraction or by PCR amplification.

4.3.2 Minimal gene-expression-cassette

Initially, the gene-expression-cassettes were generated only by restriction digestion of the plasmid followed by gel extraction of the fragment of interest. Yield of the gene-cassette fragment, on restriction digestion with *Sma*I-*Mlu*I followed by the gel extraction, was 4.3 µg in case of pSKPBA-7Z; 3.2 µg in case of pSKMAa-7Z and 4 µg in case of pSKP1B-7Z from 10 µg of the respective plasmid DNA. The yield of hygromycin resistance gene-cassette fragment (2x CaMV35S promoter-*hpt*-CaMV35 polyA) on restriction digestion with *Hin*dIII - *Sac*II enzymes followed by gel extraction, was 1.5 µg from 10 µg of pCAMBIA1305.1.

The restriction digestion and gel extraction of gene-cassette fragments was not only cumbersome and time consuming but also a low yielding process. Considering the problems involved in generating sufficient quantities of gene-cassette fragment DNA, an alternative approach of generating sufficient quantities of the minimal gene-expression-cassette DNA was investigated. This involved PCR amplification of gene-cassette using DNA polymerase with 3'-5' proof reading activity. In a pilot experiment, the quantity and quality of amplified product was assessed by comparison with that of the gene-cassette generated by restriction digestion and gel extraction process.

In this experiment, GUS reporter gene was used as a test system. The GUS gene-expression-cassette DNA was PCR amplified using high fidelity DNA polymerases. Similar fragments were also generated by restriction digestion and gel extraction. The quantity of GUS gene-cassette DNA obtained through the process of restriction digestion and gel extraction was less than one-sixth of the pCAMBIA-1305.1 plasmid DNA used. Each column of the plasmid mini-prep kit yielded about 10μg of the plasmid DNA. On restriction digestion of the plasmid with *Sph*I and subsequent gel extraction, only 1.5μg GUS fragment (3227 bp) DNA was obtained. The quantity of 'clean DNA' so obtained was just sufficient for six bombardments. Due to limitations of restriction sites, 349 bp of the vector backbone sequences was retained along with the GUS gene-cassette fragment. On the other hand, PCR amplification using specific primers yielded the GUS gene-cassette fragment (2878 bp) having no vector backbone sequences. Moreover, just one PCR reaction yielded a quantity (3 μg) of GUS fragment DNA that was sufficient for 12 bombardments.

4.3.3 Particle bombardment

Five-day-old scutellar calli of PB-1 were bombarded with the minimal gene-expression-cassette DNA generated by restriction digestion and gel extraction. The microprojectile delivery system was monitored by using the GUS reporter gene. A large number of well spread blue spots were observed on the calli bombarded with GUS gene-cassette (Fig. 36).

When the GUS fragment DNA generated by PCR was used for bombardment to assess the quality of amplified product, the results of transient GUS expression did

not show significant differences except for *Taq* polymerase (Fig. 37). Significantly fewer blue spots were seen on the calli bombarded with *Taq* polymerase amplified DNA. Differences for GUS spots, among the different polymerases with proofreading activity, were not significantly different at 5% level by DMRT (Fig. 38).

In comparison to the restriction digestion process, PCR amplification of the gene-cassette DNA was simple, relatively less time consuming and also much less expensive. For isolation of 50µg gene-cassette DNA, comparison of cost estimates (for the consumables only) indicated that the process of restriction digestion and gel extraction is several times more expensive than PCR amplification (Table 8). Cost difference was more pronounced when miniprep rather than a midiprep kit was used for DNA isolation from bacteria.

4.3.4 Selection of bombarded calli

The bombarded calli were transferred onto hygromycin containing medium (selection medium), 24 hrs after bombardment for selection of the cells transformed with *hpt* gene and the gene(s) of interest. Most of the untransformed calli could not survive on the hygromycin selection, as evident from the control where unbombarded calli were plated on selection medium. A few unbombarded calli were found to be proliferating on the selection medium, escaping the hygromycin selection system (Fig. 39a). By the end of third round of selection, most of the escapes were eliminated (Fig. 39b). The selected calli were subsequently transferred to regeneration medium.



Fig. 36. Transient expression of *GUS* gene in PB-1 calli, 24 hrs after bombardment.

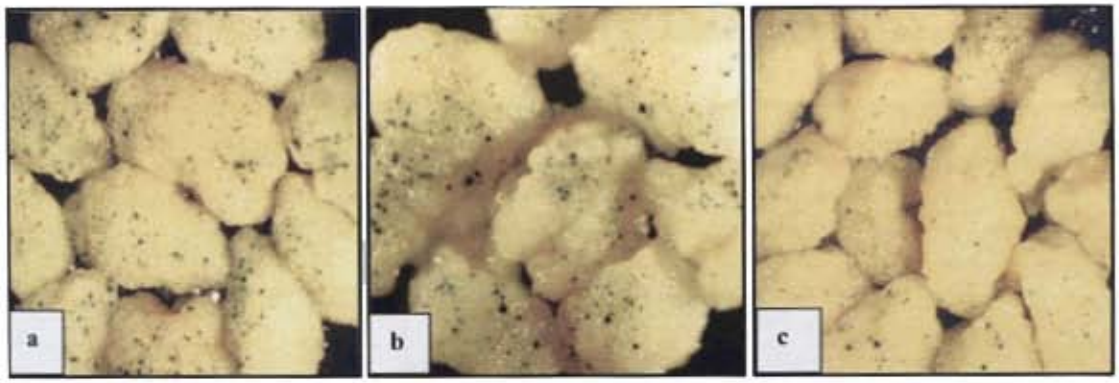


Fig. 37. GUS spots on scutellar-calli (cv IR-64) bombarded with minimal GUS gene-expression-cassette. The linear DNA fragment was generated by: **(a)** restriction digestion and gel extraction, **(b)** PCR amplification using XT-5 DNA polymerase, and **(c)** PCR amplification using *Taq* DNA polymerase.

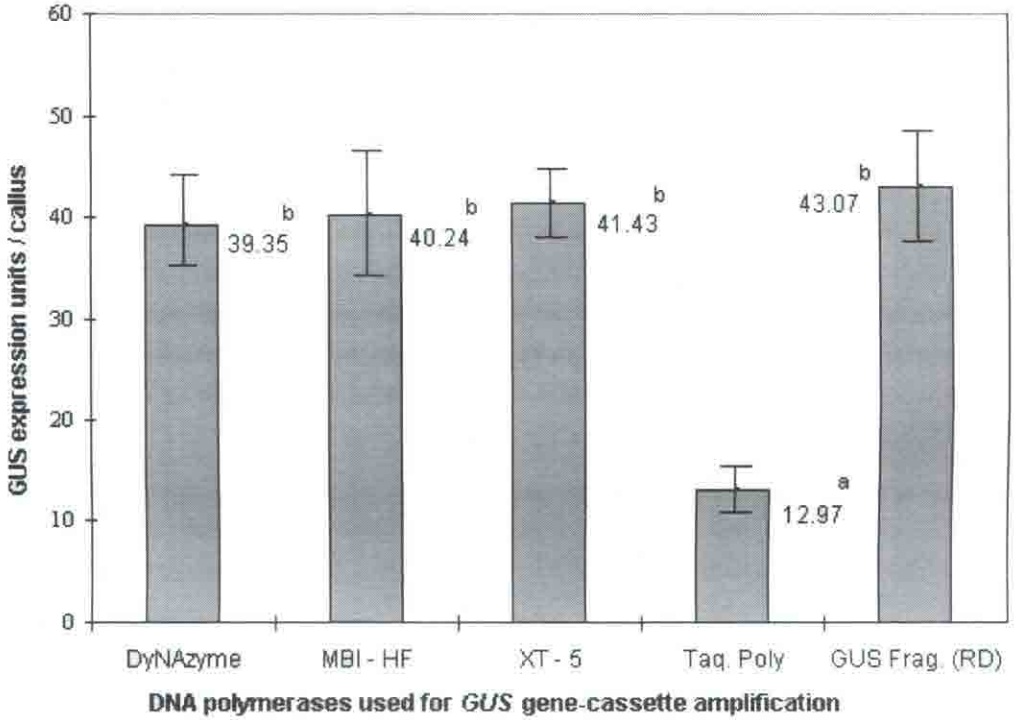


Fig. 38. Comparative profile of GUS expression units (GEU) per callus, following particle bombardment with GUS gene-fragment generated by PCR amplification or restriction digestion and gel extraction. PCR amplification was carried out using DNA polymerases, with proofreading activity (DyNAzyme, MBI-HF and XT-5) or without (*Taq* polymerase). GUS fragment (RD) column represents GEU recorded with the fragment (GUS DNA) generated by restriction digestion of the plasmid. Means of three replications followed by a common letter are not significantly different at 5% level by DMRT.

Table 8. Cost estimates of consumables used for generation of 50 µg linear gene-expression-cassette DNA.

Restriction digestion and gel extraction			PCR amplification	
Requirements	Approx. cost (US$)*		Requirements	Approx. cost (US$)*
	Miniprep Kit	Midiprep Kit		
Plasmid isolation	76.70	37.30	Primers	6.10
Restriction digestion	23.80	23.80	dNTP mix	2.60
Gel electrophoresis	22.30	22.30	Enzyme	13.50
Gel extraction	61.30	61.30	Template	0.60
Miscellaneous	1.90	1.30	Miscellaneous	1.20
Total	186.00	146.00	**Total**	24.00
Cost/bombardment	**0.93**	**0.73**	**Cost/bombardment**	**0.12**

* 1. As per listed prices in India.

2. Cost of each plasmid isolation reaction kit (column, solutions, etc.) for miniprep/midiprep is US$ 2.3/11.3. Each reaction gave 10/100µg high copy number plasmid DNA. On restriction digestion, 10µg plasmid DNA was expected to give 2.5µg linear fragment of the *GUS* gene expression cassette, since the fragment is approximately one-fourth of the plasmid. However, after gel extraction only 1.5µg linear fragment DNA was obtained. For 200 bombardments ≈ 50µg linear DNA is needed, which can be obtained by restriction digestion of about 330µg plasmid DNA at a cost of US$ 76.70 (mini kit) or 37.30 (midi kit). For gel elution of the DNA fragments, 30 reactions of QIAEX II Gel Extraction Kit are needed. The miscellaneous expenditure (Eppendorf tubes, tips, etc.) is estimated at US$ 1.90 (mini kit) or 1.30 (midi kit). About 60ng/µl of amplified DNA was obtained when DyNAzyme EXT DNA polymerase was used for PCR amplification of the gene-cassette DNA.

4.3.5 Regeneration of putative transformants

4.3.5.1 *Shoot organogenesis*

When hygromycin selected calli were transferred onto regeneration medium greening of the calli was observed after 3-4 weeks of incubation and shoot organogenesis was observed after 6-7 weeks (Fig. 40b).

4.3.5.2 *Root organogenesis*

The regenerated plantlets, of 4-5 cm 'height', were transferred to rooting medium in culture tubes for root organogenesis. Roots developed within 2 weeks of incubation (Fig. 41) and plant growth was faster after root development (Fig. 42).

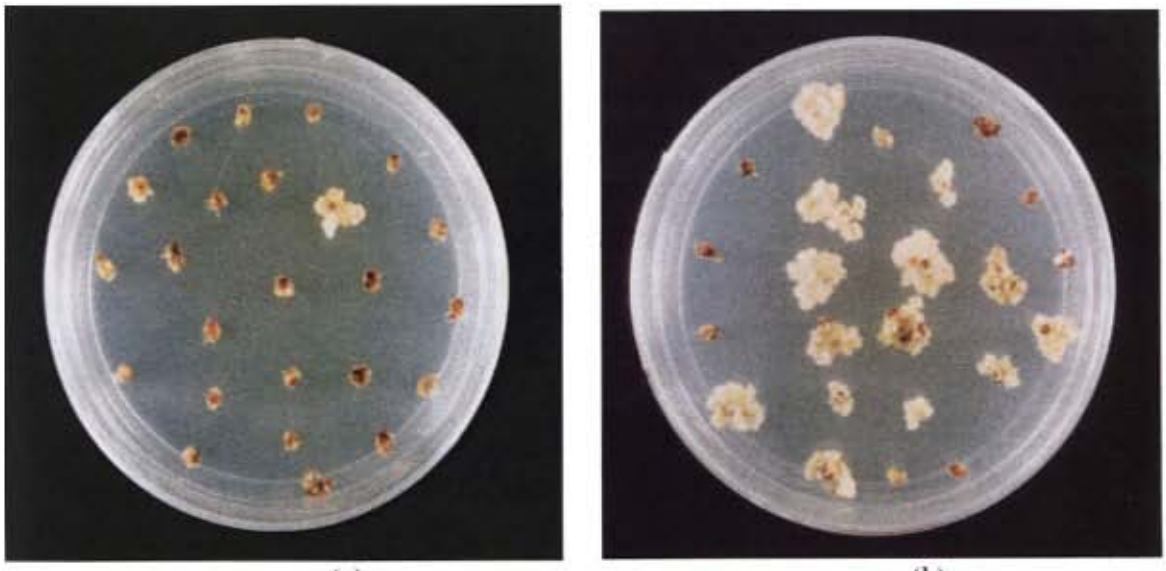
4.3.5.3 *Hardening of transformants*

Well-developed plants, of which leaves touched the cap of culture tube, were transferred and hardened in liquid hardening medium. The hardened plants were transferred to phytotron glasshouse and transplanted into pots. Most of the regenerated plants survived through the hardening process.

4.3.6 Growth and development of transgenic plants

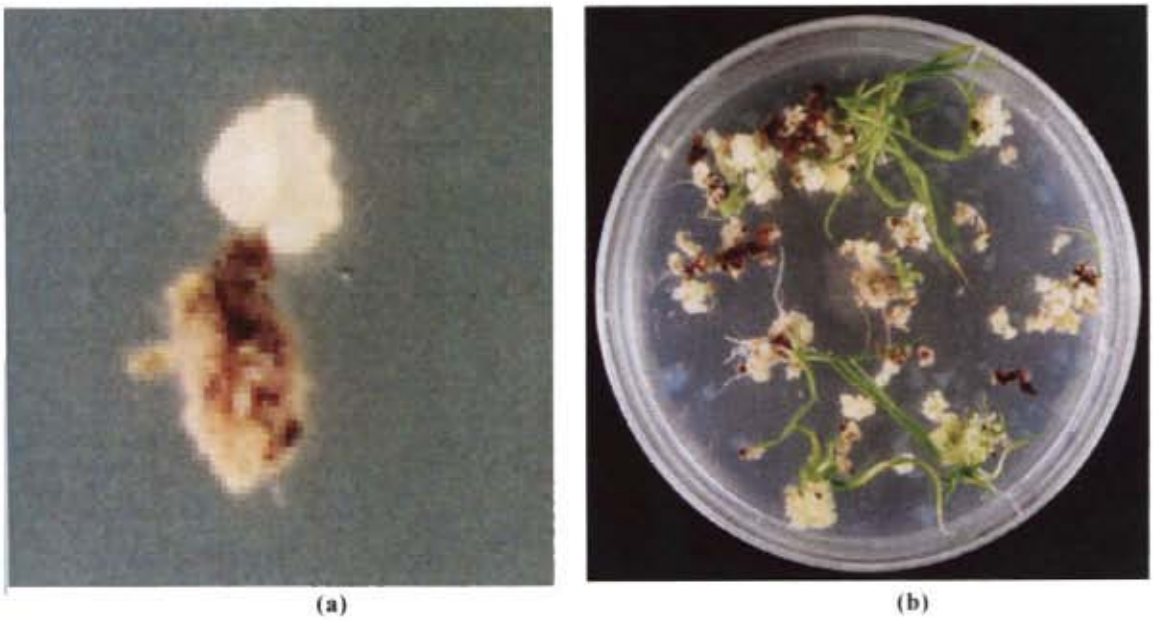
4.3.6.1 *Vegetative growth*

Although normal vegetative growth was observed for many of the regenerated plants (Fig. 44), some of the regenerants showed retarded growth and remained in the vegetative stage for a comparatively longer period (Fig. 43).



(a) (b)

Fig. 39. Selection of calli on hygromycin containing medium. **(a)** Un-bombarded calli (control) on hygromycin containing medium **(b)** Calli selected after third round of selection on hygromycin containing medium.



(a) (b)

Fig. 40. Selection and regeneration of transgenic calli. **(a)** Secondary callus emerging from one of the calli selected on hygromycin containing medium **(b)** Shoot organogenesis from hygromycin selected putative transgenic calli.



Fig. 41. Root development of plantlets regenerated on transfer to rooting medium.



Fig. 42. Well developed plantlets with roots and shoots.



Fig. 43. A putative transgenic plant showing retarded vegetative growth in comparison to other showing normal growth.

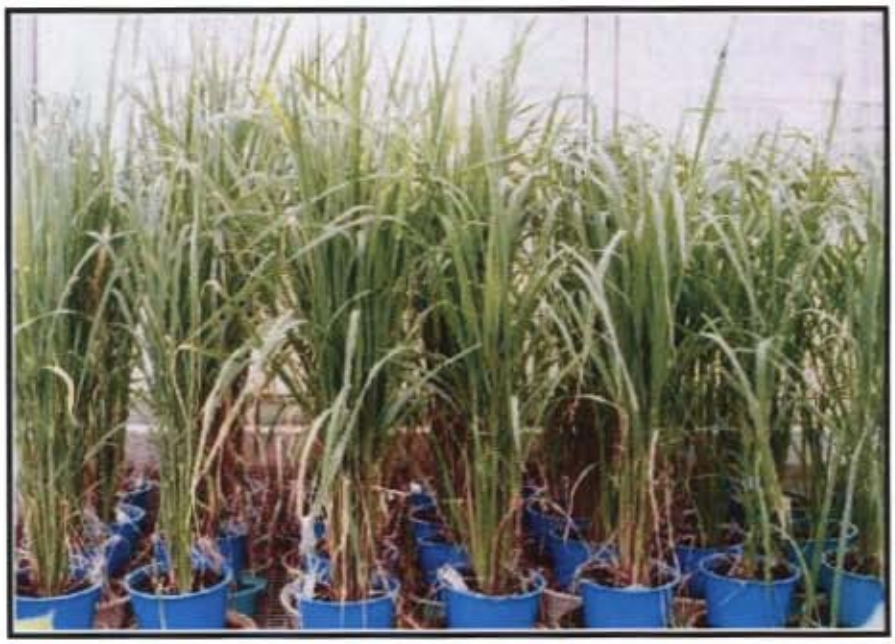


Fig. 44. Vigorous growth of transgenic plants in the Phytotron glasshouse.









Fig. 45. & 46. Transgenic plants showing normal flowering (Fig. 45) and seed setting (Fig. 46).



(a)



(b)

Fig. 47 Transgenic plants that remained in vegetative phase only **(a)**, and another one that did flower but did not have any seed set **(b)**.

4.3.6.2 *Reproductive growth*

Normal flowering and seed setting was observed in several transgenic plants (Fig. 45 & 46). But some of the transgenic plants showed continuous vegetative growth and did not enter into reproductive stage (Fig. 47a), while a few did flower but did not had any seed set (Fig. 47b).

4.4 Molecular characterization of transgenic plants

4.4.1 PCR screening

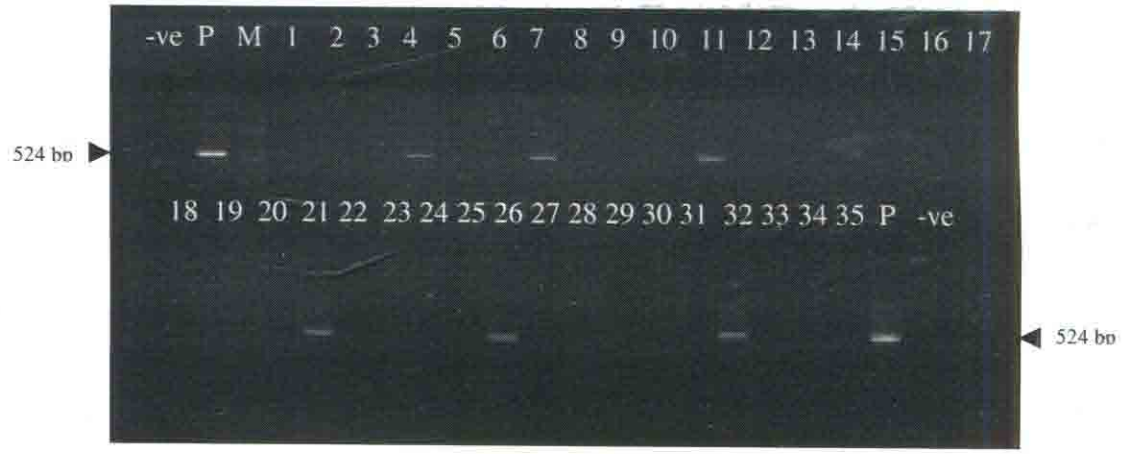
All the 98 putative transgenic plants (67 plants for *cry1B-1Aa* translational fusion gene and 31 for *cry1Aa* + *cry1B*) were screened using PCR for detecting transgenes. The amplification product of expected size was seen in case of the plants harbouring transgene (Fig. 48). Among the 67 putative transgenic plants for translational fusion genes, 17 were found positive for both the genes, while *hpt* gene was detected in 38 plants including the 17 Bt positive plants (Table 9).

Among the 31 putative co-transformants for *cry1Aa* + *cry1B* genes, 7 tested positive for *cry1B* gene and 5 for *cry1Aa*, while only 2 were tested positive for both the genes. The *hpt* was detected in 23 plants including 14 plants positive for *Bt* gene(s) (Table 9). However, out of the 98 tested, 37 plants were non-transformants, since they neither tested positive for any of the *Bt* genes or selectable marker *hpt*.

Table 9. PCR screening of PB-1 putative transgenic plants using gene-specific primers.

Gene-cassettes used for co-bombardment	Putative transformant #	Positive for *cry1B* #	Positive for *cry1Aa* #	Positive for *hpt* #	Negative for *hpt* #
*PEP-C--cry*1B-1Aa + *hpt*	67	17*	17*	38 (17*+21**)	29
*PEP-C--cry*1B + *MPI--cry*1Aa + *hpt*	31	9 (2*+7)	7 (2*+5)	23 (5+2*+7+9**)	8

* Plants PCR positive for *cry1B*, *cry1Aa* as well as for *hpt* genes.
** Plants PCR positive for *hpt* gene only.



Lane No.	Description
1 & 40	- ve control (no template)
2 & 39	Plasmid +ve control
3 (M)	Marker (100 bp ladder)
4 - 38	Amplification product (534 bp)

5 µl of amplification product was loaded on 1.2% agarose gel, run at 75 volt for 25 minutes.

Fig. 48. PCR screening of PB-1 putative transformants for *cry1B-1Aa* translational fusion gene, using *cry1B* gene-specific primer.

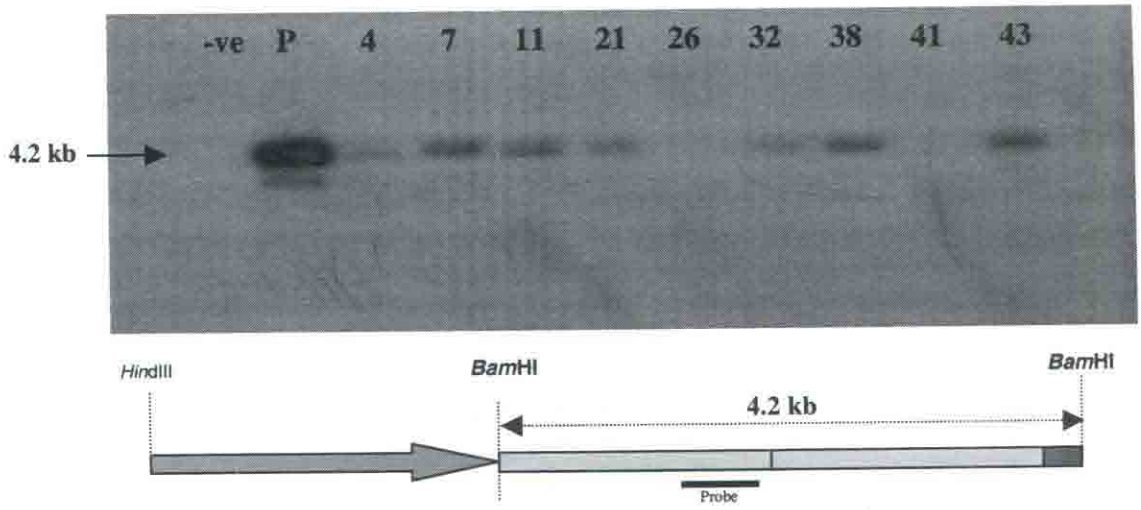
4.4.2 Southern analysis

Integration of the transgenes in the host genomic background was confirmed by Southern hybridization, using gene specific radiolabelled probes. Most of the PCR positive plants were found to be Southern positive, except for a few cases (Figs. 49-51; Table 10). Four plants out of 17 (tested PCR positive for translational fusion of *cry1B-1Aa* genes) were found to be Southern negative, while 1 out of 7 and 2 out of 9 were found to be Southern negative for *cry1Aa* and *cry1B* gene, respectively (Table 11).

Sites of integration of the transgene was detected in the T_0 plants (Nos. 7, 11, 38, 47 and 64), which showed better response in the cut-stem and the whole plant bioassay. Two to five sites of integration of the transgene was detected (Fig. 52). Variation in the intensity of bands was also observed. At least in one case (T_0 plant No. 38), where minimum of five sites of integration of the transgene was detected, complex pattern of transgene integration was observed.

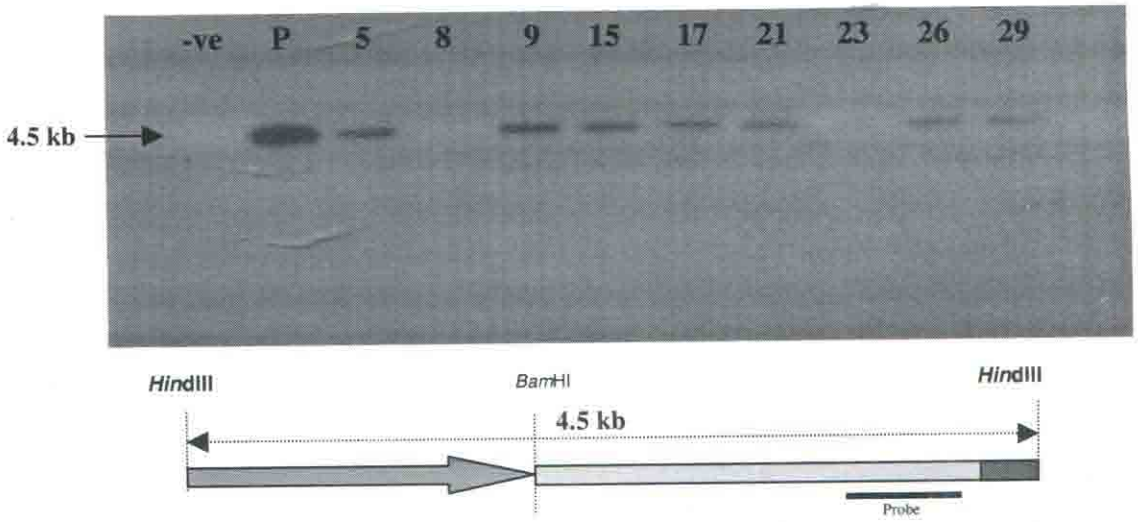
4.4.3 RT - PCR

Transcription of the introduced gene was detected by reverse transcription polymerase chain reaction (RT-PCR) in the Southern positive plants for *cry1B-1Aa* translational fusion genes. Out of the 13 such Southern positive transgenic plants, 11 showed RT-PCR bands. When *cry1B* specific primers were used in the RT-PCR, 534 bp band was observed in 11 transgenic plants (Fig. 53). Only two transgenic plants were detected negative for RT-PCR.



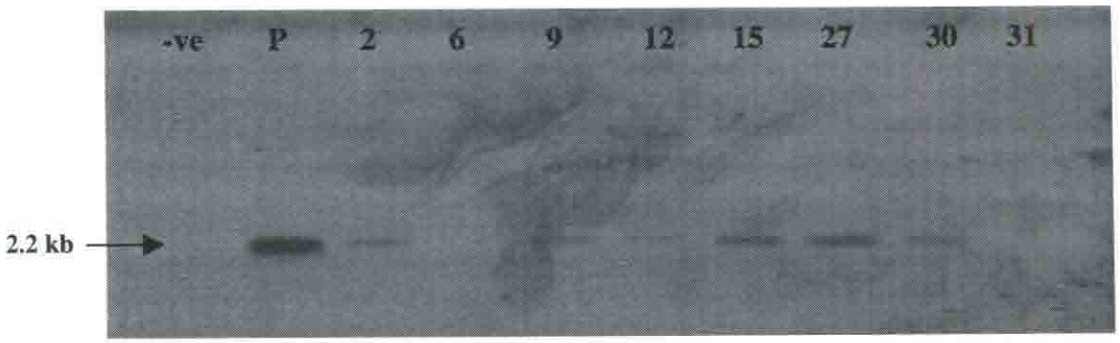
Lane No.	Description
1	- ve control plant
P	Plasmid +ve control
3-11	PCR +ve transgenic plants

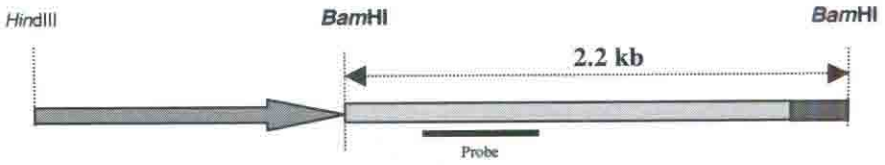
Fig. 49. Southern analysis of transformants for *cry1B-1Aa* translational fusion genes. Genomic DNA was digested with *Bam*HI and probed with *cry1B* gene-specific probe. The solid bar in the coding region indicates position of the probe used.



Lane No.	Description
1	- ve control plant
P	Plasmid +ve control
3-11	PCR +ve transgenic plants

Fig. 50. Southern analysis of transformants for *cry1B* gene. Genomic DNA was digested with *Hin*dIII and probed with *cry1B* gene-specific probe. The solid bar in the coding region indicates position of the probe used.





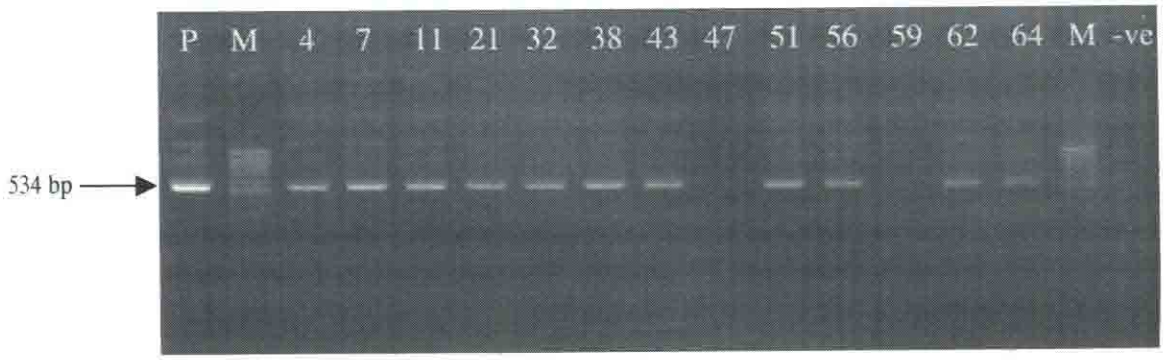
Lane No.	Description
1	- ve control plant
P	Plasmid +ve control
3-9	PCR +ve transgenic plants
10	Non-transgenic plant (PCR –ve)

Fig. 51. Southern analysis of transformants for *cry1Aa* gene. Genomic DNA was digested with *Bam*HI and probed with *cry1Aa* gene-specific probe. The solid bar in the coding region indicates position of the probe used.



Lane No.	Description
1 (P)	Plasmid restricted with *Sac* II (9.5 kb)
2 (-ve)	Untransformed control plant
3-7	Transgenic plants

Fig. 52. Southern analysis for detection of sites of integration of the transgene (*cry1B-1Aa* translational fusion gene). Plant genomic DNA and plasmid DNA was digested with *Sac*II and probed with *cry1B* gene-specific probe. The solid bar in the coding region indicates position of the probe used.



Lane No.	Description
1 (P)	Plasmid +ve control
2 & 16 (M)	Marker (100 bp ladder)
3-15	Amplification product (534 bp)
17	- ve control (no RNA)

10 µl of amplification product was loaded on 1.2% agarose gel, run at 65 volt for 40 minutes.

Fig. 53. RT-PCR of PB-1 transformants for *cry1B-1Aa* translational fusion genes driven by *pepc* promoter using *cry1B* gene-specific primer.

4.5 Evaluation of transgenic plants

Insect bioassay was conducted mainly on the transgenic plants for translational fusion gene.

4.5.1 Cut-stem bioassay

PCR positive plants, showing normal growth, were evaluated for YSB resistance. Mortality of YSB larvae was significantly higher (ranging from 31% to 71%) in comparison to the mortality (18%) in control plants (Table 12). The larvae recovered from control plants were healthy and well developed, while the larvae recovered from most of the putative transgenic plants were dead, if alive they were sluggish, weak and underdeveloped (Fig. 54).

Table 10. Southern analysis of PCR positive transformants for translational fusion genes.

T_0 plant #	PCR screening (+/-)	Southern hybridization (+/-)
Untransformed control	-	-
4	+	+
7	+	+
11	+	+
21	+	+
26	+	-
32	+	+
38	+	+
41	+	-
43	+	+
47	+	+
48	+	-
51	+	+
56	+	+
59	+	+
61	+	-
62	+	+
64	+	+

Untransformed control = Plant grown from normal seed

Table 11. Southern analysis of PCR positive transformants for *cry1Aa* and *cry1B* genes.

cry1Aa

T_0 plant #	PCR screening (+/-)	Southern hybridization (+/-)
Untransformed control	-	-
2	+	+
6	+	-
9	+	+
12	+	+
15	+	+
27	+	+
30	+	+
31 (Non-transgenic control)	-	-

Untransformed control = Plant grown from normal seed

cry1B

T_0 plant #	PCR screening (+/-)	Southern hybridization (+/-)
5	+	+
8	+	-
9	+	+
15	+	+
17	+	+
21	+	+
23	+	-
26	+	+
29	+	+

Non-transgenic control = Plant regenerated from bombarded callus

Table 12. Mortality (%) of YSB neonate larvae under cut-stem bioassay of PB-1 Bt transgenic rice plants.

T_0 plant #	PCR status (+/-)	Mortality (%)
Untransformed control	-	18.75
4	+	70.83
7	+	63.63
11	+	51.16
21	+	41.30
26	+	48.98
32	+	41.30
38	+	70.83
41	+	44.56
43	+	55.75
47	+	63.63
48	+	51.16
51	+	31.32
56	+	51.16
59	+	46.45
61	+	48.98
62	+	56.50
64	+	63.63
66 (Non-transgenic control)	-	17.5
67 (Non-transgenic control)	-	19.25

Untransformed control = Plant grown from normal seed

Non-transgenic control = Plants regenerated from bombarded calli

Table 13. Evaluation of PB-1 Bt transgenic rice plants for protection against YSB attack in whole plant bioassay.

T_0 plant #	PCR status (+/-)	Southern status (+/-)	Symptom (No. of white heads / hill)
Untransformed control	-	-	4
7	+	+	0
11	+	+	0
38	+	+	0
47	+	+	1
59	+	+	1
64	+	+	0
67 (Non-transgenic control)	-	-	3

Untransformed control = Plant grown from normal seed

Non-transgenic control = Plant regenerated from bombarded callus

4.5.2 Whole plant bioassay

Only Southern positive transformants were tested by whole plant bioassay. Observations were recorded for the typical symptoms (dead-heart / white heads) of YSB attack. Up to 4 white heads per plant were recorded for control plants (Fig. 55a), while none in case of most of the transgenic plants (Fig. 55b; Table 13). A number of well-developed larvae were recovered in the control plants. A single whitehead was observed in the case of a few transgenic plants (No. 47 and 59) which were tested PCR and Southern positive but RT-PCR negative. Three whiteheads were observed in case of PCR and Southern negative non-transgenic plant (No. 67).



Fig. 54. YSB larvae recovered from **(a)** control and **(b)** transgenic plants after cut-stem assay.

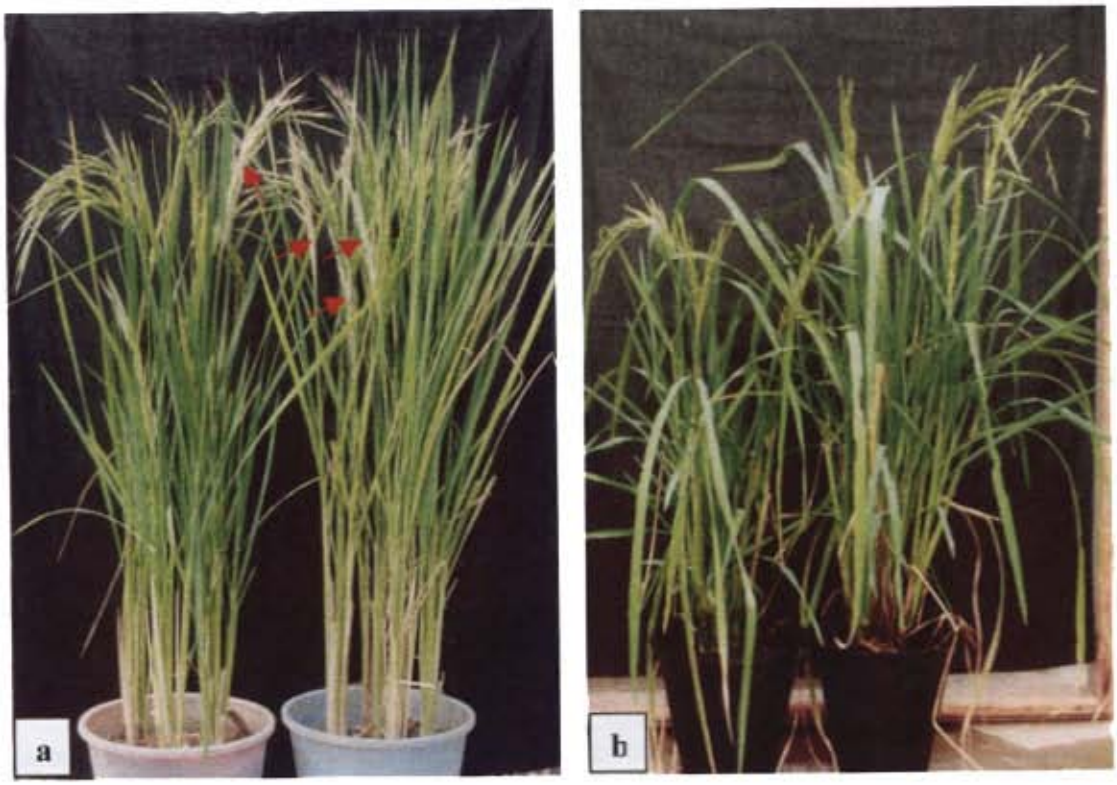


Fig. 55. White heads observed on control plants **(a)**, but not in case of transgenic plants **(b)**, in whole plant bioassay for YSB resistance.

DISCUSSION

Bt transgenic crops have the potential to significantly enhance productivity and sustainability. However, the long-term impact can only be sustained if effective and responsible deployment strategies are adopted to maintain the durability of resistance offered by *Bt* genes. One such strategy is to use refugia so as to ensure abundance of susceptible moths. While this approach has been practiced satisfactorily in the large commercial farms of the Industrialized countries, the small-scale farmers of developing countries have too small land-holdings to maintain 20% of the crop as refugia (Krattiger, 1997).

The present study was undertaken to generate two-gene Bt transgenic of *indica* rice so as to delay development of resistance in the insects. Rather than employing a strong constitutive promoter such as CaMV35S, known to show high level Bt protein expression in unwanted tissues such as seeds and roots, we have used a green tissue-specific and a wound-inducible promoter. Furthermore, so as to address biosafety concerns relating to undesirable integration of vector backbone sequences 'clean DNA' transformation method was used.

A large number of Pusa Basmati-1 transgenic plants were generated using microprojectile-mediated 'clean DNA' transformation procedure. Transformants were analyzed for the integration and expression of the transgenes using PCR, Southern hybridization and RT-PCR. The effectiveness of the *Bt* genes against manual infestation of the neonate larvae of yellow stem borer was assessed

through cut-stem bioassay and whole plant bioassay, under controlled conditions. The results are discussed.

5.1 Sequencing of genes and promoters

Base sequence information and availability of restriction sites of the genes and promoters were needed for the construction of appropriate gene-expression-cassettes. The whole exercise of sequencing genes and promoters afresh not only provided the necessary information but also helped in establishing accuracy and convincing identity of the tissue-specific and wound-inducible maize proteinase inhibitor promoters. Accurate sequence information helps in using molecular handles for (PCR, Southern hybridization and RT-PCR) characterization of the putative transgenic plants.

5.2 Cloning genes and promoters

Initially the genes and promoters were cloned in CAMBIA's new compact binary vector pCAMBIA-1305.1, which has pBR322 *ori* for high copy replication in *E. coli*. The vector has many improved features, such as a superior reporter gene (GUS*Plus*™) from *Staphylococcus* sp. and an intron from the castor bean catalase gene to ensure detection of plant-specific glucuronidase expression. According to CAMBIA (http://www.cambia.org), this new vector has been tested extensively in transgenic rice.

However, in the present study, the large size of pCAMBIA-1305.1 (11846 bp) had to be replaced since the large vector backbone was a drawback in getting sufficient

yields of the gene-expression-cassette fragments. Low yield of the desired fragment DNA was a major limitation in using this construct for 'clean DNA' transformation. Therefore, the whole exercise of cloning the desired gene construct was conducted afresh by using pGEM-7Z, a relatively very small (3 kb) high copy number cloning vector. Cloning of genes and promoters in this plasmid considerably increased the yield of gene-cassette DNA.

5.3 Generation of Bt transgenic plants

One of the major advantages of particle bombardment-mediated transformation procedure is the ability to transform plants simultaneously with several different constructs (Maqbool and Christou 1999; Maqbool *et al.*, 2001; Loc *et al.*, 2002). In the present study, using minimal gene-expression-cassettes, two or three different constructs were co-bombarded; one carrying selectable marker gene (*hpt*) while the insect-resistance (*cry1Aa* and *cry1B*) genes were carried on other constructs. Similar co-bombardment strategy was adopted by Loc *et al.* (2002).

5.3.1 Minimal gene-expression-cassette and 'clean DNA' transformation

The commonly used rice transformation techniques generally lead to the integration of vector backbone sequences into the host genome, along with the desired transgene(s) (Kononov *et al.*, 1997). The integration of vector backbone sequences may exert undesirable effects in *cis* (Artelt *et al.*, 1991), promote transgene rearrangements, and serve as recombination hot-spot that may stimulate illegitimate recombination (Muller *et al.*, 1999). In view of such concerns, Fu *et al.* (2000) and Loc *et al.* (2002) used minimal gene-cassette DNA lacking vector backbone sequences, to transform rice by particle bombardment.

In the present study, to begin with, minimal gene-cassette DNA fragments were generated by restriction digestion and gel extraction for plant transformation experiments. Although, yields of gene-cassette DNA improved considerably by cloning promoters and genes in a smaller plasmid vector pGEM-7Z, however, an alternate method for getting even better yields of gene-cassette DNA was still desirable. All the more so, since restriction digestion and gel extraction is not only a process yielding only limited amounts of gene-cassette DNA, but it is also a cumbersome and time consuming procedure. Therefore, an alternative and relatively simpler approach for generating minimal gene-expression-cassette DNA was investigated. The objective was to obtain relatively large amounts of the desired fragment DNA for undertaking large-scale bombardments.

The alternative approach involved PCR amplification of the gene-cassette using high fidelity DNA polymerase with 3'-5' proof reading activity. DNA polymerases are prone to error (2.0×10^{-5} errors per nt per cycle) during *in vitro* DNA synthesis. However, the rate of error is very low in case of high fidelity polymerases (1.6×10^{-6} errors per nt per cycle; Lundberg *et al.*, 1991). High fidelity enzymes are routinely used for PCR cloning of genes (Lundberg *et al.*, 1991; Cline *et al.*, 1996; Kitabayashi *et al.*, 2003). To begin with, we tested the PCR amplification approach for *cry1B* and the *mpi* promoter fragments. In both cases, suitable restriction sites were added to the respective termini. The amplified products were sequenced and in both the cases, no base substitution or any other error was noticed. Encouraged by the results, a pilot experiment was designed to test functionality of the PCR amplified gene-expression-cassette for the GUS reporter system. Since the functional aspect of the PCR amplified gene-cassette

could be tested just 24 hrs after bombardment, the GUS reporter system was an appropriate system for testing the quality of amplified product. If a considerable number of wrong bases were incorporated, during amplification (as expected in case of the normal *Taq* polymerase), the expression of *GUS* gene will be affected, which can be monitored by a comparative study of the number of GUS expression units (GUS spots).

Results of transient GUS expression did not show any significant difference between the PCR amplified and restriction digested gene-cassettes, except for *Taq* polymerase amplified gene-cassette. As expected, fewer blue spots were seen on the calli bombarded with *Taq* polymerase amplified GUS gene-cassettes. The higher error rate of *Taq* polymerase (2.0×10^{-5} errors per nt per cycle; Lundberg *et al.*, 1991) which gets multiplied during PCR, might have generated many erroneous gene-cassettes unable to produce functional gene products. The non-significant difference for GUS spots, produced by the gene-cassettes that were amplified using high fidelity polymerases, was possibly due to the fact that all the three high fidelity polymerases tested in the experiment offer a blend of high processive *Taq* DNA polymerase and *Pfu* DNA polymerase that exhibits a 3' - 5' exonuclease activity. Fidelity of such PCR enzyme mix, containing proofreading activity to remove miss-incorporated bases, may be several times greater than that of *Taq* DNA polymerase (Lundberg *et al.*, 1991).

5.3.2 Bombardment of calli

Scutellum-derived embyogenic calli from mature seeds have been extensively used in rice transformation as preferred explants due to the relative ease of availability

throughout the year (Sivamani *et al.*, 1996; Nayak *et al.*, 1997; Ghareyazie *et al.*, 1997; Alam *et al.*, 1998; Khanna and Raina, 2002). Five-day old embyogenic calli from mature seeds were bombarded with 'clean DNA' fragments precipitated on 0.6 μ gold particles in a molar ratio of 4:1 (or 2:2:1) for the gene(s) of interest and the plant selectable marker. The comparative higher ratio of the gene of interest has been shown to provide increased probability of co-transformation (Tang *et al.*, 1999). Fu *et al.* (2000) used a molar ratio of 3:1 for gene-cassettes of the gene of interest and plant selectable marker, whereas, Loc *et al.* (2002) used a molar ratio of 3:3:1 for gene-cassettes of the genes of interest and selectable marker gene.

5.3.3 Selection of transformed cells

When the bombarded calli were plated on selection medium containing hygromycin, many of the calli were found proliferating in first round of selection. The number of escapes was much lower in subsequent transfers. The reduction in number of surviving calli was likely due to the elimination of escapes. Even after three rounds of selection, obviously all the escapes were not eliminated because more than 43% of the regenerated plants were found to be negative for the *hpt* gene. More extensive selection of calli on hygromycin containing medium has not been recommended, particularly for *basmati* rice cultivar since it seriously affects regenerability of calli (Khanna and Raina, 2002). We used hygromycin only in the callusing medium during the three rounds of selection. The observed transformation frequency for the gene of interest was up to 0.90%, while much higher (3.19%) for *hpt*. Use of hygromycin in the callusing, regeneration (Alam *et al.*, 1998) and rooting medium (Ghareyazie *et al.*, 1997) might have helped in reducing the number of escapes but the observed transformation frequency was

very low. Relatively very high frequency of transformation has been reported using hygromycin B in callusing medium (Khanna and Raina, 2002).

5.3.3 Growth and development of transgenic plants

Although many of the putative transgenic plants showed normal growth and development, a few of them had stunted growth, while a few showed poor or even very poor seed set. Such abnormalities might be a consequence of somaclonal variations and/or due to transgene integration into host genome at hot-spot genomic region harbouring genes for crucial house keeping functions. However, poor seed set of an otherwise normal looking transformant may, in some cases, be only due to phytohormone 'hangover', which does not persist in the seed derived progenies (Raina 1997).

5.4 Molecular characterization of transgenic plants

PCR screening of the putative transgenic plants revealed presence of the transgene(s) in the host genome. Transformation frequency for the translational fusion (*cry1B-1Aa)* gene (as revealed by the Southern analysis) was observed to be 0.90%, while transformation frequency for co-transformation with *Bt* genes (*cry1Aa* and *cry1B*) was only 0.27%. About 43% of the regenerated plants were negative for the selectable marker gene (as revealed by the PCR analysis), whereas more than 31% of the regenerants were PCR positive for only *hpt* gene. More than 25% of the putative transgenic plants were PCR positive for translational fusion gene as well as the marker gene. Similar results have been reported with relatively higher frequency of co-transformants in a relatively smaller population of transformants by Loc *et al.* (2002) using the 'clean DNA' transformation approach.

More than 45% of the hygromycin resistant plants showed co-transformation of the non-selectable *cry* genes, which is considerably higher than that reported by Khanna and Raina, (2002). None of the *cry* positive plants were detected negative for *hpt* gene.

Southern analysis was undertaken to ascertain transgene integration and to detect the number of sites of integration of the transgene. Out of 17 PCR positive independent transformants for translational fusion genes, transgene integration was detected in 13 transformants (Table 10; Fig. 49). Similarly, the data presented in Table 11 indicate 2 independent transgenic events for individual *cry1Aa* and *cry1B* genes driven by specific promoters. The site of transgene integration was analyzed only in the transfomants showed better response in the cut-stem and the whole plant bioassays. Two to five sites of integration of the transgene were detected. Variation in the intensity of bands was also observed (Fig. 52). In case of T_0 plant No. 38, minimum of five sites (complex pattern) of integration of the transgene was detected. In other cases, 2 or 3 sites (relatively simple pattern) of transgene integration was observed. Simple to complex patterns of transgene integration have been reported using biolistic method of transformation by many workers (Datta *et al.*, 1998; Alam *et al.*, 1998; Khanna nad Raina 2002). Expression of the transgene, in terms of transcription, was confirmed by RT-PCR. Among 13 Southern positive plants tested for RT-PCR, all except two displayed amplification product. The absence of amplification product in two transgenic plants might be due to no transcription or very low level of transcription of the transgene, which could not be detected by RT-PCR. Similar observations have been reported earlier by Khanna and Raina, (2002).

5.5 Evaluation of transgenic plants

Several factors, such as, the method of transformation, the gene constructs used, and the site of integration into the host genome affect transgene expression (Schell, 1987; Perlak *et al.*, 1991; Maas *et al.*, 1992). Consequently, considerable variation may be seen among different transgenic events. Therefore, each transgenic event merit independent evaluation for morphological and physiological traits, including the transgene expression.

In the present study, inset bioassays were carried out to assess relative levels of insect mortality as a function of *Bt* gene expression. Larval mortality was in the range of 31% to 71% in cut-stem assays. This was considerably higher (at least in certain cases) than that recorded for control plants (18%). Slightly higher mortality rate of larvae in control plants may be due to handling of the larvae during bioassay. Many Bt transgenic Southern positive plants showed a low level of larval mortality indicating either lower level of Bt protein expression or post-transcriptional complexities. Larvae recovered from the transgenic plants were dead or had not developed beyond the first instar stage. Comparatively, larvae had developed to the third instar stage in the control plants. Similar results have been reported by several workers (Nayak *et al.*, 1997; Ghareyazie *et al.*, 1997; Datta *et al.*, 1998; Khanna and Raina, 2002). Lower levels of *Bt* gene expression have been reported in the leaf bases and/or the developing panicles of maize (Mellon and Rissler, 1998) and rice plants (Alinia *et al.*, 2000), where the *Bt* gene was placed under the control of *pepc* promoter.

More appropriate assessment of transgenic plants for insect resistance is the whole plant bioassay (Tu *et al.*, 2000; Khanna and Raina, 2002). In the present study, resistant or susceptible nature of the bioassayed plants was evaluated mainly on the basis of presence and the number of white heads following infestation. Up to 4 white heads per plant were observed in case of control plants, while none in most of the transgenic plants. A single white head was observed in case of transgenic plant Nos. 47 and 59, both were Southern positive but RT-PCR negative. Possibly the level of *Bt* gene expression in these transgenic plants was too low to be detected by RT-PCR and yet provides effective protection against yellow stem borers. No dead-heart but only white heads were observed on the yellow stem borer infested control plants since they were tested only at the early panicle emergence stage.

Four of the selected transgenic plants (Nos. 7, 11, 38 and 64) have shown high level of protection against neonate larvae of YSB. Transgene copy number (Fig. 52) did not correlate with the level of protection, as evidenced by the whole plant bioassay. The T_0 transgenic plants showed normal vegetative growth and development along with high level of gene expression, as evident from RT-PCR and also effective protection against YSB.

Since the results presented here are based on the characterization and evaluation of T_0 transgenic plants, progeny analysis of the transformants will be needed to select specific transgenic lines showing stable inheritance of the transgene as well as high expression. The expression level must show Bt-titre high enough to fit into the resistance management strategy while retaining the overall agronomic

characteristics. Although, genetic manipulation of any *basmati* rice cultivar, using recombinant-DNA techniques has been banned by the Govt. of India, the main objective of the present study was only to generate promising Bt transgenic *indica* rice lines which can then be used in rice breeding programmes as novel sources for enhanced resistance against yellow stem borers.

SUMMARY AND CONCLUSIONS

Stem borers have emerged as one of the three leading insect pests of rice causing serious losses to the crop in all the rice growing regions of India. During the last three decades, conventional breeding has made limited progress in developing stem borer resistant varieties, as resistance source of only moderate level have been found, despite screening of more than 30, 000 rice germplasm collections (Khan *et al.*, 1991). Bt toxins are highly specific to the target insects, non-toxic to the beneficial insects, birds and mammals, and yet are as effective as any of the conventional chemical insecticides. Considerable efforts have been made towards development of Bt transgenic rice (Cohen *et al.*, 2000).

In the present study, development of novel Bt transgenic *indica* rice plants (Pusa Basmati-1) have been reported. These transgenics encode two dissimilar *Bt* genes under the regulatory control of tissue specific and temporal specific promoters. To address the biosafety concerns relating to the integration of undesirable vector backbone sequences in the transgenic plants, 'clean DNA' transformation procedure was used. A large number of Pusa Basmati-1 transgenic plants, generated through microprojectile-mediated transformation method, were analyzed for the integration and expression of the transgenes using PCR, Southern hybridization and RT-PCR. Efficiency of the *Bt* genes against yellow stem borers was evaluated through cut-stem and whole plant bioassays. The salient features of the study and results obtained are summarized below:

- The *Bt* genes (*cry1B* and *cry1Aa* genes as well as translational fusion of the two genes) were cloned under tissue (green tissue) and/or temporal specific (wound inducible) promoters in pGEM-7Z vector.
- Mature seed-derived embryogenic calli of PB-1 were co-bombarded with the minimal gene-expression-cassettes for the *Bt* genes and selectable marker (*hpt*) gene, generated by restriction digestion and gel extraction.
- Realizing the problems encountered in generating sufficient quantities of gene-expression-cassette DNA, PCR amplification of the gene-cassette, using high fidelity DNA polymerase with proofreading activity, was tested for the GUS reporter gene and found to be an effective alternative as well as a low cost and easier approach.
- Ninety-eight putative transgenic (T_0) plants were characterized for the integration and expression of the genes of interest by PCR, Southern hybridization and RT-PCR.
- Transformation frequency ranged from 0.27%, for the transformants harbouring both *Bt* as well as selectable marker (*hpt)* genes, to 3.19%, for transformants harbouring at least selectable marker gene with/without *Bt* genes.
- Cut-stem bioassay of the selected transgenic plants for translational fusion genes indicated mortality of YSB larvae in the range of 31% to 71%, which was considerably higher than that observed in control plants (18%).
- Whole plant bioassay of the transgenic plants indicated complete protection against yellow stem borer larvae. No symptom of YSB infestation was observed on transgenic plants, while 4 whiteheads per hill were observed in case of control plants.

Progenies of selected *Bt* transgenic PB-1 rice plants need to be examined for stable inheritance of the gene of interest. Selected lines harbouring only the genes of interest but without selectable marker (*hpt*) gene shall provide marker free 'clean DNA' transformants offering enhanced resistance to yellow stem borers. The selected lines can then be used in the rice breeding programmes as a source of enhanced resistance against rice stem borers.

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Zhang, S., Chen, L., Qu, R., Marmey, P., Beachy, R. N. and Fauquet, C. M. (1996) Regeneration of fertile transgenic indica (group 1) rice plantlets following microprojectile transformation of embryogenic suspension culture cells. *Plant Cell Rep.* **15**: 465-469.

Zhang, W. and Wu, R. (1988) Efficient regeneration of transgenic plants from rice protoplasts and correctly regulated expression of the foreign gene in plants. *Theor. Appl. Genet.* **76**: 835-840.

APPENDIX

A-1 Bacterial growth media

1.1 Luria Broth :

Component	Quantity (g/l)
Trypton	10
Yeast extract	5
Sodium chloride	5

Final volume made up to 1000 ml with double distilled water. The medium was sterilized by autoclaving at 15 lbs (121° C) for 18 min

1.2 Luria Agar :

Component	Quantity (g/l)
Trypton	10
Yeast extract	5
Sodium chloride	5
Agar	20

Agar was added as solidifying agent after. Sterilized by autoclaving at 15 lbs (121° C) for 18 min and poured into sterile Petri plates under aseptic conditions under clean air laminar flow hood.

A-2 Solutions for plasmid DNA isolation

2.1 Solution I (Resuspension buffer) :

Component	Concentration
Glucose	50 mM
EDTA	10 mM (pH 8.0)
Tris-HCl	25 mM (pH 8.0)

The solution was autoclaved and stored at 4° C.

2.2 Solution II (Lysis buffer) :

Component	Concentration
NaOH	0.2 N
SDS	1% (w/v)

The above working solution was prepared by mixing equal volumes of 0.4 N NaOH and 2% SDS stock solutions. Stored at room temperature.

2.3 Solution III (Neutralization buffer) :

Component	Concentration
Sodium acetate	3 M (pH 5.2)

pH of the medium was adjusted with glacial acetic acid. The solution was autoclaved and stored at 4° C.

A-3 MS medium

Component	Quantity per lit.	Stock solution weight	Stock solution volume	Vol. of stock sol. for 1 lit.
NH_4NO_3	1.65 g	33.5 g		
KNO_3	1.90 g	38.0 g	2 lits.	100 ml of the 10X stock
$MgSO_4.7H_2O$	0.37 g	7.4 g		
KH_2PO_4	0.17 g	3.4 g		
$CaCl_2.2H_2O$	0.44 g	4.4 g	500 ml	50 ml of the 10X stock
$MnSO_4.4H_2O$	22.300 mg	446 mg		
$ZnSO_4.7H_2O$	8.600 mg	72 mg		
H_3BO_3	6.200 mg	124 mg	2 lits.	100 ml of the 10X stock
KI	0.830 mg	16.6 mg		
$Na_2MoO_4.2H_2O$	0.250 mg	5.0 mg		
$CuSO_4.5H_2O$	0.025 mg	0.5 mg		
$CoCl_2.6H_2O$	0.025 mg	0.5 mg		
$FeSO_4.7H_2O$	27.80 mg	556 mg		50 ml of the 20X stock
Na_2EDTA	37.30 mg	746 mg	1 lit.	
Nicotinic acid	0.5 mg	100 mg	200 ml	1 ml of 200X stock
Pyridoxine-HCl	0.5 mg	100 mg	200 ml	1 ml of 200X stock
Thiamine-HCl	0.1 mg	20 mg	200 ml	1 ml of 200X stock
Glycine	2.0 mg	200 mg	200 ml	2 ml of 100X stock
myo-Inositol	100 mg	Added solid		
Sucrose	30 g	Added solid		

[1] The iron stock solution was heated to boiling temperature after dissolving and mixing individual components, cooled down to room temperature before use. Individual 10,000X stock solutions were made for $CoCl_2.6H_2O$ and $CuSO_4.5H_2O$ (by taking 25 mg of each, dissolved in 100 ml) and used 2 ml of this (10,000X) stock for preparing 2 lits. of minor (10X) stock. All the stock solutions were stored at 4° C.

[2] To prepare the medium, required amount of stock solutions were taken, to which sucrose and *myo*-Inositol were added. The plant hormones, which can be co-autoclaved ,were also added as per the requirements of the experiments. The pH of medium was adjusted to 5.8 (using 1N NaOH or 1N HCl) before adding solidifying agent. Agarose 6 g/l or Phytagel 3 g/l was used as solidifying agent, whenever needed as per the requirement of the experiments. After making up the final volume with distilled water the medium was sterilized by autoclaving at 15 lbs. (121° C) for 18 min.

A-4 Culture media used for tissue culture

Medium	Purpose
Callus induction medium	MS medium, 2,4-D 2.0 mg/l, Phytagel 0.3%
Selection medium	MS medium, 2,4-D 2.0 mg/l, Agarose 0.6%, Hygromycin 50 mg/l
Pre-regeneration medium	MS medium, NAA 2.5 mg/l, 2,4-D 0.5 mg/l, Kinetin 0.1 mg/l, Agarose 0.6%
Regeneration medium	MS medium, BAP 2.0 mg/l, NAA 0.5 mg/l, Kinetin 0.5 mg/l, Agarose 0.6%
Rooting medium	$^1/_2$ MS medium (with full iron), Agarose 0.4%
Hardening medium	$^1/_{10}$ MS liquid medium (without sucrose)

[1] Hygromycin was added when the autoclaved medium cooled down to bearable warmth, mixed well and poured into petri plates under aseptic conditions.

A-5 GUS assay solution

Component	Concentration	Stock solution	Vol. of stock sol. for 1000 µl
EDTA (pH 8.0)	10 mM	100 mM	100 µl
Sod. Phosph. Buffer (pH 7.0)	50 mM	100 mM	500 µl
Triton X-100	0.1%	1%	100 µl
Pot. Ferricyanide	1 mM	50 mM	20 µl
Pot. ferrocyanide	1 mM	50 mM	20 µl
X-Gluc	1 mM	100 mM	10 µl
Methanol	20%	--	200 µl
Water	to make up the vol.	--	50 µl

[1] Potassium ferricyanide and Potassium ferrocyanide stock solutions were stored in dark at 4° C. To prepare 100 mM stock solution of X-Gluc, 52 mg of 5-bromo-4-chloro-3-indolyl glucuronide, cyclohexyl ammonium salt (FW 521.8) was dissolved in 1000 µl of DMSO and stored at - 20° C in dark. When colour of the stock solution of X-Gluc was changed to purple, it was discarded and fresh stock was prepared for use.

[2] To prepare GUS assay solution, the required amount of stock solutions, except for Methanol and X-Gluc, were mixed and filter sterilized using syringe and 0.2 µ filter disc. Then required amounts of Methanol and X-Gluc were added. The working solution, thus prepared, was stored at -20° C until used.

A-6 Gene / Promoter sequences

6.1 *pepc* Promoter :

AAGCTTCCAAGATAGGTGAAAGATAAGCTACCTTTGTCACAATTGAAGATTCGTGCAAGGTGGTCCAACTATTA
TTCTGAGATGTTTATTGAGACCATTGAGGACCTTTGAGTAATTAACTCTCAACCTAGTGGAAATTCGTTACCAA
CTAGGTTGCATAGGATTTCATGATTAAGAGTGCGTTTGGTTTAGGTGTGAGTTTTCTCCTATGAAAAACTGTTG
TGAGAAAAAATAGTTGAAAGTCGTTTAGTTGAAACTGTTGTGAGTTATCCACTGTAAACAAATTGTATATTGTT
TATATACACTCTGTTTAACTCTTAATCAGTATATATAATTAAAAAACTAATTTCACATTTCTGTTCCTAATATT
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AACAATCAAAAGCAAGGTCAAGCTGTTTTTTCAAAGTAGTTGTGGAAAAGTTGACCCCTTTTATTCAGCTTCTA
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TATCTTTGGTCATGTTTAAGAGGCTGGAATAGATCAACTGCAAGAACAAATAGCAGAGTGGACAGGGGGGGGGG
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TACTTAGCTCGATGTAATGCACAATGTTGATAGTATGTCGAGGATCTAGAGATGTAATGGTGTTAGGACACGTG
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AAAATCTTCTTTTTGTGTGGATATCCCAACCCAAATCCACCTCTCTCCTCAATCCGTGTATCTTCACCGCTGCC
AAGTGCCAACAACACATCGCATCGTGCAAATTTTTGTTGGCTTGTGCACGGTCGGCGCCAATGGAGGAGACACC
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CCTCCAAATTCTTGCGATCCCCCGAATCCAGCATTAACTGTTAAGGGACGCCCTCTCCACATCCTACTACCCAA
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AGACACCCCTCTCCACATCCCCTCCGGCCGCTAACAGCAGCAAGCCAAGCCAAAAAGAAGCCTCAGCCACAGCC
GGTTCCGTTGCGGTTACCGCCGATCACATGCCCAAGGCCGCGCCTTTCCAAACGCCGAGGGCCGCCCGTTCCCG
TGCACAGCCACACACCCGCCCGCCAACGACTCCCCATCCCTATTTGAACCCACCCGCGCACTGCATTGATCACC
AATCGCATCGCAGCAGCACGAGCAGCACGCCGTGCCGCTCCAACCGTCTCGCTTCCGTGCTTAGCTTCCCGCCG
CGGATCC

6.2 *mpi* Promoter :

AAGCTTGGTCGAGAGACGATATTTAGGTTCTACACAAAACCCTCTACGGCATCGTGGATGGTCCACCACCTACA
AGTGGACGGTCCACGCGCCTGCAGAGAAGAGCATAGTTATTTGCTCCATGGTGACCTACACGCGAACGGTCCAG
TGACCTATCACGAACCGTTCAGCACCTTGCAGAGAGGTCTCCAAGGTCTCGACTACTCATGAGCGGAATCTAGT
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CC

6.3 *cry1B-1Aa* translational fusion genes :

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GCTTATCGATAAGCTTGGATCC

6.4 *cry1B* :



6.5 *cry*1Aa :



A-7 Nucleotide BLAST

7.1 Results of *mpi* promoter sequence BLAST

When assembled base sequence of *mpi* promoter was used for BLAST, the following result was obtained:

NCBI

results of BLAST

BLASTN 2.2.9 [May-01-2004]

Reference:
Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

RID: 1097490925-7408-47015140186.BLASTQ4

Query=
(951 letters)

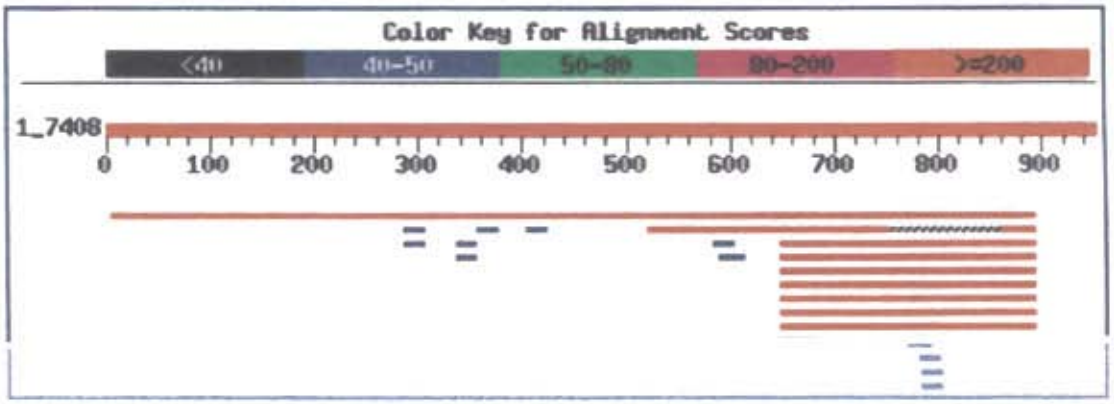
Database: All GenBank+EMBL+DDBJ+PDB sequences (but no EST, STS, GSS, environmental samples or phase 0, 1 or 2 HTGS sequences)
2,634,031 sequences; 11,969,012,090 total letters

If you have any problems or questions with the results of this search please refer to the **BLAST FAQs**

Taxonomy reports

Distribution of 130 Blast Hits on the Query Sequence

Mouse-over to show defline and scores. Click to show alignments



Sequences producing significant alignments:		Score (bits)	E Value
gb|X78589.2|ZMMPI Zea mays MPI gene		[illegible]	0.0
gb|AY104081.1	Zea mays PCO087322 mRNA sequence	358	1e-94
gb|AY549610.1	Zea diploperennis isolate d8b MP...	291	1e-75
gb|AY549609.1	Zea diploperennis isolate d8a MP...	291	1e-75
gb|AY549608.1	Zea diploperennis isolate d7b MP...	291	1e-75
gb|AY549607.1	Zea diploperennis isolate d7a MP...	291	1e-75
gb|AY549606.1	Zea diploperennis isolate d6b MP...	291	1e-75
gb|AY549605.1	Zea diploperennis isolate d6a MP...	291	1e-75

Alignments

Get selected sequences | Select all | Deselect all

>gi|9909975|emb|X78988.2|ZMMPI Zea mays MPI gene
Length = 1262

Score = 1675 bits (845), Expect = 0.0
Identities = 878/889 (98%), Gaps = 2/889 (0%)
Strand = Plus / Plus

```
Query: 6   gagacgatatttaggttctacacaaaaccctctacggcatcgtggatggtccaccaccta 65
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Sbjct: 1   gagacgatatttaggttctacacaaaaccctctacggcatcgtggatggtccaccaccta 60

Query: 66  caagtggacggtccacgcgcctgcagagaagagcatagttatttgctccatggtgaccta 125
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Sbjct: 61  caagtggacggtccacgcgcctgcagagaagagcatagttatttgctccatggtgaccta 120

Query: 126 cacgcgaacggtccagtgacctatcacgaaccgttcagcaccttgcagagaggtctccaa 185
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Query: 605 ggctgtttatcctcgtgtgcctcgcgtgcgtatatacacacacgtcctgcctgcacgcaa 664
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Sbjct: 600 ggctgtttatcctcgtgtgcctcgcgtgcgtatatacacacacgtcctgcctgcacgcaa 659


Query: 665 cgcagctctataaatacctcatcaccggtgcactcgcttaatatatatccaacaaccagc 724
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Sbjct: 720 agtgcaacaagcaagatttacctgcccgtgagtgtgcctttcaataatataatgtcttat 779


Query: 785 tttattattatgtttttgcagtgatagtagtctgtaataaatctctcctttcaatatata 844
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Sbjct: 840 taattcacatgccagatcgatcatatcgtcaactggtccggcagttcgc 888
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7.2 *mpi* promoter sequence in GenBank

The assembled base sequence of *mpi* promoter showed homology with the promoter sequence of *MPI* gene from *Zea mays*, which is as given below:




```
LOCUS       ZMMPI                   1262 bp    DNA     linear   PLN 23-AUG-2000
DEFINITION  Zea mays MPI gene.
ACCESSION   X78988
VERSION     X78988.2  GI:9909975
KEYWORDS    proteinase inhibitor.
SOURCE      Zea mays
  ORGANISM  Zea mays
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; PACCAD
            clade; Panicoideae; Andropogoneae; Zea.
REFERENCE   1
  AUTHORS   Cordero,M.J., Raventos,D. and San Segundo,B.
  TITLE     Expression of a maize proteinase inhibitor gene is induced in
            response to wounding and fungal infection: systemic wound-response
            of a monocot gene
  JOURNAL   Plant J. 6 (2), 141-150 (1994)
  MEDLINE   95004658
   PUBMED   7920708
REFERENCE   2
  AUTHORS   Breitler,J.C., Cordero,M.J., royer,M., Meynard,D., San Segundo,B.
            and Guiderdoni,E.
  TITLE     The -689/+197 region of the maize protease inhibitor gene directs
            high level, wound-inducible expression of the cry1B gene which
            protects transgenic rice plants from stemborer attack
  JOURNAL   Unpublished
REFERENCE   3
  AUTHORS   Cordero,M.J.
  TITLE     Direct Submission
  JOURNAL   Submitted (26-APR-1994) M.J. Cordero, Centro de Investigacion y
            Desarrollo de Barcelona, Consejo Superior de Investigaciones,
            Cientificas, c/Jordi Girona Salgado, 18. 08034 Barcelona, SPAIN
  REMARK    Revised by [3]
REFERENCE   4  (bases 1 to 1262)
  AUTHORS   San Segundo,B.
  TITLE     Direct Submission
  JOURNAL   Submitted (22-AUG-2000) San Segundo B., Genetica Molecular,
            Institut de Biologia Molecular de Barcelona, CID, CSIC, Jordi
            Girona 18, Barcelona 08034, SPAIN
COMMENT     On Aug 25, 2000 this sequence version replaced gi:475252.
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                     /evidence=experimental
     TATA_signal     668..674
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      361 ttttcacaca ctatcagatg ccgatcctag ctaatggcca gaatctgaga aacaaacacc
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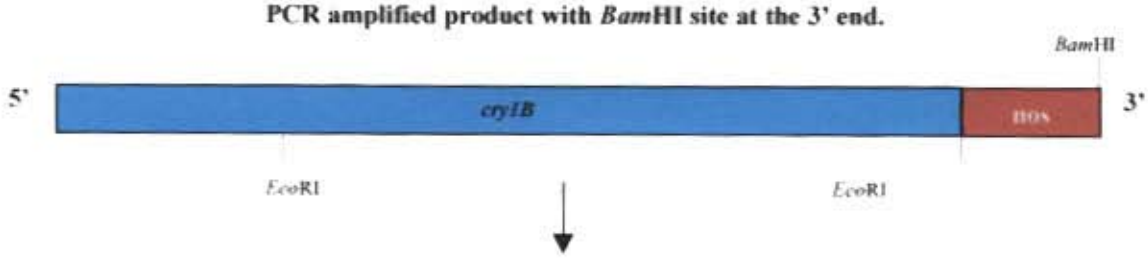
Disclaimer | Write to the Help Desk
NCBI | NLM | NIH

A-8 PCR cloning of *cry1B* gene

8.1 Primer designing :

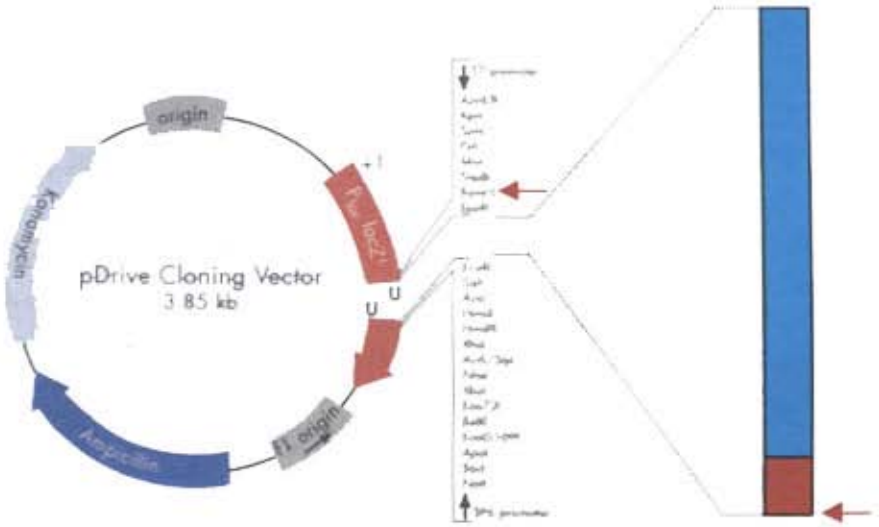


8.2 PCR amplification :

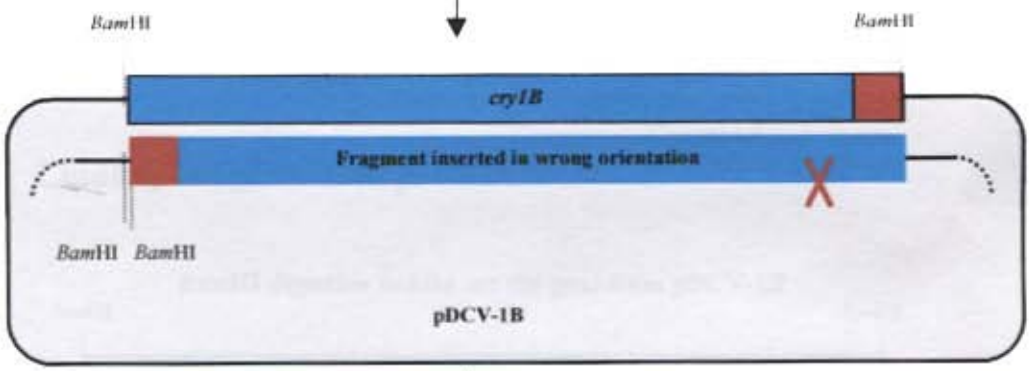


8.3 Cloning of PCR product :

Cloning in pDrive Cloning Vector



Ligation



Bacterial (*E. coli*, DH5α) transformation

Bacterial colonies were screened for the clone(s) harbouring recombinant plasmid with the gene insert in correct orientation by restriction analysis using *Bam*HI restriction enzyme

(*Bam*HI takes out 2.3 kb fragment, if the insert is in correct orientation; otherwise linearized ≈6 kb plasmid is obtained)

Confirmation by restriction digestion/mapping of the recombinant plasmid (pDCV1B) using *Eco*RI.

(*Eco*RI digestion gives fragments of 3.85 kb, 1.3 kb, 0.6 kb and 0.3 kb)

8.4 Confirmation of base sequence :

Base sequence confirmation of the PCR cloned gene by single strand non-assembled primer walking sequencing reactions

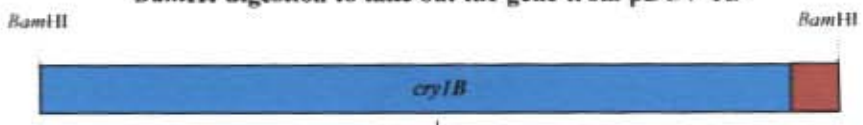
Assembled base sequences:

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CTTGTGCATCGCTGAGGGCAACAACATCGACCCATTCGTCAGCGCCTCCACCGTCCAAACCGGCATCAACATCGCTGGCCGCAT
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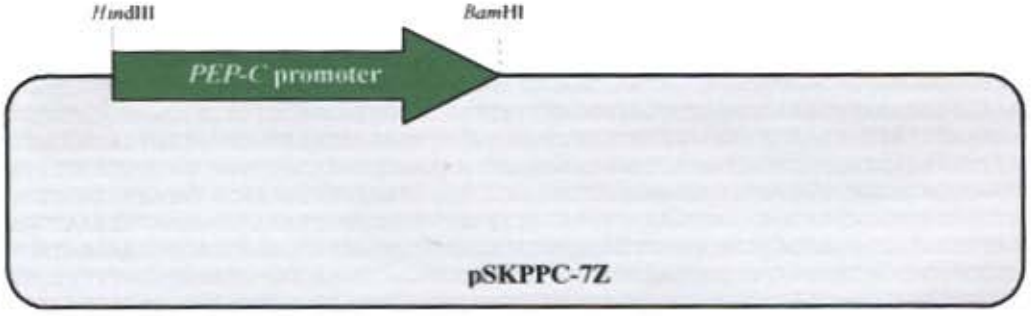
Confirmed base sequence by comparing with the original sequence

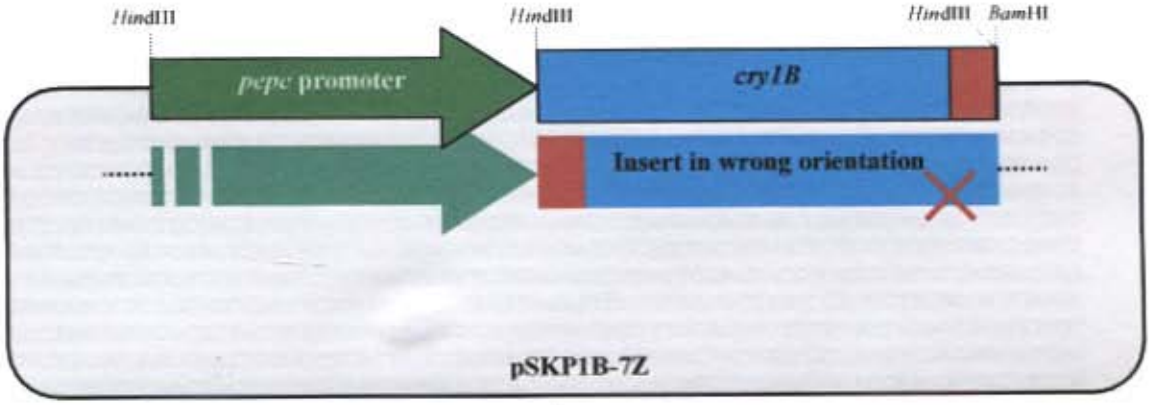
8.5 Cloning the gene with promoter :

***Bam*HI digestion to take out the gene from pDCV-1B**



Clone the gene (*cryIB*) fragment in pSKPPC-7Z at *Bam*HI site





Bacterial transformation and screening of the bacterial colonies was done for the clone(s) harbouring recombinant plasmid with the gene inserted down the promoter in correct orientation, by restriction analysis using *Hin*dIII restriction enzyme

(*Hin*dIII takes out 4.6 kb fragment, if the insert is in correct orientation; otherwise only 2.3 kb promoter fragment comes out)

***Hin*dIII restriction enzyme was used to take out the *cry1B* gene-expression-cassette fragment for clean DNA transformation.**

A-9 Plasmid base sequence

pSKPBA-7Z (9520 bp) :

Sma I

GGGCGAATTGGGCCCGACGTCGCATGCTCCTCTAGACTCGAGGAATTCGGTACCCCGGGTTCGAAATCGATAAG
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TGAGATGTTTATTGAGACCATTGAGGACCTTTGAGTAATTAACTCTCAACCTAGTGGAAATTCGTTACCAACTA
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Mlu I

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A-10 Plasmid map

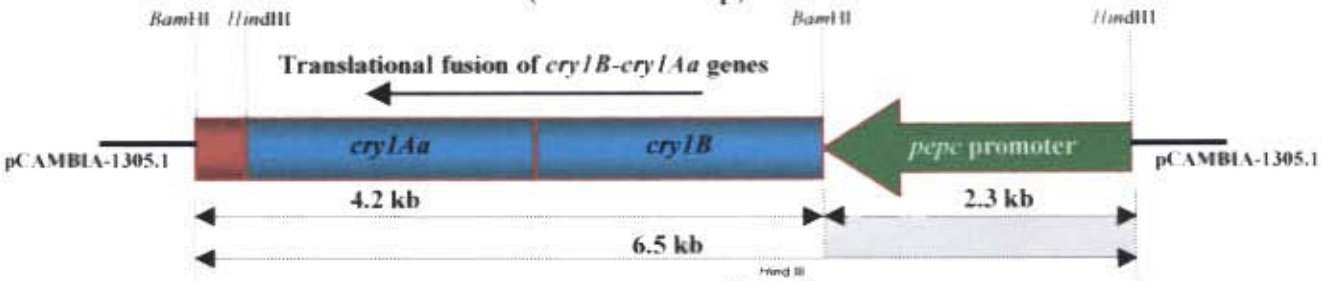
The detailed map information of the plasmid constructs made is as given below:

A-10.1 pSKPBA-05

Translational fusion of *cry1B* and *cry1Aa* genes driven by green tissue specific *pepc* promoter

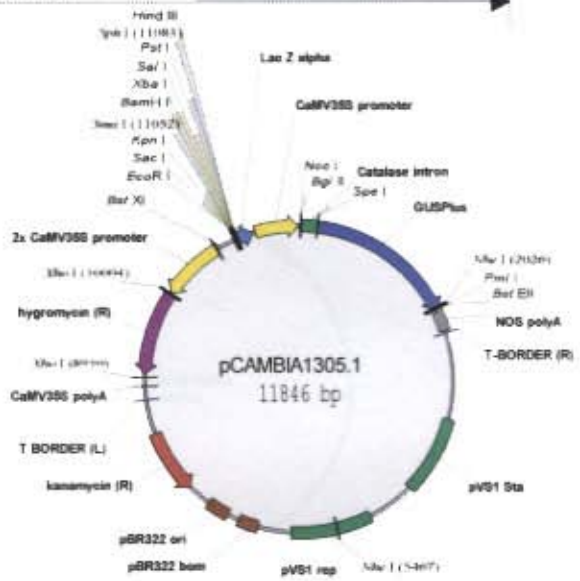
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(1) Linear map



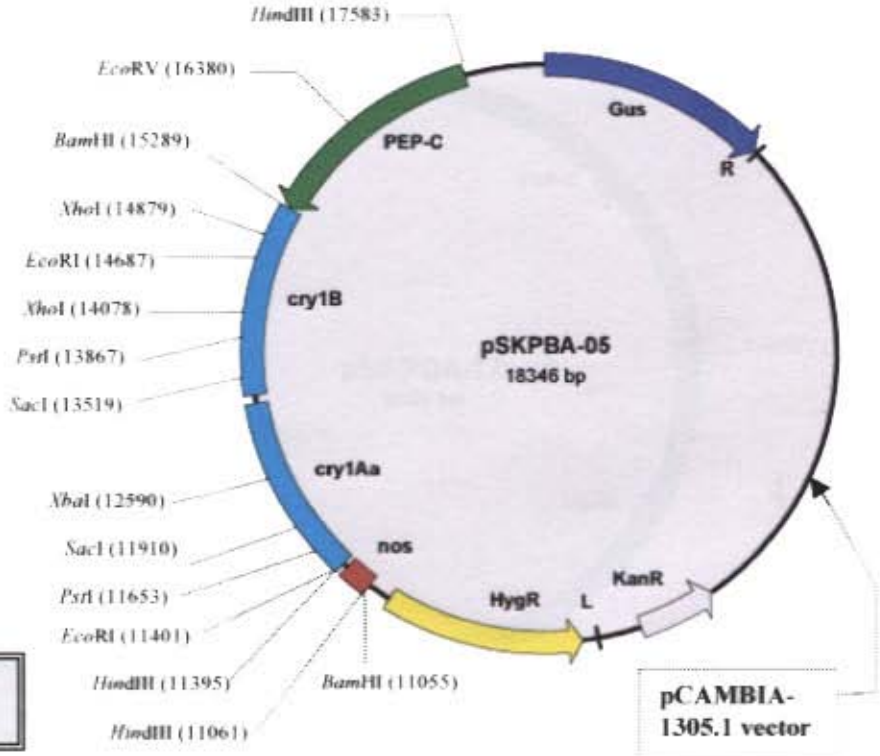
(2) pCAMBIA-1305.1, the cloning vector.

pCAMBIA-1305.1, a binary vector, having GUSPlus reporter gene, Kanamycin resistance for bacterial selection and Hygromycin resistance for plant selection.



(3) Circular map

Translational fusion gene *cry1B-cry1Aa* driven by green tissue specific *pepc* promoter, cloned in pCAMBIA-1305.1 vector in the MCS at *Hin*dIII – *Bam*HI site. Primarily suitable for *Agrobacterium*-mediated transformation.



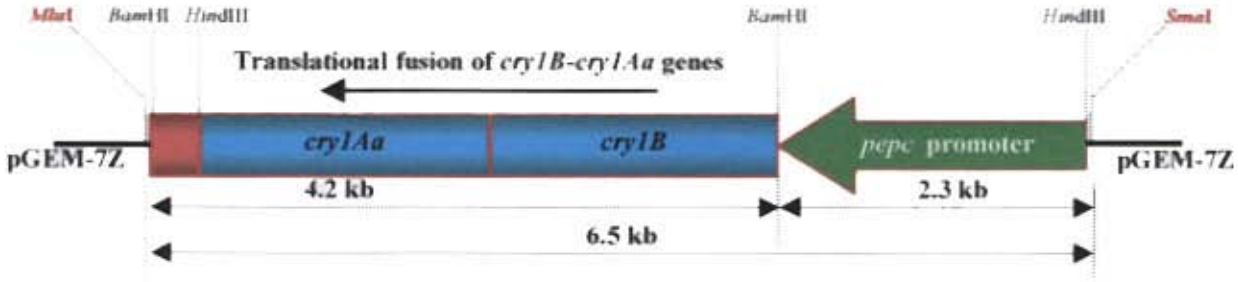
Constructed by: Suresh Kumar
Date of construction: 4th Jan. 2003.

A-10.2 pSKPBA-7Z

Translational fusion of *cry1B* and *cry1Aa* genes driven by green tissue specific *pepc* promoter

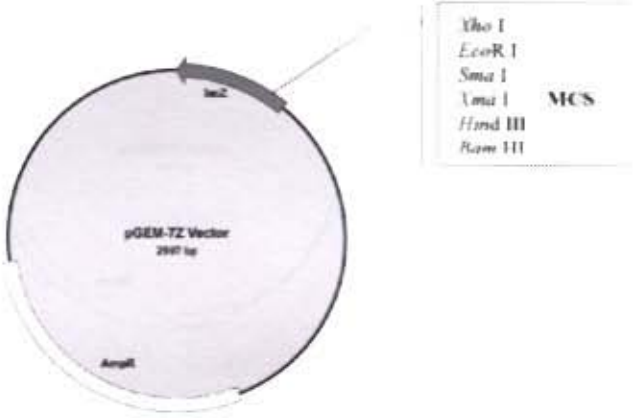
(Size: 9520 bp)

(1) Linear map



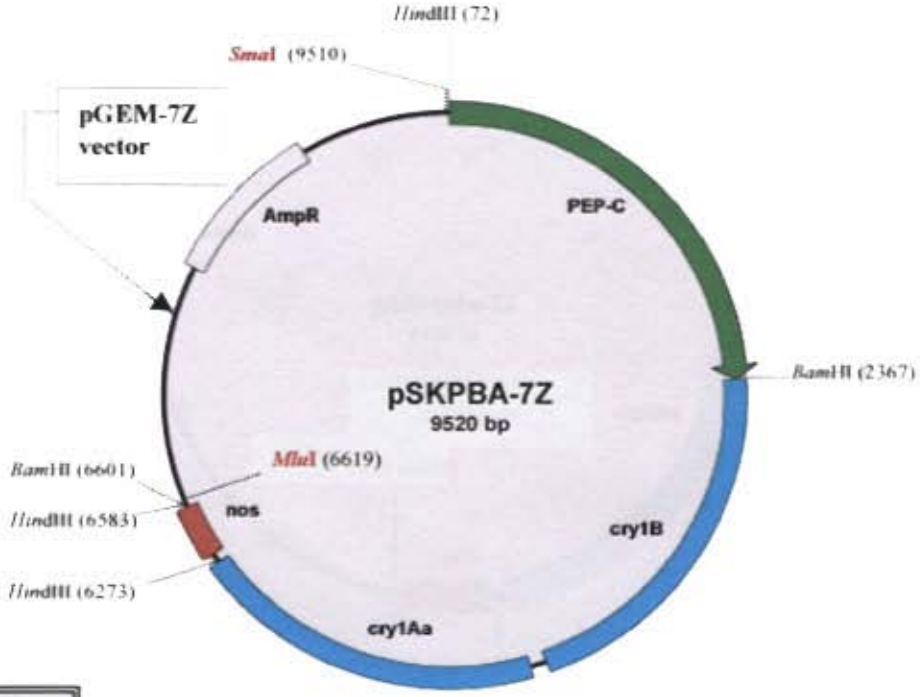
(2) pGEM-7Z, the cloning vector.

pGEM-7Z vector, having Ampicillin resistance for bacterial selection.



(3) Circular map

Translational fusion gene *cry1B-cry1Aa* driven by **green tissue** specific *pepc* promoter, cloned in pGEM-7Z vector in the MCS at *Hin*dIII – *Bam*HI site. Particularly designed for Biolistic-mediated clean DNA transformation. ***Sma*I – *Mlu*I** digestion gives 6.5 kb fragment of the gene expression cassette for bombardment.



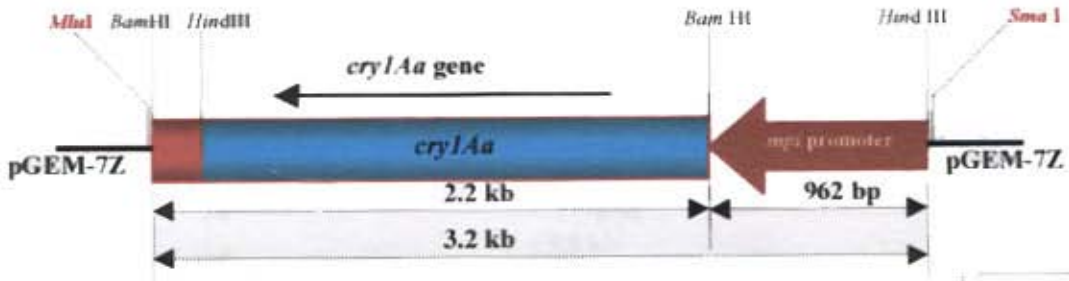
Constructed by: Suresh Kumar
Date of construction: 6th Sept., 2003.

A-10.3 pSKMAa-7Z

Bt *cry1Aa* gene driven by wound inducible *mpi* promoter.

(Size: 6150 bp)

(1) Linear map



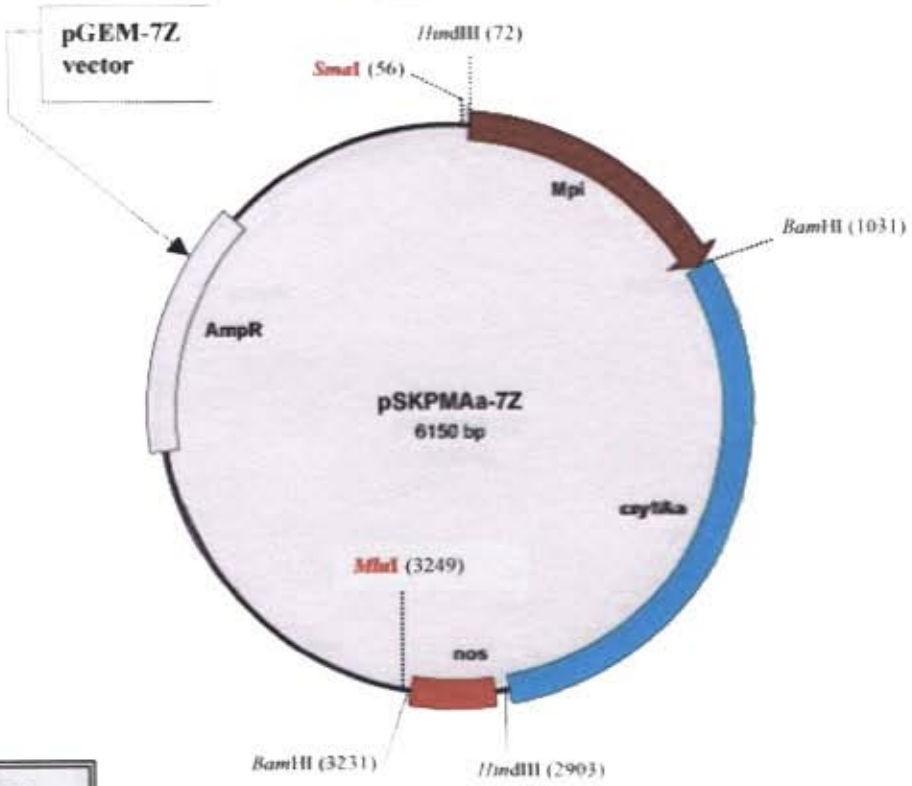
(2) pGEM-7Z, the cloning vector.





pGEM-7Z vector, having Ampicillin resistance for bacterial selection.

(3) Circular map



Bt *cry1Aa* gene driven by wound inducible maize proteinase inhibitor (*mpi*) promoter, cloned in pGEM-7Z vector in the MCS at *Hin*dIII – *Bam*HI site. Particularly designed for Biolistic-mediated clean DNA transformation. ***Sma*I-*Mlu*I** digestion gives 3.2 kb fragment of the gene expression cassette for bombardment.

Constructed by: Suresh Kumar
Date of construction: 16th Oct., 2003.

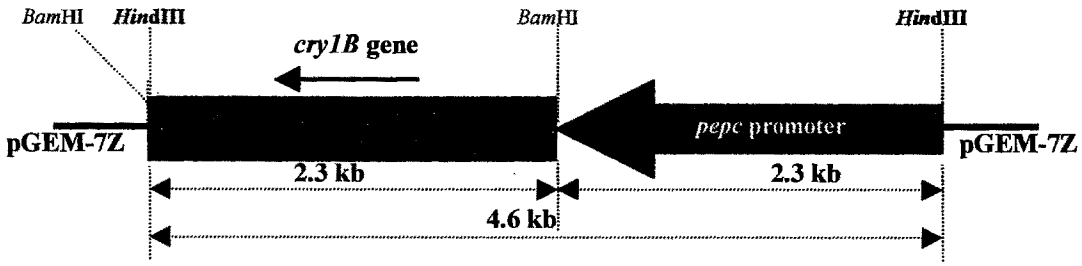
A-10.4

pSKP1B-7Z

Bt *cry1B* gene driven by green tissue specific *pepc* promoter

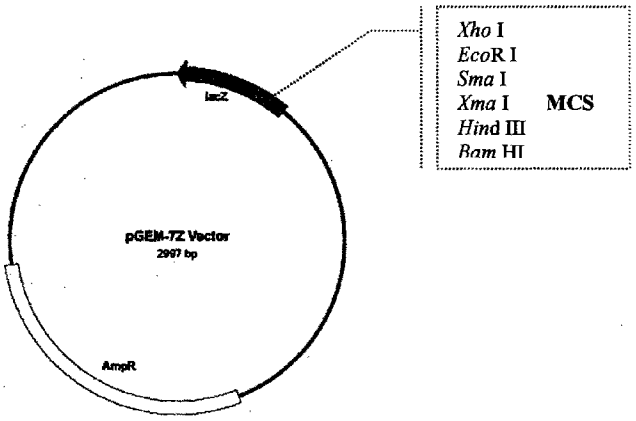
(Size: 7566 bp)

(1) Linear map



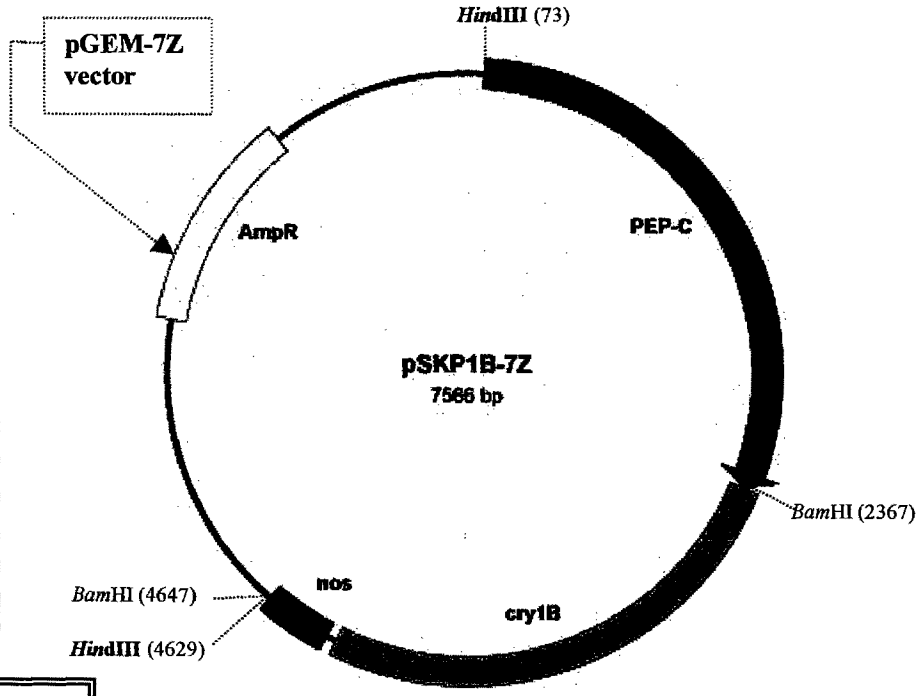
(2) pGEM-7Z, the cloning vector.

pGEM-7Z vector, having Ampicillin resistance for bacterial selection.



(3) Circular map

Bt *cry1B* gene driven by **green tissue** specific *pepc* promoter, cloned in pGEM-7Z vector in the MCS at *Hin*dIII – *Bam*HI site. Particularly designed for Biolistic-mediated clean DNA transformation. ***Hin*d III** digestion gives 4.6 kb fragment of the gene expression cassette for bombardment.



Constructed by: Suresh Kumar
Date of construction: 26th Oct., 2003.

Rice Transgenics Lab., NRC-Plant Biotechnology, I.A.R.I., New Delhi -110 012.

A-11 Preparation of Gold Suspension

Weighed 6 mg gold particles (0.6μ) in a 200 μl capacity microcentrifuge tube
↓
Added 100 μl of absolute ethanol
↓
Vortexed for 30 seconds
↓
Centrifuged for 10 seconds at 10,000 rpm
↓
Pipetted off the supernatant
↓
This ethanol-washing step was repeated twice
↓
Added 100 μl of sterile double distilled water
↓
Vortex for 30 seconds
↓
Centrifuge for 10 seconds at 10000 rpm
↓
Pipetted off the supernatant
↓
The washing step was repeated twice
↓
The surface sterilized gold particles were resuspended in 100 μl of sterile double distilled water
↓
Divide into two (50 μl each)

A-12 DNA precipitation on Gold Particles

Taken 50 μl (3 mg) of sterilized gold particle suspension in microcentrifuge tube
↓
Added DNA (2.5 μg) into the tube while vortexing continuously at slow speed
↓
Added 50 μl of 2.5 M $CaCl_2$, drop by drop while vortexing
↓
Then added 20 μl of 0.1 M spermidine, while vortexing continuously at slow speed
↓
The content was vortexed at high speed for 3 min
↓
The mix was incubated at room temperature for 30 min
↓
Centrifuged for 15 seconds at 10,000 rpm
↓
Pipetted off the supernatant
↓
Washed once with 250 μl of absolute ethanol
↓
Resuspended in 80 μl of absolute ethanol
↓
Distribute 10 μl of coated gold partile suspension onto macrocarriers
↓
Allowed the macrocarrier to dry
↓
Used for the particle bombardment (PDS-1000/BioRad)

A-13 Primer sequences used for PCR

Gene / Promoter	Primer name	Primer sequence	Purpose	Anneal Temp. (°C)	Product size (bp)
cry1B	1BF-1	5' CGA GAT CAT CAA CGC TGT GTC C 3 '	Screening of transgenic plants	60	524
	1BR-1	5' CAG CCT GAG ACA CGT CCA TCA AC 3 '			
cry1B	1BF-2	5' CTC CAG TTG AAG GAC TCC GAG AC 3'	Screening of transgenic plants	59	534
	1BR-2	5' GAT GGA GGT GCG GAT GAT GTC 3 '			
cry1Aa	1AaF-1	5' CTA CGC CGA GTC CTT CAG GGA G 3'	Screening of transgenic plants	61	599
	1AaR-1	5' CGT CGG TGT AGA TGG TGA TGG AG 3'			
hph	Hyg-F	5' ACA CAG CCA TCG GTC CAG 3'	Screening of transgenic plants	59	897
	Hyg-R	5' CGA CCT GAT GCA GCT CTC 3'			
cry1B	PCR-1B-F	5' CAT GGT TAC CTC CAA CCG 3'	PCR cloning of the gene	57	2277
	PCR-1B-R	5' GGA GCT CTC CGG ATC CAA G 3'			
mpi	PCR-Mpi-F	5' CGCAAGCTTGGTCGAGAGACGATATTTAG 3'	PCR cloning of the promoter	67	970
	PCR-Mpi-R	5' CACGGATCCAGTGGTTGGACACAGCGTTG 3'			
GUS Gene cassette)	PGusN-F	5' GCT TCA TGG AGT CAA AGA TTC 3'	PCR amplification of the gene-cassette	54	2878
	PGusN-R	5' ACC CGA TCT AGT AAC ATA GAT GAC 3'			

A-14 Solution for Southern hybridization

Solution	Composition (per lit.)
Depurination solution	11 ml Conc. HCl + 989 ml distilled water
Denaturation solution	87.66 g NaCl + 20 g NaOH
Nuetralization solution	87.66 g NaCl + 60.5 g Trizma base (pH adjusted to 7.5 with Conc. HCl)
Transfer buffer (20X SSC)	88.23 g Tri-sodium citrate + 175.32 g NaCl (pH checked to be 7.8)
Prehybridization / Hybridization solution	0.5 M phophate buffer + 7% SDS + 10 mM EDTA (pH 7.2)
Wash solution (Low stringency)	2X SSC + 0.1% SDS (w/v)
Wash solution (Medium stringency)	1X SSC + 0.1% SDS (w/v)
Wash solution (High stringency)	0.1X SSC + 0.1% SDS (w/v)

A-15 Reagents for denaturing agarose (FA) gel electrophoresis

Solution	Composition
10X Formaldehyde (FA) gel buffer	200 mM MOPS 3-[N-morpholino]propanesulfonic acid (free acid) 50 mM Sodium acetate 10 mM EDTA, pH 7.0 adjusted with 1N NaOH.
Formaldehyde agarose gel (1.2%)	1.2 g agarose 10 ml 10X FA gel buffer RNase-free water to 100 ml 1.8 ml 37% (12.3M) Formaldehyde* 1 µl Ethidium bromide* (10 mg/ml stock)
1X FA gel buffer	100 ml 10X FA gel buffer 20 ml 37% (12.3M) Formaldehyde* 880 ml RNase-free water
5X RNA loading buffer	16 µl saturated aqueous bromophenol blue solution 80 µl 500 mM EDTA (pH 8.0) 720 µl 37% (12.3M) Formaldehyde* 2 ml glycerol 3084 µl formamide 4 ml 10X FA gel buffer RNase-free water to 10 ml

- Toxic and/or mutagenic chemicals, appropriate safety measures were adopted.

The mixture of FA gel buffer, agarose and water was heated to melt agarose particles. Cooled down to 65° C and added the required volume of formaldehyde & 1 µl of Ethidium bromide. The content was mixed thoroughly and poured into the gel casting tray. The gel was equilibriated in 1X FA gel-running buffer for 30 min before running the gel.

A-16 Hoagland's solution

The medium used for irrigation of tissue cultured plants in pots

Macro I	25 X stock/500 ml (gm)	Volume to be used for 10 lit. 0.1X solution
$MgSO_4(MgSO_4.7H_2O)$	25.276	10 ml
KH_2PO_4	6.804	

Macro II	25 X stock/500 ml (gm)	Volume to be used for 10 lit. 0.1X solution
$Ca(NO_3)_2$	59.056	10 ml

Iron	25 X stock/500 ml (gm)	Volume to be used for 10 lit. 0.1X solution
$FeSO_4.7H_2O$	3.336	
Na_2 E.D.T.A.	2.506	10 ml

Micro	50 X stock/500 ml (mg)	Volume to be used for 10 lit. 0.1X solution
H_3BO_3	817.6	
$MnSO4.H_20$	604.0	
$CuSO_4.5H_2O$	99.6	
$NaMoO_4.2H_2O$	12.00	5 ml
$ZnSO_4.7H_2O$	12.00	
KI	8.28	
$CoCl_2.6H_2O$	9.04	

A-17 Cut-stem bioassay data sheet

(A sample page of the cut-stem bioassay data)

Cut-stem Insect Bioassay

September, 2004

Pusa Basmati-1, Bt Transgenic Rice

(Harbouring *cry1B-1Aa* translational fusion genes driven by *pepc* promoter)

S. No.	T_0 Plant No.	No. of larvae inoculated	No. of larvae recorded			No. of larvae escaped
			Live	Dead	Total	
1.	11	5	2	2	4	1
2.	7	5	2	3	5	0
3.	4	5	1	4	5	0
4.	4	5	2	3	5	0
5.	C	5	4	1	5	0
6.	26	5	3	1	4	1
7.	4	5	1	3	4	1
8.	C	5	4	0	4	1
9.	7	5	1	3	4	1
10.	34	5	3	1	4	1
11.	11	5	2	3	5	0
12.	11	5	3	2	5	0
13.	7	5	1	3	4	1
14.	7	5	1	3	4	1
15.	26	5	3	1	4	1
15.	26	5	2	2	4	1
16.	26	5	1	2	3	2
17.	26	5	2	2	4	1
18.	11	5	1	3	4	1
19.	11	5	2	1	3	2
20.	11	5	3	2	5	0
21.	11	5	2	2	4	1
22.	4	5	2	3	5	0
23.	C	5	4	1	5	0
24.	C	5	5	0	5	0
25.	C	5	4	1	5	0
26.	26	5	2	2	4	1
27.	34	5	3	1	4	1

C = Untransformed PB-1 (control) plant

A-18 Abbreviations used

Abbreviation	Expansion
PB-1	Pusa Basmati-1
YSB	Yellow stem borer
LA	Luria agar
LB	Luria broth
cDNA	complementary DNA
hpt	Hygromycin phosphotransferase gene
MS	Murshige and Skoog
2,4-D	2,4 - dichlorophenoxy acetic acid
BAP	Benzyl aminopurine
NAA	Naphtalene acetic acid
IAA	Indole acetic acid
DMSO	Dimethyl sulfoxide
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide
EDTA	Ethylene diamine tetra acetic acid
dNTP	deoxy nucleotide triphosphate
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription – polymerase chain reaction
SSC	Saline sodium citrate
KI	Potassium iodide
DEPC	Diethyl pyrocarbonate
mpi	Promoter of the maize proteinase inhibitor gene
pepc	Promoter of the phosphoenolpyruvate carboxilase gene

A-19 Chemicals and the source companies

Chemicals	Company
Hygromycin-B	Duchefa, The Netherlands.
Plant hormones	Sigma-Aldrich, USA.
myo-inositol	Sigma-Aldrich, USA.
Agarose and Phytagel	Sigma-Aldrich, USA.
Gold particles & other consumables for bombardments	BioRad Laboratories, Richmond, CA.
Spermidine and $CaCl_2$ for bombardment	Sigma-Aldrich, USA.
EDTA, MB grade	Sigma-Aldrich, USA.
Taq DNA polymerase	MBI Fermentas and Bangalore Genei Pvt. Ltd., Bangalore, India.
Hybridization (Hybond N^+) membrane	Amersham, Pharmacia, UK.
dNTPs	MBI Fermentas, Vilnius, Lithuania.
α-^{32}P- dCTP	Bhabha Atomic Research Centre, India.
Ethanol (for bombardment)	Merck, Germany.
Ethanol (for routine use)	Bengal chemicals, India.
Restriction enzymes	MBI Fermentas, Vilnius, Lithuania.
DNase I	MBI Fermentas, Vilnius, Lithuania.
Hexal abel Plus DNA labeling kit	MBI Fermentas, Vilnius, Lithuania.
DNA & RNA isolation kits	QIAGEN GmbH, Hilden, Germany.
X-Gluc	Melford Laboratories, Suffolk, England.
DNA size markers	MBI Fermentas, Vilnius, Lithuania.
Oligonucleotides (Primers) for PCR	Microsynth GmBH, Switzerland.
Sequencing of DNA samples	Microsynth GmBH, Switzerland.
DyNAzyme EXT	Finnzymes, Espoo, Finland.
MOPS	Sigma-Aldrich, USA.



The other chemicals of regular use were procured either from Hi Media Laboratories Limited, Mumbai or Qualigens Fine Chemicals, Mumbai.