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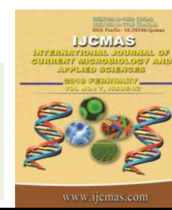

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Characterization of *Bt* Transgenic Plants: A Review

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A B S T R A C T

Insecticidal δ-endotoxins of *Bacillus thuringiensis* have acquired great significance in recent years because of their specificity to target insects and toxicity at very low concentrations. Genes from *B. thuringiensis* have been the most successful group of genes identified for use in genetic transformation of crops for pest control on a commercial scale. Successes of plant transformation include most of major economic and vegetable crops. Polymerase chain reaction (PCR) is commonly used technique for the presence of transgene in the putative transformants by DNA amplification. The Southern blot technique is used to confirm gene integration and copy numbers and Northern blot for gene expression in transgenic plants. Quantification *Bt* protein expressed in plants is performed by Enzyme Linked Immunosorbent Assay (ELISA). *Bt* transgenic plants were assessed for insect resistance against target pest/s by conducting insect bio-assays.

Introduction

Bacillus thuringiensis (*Bt*) is a gram-positive soil bacterium, which produces proteinaceuos crystalline inclusion bodies during sporulation. There are many subspecies and serotypes of *Bt* with a range of well characterized insecticidal proteins or *Bt* toxins. At present it has been estimated that over 60,000 isolates of *Bt* are being maintained in culture collections worldwide. Known *Bt* toxins kill insects belonging to the orders Lepidoptera, Coleoptera, Diptera (Hofte and Whiteley, 1989). The host range of *Bt* has expanded considerably in recent years due to extensive screening programs. Insecticidal δ-endotoxins of *B. thuringiensis* have acquired great significance in recent years because of their specificity to target insects, toxicity at very low concentrations and environment friendly nature (Kumar *et al.,* 1998).

Advances in biotechnology have provided several unique opportunities that include access to various plant transformation techniques, novel and effective molecules, ability to change the levels of gene expression, capability to change the expression pattern of genes, and develop transgenics with different insecticidal genes.

Genes from bacteria such as *Bacillus thuringiensis* and *Bacillus sphaericus* have been the most successful group of genes

identified for use in genetic transformation of crops for pest control on a commercial scale (Gill *et al.,* 1992; Charles *et al.,* 1996). Transgenic plants rarely result in 100% control, but tend to retard insect growth and development (Estruch *et al.,* 1997).

Genes coding for *Bt* δ-endotoxins have been deployed in a wide range of crop plants with considerable success (Sharma *et al.,* 1999). The first *Bt* toxin gene was cloned in 1981 and the first transgenic plant was produced by mid-1980s. Since then, several crop species have been genetically engineered to produce *Bt* toxins to control the target insect pests. Genes conferring resistance to insects have been inserted into crop plants such as cotton, maize, potato, tobacco, rice, broccoli, lettuce, walnut, apple, alfalfa and soybean (Bennett, 1994; Federici, 1998; Griffiths, 1998). The first transgenic cotton crop was grown in 1994 and large-scale cultivation was taken up in 1996 in USA (McLaren, 1998).

India, the largest cotton growing country in the world had 10.8 m ha of *Bt* cotton in 2016. The spectacular growth in *Bt* cotton is an evidence that it has consistently delivered unprecedented benefits to farmers and to the nation. *Bt* cotton has increased productivity by up to 50% while reducing the insecticide sprays by half, hence increased income to cultivators. Success achieved in cotton has served as an excellent model to emulate in many other crops such as rice, wheat, pulses and oilseeds that have the potential to make agriculture a viable profession for the peasants of India.

Mode of action of *Bt*

The crystalline protoxins are inactive, until they are solubilized by the gut proteases (Tojo and Aizawa, 1983; Milne and Kaplan, 1993). The protoxins are activated in the midgut by trypsin like proteases to toxins at alkaline pH. In general, 500 amino acids from the C terminus of 130 kDa protoxins and 28 amino acids from the N terminus are cleaved leaving a 65 to 55 kDa protease resistant toxic active core comprising the N-terminal half of the protoxin (Hofte and Whiteley, 1989). The active toxin consists of three distinct structural domains. Domain I (7 α -helices) determines toxicity and pore formation. Domain II (3 ß–sheets) determines receptor binding and specificity whereas domain III (2 ß–sheets), is involved in receptor binding and protein processing (Schnepf *et al.,* 1998). The active toxin binds to specific receptors located on the apical brush border membrane of the columnar cells in the midgut of target insect, the α -helices penetrate the membrane and lead to formation of pores (ion channels). The toxicity of *Bt* lies in the organisation of α-helices derived from domain I. The toxin induced pores formed in the columnar cells of mid gut and allow rapid fluxes of ions leading to swelling of the cells and osmotic lysis. There is a positive correlation between toxin activity and ability to bind BBMV (Brush Border Membrane Vesicles) (Gill *et al.,* 1992), and the toxicity is correlated with receptor number rather than receptor affinity (Rie *et al.,* 1989). The disruption of gut integrity leads to death of the insect through starvation or septicemia (Sneh and Schuster, 1981; Salama and Sharaby, 1985).

Transformation studies

Plant transformation is now a core research tool in plant biology and a practical tool for transgenic plant development. There are many verified methods for stable introduction of novel genes into the nuclear genomes of diverse plant species. The capacity to introduce and express diverse foreign genes in plants, first described for tobacco in 1984 (DeBlock *et al.,* 1984; Horsch, *et al.,* 1984; Paszkowski, 1984) has been extended to many plant species in at least 35 families.

Transformation successes include most major economic crops, vegetables, medicinal, fruit and pasture plants. As a result, gene transfer and regeneration of transgenic plants are no longer the factors limiting the development and application of practical transformation techniques for many plant species. But the techniques have continued to evolve to over-come a great variety of barriers experienced in the early phases of the development of transgenic plants.

Transgenic crops with *Bt* genes

The concept of creating insect resistant plants began with identifying proteins with insecticidal properties. The soil microorganism, *Bacillus thuringiensis (Bt)* has proven to be a rich source for insecticidal protein genes. Known *Bt* strains contain a great diversity of δ-endotoxin encoding genes and have proven to be the source *par excellence* of insecticidal principles to be used in transgenic plants.

An elegant and the most effective delivery system for *Bt* toxins, is the transgenic plant. The major benefits of this system are economic, environmental, and qualitative.

In addition to the reduced input costs to the farmer, the transgenic plants provide season-long protection independent of weather conditions, effective control of burrowing insects that are difficult to reach with conventional chemical sprays, and control at all of the stages of insect development.

The *Bt* genes encoding δ-endotoxins were introduced into many crops to develop insect resistant transgenic plants.

Several *Bt* toxin genes have been inserted into crop plants to provide protection against a variety of insect pests (Table 1). Many commercial crops, vegetables, cereals and forage crops are now being transformed to be protected against insect pests by *Bt* toxins (Shelton *et al.,* 2002).

Transgenic crops with single *Bt* toxin

Transgenic plants containing *Bt* genes control pests more effectively than *Bt* formulations. So far, three species of *Bt* crops (cotton, maize and soybean) have become commercially available worldwide. Commercialization of *Bt* crops has significantly reduced the use of synthetic insecticides (Ferre and van Rie, 2002). The first transgenic tobacco plants using *cry* genes were developed in 1987 (Vaeck *et al.,* 1987; Barton *et al.,* 1987; Fischoff *et al.,* 1987; Carozzi *et al.,*1992; Ranjekar *et al.,* 2003). A significant breakthrough was made in 1990 by researchers at Monsanto Company (USA) who modified the *cry* genes, *cry1Ab* and *cry1Ac* for better expression in plant cells (Perlak *et al.,* 1990).

The tobacco plants engineered with truncated genes encoding *cry1Ac* and *cry1Ab* toxins were found to be resistant to the larvae of tobacco horn worm *Manduca sexta* (Kota *et al.,* 1999). Cotton cultivar Coker 312 was first transformed by using partially modified *cry1Ac* gene. The transformed plants showed total protection against *Trichoplusia ni*, *Spodoptera exigua* and *Heliothis zea*. The maximum level of toxin protein in the plants was 0.1 per cent of the total soluble protein (Sharma and Anjaiah, 2000).

Transformed tobacco plants using *cry1Ia5* insecticidal toxin from an Indian *Bt* strain provided complete protection against *H. armigera* (Selvapandian *et al.,* 1998). Modification of natural sequences of cry toxin genes was an important approach taken to enhance the activity of the toxins in the transformed plants. The transgenic tobacco plants with the partially modified *cry1Ab* gene

had a 10 fold higher level of insect control protein and plants with the fully modified *cry1Ab* had a 100 fold higher level of *cry1Ab* protein compared with the wild type gene and exhibited 100% larval mortality of tobacco horn worm (Perlak *et al.,* 1990). Tobacco and tomato plants expressing *cry1Ab* and *cry1Ac* genes have been developed to control lepidopteran insects (Salm *et al.,* 1994).

Synthetic *cry1C* gene in alfalfa and tobacco plants resulted in the production of 0.01- 0.2% of total soluble proteins as Cry1C toxin and provided 100% protection against the Egyptain cotton leaf worm (*S. littoralis*) and the beet army worm (*S. exigua*) (Strizhov *et al.,* 1996). Expression of modified *cry1Ac* in cotton and *cry3Aa* in potato conferred considerable protection against lepidopteran and coleopteran pests respectively (Ranjekar *et al.,* 2003). Successful control of pink bollworm (*P. gossypiella*) has been achieved through transgenic cotton using a truncated *cry1Ab* gene in transgenic cotton plants (Wilson *et al.,* 1992; Arencibia *et al.,* 1997).

Scientists at the Bose Institute (Kolkata) have introduced a modified *cry1Ac* gene in rice (IR 64) for resistance to yellow stem borer (Nayak *et al.,* 1997). A synthetic *cry1Ac* gene was introduced into rice lines (Pusa Basmathi 1, Karnal Local, and IR-64) exhibiting total protection against neonate larvae of yellow stem borer.

Rice cultivars (*indica* and *japonica* types) with truncated *cry1Ab* gene caused 100% mortality of the yellow stem *borer* (*S. incertulas*; Datta *et al.,* 1998). Transgenic sugarcane plants with *cry1Ab* showed significant larvicidal activity against neonate larvae of sugarcane borer [*Diatraea saccharalis* (Fabricius)]. 'Jack', a transgenic line of soybean, *Glycine max* (L.), expressing a synthetic *cry1Ac* gene (Jack-Bt) showed 3 to 5 times less defoliation from corn earworm, *H. zea* and eight to nine times less damage from Velvetbean caterpillar, *Anticarsia gemmatalis* (Hubner) (Walker *et al.,* 2000).

Transgenic broccoli with Synthetic *cry1C* was resistant to the cabbage looper (*T. ni*), and cabbage butterfly (*Pieris rapae*; Selvapandian *et al.,* 1998). Vegetable crops like brinjal and tomato were transformed by synthetic/modified *cry1Ab* and *cry1Ac* genes respectively to confer resistance to fruit borers (*Leucinodes orbonalis*) and *H. armigera* respectively (Kumar *et al.,* 1998). The 'New Leaf' potatoes with *Bt* protein, Cry3A, are season-long resistant to Colorado potato beetle (Duncan *et al.,* 2002).

Two toxin / hybrid toxin *Bt* crops

Although no insect species resistant to *Bt* crops have been reported under natural conditions, the potential of insects to evolve resistance against *Bt* toxins is an inevitable threat to this technology. To meet this challenge, several strategies have been proposed to manage insect resistance, such as the high dose, refuge strategy, gene stacking and temporal or tissue specific expression of the toxin (Roush, 1998; Frutos *et al.,* 1999; Shelton *et al.,* 2002). Among the strategies, only the high dose or refuge strategy has been used in developed countries such as the United States and Australia.

At the same time, small farmers in Asian countries could hardly devote their land to a refuge and moreover, a high dose of a foreign protein could cause a phenotypic trade off resulting in yield penalty (Datta *et al.,* 2002). Hence, efforts are being made to develop two toxin *Bt* crops (other- wise known as the pyramiding approach), It is anticipated that two toxin cultivars require smaller refuges to achieve successful resistance management and are expected to provide sustained long term protection as against the single gene

transgenics (Cohen *et al.,* 2000). The use of multiple toxin genes with different modes of action has been proposed so that cross resistance is likely to be a less serious problem. As a result, two cry genes for toxins with different receptors or a cry gene in combination with an altogether different unrelated toxin gene, are considered the ideal options (de Maagd *et al.,* 1996; Frutos *et al.,* 1999).

Further, hybrid toxins produced through inclusion of a domain from another toxin result in increased potency of a fused protein by the shift in receptor binding (Bosch *et al.,* 1994). Alternate receptor ligand interaction may also be exploited to further broaden the host range of the *Bt* toxins (Sivasubramanian and Federici, 1994). The dual-toxin Bollgard II genotype (*cry1Ac* + *cry2Ab*) was found highly effective against lepidopteran pests, *Helicoverpa zea*, *Pseudoplusia includens* (Walker) and *Spodoptera frugiperda* compared to Bollgard I (*cry1Ac*) and conventional cotton (Chitkowski *et al.,* 2003). Rice plants expressing *cry1Ab* and *cry1Ac* genes were highly toxic to striped stem borer (*Chilo suppressalis*) and yellow stem borer (*Scirpophaga incertulas*), with mortalities of 97 to 100% within 5 days after infestation (Nayak *et al.,* 1997).

Transgenic rice IR72 lines, TT9-3 and TT9-4, carrying a fused *Bt* gene (*cry1Ab* and *cry1Ac*) demonstrated that both the transgenic lines were highly resistant against natural infestation and artificial infestation of four lepidopteran species, *viz.*, striped stem borer, *Chilo suppressalis* (Walker), pink stem borer, *Sesamia inferens* (Walker), leaf folder, *Cnaphalocrocis medinalis* (Guenee) and green semilooper, *Naranga aenescens* Moore (Ye *et al.,* 2001). The elite Vietnamese rice (*Oryza sativa* L.) cultivars transformed with translationally fused *cry* genes (*cry1Ab-1B*) exhibit 100% mortality of the neonate larvae of yellow stem borer (YSB) within a week of infestation (Ho *et al.,* 2006). Transgenic potato plants developed with a hybrid *Bt* gene *SN19* (domain I & III from *cry1Ba* and domain II from *cry1Ia*) was shown to be resistant against Colorado potato beetle, tuber moth and European corn borer (Naimov *et al.,* 2003). These are the first transgenic plants resistant to pests belonging to two different insect orders. In addition, the target receptor recognition of this hybrid protein is expected to be different from Cry proteins currently in use for these pests that makes it a useful tool for resistance management also (Naimov *et al.,* 2003).

Thus, introduction of *Bt* transgenic plants for commercial cultivation has launched a new era in Agriculture. The expression of very effective insecticidal proteins by plants delivers a remarkable level of insect control unsurpassed by any other method of insect pest management. Therefore, current and novel *Bt* δ-endotoxins are fully expected to be part of the transgenic plant approach to combat pests in the future also.

The story of *Bt* cotton in India is remarkable. With political will and farmer support in place, adoption is projected to continue increasing with *Bt* cotton planting. Coincidentally, new biotech products such as *Bt* eggplant, an important food and cash crop that can benefit up to 2 million small and resource-poor farmers, is waiting with expectations of approval in the near future in India. However, Bangladesh seems to have appreciated its value better and has officially released it for public consumption.

Characterization of transgenic plants

Molecular analysis

Development of transgenic plants is a routine exercise in plant molecular biology now-a-

days. *Agrobacterium* mediated genetic transformation is the most preferred method of gene transfer because of advantages such as its simplicity, economy and generation of single copy transgenics to overcome transgene silencing (Gelvin, 2003). Polymerase chain reaction (PCR) is the most commonly used technique to screen putative transformants (Nain *et al.,* 2005).

Polymerase chain reaction (PCR)

The presence of transgene in the transformants can be analyzed by DNA amplification. For the PCR amplification, plant genomic DNA will be extracted by various extraction methods. PCR was used as the first proof for the analysis of the putative transformants.

PCR amplification with gene specific primers of 3 modified *cry9Aa2* genes was carried out and the results confirmed that the plants were transgenic (Gleave *et al.,* 1998). A synthetic *cry1Ab* gene coding for an insecticidal crystal protein of *Bacillus thuringiensis* (*Bt*) was transferred to cabbage cultivar 'Golden Acre'. Transformed plants resistant to kanamycin were regenerated and total genomic DNA was isolated. Presence of the *cry1Ab* sequence was initially detected through PCR analysis using *cry1Ab* specific primers (Bhattacharya *et al.,* 2002).

A number of putative transgenic chickpea lines were developed using the reconstructed *Bt cry1Ac* genes for protection against pod borer *H. armigera.* The molecular analysis was done to identify homozygotes with high levels of transgene expression. PCR analysis was done with T_0 and T_1 generation plants for confirmation of the presence of the gene and amplification of both *cry1Ac* and *nptII* genes were obtained. The presence of 1.1, 0.73 and 0.5kb PCR amplicons in the transformants but not in the non-transformed control indicated the transgenic status of the plants in Vietnamese rice for *cry1Ab/cry1Ac*, *hph,* and *bar* genes respectively (Ho *et al.,* 2006). Integration of T-DNA into maize genome was confirmed by PCR (the *nptII* and *gus* reporter genes) (Chumakov *et al.,* 2006). The putative T_0 transgenic rice plants with cry2A gene were detected by PCR analysis (Chen *et al.,* 2005).

Southern analysis

Southern blot technique is a commonly used technique to confirm gene integration and copy numbers in transgenic plants. Southern analysis of *Bt*-transgenic lines with *cry1Ab* indicated independent events of transgene-integration and showed desirable single copy insertion of the transgene into the genomic DNA of transgenic cabbage (Bhattacharya *et al.,* 2002).

Southern analysis of T_0 and T_1 plants confirmed co-integration and segregation of the T-DNA bearing *cry1Ac* and *nptII* genes into the genomic DNA of transgenic chickpea plants (Sanyal *et al.,* 2005). The bands obtained in the autoradiogram indicates the co-integration of T-DNA bearing synthetic *cry2A* and *bar* gene into genomic DNA of transgenic T_1 *indica* rice plants (Chen *et al.,* 2005). The southern analysis of PCR positive plants revealed the simple integration pattern with a single 6.0 kb fragment expected for *cry1Ab-cry1B* from *Agrobacterium* mediated transformation (Ho *et al.,* 2006).

Northern analysis

Northern blot technique is generally used to confirm gene expression in transgenic plants. In Northern analysis of total RNA from Southern blot-confirmed plants, presence of a single 2.2 kb band in the autoradiogram confirmed the transcription of *cry1Ab* sequence in BT2, BT3 and BT4 cabbage lines (Bhattacharya *et al.,* 2002).

Table.1 List of transgenic crop plants developed using various *cry* genes against insect pests

Crop group	Target crop	Toxin	Target pest/s	References
Commercial crops	Cotton	*cry1Ac*	*Helicoverpa zea* (Boddie) (Lep.) *Pectinophora gossypiella* (Saunders) (Lep.)	**Bacheler and Mott, 1997**
		cry1Ab	*H. virescens* (Fabr.) (Lep.) *H. zea*	**Perlak *et al.*, 1990**
		cry 1Ac + cry 2Ab	*H. zea* *P. gossypiella* *Spodoptera exigua* (Hubner) (Lep.) *Spodoptera frugiperda* (J.E. Smith) *Pseudoplusia includens* (Walker) (Lep.)	**Adamczyk *et al.*, 2001** **Chitkowski *et al.*, 2003**
		cry 1Ac+ CpTI	*H. zea*	**Wu and Guo, 2005**
	Tobacco	*cry1Aa*	*Manduca sexta* (L.) (Lep.)	**Barton *et al.*, 1987**
		cry1Ab	*M. sexta*	**Vaeck *et al.*, 1987**
		cry1Ab + CpTI	*M. sexta*	**Perlak *et al.*, 1991**
		cry1Ab	*M. sexta*	**Williams *et al.*, 1993**
		cry1Ac	*H. virescens, H. zea* *Spodoptera littoralis* (Boisduval) (Lep.)	**McBride *et al.*, 1995**
		cry1C	*S. littoralis*	**Strizhov *et al.*, 1996**
		cry2A	*Helicoverpa armigera* (Hubner)	**Selvapandiyan *et al.*, 1998**
		cry2Aa	*H. virescens* *H. zea, Spodoptera exigua* (Hubner)	**Kota *et al.*,1999**
		Cry1Ia5	*H. armigera*	**Selvapandian *et al.*,1998**
Cereals	Corn	*cry1Ab*	*Ostrinia nubilalis* (Hubner) (Lep.)	**Koziel *et al.*, 1993**
		cry9C	***O. nubilalis***	**Jansen *et al.*, 1997**

Continued…….

Crop group	Target crop	Toxin	Target pest/s	References
Cereals	Rice	*cry1Ab*	*Chilo suppressalis* Walker (Lep.)	**Fujimoto *et al.*, 1993**
		cry1Ab and *cry1Ac*	*C. suppressalis*	**Cheng *et al.*, 1998**
		cry1B	*C. suppressalis, Cnaphalocrosis medinalis* Guenee (Lep.)	**Marfa *et al.*, 2002**
		cry1Ac, cry2A and GNA	*C. medinalis, Scirpophaga incertulas* *Nilaparvata lugens* Stal (Hom.)	**Maqbool *et al.*, 2001**
		cry3A	*Dicladispa armigera* (Oliv.) (Col.) *Sitophilus oryzae* (L.) (Col.)	**Jhonson *et al.*, 1996**
		cry2A	*S. incertulas and* *C. suppressalis*	**Chen *et al.*, 2005**
		cry1Ab / cry1Ac	*S. incertulas* *C. suppressalis* *Sesamia inferens* Walker *C. medinalis*	**Ye *et al.*, 2001**
Pulses	Soybean	*cry1Ac*	*H. virescens, H. zea* *Pseudoplusia includens* Walker (Lep.) *Anticarsia gemmatalis* (Hubner) (Lep.)	**Walker *et al.*, 2000**
	Pigeon pea	*cry1E-C*	*Spodoptera litura*	**Surekha *et al.*, 2005**
	Chickpea	*cry2Aa*	*H. armigera*	**Sarmah and Deka 2004**
		cry1Ac	*H. armigera*	**Sanyal *et al.*, 2003**
Vegetables	Tomato	*cry1Ab*	*H. virescens*	**Fischholff *et al.*, 1987**
		cry1Ac	*H. armigera*	**Mandaokar *et al.*, 2000**
	Eggplant	*cry1Ab*	*Leucinodes orbonalis* Guenee (Lep.)	**Kumar *et al.*, 1998**
		cry3A	*Leptinotarsa decemlineata* (Say) (Col.)	**Jelenkovic *et al.*, 1998**

Continued…….

Crop group	Target crop	Toxin	Target pest/s	References
Vegetables	Cabbage	*cry1Ab*	*Plutella xylostella* (L.) (Lep.)	**Bhattacharya *et al.*, 2002**
	Broccoli	*cry1C*	*P. xylostella*	**Zhao *et al.*, 2001**
Sugars and Starches	Sugarcane	*cry1A(b)*	*Diatraea saccharalis* (F.)	**Arencibia *et al.*, 1997**
	Potato	*cry1Ab*	*Phthorimaea operculella* (Zeller) (Lep.)	**Peferoen *et al.*, 1992** **Rico *et al.*, 1998**
		cry3Aa	*L. decemlineata*	**Adang *et al.*, 1993**
		Cry9Aa2	*Phthorimaea operculella*	**Gleave *et al.*, 1998**
		Cry1Ia1 (cryV)	*Phthorimaea operculella* *Symmetrischema tangolias* (Gyen) (Lep.)	**Lagnaoui *et al.*, 2000**
		Cry1Ba / cry1Ia	*Leptinotarsa decemlineata* *Phthorimaea operculella*	**Naimov *et al.*, 2003**
Other crops	Canola	*cry1Ac*	*Thrichoplusia ni* (Hubner) (Lep.) *Spodoptera exigua, H. virescens, H. zea*	**Stewart *et al.*, 1996**
	Alfalfa	*cry1C*	*S. littoralis* *Spodoptera exigua*	**Strizhov *et al.*, 1996**
	Groundnut	***Cry1AcF***	***Spodoptera litura*** ***Amsacta albistriga***	**Keshavareddy, 2009**

Lep = Lepidoptera; Col = Coleoptera; Hom = Homoptera

Screening for transcripts of transgene in leaves of soil-grown alfalfa plants carrying the transferred DNA of synthetic *cry1C* gene confirmed that transgenic plants were independent events (Strizhov *et al.,* 1996).

Enzyme Linked Immunosorbent Assay (ELISA)

The presence of the gene can be identified through PCR, but its expression level is identified and quantified by ascertaining the product of the gene using ELISA. The principle involves specific antigen-antibody reactions leading to the accumulation of the conjugate product that is identified at specific wavelength by an ELISA Reader (Sambrook *et al.,* 1989). Quantification of *Bt* protein expressed in plants is very important, as part of post transformation analysis with *Bt* genes.

The use of monoclone based technique for detection and quantification of Cry protein demonstrated as early as 1987 (Vaeck *et al.,* 1987). Polyclonal-monoclonal antibody sandwich ELISA was adopted for detecting the presence of the *cry3A* protein in transgenic *indica* rice plants (Johnson *et al.,* 1996). Quantification of the fused Cry1Ab/Cry1Ac produced in transgenic rice plants was performed using a double sandwich ELISA technique. A Cry1Ab/Cry1Ac plate kit (ENVIROLOGIX INC., Portland, Maine, USA) was used to determine nanogram quantities of the fused Cry1Ab/Cry1Ac per mg fresh leaf tissue (Ye *et al.,* 2001).

Quantitative estimation of delta Cry1Ac endotoxin expressed in transformed chickpea plantlets was performed using a double antibody sandwich ELISA (Adang *et al.,* 1993). The quantitative levels of Cry1Ac and the seasonal decline in expression differed significantly among the eight commercial Bollgard hybrids tested. Bashir *et al.,* (2004) analyzed the Cry2A protein content of a highly insect resistant *cry2A* transgenic rice line. The Cry2A protein content in transgenic *indica* rice plants was determined by ENVIROLOGIX kits (Chen *et al.,* 2005).

The Cry1Ac protein expression was found to be variable among the hybrids and also between different plant parts. The leaves of *Bt* cotton plants were found to have the highest levels of Cry1Ac expression followed by squares, bolls and flowers. The toxin expression in the boll-rind, square bud and ovary of flowers was clearly inadequate to confer full protection to the fruiting parts (Kranthi *et al.,* 2005). Sanyal *et al.,* (2005) reported that quantitative assay of Cry1Ac protein in transgenic chickpea plants indicated maximum expression of *Bt*-toxin in leaves, pods followed by green portions of stem and minimum in roots.

Bioassay studies on transgenic *Bt* plants

It is reasonable to assess insecticidal transgenic plants for insect resistance against target pest(s) by conducting insect bioassays under laboratory as well as field conditions. Under laboratory conditions various plant parts are used in insect bioassays to assess the tolerance of the transformants. Under contained or field trials, whole insecticidal transgenic plants will be compared to their control counterparts to assess their tolerance level to insect pests and also the benefits that might potentially accrue to the farmers.

In one such early example, five neonate larvae of tobacco hornworm were placed on a leaf of a plant transformed with *cry1Ab*. The plants caused 100% mortality of the hornworm and sustained no visible damage after 3 days (Perlak *et al.,* 1991).

Detached leaf bioassays of transgenic alfalfa expressing synthetic *cry1C* gene were

performed with *Spodoptera littoralis,* using ten neonate larvae placed on a moistened filter disc in petri dishes, produced 100% mortality of larvae 3 days after bioassay initiation (Strizhov *et al.,* 1996). A detached leaf bioassay of *Bt-cry1Ia1* potato transgenic plants was performed against potato tuber moth (PTM), *Phthorimaea operculella* by releasing ten neonate larvae into each petri dish and 99% mortality was observed (Lagnaoui *et al.,* 2000).

Sweet corn plants expressing *cry1Ab* gene were artificially infested with 30 *Ostrinia nubilalis* neonates per plant. Percentage of ears without larvae or damage was used to determine the percentage of marketable ears and obtained 100% marketable ears (Burkness, *et al.,* 2001).

Bhattacharya *et al.,* (2002) performed bioassay of insect resistant transgenic cabbage plants expressing a synthetic *cry1Ab* gene by releasing larvae of *Plutella xylostella.* The leaf discs (1.5 cm diameter) from young leaves were cut and placed in small petri dishes containing moistened filter paper. On each leaf disc, five late second instar (6-day old) larvae of *P. xylostella* were released and reared at room conditions. Bioassay on detached leaf discs showed significant larval mortality ranging from 51.84 to 74.06%.

Fall armyworm, *Spodoptera frugiperda* and beet armyworm, *S. exigua* exposed as second instars to leaves, squares or bolls of Bollgard II cotton plants for 6 days exhibited significantly greater mortality than larvae exposed to parts from plants of the Bollgard I variety and the conventional non-transgenic cotton variety (Chitkowski *et al.,* 2003).

Series of bioassays were performed on detached fully grown potato leaves stuck in water agar. For the Colorado potato beetle (CPB) bioassays, 10 neonate larvae were placed on the upper leaf surface. Transgenic potato leaves expressing *cry1Ba*/*cry1Ia* showed complete resistance against larvae with 100% mortality and no visible damage in a leaf feeding assay after 4 days. For adult CPB, four newly emerged insects were placed on leaves and reared for up to 10 days. The transgenic leaves were completely undamaged and most of the Colorado potato beetles which were placed on transgenic potato leaves were still alive but not feeding but smaller in size than control beetles. Potato tuber moth bioassays were performed by placing 10 neonate larvae on the back surface of potato leaves. No live PTM larvae could be recovered from the transgenic potato plant leaves. In contrast to transgenic plant leaves, leaf infestation with PTM larvae resulted in extensive tunneling by four or five live larvae in control leaves after 4 days. For testing of European corn borer (ECB) resistance, two day old larvae were used. Larvae were allowed to feed on potato leaves for two days. ECB mortality was zero after 3 days in control leaves. In contrast, transgenic potato leaves remained healthy and caused 100% mortality of the ECB larvae (Naimov *et al.,* 2003).

Five pieces of freshly cut stems (5-6 cm) with 12 first instar larvae of yellow stem borer were placed into a sealed glass bottle and incubated in the controlled environmental chamber for five days. The feeding assay of *cry2A* plants showed that all the yellow stem borer larvae in transgenic stem cuttings were killed within 5 days after infestation, whereas the larvae infesting the stem cuttings of Minghui 63 (control) grew normally and developed into second instar larvae (Chen *et al.,* 2005).

Insects reared on transgenic seeds of rice showed a high mortality rate and reduction in weight, which was found to be significantly higher than in the control. Bioassay studies

proved that the transgenic seeds showed increased resistance to rice weevil *Sitophilus oryzae* than in controls (Ignacimuthu *et al.,* 2006).

The T_0 and T_1 transgenic plants from the transgenic Vietnamese rice cultivars expressing *cry1Ab/cry1Ac* at the maximum tillering stage were bioassayed for resistance to neonate larvae of yellow stem borer using the cut stem method. Three to five stems (including sheath) of 8cm length were collected at the booting stage. These were placed on a moistened filter paper disc in a 90 mm diameter petri dish and infested with six neonate larvae of yellow stem borer. The percentage larval mortality was based on dead and alive larvae 4 days after their release. The cut stem bioassay results showed that yellow stem borer neonate larval mortality after feeding for 4 days reached 100% in more than 95% of the tested Southern and Western positive T_0 plants, whereas the mortality was 0 to 16.6% in the non-transgenic control plants (Ho *et al.,* 2006).

Insect feeding bioassay on transformed chickpea plants (T_0 and T_1) with larvae of pod borer, *H. armigera* showed high levels of toxicity to insects and protection of transgenic plants. Transformed chickpea plants expressing Cry1Ac protein above 10 ng mg^{-1} soluble protein showed 80–85% protection and high mortality (>80%) of insects (Sanyal *et al.,* 2005).

Detached leaf feeding bioassay tests were done on T_1 and T_2 generations of pigeon pea plants for insect resistance using the 1st and 2nd instar larvae of the pest *S. litura*. The highest mortality of the larvae found in the transgenic plants was 80% (four out of five larvae released). The larvae fed with leaves of transgenic plants were severely stunted in growth when compared to larvae fed with wild-type leaf (Surekha *et al.,* 2005).

Field evaluation of soybean engineered with a synthetic *cry1Ac* transgene for resistance to *Helicoverpa zea* and *Anticarsia gemmatalis* was done by planting transgenic soybean (Jack-*Bt*) in the form of hill along with wild type. Each hill consists of six plants and infested with 140 larvae. Resistance was evaluated through visual estimates of per cent defoliation of the plants in a hill and estimates were made at 2 to 3 days intervals beginning 7-8 days after initial infestation. Jack-*Bt* showed three to five times less defoliation from *H. zea* and eight to nine times less damage from *A. gemmatalis* (Walker *et al.,* 2000).

The review has amply provided evidence to the effect that transgenic technology holds many options for pest management in various crops. The production of transgenic insect resistant plants and their continuing development has been a major scientific success in terms of levels of protection afforded by expression of *Bt* toxins. However, it is important to assure that transgenic insect pest control is not compromised by the development of resistance in pest populations. The next generation transgenic plants need to be directed towards the prevention or delay of the onset of resistance and thus providing durable levels of crop protection. This would definitely be achievable using agricultural biotechnology. Efforts should be made towards realization of these goals. This could be possible by the use of fusion of proteins or even engineering plants expressing multiple toxins. Domain swapping or mutagenesis of the three domain cry toxin could be another step towards improving the efficacy of the cry toxins. There are also other forthcoming approaches involving RNAi as a tool for insect resistance. All these efforts however require proper analysis and attitude of the scientific and nonscientific community in developing insect resistant plants that can go a long way in the years to come.

Note: This is part of the review collected by first author for his doctoral work

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