2 Genetically Modified (GM) Crops Harbouring *Bacillus thuringiensis* (BT) Gene(S) to Combat Biotic Stress Caused by Insect Pests

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Abstract

Insect pests are a menace to the crop plants as they cause 15–22% annual crop loss. *Bacillus thuringiensis* (*Bt*) crystal protein toxin(s) have been observed to be effective against lepidopteran, coleopteran, dipteran and hemipteran insect pests. With the emergence of recombinant DNA technology, computational biology and plant transformation procedures, it is now possible to design, modify and transfer any gene (natural or synthetic) into crop plants especially, to cope with insect pests, herbicide tolerance, various abiotic stresses and to enhance the expression level and nutritional quality. Bt-based biopesticides are an alternative to synthetic pesticides and are insect- specific, effective, eco-friendly and cost-effective. *Agrobacterium*-mediated plant transformation technique utilizes the natural genetic engineering property of *Agrobacterium tumefaciens* which has played a pivotal role in plant genetic engineering and development of stable transgenics, over conventional breeding procedures. Several stable Bt-transgenics (potato, maize, cotton, soybean, canola, squash, rice, etc.) developed by various companies (Monsanto, Dow AgroSciences, Syngenta, Bayer cropScience, etc.) have been approved by Genetic Engineering Appraisal Committee (GEAC), Environment Protection Agency (EPA), and commercialized. The most successful story of Bt-transgenics is that of Bt-cotton (Bollgard: trade name) harbouring *Bt-cry1Ac* like gene. In order to avoid the development of insect resistance, various strategies such as use of hybrid gene, *Bt*-gene pyramiding, refugia strategies, enhanced expression of *Bt*-gene(s) and use of sterile insects are followed as and when required for maintaining the sustainability of *Bt*-technology. In the last few years, after analysing the effectiveness and promising future of this 'green

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technology,' there has been a remarkable progress in the list of countries accepting the Bt-GM crops.

Keywords
Bacillus thuringiensis · *cry* gene · *Agrobacterium tumefaciens* · Plant transformation · *Bt*-GM crops

2.1 Introduction

The sustainable plant productivity and crop yield(s) in coming years is the major constrain for food and nutritional security for the human population in developing countries, where arable land per capita is shrinking, while human and livestock population is steadily increasing. Plant and crop productivity and yield are the result of interaction of several physiological, biochemical and metabolic processes over a defined period of time, reflected in gain of total biomass or converted harvestable commodity like seeds, fruits or edible plant parts under a set of environmental conditions that consist of several physical, geo-chemical and biological components. Therefore, besides the genetic potential of plant species, the phenotypic performance of crop plants in field profoundly depends on and is influenced by several physical, abiotic and biotic parameters and is highly variable. Hence, plant yield or harvest index is dependent on several factors and several of them are beyond human control and are part of climate change and environment. Among biotic components that influence plant/crop yield perhaps infestation of plant pathogens and insect pest are major issues after the agronomic inputs and practices. The infestation of insect pests alone during field and storage condition may affect up to from 24 to 65 ± 5% loss in grain yield of major crops (Ronald 2011). Control of agricultural insect pests under field and storage conditions largely depend on the wide spread use of synthetic insecticides and pesticides which are harmful to the ecosystem and human population (Hilder and Boulter 1999; Wahab 2009). Alternative to conventional chemical insecticides, application of microbial insecticides containing different microbial preparations and delta endotoxins (Cry proteins) from *Bacillus thuringiensis* (*Bt*) have emerged as ecofriendly and sustainable method for control of agricultural insect pests in the last 50–60 years (Sanahuja et al. 2011). Attempts are being made to use alternative bioinsecticides in field as well as in storage conditions to minimize the losses in grain yield. In recent past, with the development of diverse biotechnological tools and techniques of recombinant DNA and genetic engineering, it is now possible to transfer and express a desired gene in its native or modified form into the identified organism including plants, animals and microbes. Among the battery of genetically modified organisms (GMOs), the transgenic plants, expressing genes from either trans- or cis-origin, are the latest introduction for sustainable crop and plant yield (Park et al. 2011).

The most widely used and well-documented example of transgenic plant in agriculture practice is the Bt-cotton, where Bt-toxin crystalline proteins of Cry1A family are expressed starting from the native wild *cry1Ab* and *cry1Ac* genes of *Bacillus thuringiensis* to highly modified synthetic version that are expressed in cotton followed by maize and soybean which are released for commercial cultivation (Perlak et al. 2001; James 2012). Since then, transgenics of major crop plants like cotton, maize, soybean, canola, tomato, rice, squash, potato, papaya, sugarcane and mustard have been developed for insect-pest resistance, herbicide tolerance and resistance to viruses and have been grown in more than 30 countries over 181.5 million hectares in 2016. About 17.3 million farmers over the world have been benefited by transgenic technology and are growing biotech crops. Interestingly, recently, five conservative European countries, namely Spain, Portugal, Czechia, Romania and Slovakia, have agreed to cultivate Bt-maize. Therefore, the transgenic technology has been adopted by both developed and developing countries like the United States, China, Brazil, Argentina, Canada and India and African countries, for different traits.

Bacillus thuringiensis (*Bt*) is a gram-positive soil bacterium which can produce crystalline inclusions during the second phase of sporulation. These inclusions eventually develop into hydrophobic crystalline structures consisting of several toxin proteins that are of insecticidal nature against a wide spectrum of agricultural insect pests (Whiteley and Schnepf 1986). Most of the crystal proteins are protoxins of proteinaceous nature and are proteolytically converted into smaller toxic polypeptides in the midgut region of corresponding agricultural insect. This activated toxin interacts with the midgut epithelial cells of susceptible insects (Hofmann et al. 1988; Bravo et al. 2007, 2011; Vachon et al. 2012; Pardo Lopez et al. 2013) and biochemically generate the pores in the cells of brush border membrane, thus disturbing the osmotic balance and eventually the septicemia in the target insect leading into death of the insect (Knowles and Ellar 1988; Bravo et al. 2007, 2011). Several specific high-affinity binding sites on insect membranes to *B. thuringiensis* toxins have been documented for specificity of different toxin peptides generated by different strains/species/isolates of *B. thuringiensis* owing to different genes coding for the corresponding crystal protein (Schnepf et al. 1998; Hofte and Whiteley 1989).

Since the first cloning of an insecticidal crystal protein (ICP) gene (*cry*) from *B. thuringiensis* by Schnepf and Whiteley (1981), a large number of *cry* genes from different strains/species of *Bt* have been cloned, identified and characterized (Crickmore et al. 1998; deMaagd et al. 2001). Till date, more than 500 different *cry* genes from *B. thuringiensis* have been characterized and systematically documented in the literature and enlisted in website maintained by Crickmore and his group (www. glfc.cfs.nrcan.gc.ca/bacillus). These insecticidal genes code specific toxins effective against insect orders belonging to Lepidoptera, Diptera and Coleoptera. Some are effective against other insect orders like Hymenoptera, Homoptera, Orthoptera, and Mallophaga, nematodes, mites and protozoa as well (Feitelson et al. 1992; Bravo et al. 2007). *B. thuringiensis* strains have a genome size of 2.4–5.7 million bp, and most of these bacterial strains possess both circular and

sometimes linear extra chromosal elements; however, the *cry* genes are mostly located on the large plasmid (Gonzalez et al. 1981; Gonzalez et al. 1982). A large number of *cry* genes producing insecticidal toxins effective against common agricultural insect pests have been identified, cloned and expressed in different plant species to develop insect pest resistance genetically modified transgenic plants (James 2012).

Since the first introduction of *cry* gene into model plant tobacco for expressing insect-resistant trait (Barton et al. 1987; Vaeck et al. 1987), several major crop species have been genetically modified for expression of different insecticidal *cry* genes affective against different order of insects (Fischhoff et al. 1987; Perlak et al. 1990; Perlak and Fischhoff 1993; Fujimoto et al. 1993; Koziel et al. 1993; Adang et al. 1993; Nayak et al. 1997; Sanyal et al. 2005). The initial studies with introduction and expression of native full-length *cry* genes from *B. thuringiensis* into plants have shown very poor expression of toxin production, and the produced toxin was unstable in the plant system (Perlak et al. 1990; Schnepf et al. 1998). Several biochemical and genetical reasons have been attributed for poor stability and low expression of Bt-toxins in transgenic plants.

The earlier studies with transfer of Bt-*cry* genes showing poor expression were attributed to silencing of foreign gene, instability of RNA transcripts of insecticidal crystal protein genes (Murray et al. 1989), early termination of the transcript due to existence of polyadenylation at multiple sites in coding region of native Bt-*cry* genes (Diehn et al. 1996, 1998) and rapid degradation of mRNA (Perlak et al. 1991; Adang et al. 1993; DeRocher et al. 1998). The evidence to these factors was associated to earlier reports for lack of a correlation between promoter activity and mRNA accumulation (Fischhoff et al. 1987; Vaeck et al. 1987). The analytical results of tobacco transgenics expressing full-length native *cry1Ac* showed majority of transcript shorter than anticipated full length of the gene (Barton et al. 1987). These studies based on expression of full-length native *cry1Ac* and *cry1Ab* insecticidal genes lead to characterization of several polyadenylation sequences along with cryptic termination sequences in native *Bt-cry* genes.

These early reports suggested reinvesting the *Bt-cry* gene for its structure and functioning in the plant system. Subsequently, by analysing the nucleotide sequences of several *cry* genes, it was evident that crystal protein genes of *B. thuringiensis* were destined for expression in prokaryotic cell and of typical prokaryotic architecture in having codon sequences preferable to prokaryotes and gene length for optimum expression and stability of toxin in hydrophobic state and nucleotide sequences and GC content suitable to prokaryotes. These observation lead to several modifications in Bt-*cry* genes which included truncation of 3′ end of gene to eliminate hydrophobicity of the endotoxin, removable of polyadenylation, mRNA instability and criptic termination sequences, for higher expression of Bt-*cry* genes in plants (Fischhoff et al. 1987; Vaeck et al. 1987; Perlak et al. 1991). A major modification in the *cry* gene was incorporated to modify and introduce plant-preferred codons in the truncated version of Bt-*cry* genes (Delannay et al. 1989; Perlak et al. 1990, 1991).

These studies eventually led to major modification in designing of synthetic version of truncated *cry1Ac* and *cry1Ab* genes comprising of about ~1845 bp, where maximum care was taken to possibly use plant-preferred codons, elimination of all the termination sequences and mRNA instability components (Perlak et al. 1991; Sardana et al. 1996; Cheng et al. 1998). The designed genes were successfully shown to express the Cry1Ab and Cry1Ac toxins in different plant species, and promising transgenic plants of various species were developed (Perlak et al. 1991; Stewart Jr et al. 1996; Singsit et al. 1997; Perlak et al. 2001; Sanyal et al. 2005). Based on these developments and further molecular investigation of *cry* toxin and its interaction with different receptor on susceptible insect resulted in development of hybrid and fusion *cry* genes for wider host range and enhanced toxicity against agriculturally important target insects (Datta et al. 1998; Wu et al. 2000; Naimov et al. 2003; Singh et al. 2004; Ho et al. 2006; Rajamohan et al. 2006). To enhance host range of Cry toxin and to address the growing resistance development in target insects against these toxins, several mutations have been incorporated in the toxin for effective binding to receptor (Bravo and Soberon 2008; Soberon et al. 2013). Similarly, translation fusions of two cry genes or additional sequences for wider host range have been designed (Bohorova et al. 2001; Mehlo et al. 2005).

Lepidoptera is the most devastating group of field insects causing significant damages to large number of crop plants. Among them, *Helicoverpa armigera*, *Heliothis virescens*, *Ostrinia nubilalis*, *Spodoptera* spp., *Plutella xylostella* and *Pectinovophora gossypiella* are the important insects infesting several important crops like cotton, cabbage, okra, tomato, cauliflower, chickpea, maize and soybean. Two Bt-genes, *cry1Ab* and *cry1Ac*, have been documented for coding most effective toxin showing maximum mortality in range of 20–80 ng toxin/mg of fresh weight. Both these genes are most widely used for developing insect-resistant phenotype. To make these two toxins highly effective and efficient against target insect pests, several modifications have been incorporated including truncation, codon optimization, point mutations and application of 5′ regulatory sequences for over expression of the toxins at desired level in different plant species. The mechanism for pore formation and recognition of different receptors and their affinity to these toxins have been well documented. The native and modified versions of full length *cry1Ab* (3.5 kb) and *cry1Ac* (3.5 kb) and their synthetic modified truncated versions of 1.8 kb size have been widely used for developing the transgenic plants of different species exhibiting resistance against a number of insect pests (Cheng et al. 1992; Koziel et al. 1993; Stewart Jr et al. 1996; Alam et al. 1998; Cheng et al. 1998; Perlak et al. 2001; Sanyal et al. 2005; Mehrotra et al. 2011; Sanahuja et al. 2011). Except for selection of a unique event of transgenic cotton expressing a full-length native *cry1Ac* gene with few modifications and transgenic maize expressing *cry1Ab,* which have gone for commercial cultivation (Koziel et al. 1993; Perlak et al. 2001; Ferry et al. 2004; James 2012), most of the transgenics of different plant species are limited to demonstration under laboratory conditions. Despite several modifications incorporated in native wild type *cry1Ab* and *cry1Ac* genes which share more than 94 ± 0.5% sequence homology, their over-expression, however, in different plant species to recover promising transgenic plants with sufficient level of Bt-toxin(s)

have been a matter of concern (Diehn et al. 1996; DeRocher et al. 1998). The most widely used successful transgenic event of Bt-cotton (Monsanto to 531) resistant to bollworm complex of *Heliothis virescens/Helicoverpa armigera*, *Pectinophora gossypiella* and *Helicoverpa zea* was developed with native full-length *cry1Ac* gene having some specific minor modification (Perlak et al. 2001). The event has been designated as Bollgard I and been grown commercially in large areas in several countries (James 2012). Subsequently, to check the possibility of insect developing resistance against Bt-cotton technology, a second version of transgenic cotton plant designated as Bollgard II, expressing two different *cry* genes such as *cry1Ac* and *cry2Ab*, has been developed and released for commercial cultivation (Purcell et al. 2004; Ferry et al. 2004). Interestingly, native *cry1Ac* coding gene was documented for very poor expression in higher plants owing to high AT content and presence of several pre-termination sequences. This situation necessitated the truncation and enrichment of GC content, since plants in general have a higher GC content than that found in bacterial genes (Murray et al. 1989), and particularly delta-endotoxin *cry* genes have higher AT content. Modifying the coding sequences to increase GC content, 3′ truncation and possible elimination of polyadenylation or termination sequences of the native *cry* genes resulted into dramatic increase in the expression of the insecticidal toxin proteins (Delannay et al. 1989; Perlak et al. 1991; Carozzi et al. 1992). A highly modified *cry1Ab* gene-coding toxin protein of 648 amino acid of the native proto-toxin of 1155 amino acids was expressed in maize to develop resistance against European corn borer (ECB), *Ostrinia nubilalis* (Hubner), a major pest of maize (Carozzi et al. 1992; Koziel et al. 1993). Comparative nucleotide and amino acid sequences of prominent *cry1A* group of genes (*cry1Aa*, *Ab* and *Ac*) coding insecticidal crystal proteins affective against large number of Lepidopteran insects showed distinct homology and similarities in 5′ coding sequences for toxin molecules comprising of pore forming and receptor-binding domains except for the specific changes in the sequences coding for the receptor-recognizing domains of the toxin molecules (Haider and Ellar 1987; Schnepf et al. 1998; Bravo and Soberon 2008). This comparative and exhaustive sequence analysis was further executed to other group of insecticidal crystal protein genes to reflect the diversity and evolution of different *cry* genes coding different insecticidal toxin proteins effective against specific insects (Feitelson et al. 1992; DeMaagd et al. 2001; Sanahuja et al. 2011).

Among *cry1A* group of genes, the response of the toxins against lepidopteran insects has been found in the order *cry1Ac* > *cry1Ab* and least in *cry1Aa* gene. This is further attributed to the molecular structure of insecticidal Cry toxin and its affinity to bind with a different receptor on the midgut of susceptible insects and attachment of toxin molecules with different epitopes of same or different receptors on BBMV cells (Estela et al. 2004; Bravo et al. 2007). Considering the close resemblance and high homology of nucleotide sequences of *cry1Ab* and *cry1Ac* gene and based on the architecture of toxin-coding sequences, completely synthetic version of both the 1.8 kb genes has been developed and extensively used for optimal expression in higher plants (Sardana et al. 1996; Cheng et al. 1998). The comparative sequence analyses of both *cry1Ab* and *cry1Ac* genes have shown three blocks of 668, 403 and 279 bp which are identical in both the case while the fourth block of

495 bp comprises sequence variations that seem to code for the receptor-binding domain of the toxin protein and may be the possible reason for differential toxicity. Considering the high homology and similarities between modified synthetic *cry1Ab* and *cry1Ac* genes for enhanced expression of toxin in higher plant, achieving promising number of transgenic with high level of toxin expression is not a routine process. Despite the successful commercial release of Bt-cotton expressing *cry1Ac* gene but recovery of stable transgenic plant with high level of Cry1Ac toxin is still confined to laboratory level around the globe. Only restricted plant species have been documented for high level expression of *Cry1Ac* toxin compared to number of transgenic plants developed with Cry1Ab toxin. The modified-*cry1Ab* gene has been successfully introduced and expressed to sufficient level in several plant species like maize (Koziel et al. 1993; Singh et al. 2005), rice (Fujimoto et al. 1993; Wunn et al. 1996; Wu et al. 1997; Cheng et al. 1998; Alam et al. 1998, 1999; Tu et al. 2000; Marfà et al. 2002), cotton (Perlak et al. 1990), brinjal (Kumar et al. 1998), soybean (Parrott and Clemente 2004), tomato (Kumar and Kumar 2004), sugar beet (Jafari et al. 2009) and chickpea (Mehrotra et al. 2011). However, restricted plant species have been transformed with *cry1Ac* gene to develop stable transgenic plants of cotton (Perlak et al. 1990, 2001; Rawat et al. 2011), tobacco (Barton et al. 1987; Vaeck et al. 1987), tomato (Fischhoff et al. 1987; Mandaokar et al. 2000), chickpea (Kar et al. 1997; Sanyal et al. 2005), peanut (Singsit et al. 1997) and canola (Stewart Jr et al. 1996).

2.2 Genetic Transformation

Genetic transformation is the deliberate alternation and modification of the genome of an organism (bacteria, plant, animal) by introduction of one or few specific foreign genes using other than conventional procedures, and the modified organism is termed as transformed or transgenic organism. Genetic transformation of plants is becoming an indispensable aid to plant physiologists, biochemists and biotechnologists in understanding the role of individual and application of these procedures for crop improvement with newer traits. Scientists of Calgene Inc. of Davis, California, used the antisense RNA technology to inactivate the gene (polgalacturonase [PG]) responsible for softening the tomato to produce first genetically modified tomato 'Flavr-Savr' in 1991 and was approved by the US FDA in 1994.

2.3 Gene Transfer Method

2.3.1 Direct DNA Transfer Methods

The direct DNA transfer method has been proved to be simple and effective for introducing foreign DNA into plant genomes (Fig. 2.1). Among these methods, the most frequently used one is the microprojectile bombardment procedure where

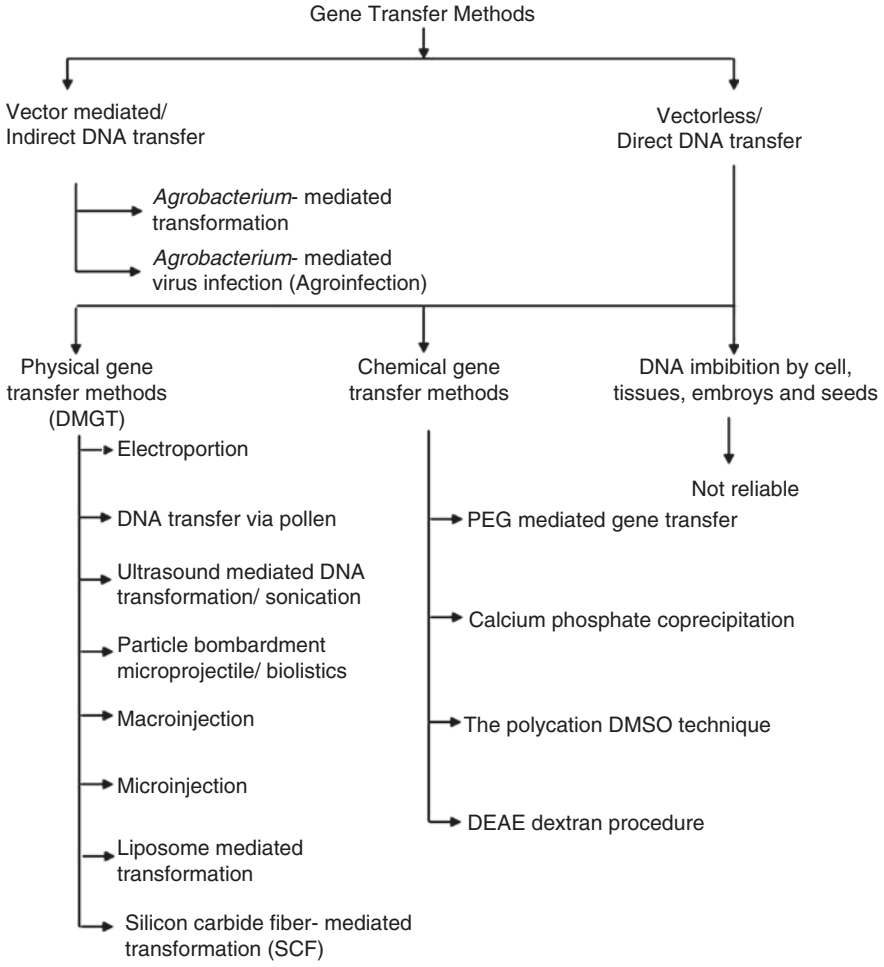

Fig 2.1 Schematic representation of the various gene transfer strategies

transforming DNA is coated onto metal microcarriers like tungsten or gold that are accelerated with high velocity either by gun powder device or through compressed inert gases. The microcarriers acquire sufficient kinetic energy to allow them to penetrate to the intact plant, animal or bacterial cell wall and plasma membrane without killing the cells.

2.3.2 Indirect DNA Transfer Method

As with other dicotyledonous crops, *Agrobacterium*-mediated transformation is the most widely used method for gene transfer. Among the various vectors used in plant transformation, the Ti plasmid of *Agrobacterium tumefaciens* has been widely used.

This bacteria is known as 'natural genetic engineer' of plants, because these bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti plasmid in place of unwanted sequences.

2.4 *Agrobacterium*-Mediated Genetic Transformation

Agrobacterium tumefaciens is a gram-negative, soil phytopathogen of family Rhizobiaceae that causes the disease 'crown gall' in a wide variety of dicotyledonous plants (Fig. 2.2). Crown gall is a plant tumour, a lump of undifferentiated tissue, which often forms at the area of crown, the junction between the root and the stem of the infected plants. The pathogenic property of this bacterium was recognized much earlier (Smith and Townsend 1907).

A. *tumefaciens*: induces crown gall disease.
A. *rhizogenes*: induces hairy root disease.
A. *radiobacter*: an avirulent strain.

During the infection at wound site, the bacterium transfers a small part of its own plasmid DNA called T-DNA (transfer DNA) into the plant cell that results in two key events.

1. The plant cell begins to proliferate and form tumours and receive the ability to grow in cultures, which even do not have any growth regulator.
2. They begin to synthesize an unusual arginine derivative called opines (octopine, nopaline, etc.) which are not found in normal tissues.

Bacteria can be classified as octopine, nopaline, agropine, succinamopine or chrysopine strains (octopine is condensation product of arginine and pyruvic acid). The metabolism of opines is a central feature of crown gall disease. The type of opine produced is not determined by the host plant but by the bacterial strain. In general, the bacterium induces the synthesis of an opine, which it can catabolize and

Fig. 2.2 (**a**) Electron micrograph of *A. tumefaciens* (**b**) A plant root with crown galls, (**c**) A plant showing symptoms of hairy roots

use as its sole energy source for carbon and nitrogen. Clearly, an interesting interrelationship is evolved, where *A. tumefaciens* subvert the plant's metabolism to make amino acids, which can be utilized only by the bacteria as a food and energy source.

2.4.1 Ti Plasmid of *Agrobacterium*

The ability of *Agrobacterium tumefaciens* to induce crown gall disease in plants is controlled by genetic information carried on a large conjugative plasmid (of about 200 kb size) called Ti plasmid for its tumour-inducing capacity. Virulence is lost when the bacterium is cured for the plasmid, and cured strains have lost the capacity to utilize octopine or nopaline. Ti plasmids have temperature-sensitive replication, i.e. high temperature (more than 30 °C) leads to curing of plasmids. Ti plasmids have regions for replication (origin of replication), conjugal transfer, virulence and T-DNA.

Three bacterial genetic elements are required for T-DNA transfer to plants.

1. 25 bp direct repeated flanking and defining the T-DNA
2. Virulence (*vir*) genes encoded by the Ti plasmid in a region outside of the T-DNA.
3. Number of chromosomal genes, of which some are important for attachment to the bacterium to the plant cell

2.4.2 Organization of T-DNA

T-DNA (transfer DNA) is about 23 kb segment of Ti plasmid, which is transferred into the plant genome during *Agrobacterium* infection. T-DNA contains the gene for constitutive synthesis of auxins, cytokinins and opines and is defined on both the sides by 24 bp direct inverted repeat called border sequences, which are required for T-DNA excision and transfer. The deletion of either border sequence completely blocks the transfer of T-DNA into the plant cell. However, mutational analysis shows that only the right repeat is absolutely required for T-DNA transfer and they function in *cis* and polar fashion. The T-DNA is organized into two distinct regions called TL (left T-DNA) and TR (right T-DNA). Both TL and TR are always transferred together in nopaline plasmids and integrated into the plant genome as a single segment. But in octopine plasmids, the TL and TR are transferred independently so that a single cell may contain one or both of these segments. T-DNA has three genes, which are involved in crown gall formation. Two of these genes, *iaa*M and *iaa*H encodes tryptophan 2-monooxygenase and indoleacetamide hydrolase, respectively, which together convert tryptophan into indole 3-acetic acid (IAA); the locus was earlier called 'shooty' locus, and the genes were designated as *tms1* (tumour with shoots) and *tms2*. The third gene, *ipt*, encodes a zeatin-type cytokinin, isopentenyl transferase; the locus was earlier designated as 'rooty' locus and

designated as *tmr* (tumour having roots). T-DNA also contains genes involved in opine biosynthesis near the right border. All the genes present in T-DNA contain eukaryotic regulatory sequences. As a result, these genes are expressed only in plant cells, and they are not expressed either in *Agrobacterium* or in *E. coli*.

2.4.3 Organization of *vir* Region

The *vir* region of the Ti plasmid contains 8 operons, which together span to about 40 kb of DNA and possesses 25 genes. This region mediates the transfer of T-DNA in both *cis* and *trans* fashion into plant genome, and hence is essential for virulence and transfer of T-DNA (Hooykaas and Mozo 1994). Among the eight *vir* operons, four operons, viz., *vir*A, *vir*B, *vir*D and *vir*G are essential for virulence, while the remaining four operons play an accessory role in transfer of T-DNA. *Vir*A and *vir*G, which are constitutively expressed, regulate the expression of other *vir* loci. Signal transduction proceeds via activation of *vir*G by *vir*A, in response to the activation of *vir*A by plant phenolics like acetosyringone and α-hydroxy acetosyringone. After activation, *vir*G dimerizes and activates the transcription of other *vir* genes (Zambryski et al. 1989). The functions of different *vir* genes are given in Table 2.1.

2.4.4 T-DNA Transfer Process

T-DNA transfer begins with the introduction of bacteria into a plant wound (Fig. 2.3). Wounding is a necessary event in the process and may, at least is part, be required for the synthesis by the plant, certain compounds that induce the expression of the vir genes. Two of the most active substances identified are acetosysingone and β-hydroxy acetosysingone. T-DNA transfer process starts by binding of *vir*D1 gene product to the right border (RB) sequence, *vir*D1 has the topoisomerase activity that facilitates the action of protein *vir*D2, as endonuclease; in nicking, at the right border and covalently binds to the 5′ end. The 3′ end produced at the site

Table 2.1 Functions of different *vir* genes

Vir region	No. of genes	Function
*vir*A	1	Encodes a sensor protein; receptor for acetosyringone and functions as an autokinase; also phosphorylates *vir*G protein; constitutive expression
*vir*B	11	Membrane proteins; role in conjugal tube formation
*vir*C	2	Helicase activity
*vir*D	4	*Vir*D1, has topoisomerase activity and *vir*D2 is an endonuclease
*vir*E	2	Single strand binding protein (SSBP)
*vir*F	1	Not well understood
*vir*G	2	DNA binding protein, induces the expression of all *vir* operon; constitutive expression
*vir*H	2	Not well known

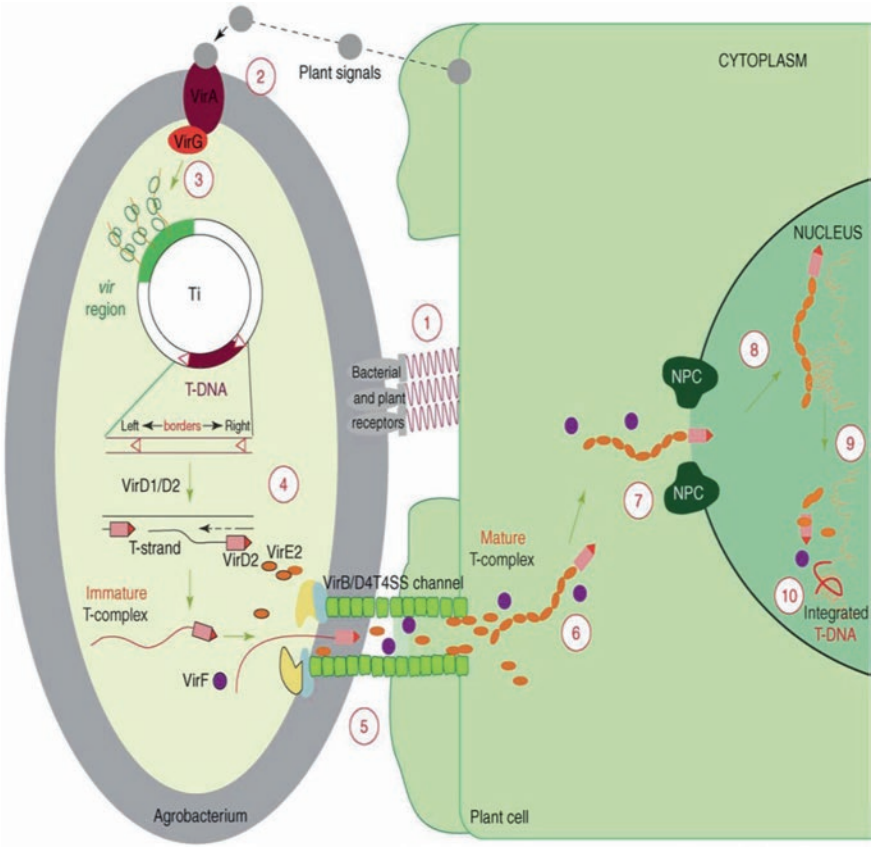

Fig. 2.3 Model for *Agrobacterium*-mediated genetic transformation of plants (Tzfira and Citovsky 2006). The transformation process comprises of 10 major steps and begins with recognition and attachment of the *Agrobacterium* to the host cells (*1*). Sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal-transduction system (*2*). Following activation of the vir gene region (*3*), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (*4*) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (*5*). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (*6*) and is actively imported into the host-cell nucleus (*7*). Once inside the nucleus, the T-DNA is recruited to the point of integration (*8*), stripped of its escorting proteins (*9*) and integrated into the host genome (*10*)

of nick serves as a primer for replacement synthesis of DNA in the 5′ → 3′ direction as a result of which the T-strand is displaced from the DNA duplex.

The *vir*E2 protein is a single-strand DNA-binding protein and about 600 copies of it binds to the single-stranded T-DNA, thus protecting it from nuclease action. *Vir*B operon encodes membrane-bound proteins, which participate in conjugal tube formation between the bacterial and plant cells to provide a channel for T-DNA transfer, whereas *vir*B11 has ATPase activity, which generates energy needed for the delivery of T-DNA into the plant cells (Zambryski et al. 1989). The nuclear

localization signals present on the *vir*D2 and *vir*E2 proteins drive the T-DNA towards the nucleus of the plant cell. This mechanism accounts for the polarity; *cis*-acting nature of the border repeat sequences also explains the importance of right border repeat in T-DNA transfer. Apart from Ti plasmid, chromosomal virulence genes (*chv*) are also involved in T-DNA transfer from *Agrobacterium* to plants. The *chv* genes are required for the synthesis of cyclic glucans, which are involved in plant cell-binding *Chv* A, *chv*B and *psc* A that are involved in the synthesis and export of cyclic β-1,2-glucan. A more direct role in attachment has been demonstrated for rhicadhesin, a calcium-binding protein located on bacterial cell surface. The induction of *Agrobacterium vir* genes in response to plant wound-specific compounds implies that a bacterial recognition system must detect the plant signal and transmit the information inside the bacterial cells. This process is mediated by products of *vir* A and *vir* G.

2.4.5 Vectors Derived from Ti Plasmids

Large size, absence of unique restriction sites and tumourigenic properties of Ti plasmids precluded the use of wild-type Ti plasmids as vectors. Presently, plant transformation vectors have been produced by replacing tumour-including genes with dominant selectable markers and desired traits. These types of vectors are known as disarmed vectors; with functional *vir* genes and T-DNA border sequences. Such non-oncogenic plant transformation vectors are either co-integrated or binary types.

2.4.6 Co-integrate Vector System

Vectors that recombine via DNA homology into a resident Ti plasmid are often referred to as integrative or cointegrative vectors. In this type of vector systems, both T-DNA and *vir* regions are present in the same Ti plasmids. Gene of interest can be inserted in between T-DNA borders by a co-integration event between the homologous sequences present in the cloning vector and T-DNA region of Ti plasmid. Efficiency of co-integrate system relies on the frequency of conjugal transfer and homologous recombination.

2.4.7 Binary Vector System

The binary vector system consists of two autonomously replicating plasmids within *A. tumefaciens* a shuttle (more commonly referred to as binary) vector that contains gene of interest between the T-DNA border and a helper Ti plasmid that provides the *vir* gene products. The *vir* gene can act in trans and encode proteins, which are required for the transfer of T-DNA. The standard components of binary vector are:

1. Multiple cloning site
2. A broad host range origin of replication functional in both *E. coli* and *A. tumefaciens*
3. Selectable markers for both bacteria and plants
4. T-DNA border sequences (although only right border is absolutely essential)

2.4.8 Selectable Markers

Selection of transformed cells is a key factor in developing successful methods for genetic transformation. This is done by certain selectable marker genes that are present in the vector along with the gene of interest. Selectable markers are an integral part of plant transformation strategies (Table 2.2).

Each selectable marker presents some favourable and some unfavourable features. Therefore, the choice of a marker should be based on the plant species and other considerations in the study. The NPT II gene from transposon *Tn5* confers resistance to the amino glycoside antibiotics kanamycin, neomycin and G 418. The NPT II gene product, *neomycin phosphotransferase*, inactivates these antibiotics through its phosphorylation (Bevan et al. 1983). This marker is a most widely used system for plant selection and screening as no endogenous level is reported so far in green plants.

2.4.9 Advantages of *Agrobacterium*-Mediated Plant Transformation

It is a natural means of DNA transfer and is perceived as a more acceptable technique over long conventional breeding procedures. It is capable of infecting intact plant cells, tissues and organs. Transformed plants can be regenerated more rapidly. It is capable of transferring large fragments of DNA very efficiently without substantial rearrangements of the transgene. Integration of DNA is relatively a precise

Table 2.2 Selectable markers genes used for gene transfer

Selectable marker genes	Substrates used for selection
Neomycin phospotransferase (*npt*II)	G 418, kanamycin, neomycin, paromycin
Hygromycin phospotransferase (*npt*)	Hygromycin B.
Gentamycin acetyl transferase	Gentamycin
Streptomycin phospotransferase	Streptomycin
Dihydrofolate reductase (*dhfr*)	Methotrexate
Phosphinothricin acetyl transferase	L-Phosphinothricin (PPT)
5-Enolpyruvyl shikimate 3 phosphate (EPSP) syhthase (*aro*A)	Glyphosafe
Acetolactate synthase mutant form (*als*)	Sulphonyl urea, imidazolinones
Bromoxynil nitrilase (*bxn*)	Bromoxynil

process; it serves as an ideal insertional mutagenesis vehicle as it introduces one to several copies of the transferred DNA into the intact genome at one or few loci. The integrated DNA gives consistent maps and appropriate segregation ratios. The stability of the gene(s) and the respective trait(s) have been found to be stable over many generations. All of these features make this technique reliable for commercialization of transgenic plants. A wide range of explants have been successfully transformed using Agrobacterium, although cotyledons have been most commonly used (McCormick et al. 1986). Other explants like vegetative leaves and hypocotyl (McCormick et al. 1986) stem have also been used with high transformation frequency both with binary as well as co-integrate Ti plasmid vectors used in these experiments.

2.4.10 Disadvantages of *Agrobacterium*-Mediated Plant Transformation

There is limitation of host range as it cannot transform many important food crops. Cells and tissues that are able to regenerate are difficult to transform. The embryogenic cells are placed in deeper layers and are thus not amenable to T-DNA transfer.

2.5 Factors Affecting Plant Transformation

A successful gene transfer procedure is mainly dependant on the following factors: (1) simple, reproducible, genotype-independent and cost-effective regeneration protocol for (2) target tissues, which are both competent for transformation and regeneration, (3) an efficient DNA delivery method, (4) procedure to select for transgenic tissues and (5) the ability to recover fertile plants avoiding somaclonal variation in transgenic plants (Velcheva et al. 2005; Thi Van et al. 2010).

Availability of high-frequency genotype-independent in vitro regeneration system amenable to *Agrobacterium*-mediated transformation is the major pre-requisite for developing transgenic lines (Birch 1997). A number of factors influencing genetic transformation such as genotype, type of explant, explant orientation, wounding procedure, co-cultivation duration, the role of phenolic compounds, *Agrobacterium* strain, bacterial cell density, etc. play an important role in determining overall transformation efficiency. The optimization of selection and screening procedures are crucial for improving transformation efficiency and most importantly developing non-chimeric transgenic plants.

2.6 *Bacillus thuringiensis* (*Bt*) Endotoxin Crystal Protein Genes for Insect Resistance

Agricultural pests are mostly controlled by the use of synthetic pesticides and rarely by cultural practices. Therefore, the excessive and reckless use of agrochemicals has been a subject of public concern as it has led to harmful consequences on the environment and carcinogenicity to non-targets organisms.

The reliance on gene transfer technology to transfer insect-resistance genes of diverse origin into crop plants provides an economical, feasible and eco-friendly alternative to the extensive use of chemicals pesticides. Insect-resistant transgenic plants may be raised by introducing foreign genes encoding either δ-endotoxin, protease inhibitors (PI), lectins, amylase inhibitors, etc. (Boulter 1993; Gatehouse et al. 1997). The most widely used, well-documented and reliable approach in this context is the insecticidal crystal protein (ICP) genes of *Bacillus thuringiensis* (*Bt*) which code for δ-endotoxin (Whiteley and Schnepf 1986). Gram-positive spore-forming entomopathogenic bacteria of Bacillaceae family particularly *Bacillus thuringiensis* produce a large variety of protein toxins to aid them to invade, infect and kill their hosts. This bacterium produces an insecticidal crystal protein which forms inclusion bodies of bipyramidal, cuboidal, flat rhomboid or a composite with two or more crystal types during sporulation (Bajwa and Kogan 2001). ICPs are one of the several classes of endotoxins produced during sporulation, and δ-endotoxins (delta endotoxins) are the most effective than other classes of α-, β- and γ-endotoxins (alpha, beta and gamma) to agricultural insect pests. The genes coding these toxins are called *cry* genes.

Although the Cry proteins exhibit diversity, they are specific to the target insect orders: lepidoptera (moths and butterflies), diptera (mosquitoes and flies) and coleopteran (weevils and beetles), and few new toxins have been identified to kill hymenopterans (bees and wasps) and nematodes (Schnepf et al. 1998; Pigott and Ellar 2007; Bravo et al. 2007). Considering a large number of *cry* genes and diversity of encoded toxins against different groups of insects and microbes, several nomenclatures and classification of ICP genes have been proposed (Hofte and Whiteley 1989; Sanchis et al. 1988; Crickmore et al. 1998; Crickmore et al. 2011).

Table 2.3 Classification of *cry* genes on the basis of their activity spectrum[a]

Protein	Subspecies (strain)	Activity spectrum	Protoxin/active molecular mass in kDa
Cry I	*Cry*I *Kurstak*i (HD-1), *aizawai*, *sotto*	Lepidopteran	130–160/ca.60
*Cry*II	*Cry*II *Kurstak*i (HD-1), *Kurstak*i (HD-263)	Lepidopteran and dipteran (mosquito)	70–71/ca.65
*Cry*IIIA	*Tenebrionsis*	Coleopteran (chrusomelids)	73/ca.65
*Cry*IIIB	*Japonicus*	Coleopteran (scrarabaeids)	73/ca.55
*Cry*IV	*Israelensis*	Diptera (mosquito, black flies and nematodes)	72–134/ca.46–48

[a]Hofte and Whiteley (1989); Rukmini et al. 2000)

However, new toxin-encoding genes are being identified and the number is increasing therefore, nomenclature and name of the new *cry* genes is assigned according to the extent of evolutionary divergence, as projected by phylogenetic tree algorithms. The large and variable family of insecticidal proteins of BT was earlier classified on the basis of their activity, into five major classes, as shown in Table 2.3. Later, Crickmore et al. (1998) suggested a common platform for nomenclature of Bt-*cry* genes and broadly classified them into 22 groups of *cry* genes and two groups of cytolytic (*cyt*) parasporal inclusion protein genes that exhibited hemolytic activity.

According to Crickmore et al. (2011), Cry toxins have been classified on the basis of their primary amino acid sequence and more than 500 different *cry* gene sequences have been classified into 70 subgroups. These *cry* gene sequences have been divided into four phylogentically unrelated protein families with different modes of action: three domain Cry toxins (3D), mosquitocidal Cry toxins (Mtx), binary-like (Bin) and the Cyt toxins. Among these toxins, the family of three-domain Cry toxins represents the largest group with more than 53 different subgroups.

As mentioned before, Bt-toxins are extremely specific to the target insect pests, non-toxic to animals including non-target insects and human beings, non-hazardous and eco-friendly (DeMaagd et al. 2001). These characteristics led to the advancement of bioinsecticides, and formulations based on Bt-spores to control agricultural insects have been developed and used extensively. Besides production of insecticidal δ-endotoxins by *B. thuringiensis*, some of the bacterial species are documented to express toxins during the non-sporulating state called 'Vip,' or vegetative insecticidal protein, which are toxic to insects and microbes (Gatehouse 2008). Both Cry and Cyt toxins interact with very specific receptors on susceptible insect pests. The primary mode of Cry protein is to recognize the receptor on insect midgut epithelial cells and lyse the cells by inserting the domain I and resulting into pore formation.

The three-domain Cry toxins are globular molecules harbouring three distinct domains connected by single linkers. The domain I at the N-terminal end comprises a series of α-helices arranged in a cylindrical formation while domain II comprises a triple β-sandwich for receptor binding. Most of the *Bt*-toxins are expressed as protoxin of higher molecular weight and are non-toxic; however, their proteolytic products are of smaller size and are highly toxic to the susceptible insects. The main difference between the 65 and 130-kDa three-domain Cry toxin is a C-terminal extension that is found in the 130-kDa protoxins, which is cleaved by proteases present in the larval midgut and is therefore dispensable for toxicity (DeMaagd et al. 2001). The N-terminal region of all three-domain *cry* genes codes for the N-terminal fragment of protoxin which comprises 20–60 residues, while the active toxin is composed of approximately 600–620 amino acid residues. The X-ray crystallographic studies of different trypsin-activated Cry toxins, such as Cry1Aa (Lepidopteran specific), Cry3Aa, Cry3Bb and Cry8Ea (Coleopteran specific), Cry4Aa and Cry4Ba (Dipteran specific) and Cry2Aa protoxin (Dipteran-lepidopteran specific), have been determined (Li et al. 1991; Grochulski et al. 1995; Galitsky et al. 2001; Morse et al. 2001; Boonserm et al. 2005, 2006; Guo et al. 2009).

Cry proteins are modular in structure, consisting of three different functional domains as I, II and III (Schnepf et al. 1998). N-terminal part of the toxin fragment comprising six amphipathic helices (α-1, 2, 3, 4, 6, 7) with a central hydrophobic helix (α-5) makes the domain I of δ-endotoxins (Li et al. 1991; Grochulski et al. 1995). Two alternative models, viz. 'Penknife Model' (Hodgman and Ellar 1990) and 'umbrella model' (Li et al. 1991), were proposed to explain the pore-forming mechanism of domain I of δ-endotoxins. Following insertion of the toxin, helix α-1 is removed due to protease digestion, and it is the only helix that does not bind to BBMV vesicles as synthetic peptide mimicking studies show that α-5 helix and α-4-α-5 helix loop is important for toxin aggregation and ion channel formation (Gerber and Shai 2000). It has been proposed that after the toxin binds to the receptor, there occurs a change in the conformation of this domain allowing the hydrophobic surfaces of the helices to face the exterior of the bundle, leading to insertion into the membrane and the formation of ion channels (Knowles 1994). Domain II is made of three antiparallel β-sheets, oriented parallel to the α-helices of domain I. Domain III is made of two antiparallel β-sheets into β-sandwich structure which is involved in several functions such as stability, as receptor binding, specificity determination and ion channel gating (Schnepf et al. 1998). Arginine-rich block in domain III of δ-endotoxin is called 'arg face,' through which domain III makes contact with domain I and regulates ion channel conductance (Saraswathy and Kumar 2004).

The results of phylogenetic analysis suggest that domain I sequences seem common only for a subgroup of toxin proteins. Shuffling of the functional domains was observed only for domain II and III in some toxins. Toxins with dual specificity for lepidopteran and coleopteran insects are examples of domain III shuffling among coleopteran and lepidopteran-specific toxins. The phylogenetic analysis of the Cry toxin family shows that the great variability in the biocidal activity has resulted from two fundamental evolutionary processes: (i) independent evolution of the three functional domains and (ii) domain swapping among different toxins. These two processes have generated toxin proteins with similar modes of action but with diverse specificities. It is suggested that sequence divergence in combination with domains swapping by homologous recombination might have caused extensive range of specificities and evolution of different Bt-toxins (DeMaagd et al. 2001; Bravo et al. 2007).

After ingestion of Bt-ICP by a target insect, Bt-protoxin first passes through the peritrophic matrix (PM) diffusing into the midgut brush border, where it is digested to yield toxin of smaller molecular mass that mediates insect death (Gill et al. 1992; Knowles 1994). The PM is a single semiporous tube consisting of several layers of mucin like glycoproteins and chitin microfibrils (Nation 2002; Ma 2005). It serves as a barrier against the entry of virus, bacteria and bacterial products, such as Bt-protoxin (Nation 2002). Receptor binding is a key factor for specificity, specific binding involves two steps: one that is reversible and other is irreversible. Recent data suggested that toxicity correlates with irreversible binding (Aronson and Shai 2001). Irreversible binding might be related to insertion of the toxin into the membrane but could also reflect a tighter interaction of the toxin with the receptor. The delta endotoxin-binding receptors in the larval midgut are identified as glycoprotein. Domain II

loops showed immunoglobulin-like structural folds, and carbohydrates are used as recognition epitopes by these folds (Li et al. 1991). CryIAc toxin specifically binds to a 120 kDa aminopeptidase-N (APN) receptor and binding interaction is mediated by Gal NAc, presumably covalently attached to the APN. Knight et al. (1994) have shown that O-glycans associated with a C-terminal O-glycosylated 'Stalk' structure in the APN molecule are the most likely site for CryIAc toxin binding determined by lectin binding and carbohydrate compositional analysis.

Cadherin-like proteins also serve as receptors for CryIAc toxins in lepidopteran insects. Cadherin is critical for initial binding with toxin followed by further proteolytic changes, oligomerization, binding to APNs in lipid rafts and insertion into the cell membrane for forming pores (Hua et al. 2004). Regions of domain II of CryIA toxins bind to specific sites on Bt-R_1 Cadherin-like protein. Three CryIAb toxin-binding regions in *Manduca sexta* Bt-R_1 have been mapped to aa^{865}–aa^{875} (Site 1), aa^{1331}–aa^{1342} (Site 2) and aa^{1363}–aa^{1464} (Site 3). The first site ^{865}NITIHITDTNN^{875} is involved in binding loop 2 and second site ^{1331} IPLPASILTVTV^{1342} binds to loop α-8 located on CR11. Ectodomain CR12 (Site 3) is a critical Cry1Ab receptor epitope and is the minimum region found to be crucial to confer cell susceptibility to Cry1Ab to the same level as full-length Bt-R_1 (Hua et al. 2004; Xie et al. 2005).

2.7 Mechanism of Action of Three-Domain Cry Toxins in Lepidoptera

The activated toxin of 60 kDa goes through a complex sequence of binding events with different insect gut Cry-binding proteins (receptors), leading to membrane insertion and pore formation (Bravo et al. 2004; Pigott and Ellar 2007; Pacheco et al. 2009). Two models have been proposed which demonstrate the series of events that occur during receptor–Bt-protein interaction: [A] pore formation model and [B] signal transduction model.

2.7.1 Pore Formation Model

According to the pore formation model, binding to Bt-R_1 (receptor) is possibly the first event in the interaction with the microvilli membrane. This initial binding promotes a conformational change in the toxin-facilitating proteolytic cleavage of helix α-1, by a membrane-bound protease followed by formation of pre-pore oligomeric structure. The oligomeric toxin then binds to the APN which induces a conformation change and a molten globule state of the toxin which is inserted into lipid rafts inducing pore formation and cell swelling (Bravo et al. 2007). After insertion into the membrane bilayers, the toxin inhibits k^+ transport and amino acid assimilation in the gut lumen, causing imbalance in pH, ion and other macro molecules and culminate into insect death (Ma 2005). According to a recent report of Pardo Lopez et al. (2013), which is an extension of pore-formation model, the first binding/interaction of activated Cry1A toxins is a low-affinity interaction with

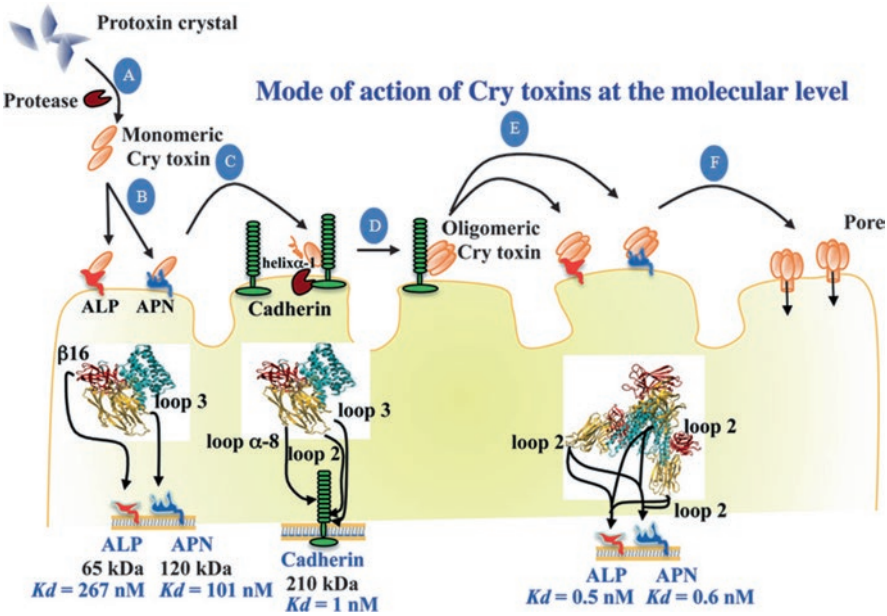

Fig. 2.4 Schematic representation of the mechanism of action of three-domain Cry toxins in Lepidoptera at the molecular level. (*A*) the larvae ingest the three domain-Cry protoxin, which is solubilized in the midgut lumen due to high pH and reducing conditions and get activated by gut proteases, thus generating the toxin fragment. (*B*) the monomeric three domain-Cry toxin binds ALP and APN receptors, in a low-affinity interaction, the toxin is then located in close proximity to the membrane (*C*) the monomeric three domain-Cry toxin binds the cadherin receptor in a high-affinity interaction and this interaction induces proteolytic cleavage of the N-terminal end of the toxin, including helix α-1 of domain I (*D*) the cleaved three domain-Cry toxin is then able to oligomerize in a toxin pre-pore oligomer (*E*) the oligomeric three domain-Cry structure binds to ALP and APN receptors with high affinity (*F*) the pre-pore inserts into the membrane causing pore formation

ALP (alkaline phosphatase) and APN receptors (aminopeptidase-N) (K_d = 101 nM for APN and 287 nM for ALP). The interaction with APN occurs through exposed loop 3 of domain II and interaction with ALP through strand β-16 of domain III (Masson et al. 1995; Pacheco et al. 2009; Arenas et al. 2010). ALP and APN are highly abundant proteins anchored to the membrane by a glycosyl phosphatidylinositol anchor (Upadhyay and Singh 2011). The interaction with ALP and APN concentrates the activated toxin in the microvilli of the midgut cells due to which the toxin is able to bind in a high affinity interaction to the cadherin receptor (K_d = 1 nm; Vadlamudi et al. 1995; Gómez et al. 2006, Pacheco et al. 2009; Arenas et al. 2010). A schematic representation of mechanism of action of three-domain Cry toxin in Lepidopterans at the molecular level has been shown in Fig. 2.4.

2.7.2 Signal Transduction Model

Another model which is signal transduction suggests that Bt-toxicity could be related to G-protein-mediated apoptosis following the receptor binding (Zhang et al. 2006). Binding of Cry toxin to Bt-R_1 mediates cell death by activating a signalling pathway involving stimulation of the stimulatory G-protein-α-subunit (G-αs) and adenylyl cyclase (AC), which increases the cyclic adenosine monophosphate (AMP) levels, and activation of protein kinase A (PKA). Activation of AC/PKA signalling pathway initiates a series of cytological events that include membrane blebbing, appearing of nuclear ghosts and cell swelling followed by cell lysis (Zhang et al. 2006). Diagrammatic view of the two models of Cry toxin action has been shown in Fig. 2.5.

Broderick et al. (2006) have put up an interesting observation that *B. thuringiensis* toxicity depends on the interaction with microorganisms of the normal gut community. Elimination of gut microbial community by oral administration of antibodies abolished insecticidal toxicity, and re-establishment of an enterobacter sp., that normally resides in the midgut microbial community has restored *B. thuringiensis*-mediated killing.

Transgenic plants expressing *B. thuringiensis* toxins have been used successfully to provide resistance against selected agricultural insects. Since the development of first transgenic tobacco and tomato plants with native Bt-*cry* gene (Vaeck et al. 1987; Fischhoff et al. 1987; Barton et al. 1987) considerable progress has been made to develop promising transgenic plants with highly modified Bt-*cry* genes for stability of mRNA and high-level expression (Gatehouse 2008). A large number of stable transgenic plants of different families, expressing various Bt-*cry* genes have been developed which exhibit significant protection to insect damages in lab and field (Hilder and Boulter 1999; Sharma et al. 2000; Tabashnik et al. 2003).

2.8 BT-GM Crops

A large number of crop plants expressing Bt-insecticidal endotoxin have been successfully transformed by *Agrobacterium*-mediated approach. Major reports on development of insect-resistant plants are summarized in Table 2.4.

Stable transgenic plants of tobacco (Barton et al. 1987), tomato (Delannay et al. 1989; Gordon-Kamm et al. 1990), rice (Koziel et al. 1993; Datta et al. 1998;), soybean (Parrott et al. 1994; Stewart Jr et al. 1996), groundnut (Singsit et al. 1997), pigeonpea (Surekha et al. 2005) and chickpea (Kar et al. 1997; Sanyal et al. 2005) have been developed. Recently, very high level of expression of Bt-cry2Aa2 protein in chloroplast up to 35.5% of total protein (DeCosa et al. 2001) and expression and inheritance of multiple transgenes (gene pyramiding) in rice (Cheng et al. 1998; Maqbool et al. 2001) and cabbage (Cao et al. 2001; Zhao et al. 2003) have been documented, for efficient management of insects and as insect-resistance management strategy. The global status of approved and commercially available Bt-GM crops is shown in Table 2.5.

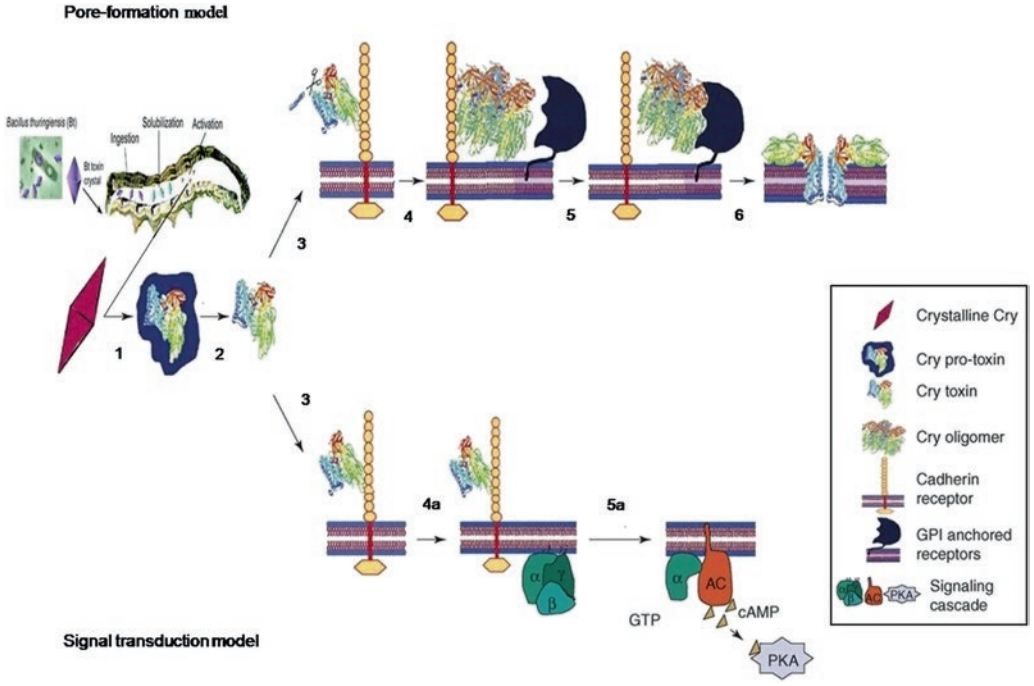

Fig. 2.5 Models of the mode of action of Cry toxins and resulting mechanism for resistance (Bravo and Soberon 2008). Two different mechanisms can be distinguished: the pore formation model (top) and the signal transduction model (bottom), which both include similar initial steps for toxin solubilization in midgut lumen (*1*) activation by midgut proteases (*2*) and binding to primary receptor cadherin. In the pore formation model (top), step (*3*) induces the cleavage of helix α-1 and triggers toxin oligomerization (*4*) the toxin oligomer then binds to a secondary receptor, such as aminopeptidase or alkaline phosphatase, which are anchored by a glycosylphosphatidylinositol anchor in the membrane, and (*5*) finally, the toxin inserts itself into the membrane, thereby forming a pore that kills the insect cells. (*6*) The signal transduction model (bottom) proposes that the interaction of the Cry toxin with a cadherin receptor triggers an intracellular cascade pathway that is mediated by activation of protein G (*4a*) which, in a subsequent step (*5a*), activates adenylyl cyclase. This signal then increases the levels of cyclic adenosine monophosphate, which activates protein kinase A and leads to cell death

Table 2.4 Agriculturally important plants transformed with *Bt*-genes for insect resistance

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
	Arachis hypogea	*cry1Ac*	Efficacy against lesser cornstalk borer	0.18%	Singsit et al. (1997)
	Brassica napus	*cry1Ac*	Resistance to *H. zea* Boddie and *S. exigua* Hubner	0.4%	Stewart Jr et al. (1996)
	Brassica oleracea	*cry1Ab*	Resistance to diamond back moth larvae	0.5.ng g^{-1} f.w.	Cao et al. (2001), Bhattacharya et al. (2002)
		cry1C	*Plutella xylostella*	–	Zhao et al. (2001)
	Cajanus cajan	*cry1EC*	Resistance to *Spodoptera litura*	–	Surekha et al. (2005)
		cry1Ab	Protection from *Helicoverpa armigera*	–	Verma and Chand (2005)
		cry1Ab	Protection from *H. armigera*	–	Sharma et al. (2006)
	Cicer arietinum	*cry1Ac*	Resistance against pod borer *Heliothis armigera*	0.003%	Kar et al. (1997)
		cry1Ac	Pod borer insect *H. armigera*	14.5–23.5 ng. Mg^{-1}	Sanyal et al. (2005)
		cry1Ac	Protection from *H. armigera* and *S. litura*	6–20 ng.Mg^{-1}	Indurker et al. (2010)
	Coffea canephera/ Coffea arabica	*cry1Ac*	Resistance to leaf miner	>0.1%	Leroy et al. (2000)
	Glycine max	*cry1Ac*	Resistance to bollworm (*H. zea Boddie* Boddie) and bud worm (*H. virescens* F.)	0.02%	Stewart Jr et al. (1996)
	Gossypium hirsutum	*cry1Ab cry1Ac*	Resistance to cotton bollworm (*H. armigera* Hubner)	0.05–0.1%	Perlak et al. (1990)
		cry2Ab	Resistance to pinkboll worm (*Pectinophora gossypiella*)	–	Tabashnik et al. (2002)

(continued)

Table 2.4 (continued)

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
	Ipomoea batatas	*cryIIIA δ-endotoxin*	Resistance against sweet potato weevil (*Cylas formicarius*)	–	Morán et al. (1998)
	Lycopersicon esculentum	*cry1Ac*	Resistance to tobacco hornworm (*Manduca sexta* L.)	0.001%	Fischhoff et al. (1987)
		Bt(k)	Resistance to tobacco hornworm (*M. sexta* L.), tomato pinworm (*Keiferia lycopersicella*) and tomato fruit worm (*Heliothis zea*)	1 ng mg^{-1} TSP	Delannay et al. (1989)
		cry1Ac	Resistance to fruitworm (*H. armigera* Hubner)	0.06–0.42%	Mandaokar et al. (2000)
		cry1Ab	Protection against fruitborer (*H. armigera* Hubner)	–	Kumar and Kumar (2004)
	Meidcago sativa	*cryIC*	Resistance to *S. litura* and *S. exigua*	0.01–2%	Strizhov et al. (1996)
	Nicotiana tabacum	*cry1Aa*	Resistance to tobacco hornworm (*M. sexta* L.)	–	Barton et al. (1987)
		δ-Endotoxin var. kurstaki HD1	Resistance to lepidopteran insects	–	Barton et al. (1987)
		cry1Ab	Resistance to tobacco hornworm (*M. sexta* L.) and budworm (*H. virescens* Fabricius)	0.001%	Vaeck et al. (1987)
		cry1Ac	Resistance to tobacco hornworm (*M. sexta* L.)	0.03%	Perlak et al. (1991)
		cry1Ab	Resistance to lepidopteran pests	400 ng^{-1}µg g^{-1}f.w.	Carozzi et al. (1992)
		cry1Ac	Resistance to tobacco budworm (*H. virescens* Fabricius)	3–5%	McBride et al. (1995)
		cry1C	Resistance to *S. litura* and *S. exigua*	0.01–0.2%	Strizhov et al. (1996)

(continued)

Table 2.4 (continued)

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
		cry1Ia5	Protection against *Heliothis armigera*	0.06%	Selvapandiyan et al. (1998)
		cry1Aa2	Resistance to *H. virescens, H. zea, S. exigua*	2–3%	Kota et al. (1999)
		cry2Aa2	Resistance to cotton bollworm (*H. zea* Boddie)	35.5%	DeCosa et al. (2001)
		δ-Endotoxin	Control of polyphagous pest *S. litura*	–	Singh et al. (2004)
		cry2Aa2	Effective control of *H. virescens*	0.21%	Zaidi et al. (2005)
		cry1Ac	Control of *H. virescens* and *M. sexta*	0.083%	Gulbitti-Onarici et al. (2009)
	Oryza sativa	*cry1Ab*	Resistance to striped stem borer (*Chilo suppressalis* Walker), and leaf folder (*Cnaphalocrocis medinalis* Guenee)	0.05%	Fujimoto et al. (1993)
		cry1Ac	Resistance to yellow stem borer (*S. incertulas* Walker)	–	Nayak et al. (1997)
		cry1Ab	Resistance to yellow stem borer (*S. incertulas*)	–	Wu et al. (1997)
		cry1Ab/Ac	Resistance to striped stemborer & yellow stem borer	3%	Cheng et al. (1998)
		cry1Ab	Resistance to yellow stem borer (*S. incertulas* Walker)	–	Datta et al. (1998)
		cry2A	Effective control of yellow stemborer and rice leaf folder	5%	Maqbool et al. (1998)
		cry1B	Resistance to striped stem borer	0.01–0.4%	Breitler et al. (2004)

(continued)

Table 2.4 (continued)

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
		cry1Ab	Resistance to 8 lepidopteran rice pests	1%	Shu et al. 2000
		cry1Ab cry1Ac Hybrid	Resistance to leaf folder (*C. medinalis* Guenee) and yellow Stem borer (*S. incertulas* Walker)	0.01–0.2%	Tu et al. (2000)
		cry1Ab	Resistance to eight lepidopteran rice pests	–	Shu et al. (2000)
		cry1Ac	Resistance to yellow stem borer (*S. incertulas*)	0.1%	Khanna and Raina (2002)
		cry1Ab	Resistance to rice leaffolder *C. medinalis*	–	Ye et al. (2003)
		cry1Ab/Ac	Resistance to stem borer	–	Ramesh et al. (2004)
		cry2A	Resistance to lepidopteran rice pest	9.65–12.11 μg g^{-1}f.w.	Chen et al. (2005)
	Populus tremuloides	*cry1Aa*	Resistance to forest tent caterpillar (*Malacosoma disstria* Lasiocampidae) and gypsy moth	–	McCown et al. (1991)
		cry3A	*Chrysomela tremulae* F. (Col.)	–	Cornu et al. (1996)
	Saccharum officinarum	*cry1Ab*	Resistance to stem borer (*Diatraea saccharalis* F.)	–	Arencibia et al. (1997)
		cry1Ac	Control against stemborer in field trials	50 ng mg^{-1} TSP	Weng et al. (2010)
	Solanum melongena	*cryIIIb*	Resistance to Colorado potato beetle (*Leptinotarsa decemlineata* Say)	–	Arencibia et al. (1997)
		cryIIIA	Resistance to Colorado potato beetle (*L. decemlineata* Say)	–	Jelenkovic et al. (1998)

(continued)

Table 2.4 (continued)

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
		cry1Ab	Significant insecticidal activity against *Leucinodes orbonalis*	0.02%	Kumar et al. (1998)
	Solanum tuberosum	*cry1Ab*	Resistance to tuber moth (*Phthorimaea operculella* Zeller)	–	Peferoen et al. (1990); Rico et al. (1998)
		cryIIIA	Tolerance to Colorado beetle (*L. decemlineata* Say)	–	Adang et al. (1993); Perlak and Fischhoff (1993); Coombs et al. (2002)
		*cry*V Bt *cry1Ab*	Resistance to potato tuber moth (*P. operculella* Zeller)	–	Douches et al. (1998)
		cry9Aa2	Resistance to potato tuber moth	–	Gleave et al. (1998)
		cry1Ab	Resistance to *H. armigera*	0.005–0.04%	Chakrabarti et al. (2000)
		cry1Ac	Resistance to *Tecia solanivora*	0.02–17 μg g^{-1} f.w.	Valderrama et al. (2007)
	Vigna aconitifolia	*cry1Ac*	Protection from *H. armigera*	–	Kamble et al. (2003)
	Zea mays	*cry1Ab*	Resistance to European corn borer (*Ostrinia nubilalis* Hubner)	0.4%	Koziel et al. (1993)
		cry1Ab	Resistance to *O. nubialis*	14–213 ng g^{-1} f.w.	Fearing et al. (1997)
		cry1Ab	Protection against *S. littoralis*	46.8–85.3 $ngcm^{-2}$	Dutton et al. (2005)

Table 2.5 Global status of approved and commercially available Bt-crops

Crop	Transgenic event(s)	Trade name	Company	Trait genes	Trait targets
Cotton	LLCotton25, MON 15985	FiberMax Libertylink Bollguard II∗	Bayer cropScience	*bar*, *cry 1Ac*, *cry2Ab*	Lepidopteran pests, weeds
Cotton	DAS-21023-5, DAS-24236-5	Wide strike∗	Dow AgroSciences	*pat*, *cry1Ac*, *cry1Fa*	Lepidopteran pests, weeds
Cotton	DAS-21023-5, DAS-24236-5, MONO1445-2	Wide strike∗ Roundup/Ready∗	Dow AgroSciences	*pat*, *cry1Ac*, *cry1Fa*, *CP4EPSPS*	Lepidopteran pests, weeds
Cotton	DAS-21023-5, DAS-24236-5, MON88913-8	Wide strike∗/ Roundup Ready∗ Flex	Dow AgroSciences	*pat*, *cry1Ac*, *cry1Fa*, *CP4EPSPS*	Lepidopteran pests, weeds
Cotton	MON531, MON1445-2	Roundup Ready∗, Bollguard	Monsanto	*cry1Ac*, *CP4EPSPS*	Lepidopteran pests, weeds
Cotton	MON88913-8, MON15985	Bollguard II∗, Roundup/Ready∗ Flex	Monsanto	*CP4EPSPS*, *cry1Ac*, *cry1Ab*	Lepidopteran pests, weeds
Maize	TC1507	Herculex CB	Dow AgroSciences and Pioneer Hi Bred	*cry1Fa*, *pat*	Lepidopteran pests (European corn borer), weeds
Maize	TC1507	Herculex CB	Dow AgroSciences and Pioneer Hi Bred	*cry1Fa*, *pat*	Lepidopteran pests (European corn borer), weeds
Maize	DAS-59122-7	Herculex RW	Dow AgroSciences and Pioneer Hi Bred	*cry34Ab*/*cry35Ab1*, *pat*	Coleopteran pests (corm rootworms), weeds

Crop	Transgenic event(s)	Trade name	Company	Trait genes	Trait targets
Maize	TC1507, DAS-59122-7	Herculex XTRA	Dow AgroSciences and Pioneer Hi Bred	*cry1Fa*, *cry34Ab1*, *cry35Ab1*, *pat*	Lepidopteran and coleopteran pests, weeds
Maize	DAS-59122-7, TC1507, NK603	Herculex XTRA/ Roundup/Ready∗ 2	Dow AgroSciences and Pioneer Hi Bred	*pat*, *CP4EPSPS*, *cry34Ab1*, *cry35Ab1*, *cry1Fa 2*	Lepidopteran and coleopteran pests, weeds
Maize	MON89034	Yield guard VT pro	Monsanto	*cry1A105*, *cry2Ab2*	Lepidopteran pests
Maize	MON88017	Yield guard VT	Monsanto	*CP4EPSPS*, *cry3Bb1*	Coleopteran pests (corm rootworms), weeds
Maize	MON810, MON88017	Yield guard VT Triple	Monsanto	*cry1A105*, *cry2Ab2*, *cry3Bb*	Lepidopteran and coleopteran pests, weeds
Maize	MON89034, MON88017	Genuity VT Triple Pro	Monsanto	*cry1A105*, *cry2Ab2*, *cry3Bb*	Lepidopteran and coleopteran pests, weeds
Maize	MON89034, TC1507, MON88017, DAS-59122-7	Genuity Smartstax TM	Monsanto and Dow AgroSciences	*pat*, *CP4EPSPS*, *cry 1Fa2*, *cry1A105*, *cry2Ab*, *cry3Bb1*, *cry34Ab1*, *cry35Ab1*	Lepidopteran and coleopteran pests, weeds
Maize	BtII, GA21	Agrisure GT/CB/ LL	Syngenta	*cry1Ab*, *pat*, mutant maize *EPSPS*	Lepidopteran pests (European corn borer), weeds
Maize	BtII, MIR604	Agrisure CB/LL/ RW	Syngenta	*cry1Ab*, m*cry3Aa*, *pat*	Lepidopteran and coleopteran pests, weeds
Maize	GA21, BtII, MIR604	Agrisure 3000GT (GT/CB/LL/RW)	Syngenta	*pat*, *cry1Ab*, m*cry3Aa*, mutant maize *EPSPS*	Lepidopteran and coleopteran pests, weeds

2.9 Management of Resistance Development

Since most of the insect-resistant transgenic plants released for commercial cultivation harbour single insecticidal Bt-*cry* gene and the target insect populations are consistently being exposed to the single toxin protein, the possibility of insects evolving resistance to single Bt-toxin is high (Zhao et al. 2005; Gunning et al. 2005). There are reports on development of resistance to *cry1Ab* in open field populations of the diamond black moth, *Plutella xylostella* (Tabashnik et al. 1993; Ballester et al. 1994) and resistance to *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry1F* have been reported in laboratory selection experiments (Tabashnik et al. 1997). In recent years, several Bt-cotton hybrid lines expressing *cry1Ac* have been approved for commercial cultivation in India, and due to small farm holdings, diverse cropping system and immigration of insects to alternative hosts, the possibility of developing heterogeneous insect population is very high (James 2012). Moreover, pink bollworm resistant to Bt-cotton harbouring the Bt-*cry1Ac* gene has been reported in the fields in India, where farmers rarely follow the refugia strategy (Tabashnik et al. 2010). Several strategies have been proposed for the management of resistance development in field insects, including the application of diverse mixture of toxins, high expression of Bt-toxin, weedy refugia, hybrid and pyramiding of different Bt-toxin genes and use of sterile insect (Gatehouse 2008; Tabashnik et al. 2010). Few key reports that have demonstrated the beneficial aspect of gene pyramiding in transgenic plants have been summarized in Fig. 2.6.

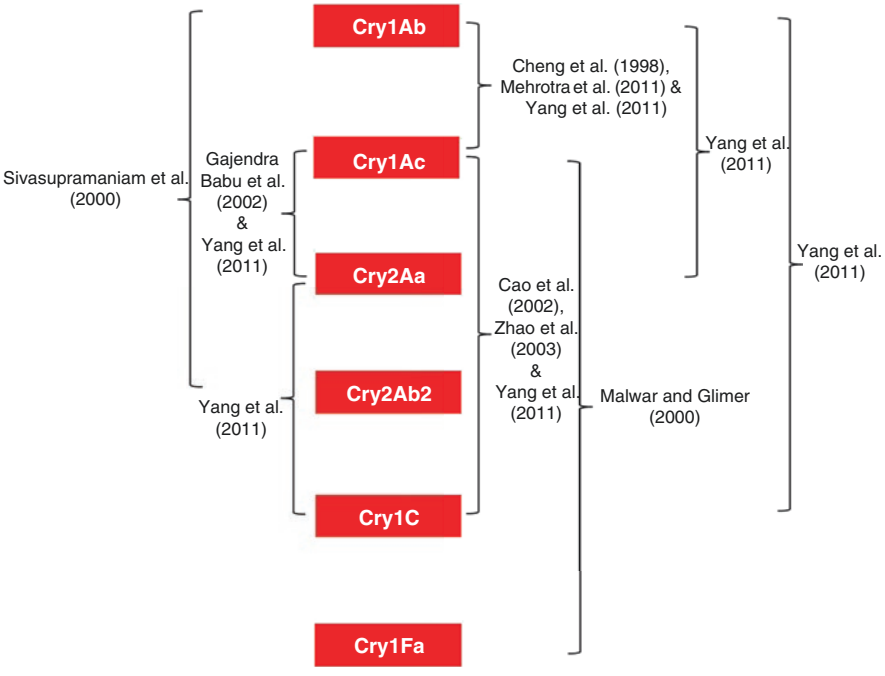

Fig. 2.6 Bt-gene pyramiding as a preventive and resistance management strategy

In recent years, transgenic plants expressing two dissimilar insect toxins have been developed, and the most successful example is Bt-cotton 'Bollgard II' expressing *cry1Ac* and *cry2Ab2* genes (Perlak et al. 2001; Zhao et al. 2005). The efficacy and sustainability of transgenic plants towards prevention of resistance development in insects rely on the pyramiding and co-expression of two or more diverse transgenes, without affecting the yield parameters (Zhao et al. 2003; Gatehouse 2008).

2.10 Conclusions

The transfer of Bt-*cry* gene(s) into plants has provided potentially powerful alternative strategies for the protection of crops against major agricultural field insects. The toxin encoded by *cry1A* gene(s) is highly effective against Lepidopteran group of insects that causes major damages to crop plants. A comparative interaction of Bt-toxins Cry1Aa, Cry1Ab and Cry1Ac encoded by corresponding genes with larval midgut binding sites (receptors) of *Helicoverpa armigera* has shown their competition for common binding sites to different epitopes of the receptors in the order of Cry1Ac > 1Ab > 1Aa and exerting corresponding toxicity (Estela et al. 2004; Bravo et al. 2007).

The *cry1Ac* gene has been extensively modified and codon optimized along with other modifications for over-expression in different plant species like tobacco, cotton, tomato, potato, chickpea and rice (Sharma et al. 2004; Ferry et al. 2004). The most successful story is the commercialization of transgenic cotton expressing the *cry1Ac* gene as Bollgard I in 1996 and Bollgard II with *cry1Ac* and *cry2Ab* in the year 2000 that has offered significant benefits over the application of synthetic insecticides and yield to the farmers (Perlak et al. 2001). The expression of native (wild type) full-length *cry1Ac* gene in plants was very low due to instability and premature termination of transcript (DeRocher et al. 1998; Perlak et al. 1990). Several modifications have been incorporated in the *cry* gene for over-expression, and the major breakthrough has been in the designing of synthetic versions of the gene with codon modifications to remove the putative polyadenylation sequences and use of plant preferred codons for high-level expression in plants (Perlak et al. 1990).

The 5′ and 3′ UTR leader sequences play an important role in transgene expression by regulating transcription and translation initiation of the foreign gene (Tyc et al. 1984; Lu et al. 2008). In particular, the use of viral leaders 5′ UTR has shown to greatly increase the accumulation of recombinant proteins (Dowson Day et al. 1993). The most preferred are tobacco mosaic virus Ω sequence (TMV), tobacco etch virus (TEV) and alfalfa mosaic virus (AMV) leader sequences (Datla et al. 1993; Gallie et al. 1995; Wang et al. 2001) which have been used for optimization of expression of several foreign proteins in plants (Haq et al. 1995; Agarwal et al. 2008; Wang et al. 2008). The 3′ UTR contains message for transcript polyadenylation that directly affects mRNA stability (Chan and Yu 1998). Heterologous 3′ UTR from plant or plant viruses have been used to stabilize the transcript formation (Hood et al. 1997; Staub et al. 2000; Ko et al. 2003).

The use of a synthetic truncated version (1.85 kb) of the *cry* genes coding toxin portion has been demonstrated to be most effective for Bt-transgenics against Lepidopteran insects (Perlak et al. 1990; Sardana et al. 1996). However, the most promising transgenic event of cotton (Monsanto 531) which has been commercialized is developed with full-length modified *cry1Ac* gene (Perlak et al. 2001; Purcell et al. 2004). All insect-resistant transgenic cotton varieties derived from this single event are performing well under field conditions in different agroclimatic regions across the globe (James 2012). Interestingly, development of stable transgenic plants of tomato expressing Cry1Ab toxin has been documented for insect protection (Kumar and Kumar 2004; Fischhoff et al. 1987; Srivastava 2007). Moreover, frequency and recovery of promising transgenic plant expressing Bt-toxin coded by full-length gene is also extremely low. But a question arises as to why the full-length synthetic gene was used, while the initial trials were performed with its truncated version? The answer to this is the modified full-length Cry1Ac toxin, although exhibits lower expression levels, efficiently induces oligomerization, prepore formation and insecticidal activity compared to modified truncated Cry1Ac toxin, at higher expression levels. These results suggest the importance of modified full-length *cry1Ac* gene for stability and integrity of the insect-resistance trait compared to truncated version of *cry1Ab* or *cry1Ac* gene(s) alone (Koul 2013). In reality, the commercially released Bt-cotton was developed with full-length *cry1Ac*-like gene whose nucleotide alignment study revealed that 'Monsanto 531' *cry* gene sequence is a hybrid gene where the sequence 1–1398 bp is that of *cry1Ab* gene. It was done in order to provide a blend of binding characteristics offered by Cry1Ab as well as pore formation characteristics offered by Cry1Ac, in the aforementioned successful *cry1Ac*-like gene, for raising transgenic cotton and its commercialization.

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