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Genetic engineering and insect resistance

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Abstract
In agriculture the *Bacillus thuringensis* as insecticidal toxins have been used for insect pest management for decades. The specific molecular interaction between the toxin and the insect midgut receptors makes the *Bt* a popular choice for pest control. This specificity of the action of *Bt* toxins reduce the concern of adverse effect on the non-target species, a concern which remains with the chemical insecticides since centuries. Different class of toxins are produced by *Bt* and to maximise the effect the diferent toxins are often combined which makes the Bt expression in then transgenic more effective. Although, the *Bt* is very effective, but there are concerns over the resistance development by insects species and also the narrow spectrum of activities of individual toxins. Gene flow, cross- resistance and hazards to the environment are of major concern in developed countries. Research is in progress to improve the toxicity of the Bt-toxins so as to control the resiatnce development. Therefore the aim of present revive is to explain the genetic engineering of the plants for insect control, disadvantages and strategies for the continuous research, development and improvement of *Bt* and non-*Bt* resistance products to tackle the insect pests problems without any harmful impact on the environment.

Keywords: Insect, resistance, transgenic, toxin

1. Introduction
Agricultural productivity is highly influenced by insect pests and diseases; known as the most harmful factors concerning growth and productivity of crops worldwide [1], causing the loss of 15% worldwide [2]. Recently, in Brazil the Velvetbean caterpillar has been successfully controlled by introduction of *Bt* soyabean [3].Conventional breeding methods are being used to develop the varieties more resistant to biotic stresses, at the same time are time taking, resource consuming and germplasm dependent. Besides, it requires evaluation at hot spot area and does not give consistent results, but improves flavor and nutrition. More often, the traditional crop breeders are focused on disease and insect pest resistance, these efforts are regional in nature [4]. On the other hand, insect pest management by chemicals obviously has brought about considerable protection to the crop yields over the past few decades. Regrettably, extensive and very often indiscriminate usage of chemical pesticides has resulted in the eradications of beneficial insects and development of pest resistant insects. At this situation, tool of the genetic engineering has provided humankind with unprecedented power to manipulate and develop the novel crop genotypes towards a safe and sustainable agriculture in the 21st century. One of the best modern agricultural defenses against plant-eating insects is *Bacillus thuringiensis* [5]. In recent times, it has become a source of agriculture innovations, providing a new solution to the age of old problems [6]. Biotechnology is often equated with genetic engineering and the support or opposition to genetically engineered crops is often distilled down to being for or against 'science' [7]. Plant genes are being cloned, genetic regulatory signals deciphered and genes transferred from the entirely unrelated organisms to confer new agriculturally useful traits on crop plants. Recent advances in genetic engineering have emerged a powerful modality for battling some of the important insect pests. It is chemical free and economically viable approach for insect pest control in plants. Negotiate exchange of this transgenic technology to the developing countries at easy terms and its integration with the conventional approach for resistance breeding will ensure evergreen revolution crucial for global food security. In this revive we mainly discussed on the role of genetic engineering in crop protection, global status, benefits, hazards and the future of transgenic insect resistant crops.

2. Conventional host plant resistance – A traditional strategy

Plant secondary metabolites influence the growth, survival and reproduction of the organisms, more specifically insects [8], besides varied in their constituents, are known to perform the diverse ecological functions notably in defense against herbivores, pathogens and abiotic stress and in interaction with competitors and mutualists[9]. Further these decrease the reliance on the synthetic pesticides and improving the ecological environment [10]; besides improving the fitness of the herbivour species on the particular plant [11]. Generalist herbivours usually cannot detoxify or sequester specific secondary pant compounds; Whileas, Specialist herbivours may overcome the natural plant defence easily. However, the *nicotana attenudata* is attacked by larvae of both specialist (*Munduca sexta*) and generalist (*Spodoptera exigua*) lepidopteran herbivours in its native habitat. Nicotine is the major defence metabolite and *M. sexta* is highly tolerant to it, in which cytochrome P450-mediated oxidative detoxification and rapid excretion is responsible for its exceptional tolerance [12]. When very large amount of allelochemicals are produced by a new cultivar, specialist herbivours may be negatively affected by these compounds. Chemical analysis of plants varying in the phenolics show that all the positive and negative effects on the insect tallow tree specialist beetle *Bikashs collaris* larvae are related to the tannin concentration, present in the roots and shoots. For specialist aphids on the *A. syriaca* show a negative correlation between jasmonic acid and salicylic acid when infested by both caterpillars and aphids. These acids were produced when the host plant is attacked by caterpillars but were attenuated by aphids [13]. Therefore specific plant volatiles allow herbivours to provide either facilitation or inhibition for feeding and oviposition [14], e.g rice (*Oryza sativa*) show strong metabolite influence to the insects.

The metabolites like *Bt* toxins in transgenic plants may be passed on to biological control agents either directly or indirectly, and affect the natural enemies at the end.

The changes of plant characteristics in breeding programme have positive, negative or neutral effect on insects. The specific breeding programmes have aimed for physical plant barriers, such as an altered wax layer or glandular trichomes or plant allelochemicals that negatively affect herbivore performance, specifically in cotton and other nectariless cultivars were nectar attract herbivore. An example of positive effects of host plant characteristics on carnivores are plant digestibility reducer (antibiosis) that slow down herbivour development. Because, the herbivores insects remain in a stage that is susceptible to their enemies for a longer period of time, this may increase carnivore effectiveness. Plant structures, such as domatia that are used by ants or mites to provide shelter may also enhance the effectiveness of carnivores. Negative effects of the conventional host plant resistance on carnivore may be found when herbivours sequester plant toxins (physiological resistance) and subsequently employ them in their defence against their carnivores enemies (bioagents), especially in specialists herbivours. Specialist carnivores in turn are generally better able to cope with herbivour, sequestered plant toxins than generalist carnivores. Natural enemies may also come into contact directly with plant toxins because they may use various plant tissues and plant products such as floral and extra floral nectars, plant sap and pollens, as source of nutrition. With the specific insectidal effect on the orders Coleoptera (beetles and weevils) [15, 16], Diptera (flies and mosquitos), Hymenoptera (bees and wasps), and Lepidoptera (butterflies and moths) [17, 18] and to non-insect species such as nematodes [19].

Bt toxins have taken centre stage as the major biological control agent and widely preferred to chemical insecticides. The efficiency against the insect pets is mainly due to the multiple site of action. Various assessments has shown that *Bt* is mostly environmentally friendly without significant adverse effects [20, 21]. The increased popularity of this biological control agents over synthetic chemicals is because of the non-selective lethal effects of the latter agents [22] and the rapid development of the resistance by insect pests to synthetic insecticides. While as, the transgenic crops play a central role in protecting the crop from its major insect pests and provide a valuable resource for insect pest suppression [23]. The production of the tolerant or resistant plant has been major success for the scientists at the same time the efficiency of the transgenic crops depends upon very much on whether they are viewed from the perspective of reducing chemical pesticides or from that of additional protective intervention and increasing crop yield. At the same time, there are many insect pests which are not susceptible to available range of insecticidal crystal protein genes. Many serious pests of local crop specific importance have received little or no attention from this technology. There is the need to broaden the pool of genes which are available to cover these pests which are currently untreatable or have developed resistance [24]. Transgenic crops are used worldwide to control major pests. Development of strategies to delay the evolution of pests' resistance to *Bt* crops requires an understanding of the factors affecting responses to natural selection, which include variation in survival on the Bt crops, heritability of resistance and fitness advantages associated with resistance mutation. The two strategies adopted for delaying the resistance are the refuges and gene pyramid. Both can reduce the heritability of resistance, but gene pyramiding can also delay resistance by reducing genetic variation for resistance. In nutshell, this mechanism of resistance is not so much effective and broad as compared to the resistance offered by transgenic crops itself.

3. Evolving genetic engineering approach in plants

Genetic engineering as an evolving approach in plants, mostly involves the addition and integration of genetic material (single or multiple genes) into a recipient plant, leading to the modification of the plants genome. The plant with modified genome is known as transgenic plant or genetically modified plants [25]. Useful traits responsible for resistance against the insect pests have been transferred to crop varieties from non-cultivated plants, since decades. Plant improvement whether as a result of natural selection or the efforts of plant breeder, has always relied upon evolving, evaluating and selecting the right combination of alleles. In 2013, GE crops were planted on more than 95% of sugarbeet, 93% of soyabean and 90% of all cotton and corn acres in the United States [26]. Advancement in the field of genetic engineering have proved new technologies for gene identification and gene transfer into plants that offered resistance to agricultural pests without altering plant critical quality traits[27]. Moreover, the transgenic research has made significant progress not only just widening the genetic pool of useful genes but, also permitting the introduction of a number of different desirable genes at a single event. Besides, the introduction of molecular changes by genetic engineering takes less time compared to conventional genetic methods. Hence, genetic engineering for developing insect pest tolerant plants based on the

introgression of gene might be a faster track towards improving crop varieties, not only interns of yield parameters but also in offering resistance to insect pests [24].

3.1. Cry genes from bacillus thuringiensis

Transgenic crops modified by cry (Bt) genes obtained from bacterium *Bacillus thuringiensis*, are the firstly used insecticidal genes for plant transformation (Table 1). *B. thuringiensis* is a gram-positive bacterium producing highly insecticidal protein crystals toxins during sporulation. Digestive system of the insect is the first target of this toxin. After ingestion by susceptible insects, toxins bind to specific receptors in the gut and are solubilized and activated by proteinases in the insect midgut epithelium. More than 400 genes encoding toxins from wide range of *B. thuringiensis* have been identified so far. Many of the identified cry genes (e.g, cry1Aa, cry1Ab, cry1Ac, cry1Ba, cry1Ca, cry1H, cry2Aa, cry3A, cry6A, cry9C, cry1F) have been engineered into plants against insect pests. Most cry proteins, even within cry1A subfamily have a distinctive insecticidal spectrum. While most crystal toxins are specific to larvae of Lepidopteran pests, some others are toxic to Coleopteran, or Dipteran pests. The level of expression of wild-type *Bt* toxins in transgenic plants is low compared to many other heterogonous genes, but become sufficient to cause high mortality of target pests in the field. Transgenic crops are used worldwide to control major pests of the cotton, corn and soyabean. The first planted cultivars were corn producing *Bt* toxin Cry1Ab and cotton producing Bt toxin Cry1Ac [28].

Table 1: Successful examples of B. thuringiensis genes integration for insect pest resistance in plants

S.no	Gene	Target pest	References
1	cry 1 a (b)	yellow stem borer & pink borer	Prashantya *et al.* [29]
1	cry 1 a (b)	yellow stem borer & striped stem orer	Wunn *et al.* [30]
2	cry 1a(b)	yellow stem borer & striped stem borer	Ghareyazie *et al.* [31]
3	cry 1a(b)	yellow stem borer	Datta *et al.* [32]
4	cry1a(b)/ cry1a(c)	leaf folder & yellow stem borer	Tu *et al.* [33]
5	cry 1a(b)/ cry1a(c)	yellow stem borer	Ramesh *et al.* [34]
6	cry 1a(c)	yellow stem borer	Nayak *et al.* [35]
7	cry 1a(c)	yellow stem borer	Khanna and Raina [36]
8	cry 1a(c)	striped stem borer	Liu *et al.* [37]
9	cry 2a	leaf folder & yellow stem borer	Maqbool *et al.* [38]
10	cry 2a/ cry 1a(c)	leaf folder & yellow stem borer	Maqbool *et al.* [39]
11	cry 1le	corn borer	Liu *et al.*[40]
12	cry 1a(b)	striped stem borer & leaf folder	Fujimoto *et al.* [41]

4. Resistance genes from other microorganisms

The closest to the commercial used cry genes against insect pests are the VIP genes from B. thuringiensis and *B. cereus*. Unlike Bt toxins, whose expression is restricted to sporulation, Vip insecticidal proteins are expressed in the vegetative stage of growth starting at mid-log phase as well as during sporulation. More than 50 Vip proteins have been identified so far. It is known that ingestion of Vip proteins causes swelling and disruption of the midgut epithelial cells and peritrophic membrane by osmotic lysis in the target insects [42]. Vip toxins consist of binary toxins that are made of two components, Vip1 and Vip2. And the combination of Vip1 and Vip2 is highly insecticidal to an insect e.g, western corn rootworm (*D. virgifera*), but does not show any insecticidal activity to lepidopteran insects. The other group consists of Vip3 toxins, and the first-identified Vip3 toxin, Vip3Aa1, is highly insecticidal to several major lepidopteran pests of maize and cotton. A highly effective protein (cholesterol-oxidase) that killed the larvae of the boll weevil (*A. grandis*) was discovered in Streptomyces culture filtrate. Morphological changes induced by ingestion of cholesterol oxidase suggest that enzyme has a direct effect on the midgut tissue of boll weevil larvae (Table, 2), and disrupted the midgut epithelium at low doses and lysed its cells at higher doses. Transgenic leaf tissues in transformed tobacco (*Nicotiana tabacum* L.) plants expressing cholesterol oxidase choM gene exerted insecticidal activity including severe developmental aberrations against boll weevil larvae. Bowen *et al*.[43] observed a toxin secreted by bacterium *Photorhabdus luminescens*, which lives in the gut of entomophagous nematodes. Insects infected with the nematode, the bacteria are released into the insect hemocoel; the insect dies and the nematodes and bacteria replicate in the cadaver. The toxin consists of a series of four native complexes encoded by toxin complex loci tca, tcb, tcc and tcd. Both tca and tcd encode complexes with high toxicity to tobacco hornworm (*Manduca sexta*) and therefore represent potential alternatives to Bt for transgenic deployment (Table, 2). Liu *et al*. [44] introduced the tcdA gene of Photorhabdus luminescens encoding a 283-kDa protein; into *Arabidopsis thaliana* L. Toxin A is highly toxic to a variety of insects, and toxin A expression above 700 ng/mg showed highly toxic effect to tobacco hornworm and southern corn rootworm (Fig 1). Similarly, transgenic expression leads to the production of several protein inhibitors in field crops exhibit resistance to agriculturally important pests (Table, 2). The activated toxins induce the formation of a lytic pore in the midgut epithelial membrane that results in cell lysis, cessation of feeding, and death of the larva. Further, the transgenic crops produce the digestive inhibitor proteins which are responsible for causing antibiosis and retard the insect larval development. The two types of proteins namely lectins and lipoxidase and polyphenol oxidase did show deleterious effects to herbivours. The toxicity of lectins varied considerably from those that had very little effect on the corrected mortality at the concentration of 0.1%w/v to the lectin from garden pea and snowdrop with corrected mortality values of nearly 90%. The lectin bound to gut epithelial cells and cause effects on growth of tissues, particularly in terms of effects on the normal structure of villi. About 3000 strains known produce a variety of crystal toxins.

Table 2: Transgenic plants expressing introduced protease inhibitor genes against insect pests

Inhibitor	Plant	Insect	Effect	Reference
Plant derived				
Cowpea-TI	Tobacco Potato Strawberry Cabbage Rice	*H. virescens* *L. oleracae* *O. sulcatus* *P. rapae* *C. suppressalis*	+ + + (Field) +	Hilder *et al.*[45] Gatehouse *et al.* [46] Graham *et al.* [47] Hao and Ao [48] Xu *et al.* [49]
Sweet potato-TI	Tobacco	*S. litura*	+	Yeh *et al.* [50]
Potato I (CI)	Tobacco	*S. litura*	-	McManus *et al.*[51]
Potato II (T/C-I)	Tobacco Rice	*M. sexta*	+ +(Field)	Johnson *et al* [52] Jongsma *et al* [53]
Tomato I (CI)	Tobacco	*M. sexta*	-	Johnson *et al.* [54]
Tomato II(T/C-I)	Tobacco	*M. sexta and A. grandis*	+	Johnson *et al.* [55]
Soyabean KTI	Tobacco	*H. virescens*	+	Gatehouse *et al.* [56]
Rice OZC-1	Poplar	*C. tremulae*	+	Leple *et al.* [57]
Insect-derived				
Manduca serpin	Cotton	*B. tabaci*	+	Thomas *et al.* [58]
Manduca E-I	Alfalfa	Thrips	+	Wasmann *et al.* [59]

Lectins show systematic effects by crossing the gut wall and passing into the circulatory system. The attachment of microflora to the gut epithelium, lead to breakdown of the gut wall and finally the bacterial invasion of the gut tissues lead to insect paralysis and death e.g, in *N. lugens*.

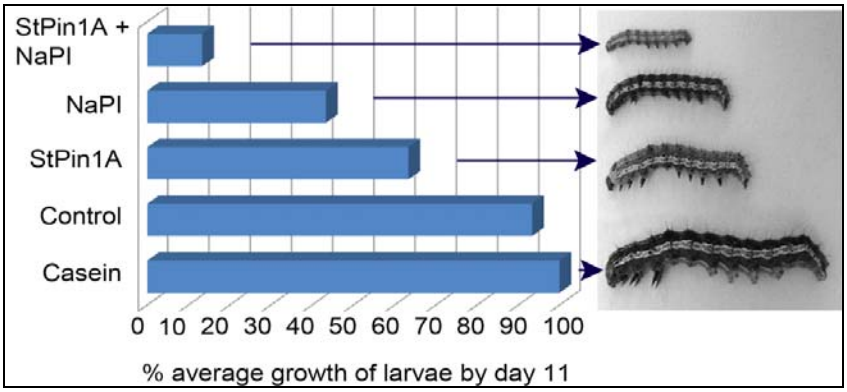

Fig 1: Proteinase inhibitors from transgenic *Nicotiana alata* as defense molecules against insect pests causing the antibiosis and retard the Tobacco catterpiller (*Spodoptera litura*) larval development

Table 3: Mortality Effects (LT_{10}, LT_{50} and LT_{90}), per cent survival rate and weight gains of 2nd instar larvae *Helicoverpa armigera* fed on T1 progeny from transgenic tobacco lines [60]

	Effects in tobacco lines						
	Wild Type	**Tb 2# 8**	**Tb 2# 11**	**Tb 2# 16**	**Tb 5# 4**	**Tb 5# 10**	**Tb 5# 17**
Lethal Effect*							
LT_{10} (days)	NA	1.5±0.29	1.4±0.62	2.8±0.21	1.2±0.09	2.3±0.28	1.9±0.26
LT_{50} (days)	NA	3.7±0.36	4.9±0.11	4.8±0.37	5.8±0.32	5.8±0.51	5.6±0.13
LT_{90} (days)	NA	8.2±0.47	17.3±0.19	8.3±0.22	20.8±0.13	15.0±0.26	15.3±0.16
Larvae weight†(mg)	32.3 ± 1.7^{d}	18.0 ± 1.5^{a}	23.0 ± 4.6^{b}	22.0 ± 1.2^{b}	17.0 ± 1.1^{a}	24.0 ± 3.6^{b}	22.0 ± 1.2^{bc}
Survival rate (%)	100 ± 0.0^{d}	20.7 ± 1.7^{a}	17.7 ± 1.7^{bc}	16.2 ± 2.1^{b}	22.7 ± 2.2^{a}	14.0 ± 1.4^{c}	15.0 ± 2.5^{c}

*Lethal time (LT) values based on 10%, 50% and 90% mortality of the exposed larvae determined by probit analysis. Wild type nil mortality. NA, not applicable. †Larvae weights and Survival rate were recorded 7 days after feeding. Values represent means (±SE) of three replicates. Means within a row followed by the same superscript alphabets are not significantly different ($P < 0.05$) by one-way ANOVA test.

Trypsin inhibitory activity in T_0 and T_1 transgenic tobacco showed a clear zone of hydrolysis on the gel, however, low or no significant activity of Wild Type (WT) against trypsin was observed. Inhibition percentage was measured against trypsin and papain. A significant high level of inhibition was noticed in transgenic lines Tb 2# 8, Tb 5# 17; Tb 2# 8 and Tb 5# 4 (Table 3). The median lethal time value (LT_{50}) for larvae of Helicoverpa armigera reared on transgenic lines ranged from 3.7 to 5.8 days whereas, all larvae on WT plants survived for at least 7 days. The amount of leaf area consumed by larvae varied between WT and transgenic tobacco plants. WT tobacco plants were severely damaged by H. armigera, whereas transgenic lines over-expressing stacked protease inhibitor genes exhibited only minor damage on the leaves. The larvae recovered from the T1 transgenic tobacco lines showed growth retardation and finally became brown-colored, as compared to the 90% of larvae recovered from the control plants were alive larva caused considerable damage.

5. Disadvantage of adoption of biotech crops

5.1 Basis of insect resistance

Bt technology has many potential benefits in terms of insect control, but there will continue to be potential problems with stink bugs and other arthropod pests that are not controlled by

Bt toxins and exhibited the resistance [61]. Insect resistance to the *Bacillus thuringiensis* crystal protein is a major threat to the long term use of the transgenic crops [62]. There have been a number of proposed modes of resistance of the insect pests to *Bt* toxins including reduced binding of toxins to the receptors in the midgut of the insects, reduced solubilisation of protoxin, alteration of proteolytic processing of protoxins and toxin degradation and precipitation by proteases. The understanding of the mechanism of action of Bt toxins have enhanced the verification of some of the modes, and the most studied and experimentally verified mode of resistance is 'mode 1' which is characterized by the recessive inheritance, reduced binding at one Cry1A toxin, and negligible cross-resiatnce to the Cry1C. Alteration of the protease profile in the midgut of the Cry1Ac resistant *Helicoverpa armigera* affected the proteolytic processing of the Cry1Ac resulting in the production of 95 and 68kDa toxin by midgut protease from susceptible population suggesting a link between improper processing of *Bt* toxin and development of resistance. Sayyed *et al*. [63] also demonstrated that a field collected resistant population of *Plutella xylostella* which was subsequently selected in the laboratory using Cry- 1Ab and named Cry1Ab-SEL was more sensitive to trypsin-activated Cry1Ab compared to Cry1Ab protoxins. Brush border membrane vesicles from the selected populations of the European corn borer (*Ostrinia nubilalis*) resistant to the Cry1F were found binding the toxin as well as those from a susceptible population and no differences in the activity of luminal gut proteases or proteolytic processing of toxin were observed. This failure to implicate defects in binding of toxin or toxin processing in the resistant strain indicates both alternative and strong resistance mechanisms in the insects.

5.2 Increasing resistance of insect pests

The widespread planting of crops genetically engineered to produce insecticidal toxins derived from the bacterium *Bacillus thuringiensis* (Bt) places intense selective pressure on pest populations to evolve resistance [64]. One potential risk was that target pests would become resistant to toxins produced by pest resistant GM crops, such as *Bt* maize and Bt cotton. A new scientific publication co-authored by Monsanto employees, is warning that the cultivation of the genetically modified soybean Intacta (MON 87701 × MON 89788) promote the spread of specific pest insects. The effects are likely to be caused by unintended effects in the plants arising from the insertion of the additional DNA [65]. The continued relevance of *Bt* toxins in the control of insect and non-insect pests is threatened by the development of resistance by the pests in the field and laboratory also. Moreover, studies suggested that target pests reared under laboratory evolve resistance more rapidly than had previously been thought possible. However, studies from Arizona and Australia indicated that, contrary to these prognostications, bollworm (*H. armigera* and *P. gossypiella*) did not increase its resistance to *Bt* toxin produced by a Bt cotton. One of the strategies conceived by the scientists is to preserve refuge areas planted with non-transgenic plants next to the fields where the transgenic crop is growing. In refuge areas the insects sensitive to the toxins could survive and mate with those which could stand the high doses of toxins produced in the transgenic plants. This result in the "dilution" of eventual resistance, the following generation of insects still harboring the genes for toxin sensitivity. The high-dose refuge strategy, recommended the farmers to leave aside 4 per cent of the acreage as plots planted with conventional plants, it could be increase to 20 per cent if they would like to treat them with conventional pesticides. However, some scientists considered that this acreage proportion could be as high as 50 per cent. Before the introduction of *Bt* maize, only 5 per cent of the acreage were used against the stem borer. According to the US Environmental Protection Agency (EPA), the 92 per cent of the farmers that grew Bt maize fulfilled the requisite of having refuges in their fields, and the farmers growing Bt maize had significantly decreased the use of pesticides. On average, GM technology adoption has reduced chemical pesticide use by 37%, increased crop yields by 22%, and increased farmer profits by 68%, even higher than for herbicide-tolerant crops. Yield gains and pesticide reductions are larger for insect-resistant crops than for growing herbicide-tolerant crops [66]. Transgenic crops promise more food with less land. GMO crops have been found to increase yields and overall increase in productivity (USDA) [65].

5.3 Cross resistance

Some insect populations resistant to a particular toxin exhibiting resistance to other toxins to which they have not previously been exposed, a term known as 'cross resistance. There have been a number of mechanisms responsible for resistance of the insect to the *Bt* toxins including reduction of binding of toxins to the receptors in the mid-gut of the insects, reduced solubilisation of the protoxins[23]. Further, Benjamin *et al*.[23] found a significant correlation among populations of the western corn rootworm for survival on Cry3Bb1 maize and mCry3A maize, indicating cross-resistance between mCry3A and Cry3Bb1, however; reported no cross resistance between Cry34/35Ab1 maize and Cry3Bb1/mCry3A maize. The resistance development and cross-resistance to other *Bt* toxins such as Cry1Ab, Cry1Ac and Cry1Fa after 14 generations of selection were determined by Xia *et al*. [67]. The widespread use of the transgenic Cry1Ie maize could lead to the development of resistance in the target pest *O. furnacalis*. However, luckily lack of cross-resistance between Cry1Ie and Cry1Ab, Cry1Ac or Cry1Fa showed that maize hybrids expressing the toxins viz.Cry1Ie and Cry1Ab, Cry1Ac or Cry1F are likely to be compatible for resistance management of *O. furnacalis*[68]. The classifications of the toxins are based on the phylogenetic similarity: the toxins that shear the same numeric values (e.g., Cry1A and Cry1F) have more recent common ancestral values. Likewise, cross-resistance is found more often among toxins with the same toxins numeric value than among toxins with different numeric values consequently, the greater phylogenetic similarity of Cry3Bb1 and mCry3A greater is the similarity in the mode of the action in these toxins as compared with Cry34/35Ab1. Toxins Cry3Bb1 and mCry3A belong to the three domain Cry family, whereas Cry34/35Ab1 belongs to the binary like family of cry toxins[69].The structural similarity among three-domain toxins results in the potential similarities in the binding sites in the insect mid-gut, whereas competitive binding analysis with Cry34/35Ab1 and mCry3A exhibits some discordance in binding sites[69,70]. Proteins in the insect mid-gut involved with the mode of action for the three-domain *Bt* toxins include alkaline phosphatase, aminopeptidase, and cadherin, and these proteins have been isolated from the midgut of the Chrysomelidae. Thus, structural change of *Bt*-binding sites may confer resistance to Cry3Bb1 and mCry3A simultaneously and not affecting susceptibility to Cry34/35Ab1.

5.4 Narrow spectrum of activity

Bacillus thurangensis is specific to six insect orders even then; it has only 2% of total market [5] at present. However, it has several limitations, such as a narrow activity spectrum, instability in rain and sunlight, and inefficiency against the pests feeding on the internal tissues of the plant. The first step towards improving *Bt* activity involve the isolation of the new strains with higher and broder insecticidal activity against target insect pests and cloning of cry genes encoding new insecticidal crystal proteins. A strategy aimed to increase the persistence of its toxins in the field is encapsulation in recombinant asporogenic *Bt* strains or other heterologous recombinant microbial hosts; this protected the toxin against the UV degradation and have the advantage of increasing the viability as the transgenic microorganisms released into the environment were non-viable. Apart from resistance by pests being a major threat to the future of *Bt* products, the problem of efficacy and spectrum of activity remain a challenge. In contrast to many synthetic insecticides most *Bt* toxins cloned have a narrow spectrum of activity. Only a small number of toxins (such as *Cry*1Ba) show activity that spans two to three insect orders.

5.5 Hazards to wildlife- migration

The monarch butterfly named as *Danaus plexippus* by Linnaeus. The migration takes the insects over 4,200 km, from the Canadian border during summer to the centre of Mexico during winter[71].This genetically modified plant produces a milky toxic sap and is not grazed by *D. plexippus*. The monarch can tolerate the poison, which accumulates in the hard parts of its wings, the exoskeleton; making the butterfly unpalatable to most birds. The monarchs born in Canada feed greedily for a few days in order to accumulate sufficient fat and start their migration before the arrival of the very cold winter [71].

5.6 Impact on biological diversity

Agricultural organization of the United Nations referring to the scale of loss as “extensive,” found that 75% of the plant genetic diversity has been lost since 1900 as the farmers in the developed and industrial countries turn to the genetically uniform mass produced crop varieties [72]. As most of the insect herbivours feed on these crops therefore directly or indirectly the effecting their biodiversity. Transgenes is not only alters the modified genome’s structure but also makes it unsuitable through time, produces the undesired disruptions or activations of the host’s genes and directly or indirectly affecting the operational state of the entire genome and regulatory networks that maintains its dynamic balance, as demonstrated by the viration in the phenotype response of the same genotype to the environmental changes [73]. The dissemination of the pesticide resistant genes or toxins encoding genes is harmful to the environment. Because it increase the risk of ‘bio-envasions’, considered a threat to the insect biological diversity. Bioinvasions involve the proliferation of the species which are transported from their original ecosystems and multiply in the host ecosystem and eliminate the autochthonous species. Genes from the genetically modified organisms could be disseminated towards related species which would acquire pesticide-resistant genes and become "superweeds"; and towards varieties of the same species. This phemenone is not only related to the plants, but a study published on the 23 November 1999 revealed that a transgene introduced into the population of the fishes could be transmitted to the whole population and eliminate the non-transgenic populations over a few generations. These studies suggested that these transgenes have a direct effect on the aquatic insects.

5.7 Gene flow

Gene flow is one of the major concerns with the release of transgenic plants in the environment. The potential environmental risk associated with the rice is the transgene flow [74, 75] and it is of the major challenge for the plant breeders. Bees play a key role in the agricultural production through their pollination activities; particularly in the United States about 60% of the total food consumed has a bee connection. Without bees the US would lose about $20 in the crops. Some of these studies reported that GM pollen donor rice transferring GM traits to conventional rice and red rice weed under field conditions [76]. Unrestricted gene flow can results in super weeds, reduction in species fitness and genetic and biological diversity, and contamination of traditional plants and foods. The insect resistant and herbicide-tolerant gene flow from transgenic cotton was determined by Pan [77]. A widely-publicized example of possible gene flow from transgenic plants to conventional crops was the discovery of gene constructs in native Mexican maize plants by Ignacio and David [78]. Regression analysis showed that there is a positive relationship between cross-pollination frequency and flowering synchronization and insect pollinators. For example, when genetically resistant soyabean were released in the field; in order to control the gene flow it was critically separated from conventional soyabean in space and time with efficient insect control during the flowering [79].

6. The strategies for continuing the use of *bt* and its products in agriculture

6.1 Search for *Bt* strains expressing the improved activity

The formulated and sporulated cultures of Bt have been widely used against the insect pests, but after the advent of genetically modified plants expressing the δ-endotoxins, bioavaibility of *Cry* proteins has been increased [80]. The major problem to face by *Bt* is inability of many existing Bacillus Thuringensis toxins to overcome the resistance developed by insect species in the field [63] and in laboratory reared populations; therefore, the efforts are continuing to search for Bt strains expressing novel toxins with improved activity. The protein Cry1AbMod, a mutant; kills the Cry toxin resistant insect population, but loss potency against the susceptible insects. As, the Cry1AbMod- protoxin efficiently induce oligomerization; thereafter, induce the pore formation in the midgut and finally kills the insect, while as; Cry1AbMod-toxin, fail to induce the oligomerization which explains the loos of potency even against the susceptible insects. More than 600 insecticidal genes have been cloned from various *Bt* strains, among these a large number have been heterologously expressed either independently or in combination with toxic against a specific insect species in one or more orders.

6.2 Use of synergism between *Bt* products or between *Bt* and other substances

Insect specific toxins derived from *Bt* provides a valuable source for the pest suppression. Modifications including toxin truncation, modification of protease cleavage sites, domain swapping, site directed mutagenesis, peptide addition and the toxin mutation by different synergizing strategies have been done to enhance the activity of the toxins. These strategies have been employed to enhance the toxicity against the

specific insect pest including those that have developed the resistance against to *Bt* or to modify the host range of the Bt crystal (*Cry*) and cytolytic (*Cyt*) toxins [23]. *Bt* toxins cloned have a narrow range of activity, while; few expressed toxins like *Cyt*1Aa expressed a weak toxicity to mosquitos on their own but show synergistic activity when combined with other toxins like *Cry*4Ba and *Cry*11Aa. To increase the efficiency of the Bt insecticidal toxins and to overcome resistance posed by insect pests, the use of other proteins like cadherin fragments have been shown to be a successful strategy. The use of a toxic compound, gossypol derived from the cotton plant has also been used in combination with *CryAc* to boost its efficiency against a resistant population of *Helicoverpa zea*. Co-expression of chitinase an enzyme that is known to disrupt chitin present in the midgut of the insect has been shown to have an enhanced effect on the efficiency of the *Cry*1Ac against *Helicoverpa armigera* and Cry1C against *Spodoptera littoralis*. Also combinations of *Cry* toxins have proven to be very useful strategy employed in boosting efficiency and fighting resistance. In Colorado potato beetle the midgut membrane metalloproteases were found to be involved in the proteolytic processing of Cry3Aa [81]. The combination of Cry2Ac and Cry2Ab exhibited have a synergistic effect against *H. armigera*. The mixture of crystal proteins and spores from the same strain can result in a synergistic insecticidal activity.

6.3 Remodeling of the *Bt* stains and toxins

Cleavage was demonstrated to occur in the protease accessible regions of the domain III and was specifically inhibited by the metalloprotease inhibitors 1, 10-phenanthroline and acetohydroxamic acid [81] in Colorado potato beetle. In domain II replacing the single residues in the loop 2, 3 and in domain III replacing the residues 541-544 of the Cry1Ca with alanine resulted in lower toxicity to the Spodoptera exigua, while its toxicity to the Manduca sexta were not affected.

The domain II of the Cry4Ba were remoulded to resemble that of the Cry4Aa and generated mutant showed the improved toxicity to the Culex quinquefasciatus and showed 700 fold increased toxicity as compared to the 285 fold in *Culex pipens*. Cry19Aa is a mosquitocidal toxin with specificity towards *Anopheles stephensi* and *Culex pipens* but with no measurable toxic activity against the *Aedes aegypti*. *Cry*19Aa exhibited more than 42,000 fold increase activity to *A.aegypti* by engineering the domain II loop1 and 2 to resemble that of the *Cry*4Ba. Similarly the rational design to the sequence of loop 1 and 2 based on the alignment with the Cry4Ba (naturally occurring mosquitocide), resulted into the increased mosquitocidal activity to the Cry1Aa. Site directed mutagenesis has great potential to alter the toxin encoding genes particularly when sufficient structural informations is available to inform the choice of the mutation. Understanding of the domain structure and function of the Cry1Ac enhanced the use of site directed mutagenesis to effect the changes to the domain I and II that resulted in the mutant showed the improved activity to the *Ostrinia furnacalis* and *Plutella xylostella*. The biotechnological technique like gene shuffling has been used in artificially directing the evolution of the new genes with novel characteristics. Shah *et al*[82] used the error-prone PCR and staggered extension process (St EP) shuffling combined with Red/Et homologous recombination to investigate the insecticidal activity of the CryAc and isolate a toxin variant designated as T524N which has increased insecticidal action against the *Spodoptera exigua* larva; while its original insecticidal activity against the *Helicoverpa armigera* larva were retained.

6.4 Use of the gene stacking

Gene stacking is readily deployable strategy to delay the development of insect resistance, while it may also broaden the insecticidal spectrum. Study suggested that 2A peptide can be utilized to express multiple *Bt* genes at high levels in transgenic crops [62]. Expression of multiple genes using conventional approaches has several potential limitations, most notably imbalanced expression among different genes and a large TDNA size required to include multiple genes. Gene stacking approach involves the expression of the two or more *Bt* toxins with different spectrum of activities and mechanisms of action in transgenic plants to control insect pests which has the advantage of controlling pests from many orders as opposed to the narrow spectrum that a single toxin can control. It also has the advantage of reducing the development of resistance because if the toxins used are such that there is little potential for cross-resistance between them, there has to be the resistant alleles at independent loci before an insect can develop resistance to the stacked toxins, which is a rear event. Two potential crystal proteins *viz*. Cry1Ab and Cry2Ab have diferent receptors in the insect midgut, were expressed by stacked polycistronic transgene using 2A peptide. Further it is the first appilication of the 2A peptide for expressing two *Bt* genes in the transgenic crop [62]. The effectiveness of the method of the gene stacking relies on the fact that development of resistance by the insect to the stacked toxins were through similar mechanism e.g. reduced toxin binding and therefore if stacked toxins have different binding sites, it will be difficult for an insect to develop resistance to all the stacked toxins. This assumption has been challenged as; the two toxins Cry1Ac and Cry2Aa are believed to have different binding sites in *Heliothis virescens*, but had shown to have cross-resistance which provokes a rethink on a advantages of gene stacking.

7. Future prospects

The transgenic crops have clearly revolutioned pest management in an agronomic agriculture. After more than 17 years of the safe use, virtually all the transgenes for the pest management revolve around glyphosate resistant crops, weed and *Bt* crops for insects. *Bt* crops are not encountering widespread evolved insect resistance, perhaps because of the mandated resistance management. The prevention and mitigation strategies are well understood but there has been little will to implement them. Therefore, the strategy of research should be upon the heavy thrust to implement them besides keeping in view the present and future environmental concerns.

8. Conclusion

The advances in genetic engineering have emerged a powerful modality against the important insect pests. Being chemical free and economically viable approach for insect pest control in plants. It is necessary to negotiate exchange of this transgenic technology at easy terms. Further its integration with the conventional approach for resistance breeding will ensure evergreen revolution crucial for global food security. Therefore, aim to discuss the role of genetic engineering in crop protection.

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10. References

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