Chapter 15
Bacillus thuringiensis-Based Gene Pyramiding: a Way Forward for a Combined Horizontal and Vertical Resistance in Plant

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15.1 Introduction

Crop loss due to pests is a major concern worldwide which could reach as high as 70% if preventive measures with either pesticide, natural enemies, host plant resistance or other controls are not utilized. About 67,000 pest species damage crops, of which 9000 contribute to insect species and mites (Ibrahim and Shawer 2014). Furthermore, insects are the primary direct cause of crop losses, whereas the indirect object is by the impaired quality of the products and their roles as vectors of various plant pathogens (Kumar et al. 2006). Apart from that, crops contribute a significant part of the world food supply to maintain the growing human population (Osman et al. 2015; Oerke 2006). Most developing countries still rely on agriculture as their primary source of food. Hence, the development and protection of agriculture are very critical in sustaining the growing human population worldwide.

Crop protection could be achieved, though not sustainably, through conventional methods like application of chemical pesticides and other cultural approaches. Yet, bioprotection of crop plants from insect pests via application of natural enemies is relatively sustainable and environmentally friendly. *Bacillus thuringiensis* (Bt)-based technologies like Bt crops have widely been used with relative success. However, recent reports indicated that Bt crops are losing sustainability as insects pests are learning to somehow develop resistance to withstand pressure exerted by Bt crops, thereby compromising Bt crop's resistance to pests [for further reading

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you may refer to a review by Feto (2016)]. Hence, the decades old ‘single-gene-Bt crop’ technology started to fail. Thus, there is a need to come up with an alternative sustainable tool to fill the void.

On the other hand, the novel Bt-based gene pyramiding could serve as an alternative to ‘single-gene-Bt crop’. Though the tool has yet to go mainstream, success has already been reported (Jain et al. 2017).

Therefore, this review will explore further on Bt as the most suitable natural source of genes that could be used to provide durable resistance in crops, since the multiple genes that make up the pyramid will render the transgene resistant to multiple pests.

Therefore, in this chapter different crop protection strategies are compared, and efforts have been made to underline that the gene pyramiding could be a better alternative if not the only one for sustainable pest management.

15.2 Prevalence of Crop Loss Worldwide

The incidence of crop loss worldwide is mainly due to insect attacks which can be as high as 70% if preventive measures are not used (Maxmen 2013). Previous reports have summarized the loss of crops to various insects and bacterial and fungal pests (Table 15.1). Most affected crops are wheat (Bahri et al. 2011), rice (Niu

Table 15.1 Percentage yield loss of crops mainly attributed to insect, bacterial, viral or fungal pests

Crop	Yield loss (%)	Pest	Origin	References
Wheat	70	Yellow rust (*Puccinia striiformis*)	Pakistan	Bahri et al. (2011)
Rice	5–10 and can reach 60% if the conditions are favourable	Rice stem borer (*Helicoverpa zea*), Brown planthopper (*Nilaparvata lugens*)	Pakistan	Liu et al. (2016)
Cowpea	40–68 if heavily infected	*Xanthomonas axonopodis pv. Vignicola*	Nigeria	Okechukwu et al. (2010) and Neya et al (2015)
Soybean	61	*Helicoverpa zea* (Corn earworm)	USA	Abudulai et al. (2012)
	25.8–42.8	*Aspavia amigera* (Stinkbug)	Ghana	Musser et al. (2016)
		Nezara viridula (Southern green stinkbug)		
Maize	Total of 57	Insects and pathogens	Kenya	Grisley (1997) and Anderson et al. (2016)
	47	Stalk borers (*Busseola fusca*) and weevils (*Sitophilus oryzae*)		
	10	Head smut *(Sporisorium reilianum)*, *MSV* (Maize streak virus)		

et al. 2017; Liu et al. 2016), cowpea (Okechukwu et al. 2010), soybean (Musser et al. 2016; Abudulai et al. 2012) and maize (Anderson et al. 2016; Grisley 1997). These crops are usually affected by pests such *Xanthomonas axonopodis pv. Vignola* (Xav), *Nezara viridula* and many more which require immediate attention.

15.3 Crop Protection Methods Against Pests

In the past, humans have searched for crops that can survive and produce under different biotic and abiotic stresses. Furthermore, farmers avoided yield loss through searching pest-resistant crops by collecting the seeds from only the highest yielding crops in their fields (Ibrahim and Shawer 2014) or through the application of chemical pesticides. Although chemical pesticides do result in reduced crop yield loss, more money is spent each year globally for inadequate control measures. Hence there is still a need to search for adequate protection of crops against pests.

15.3.1 Conventional or Traditional Methods and Its Drawbacks

Traditional crop protection method such as chemical control of pests was used as the most effective and attractive strategy in the previous century, during the 1940s and 1950s (Malav et al. 2016; Oerke 2006; Graves et al. 1999). Moreover, conventionally grown crops use more pesticides, and that represents the worst effect of chemically dependent agriculture. Although chemical pesticides are effective and had guaranteed a production increase in agriculture during the last 40 years, their continuous use is a primary cause of resistance and environmental concern (Mekonnen et al. 2017; Oerke 2006; Graves et al. 1999). These led to contamination of water and food sources, as well as the poisoning of nontarget beneficial pests and development of pests that are resistant to the chemical pesticides (Kumar et al. 2008; Scheyer et al. 2005).

Thus, the global public concern to seek alternative methods to control pests such as insects and fungal pathogens has increased due to the adverse effect of the application of chemical pesticides (Ibrahim and Shawer 2014). Furthermore, one approach could be the use of biological control methods such as biopesticides and entomopathogenic microorganisms like bacteria, fungi and viruses that include the development of technologies that would allow the insertion and functional expression of foreign genes in plant cells (Malav et al. 2016; Danny et al. 1992). Moreover, biological control reduces expenses and health hazards associated with pesticide formulations (Kouser and Qaim 2011). Hence, Bt has been used for several years as an alternative crop protection method to conventional methods.

15.3.2 Bt Biopesticide Methods

Currently, the application of Bt as a biological control method has increased crop production. Furthermore, Bt biopesticides are more effective as compared to the use of chemical pesticides which attribute more adverse effect due to contamination of the environment and food products and lead to human health problems (Tu et al. 2000). The insecticidal activity of Bt bacterium is due to the presence of the parasporal crystals (*cry*) which are formed during the sporulation phase of the bacterium and are assembled by the *cry* proteins that are expressed by the *cry* genes (Crickmore et al. 2017; Schnepf et al. 1998).

The Bt species has considerable variability due to the number of strains isolated around the world (Palma et al. 2014; Roh et al. 2007), the number of serotypes known to date (Roh et al. 2009) and the high number of crystal (*cry*) gene sequences accumulated so far. Despite the variability observed within this species, there is some uniformity in at least part, which shows some reflection on the five conserved blocks in the gene structure that is present in almost all the *cry* genes (De Maagd et al. 2001). Bt toxins create a heterozygous family of 74 different types of proteins (*cry*1–*cry*74) that are toxic to numerous insect pests such as *lepidopteran*, *coleopteran*, *dipteran*, *hemipteran*, some nematodes and snails' species that cause a severe damage to economically important crops (Crickmore et al. 2017; Palma et al. 2014).

The *cry* toxins belonging to three domain families share similar and conserved three domain structures (Fig. 15.1) which display their differences in amino acid sequences (Pardo-Lopez et al. 2013). Also, domain I constitutes of seven α-helix clusters that are subjected to proteolytic cleavage in all three-domain *cry* proteins during toxin activation (Fig. 15.1). It is usually referred to as perforating domain and is located towards the N-terminus which may be responsible for toxin membrane insertion and pore formation (Ben-Dov 2014; Xu et al. 2014). Moreover, domain II or middle domain is responsible for toxin-receptor interactions, and it consists of three antiparallel β-sheets (Xu et al. 2014; Jenkins and Dean 2000). Besides, domain III which is usually referred to as the galactose-binding domain has two antiparallel β-sheet sandwiches (Fig. 15.1), which are also involved in receptor binding and pore formation (Xu et al. 2014). In addition, domain IV

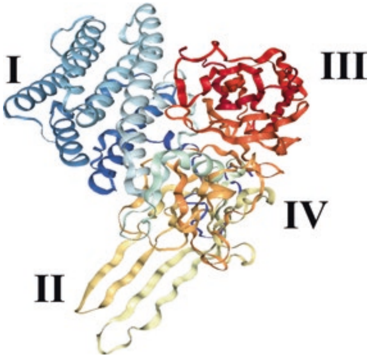

Fig. 15.1 The 3-D structure of Cry2Aa toxin (PDB accession number 1I5P) showing the four domain toxins (I–IV) produced by Bt (Soberon et al. 2016)

(Fig. 15.1) is mainly composed of alpha helices which resemble structural domains such as spectrin- or fibrinogen-binding complement inhibitor (Soberon et al. 2016).

15.3.2.1 Mechanism of Bt Biopesticides

The processing of the crystals relies on the solubilization of the toxins in the alkaline midgut of the insect pest and then activated by proteolytic digestion of the specific serine proteases (Palma et al. 2014). Interestingly, consumption of Bt toxins is found to be safe to humans because the intestinal walls of mammals do not have endotoxin receptor necessary for the toxic effect mainly due to the acidic conditions, and thus, the proteins tend to get degraded quickly in the stomach (Mekonnen et al. 2017). Some reports showed that *cry* gene specificity and activity could be influenced by other factors such as associated with toxin processing or stability in the insect midgut apart from the receptor binding (Jurat-Fuentes and Crickmore 2017). Moreover, *cry* genes are co-localized with other genes such as vegetative insecticidal proteins (*vip*) forming the insecticidal pathogenicity island (PAI) (Zhu et al. 2015).

15.4 Possible Challenges of Bt Toxins and Resistant Breakdown of Bt Crops

Bt has been studied for decades and is a used bacterial control agent to date. Nevertheless, several pest species have acquired field resistance to the most used Bt toxins and more severely to those included in transgenic crops (Peralta and Palma 2017). The currently used Bt toxins have not provided durable resistance due to the observed Bt resistance breakdown in the current Bt crops (Table 15.2) (Peralta and Palma 2017). An explanation for this may be due to the insects and pathogenic

Table 15.2 Resistance breakdown in the Bt crops

Bt crop	Insect pest	Resistance type	Origin	References
Cotton	Corn earworm (*Helicoverpa zea*)	A mild resistance that led to reduced efficacy of 2nd generation of crops	Tucson (USA)	Brévault et al. (2013)
	Pink bollworm (*Pectino gossypiella*)	Field resistance to Cry1Ac Bollgard® I	India	Tabashnik and Carrière (2010) and Dhurua and Gujar (2011)
Corn	Fall armyworm (*Spodoptera frugiperda*)	A mild resistance that led to reduced efficacy of 2nd generation of crops	Brazil	Santos-Amaya et al. (2015)
Maize	African caterpillar (*Busseola fusca*)	Field resistance and is dominant	South Africa	Campagne et al. (2013)

diversity displayed by most pests which lead to a rapid breakdown of specific resistance genes (Peralta and Palma 2017; Geffroy et al. 1999). Even though numerous reports involve the insect resistance over Bt-based formulations, field-evolved resistance has occurred which is promoted by the selective pressure applied over some insect populations (e.g. the *lepidopteran Trichoplusiani*) (Song et al. 2015). Furthermore, this occurs frequently in the most used Bt crops in agriculture, especially those from the first generation that express only one protein and plants that have multiple genes which have similar toxins.

Previously, some reports indicated the reduced efficacy of second-generation Bt cotton and corn harbouring *cry*1Ac + *cry*2Ab and *cry*1A.105 + *cry*2Ab against *Helicoverpa zea* and *Spodoptera frugiperda*, respectively (Table 15.2) (Santos-Amaya et al. 2015; Brévault et al. 2013). Furthermore, some authors reported on field resistance of Bt spray (Table 15.2) containing *cry*1C or *cry*1Ac observed in maize and cotton (Brévault et al. 2013; Campagne et al. 2013). Hence, there is a need in continuous search for novel Bt strains that have a broad spectrum range and could potentially circumvent the resistant issue, thus requiring the novel strategies that could anticipate the evolutionary responses of insects pests (Peralta and Palma 2017). Therefore, the best possible crop protection strategy could be pyramiding genes in such a way that could address both vertical and horizontal resistance in plants.

15.5 Transgenic Bt Crops

Genes from Bt have currently received increased attention due to their broad range of biotechnological applications, especially in agriculture for biocontrol of harmful insects and fungal pathogens (Kuddus and Ahmad 2013). These single or multiple *cry*-based genes could be inserted into crops, resulting in transgenic crops that are resistant to insects and fungal pathogens.

15.5.1 Single Cry-Based Bt Crops

Single *cry*-based Bt crops are crops incorporated with only a single *cry* toxin. However, single *cry*-based Bt crops are most likely prone to resistant breakdown than multiple *cry*-based Bt crops (Keshavareddy and Kumar 2018). These might be due to the pest developing resistance towards the crop mainly because the pests tend to adapt to the treatment conditions very quickly than in multiple *cry*-based crops. Moreover, commercialization of Bt crops such as maize, cotton and soybean worldwide has significantly reduced the application of synthetic pesticides (Keshavareddy and Kumar 2018; Ferré and Van Rie 2002). In addition, some reports showed the effective control of Bt rice such as KMD (*cry*1Ab), T1c-9 (*cry*1C) and T2A-1 (*cry*2A) to target *lepidopteran* insects (Table 15.3) including stem borers and leaf folders (Wang et al. 2016; Zheng et al. 2011; Chen et al. 2005).

15.5.2 Multiple Cry-Based Bt Crops

The multiple *cry*-based Bt crops are crops incorporated with two or more *cry* toxins. Even though multiple *cry*-based genes were used in transgenic crops before such as chickpea and brassica (Table 15.3), the broad-spectrum range has not been considered, or the incorporated multiple genes shared similar toxins. Hence, there is a resistant development as well (Meenakshi et al. 2011; Cao et al. 2008). Therefore, a wide range of sequences known to date is attributed to intense interest in finding novel *cry* proteins with alternative toxins that has a broad spectrum to manage the resistant breakdown observed in the current Bt crops (Ibrahim and Shawer 2014). These could be done with the application of Bt-based gene pyramiding as a genetic tool for inserting multiple genes that do not share the same toxins. As studies show Bt crops consisting of a single *cry*-based gene or multiple *cry*-based genes sharing the same toxins tends to be more prone to pest resistance (Keshavareddy and Kumar 2018).

Table 15.3 Successful genetically engineered crops with Bt genes with broad spectrum that provide durable resistance

Crop type	Trait	Engineered genes	References
Corn	Asian corn borer	*Cry1Ie* and *Cry1Ac*	Jiang et al. (2016)
Rice	Yellow stem borer resistance	*Cry1Ab*/*Cry1Ac*	Datta et al. (2002) and Cheng et al. (1998)
	Striped stem borers resistance	*Cry1Ab*, *Cry1Ac*, and *Cry2A*	Wang et al. (2016)
	Stem borers and bacterial blight disease resistance	*Cry1Ab*/*Cry1Ac* and *Xa21*	Jiang et al. (2004)
	Leaf folder, yellow stem borer, and brown planthopper resistance and increasing lectin content	*Cry1Ac, Cry2A*, and *gna*	Maqbool et al. (2001)
	Lepidopteran resistance and increasing lysine content	*Cry1Ac* and *LRP*	Liu et al. (2016)
Cotton	Insect pest resistance	*Cry1Ac*, and *Cry2Ac*	Gahan et al. (2005)
	Bollworm resistance	*Cry1Ac*, and *Cry2Ab*	Jackson et al. (2004)
Broccoli	Diamondback moth resistance	*Cry1Ac* + *Cry1C*	Cao et al. (2002)
Brassica	Diamondback moth larvae and lepidopteran insect resistance	*Cry1Ac* + *Cry1C*	Cao et al. (2008)
Chickpea	Lepidopteran resistance	*Cry1Ac* + *Cry1Ab*	Meenakshi et al. (2011) and Ahmed et al. (2017)
Soya bean	Lepidopteran resistance	*Cry1Ac + corn earworm QTL*	Walker et al. (2002) and Malav et al. (2016)

15.6 Gene Pyramiding Method

Gene pyramiding is a method of assembling or stacking multiple genes to improve durable resistance in crops against insects or diseases which is crucial for stable food production. Moreover, breeding resistance crops with either single or multiple Bt-based *cry* genes is the most cost-effective and environment-friendly strategy for resistance management. The advantage of gene pyramiding is that it uses the same strategy as that of the pesticidal mixture to broaden the resistance spectrum in crops. In addition, if two or more resistant genes are incorporated in a crop, it is less likely for the crop to be attacked by a pathogen race resistant to both genes or for the plant to lose both genes at the same time (Meenakshi et al. 2011). Furthermore, due to biotic factors, gene pyramiding is a cost-effective and environmentally friendly method used to manage crop production. Hence it has become the most used method for developing durable resistance in crops against pests (Meziadi et al. 2016; Fukuoka et al. 2015).

Previous reports on Bt-based gene pyramiding have shown an outstanding performance against insects where Bt toxins were incorporated in rice (Ye et al. 2009; Chen et al. 2008). Moreover, the integrated genes into elite cultivars with different genetic background were introduced by sexual crossing. Hence, in field evaluations, the improved lines also showed excellent efficacy against the target insects (Liu et al. 2016; Yang et al. 2011). So recently, different types of gene pyramiding such as conventional gene pyramiding and molecular gene pyramiding are widely used to obtain durable resistance in crops (Meenakshi et al. 2011).

15.6.1 Conventional Gene Pyramiding

Conventional gene pyramiding also known as serial gene pyramiding is a method where genes are arranged in the same plant one after another. These include pedigree crossing, backcross breeding and recurrent selection (Table 15.4). The identification of sources of useful genes is very slow using traditional methods. Hence, breeders' capability to trace the presence or absence of the target genes is limited, thus resulting in the limited number of genes incorporated into selected cultivars (Malav et al. 2016).

15.6.2 Molecular Gene Pyramiding

Molecular gene pyramiding also referred to as simultaneous gene pyramiding is a method where genes are arranged at the same time in a plant (Srivastava et al. 2017). These include marker-assisted selection and transgenic methods (Table 15.4). The differences among the two gene pyramiding methods are summarized below

Table 15.4 Differences between conventional and molecular gene pyramiding

Conventional gene pyramiding	Molecular gene pyramiding
Pedigree: *Is suitable when resistance is administered by the significant genes (Malav et al. 2016).*	Marker-assisted: *It involves the use of molecular markers for the selection of desired traits and identification of genomic regions associated with different major diseases (e.g., blast resistance). These markers are highly precise and reduce the selection time which makes this approach outstand conventional approach (Mekonnen et al. 2017; Srivastava et al. 2017).*
Backcrossing: *It involves the substitution of the desired gene from the donor parent to the recipient parent. It is mainly used to decline the donor genome content into the progenies (Allard and Allard 1999; Mekonnen et al. 2017).*	Transgenic: *It involves methods such as Agrobacterium transformation which is used to transfer a gene/s of interest into plant cells. This method ensures the stable integration of DNA of the desired gene into the genome (Srivastava et al. 2017).*
Re-current selection: *It allows for shorter breeding cycles. Besides, more specific follow-up of genetic gains is involved and provides an opportunity to develop a broad range of genetic diversity in breeding lines (Srivastava et al. 2017).*	

(Table 15.4). In addition, molecular gene pyramiding such as transgenic method is more advantageous over other pyramiding methods (Keshavareddy and Kumar 2018). Although there has been a success in Bt crop production, there are some drawbacks concerning Bt-based gene pyramiding.

15.7 Potential Challenges of Gene Pyramiding

Although gene pyramiding is a widely adopted strategy for improvement of crops against resistant effects, there are certain drawbacks associated with this strategy. In addition, the reliability of phenotyping at an individual level is minimal since the presence of target traits must first be confirmed. Phenotyping influences the inheritance model of genes for the target traits, linkage and pleiotropism between the target traits at an individual level (Malav et al. 2016; Riaz et al. 2006).

Another drawback involves the limitation of successfully pyramided transgenic crops for enhanced fungal and bacterial resistance (Summers and Brown 2013; Punja 2006; Schnepf et al. 1998). These may be due to two primary life strategies of pathogens, namely, biotrophy and necrotrophy. Thus, biotrophic pathogens essentially act as a sink for the hosts' anabolic assimilates which keep it alive, while necrotrophic pathogens consume the hosts' tissues as invaded. As a result, plants developed different approaches to deal with these two strategies (Summers and Brown 2013; Punja 2006) which are not obtained through genetic engineering.

Lastly, to avoid recognition by host (R) genes, the pathogen avirulence (Avr) gene undergoes strong diversifying selection or mutation (Ferry et al. 2004). The low level of pathogenic resistance by some transgenic crops coupled with a negative perception of genetic engineering-modified crops has resulted in few transgenic crops (Palma et al. 2014) being brought to the market due to the relatively small number of transgenic crops available (Mekonnen et al. 2017).

Apart from those resistant to fungal and bacterial pathogens, virus-resistant crops are not commercially available (Collinge et al. 2007). Hence, many transformation strategies have been used to increase fungal, bacterial and viral resistance in crops (Mekonnen et al. 2017). In addition, this includes introgressing R genes and introducing genes coding for antimicrobial compounds such as chitinase and glucanase enzymes that break down the fungal cell walls (chitin or glucan) and also upregulating defence pathways through promoter transfer, disarming host susceptibility genes, detoxifying pathogen virulence factors (toxins), increasing structural barriers and silencing essential pathogen genes (RNA silencing, RNA interference or RNAi) (Vincelli 2016; Collinge et al. 2007; Schnepf et al. 1998). Hence, two R genes were introgressed to develop rice cultivars resistant to bacterial blight and bacterial streak diseases in a study conducted by Zhou et al. (2008).

15.8 Conclusion and Future Prospects

The addressed reports presented insights into the fundamental basis of Bt isolates with broad spectrum, subjected to screening programmes to evaluate their insecticidal activity. The current review shows that the production and continuing development of Bt crops has been a major scientific success up to date which is deployed by the expression of Bt toxins. However, several studies documented that pests are developing resistance to Bt crops or Bt biopesticides. This situation is mostly observed in the current Bt crops that are incorporated with *cry*2 genes and lower. Hence, there is still a need to explore other effective strategies that could stand on its own or could be integrated with other control measures to diversify the resistance management tools.

Therefore, in this chapter, we tried to compare different crop protection strategies and make a point that gene pyramiding could be a better alternative if not the only one. These could include the involvement of pyramiding Bt toxins with other genes such as phytase, *vip*3 and other genes to broaden the spectrum. Another management tool could consist of the crop rotation method of cultivating Bt crops with other non-Bt crops to try and confuse the pests. However, there must be an assurance that the development of pest resistance genes does not compromise the protection of produced Bt crops. Furthermore, there are commercially available Bt crops with single or multiple toxins which reduced the application of chemical pesticides.

This review stipulated possible challenges that can inhibit the efficiency of gene pyramiding. Hence, extensive and precise phenotyping is required to counteract the difficulties in gene pyramiding. These involve the dissection of phenotypes into components that can improve the heritability, thus aiding the understanding of biological systems causing the phenotype (Varshney et al. 2005). Another strategy is phenotyping characterization of large mutagenized populations and tilling populations which could link a gene with phenotype. Apart from the challenges of gene pyramiding and current resistant breakdown in Bt crops, Bt will continue to play a significant role as a candidate bacterium for pyramiding multiple toxin genes into crops for resistant management due to its broad spectrum of resistance from the natural origin. Very recently some studies have been carried out to pyramid different Bt-sourced *cry* genes. Such kind of strategy is a relatively recent advancement that should be explored further.

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