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Helicoverpa Resistant Chickpea Plants: From Bt Toxins to Plant-Mediated RNAi

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ABSTRACT

Helicoverpa armigera, the pod borer is a major constraint to global chickpea production. Genetic improvement of chickpea for insect resistance by traditional methods has been hampered by narrow genetic diversity in the elite gene pool. *Bacillus thuringiensis* (Bt) chickpea plants expressing Bt genes as well as pyramids also have been developed already and many are in field trials. But, already available Bt crops like cotton have increased the insect resistance to transgenic plants in *H. armigera.* Although Bt chickpeas have yet to be commercialized, but the sustainability of Btcrops is vulnerable to the insect resistance in *Helicoverpa*. The next generation approach for crop protection against *Helicoverpa* is to knock down the crucial physiology-related genes of insect pests using transgenic plants, which is called Plant-mediated RNAinterference (RNAi). Common small interfering RNAs (siRNAs) for the target genes of *H. armigera,* designed *in silico* could be used to study the lethal effect of down-regulating crucial target genes in chickpea. This review describes the progress of developing resistance to *H. armigera* in chickpea using Bt toxin genes and the future prospects of using plant-mediated RNAi for *H. armigera* resistance. The plant-mediated RNAi approach holds great promise for future development but further studies will be required to optimize RNAi-based strategies for chickpea protection against *H. armigera* using integrated pest management strategies.

Keywords: Chickpea, Bt toxin, Plant-mediated RNAi, siRNA, *Helicoverpa*.

Introduction

Chickpea (*Cicer arietinum* L.), a self-pollinating diploid and world's second most widely grown annual legume crop. Chickpea production is of prime importance to world food security and in diversifying the cereal-based cropping system, owing to its capacity for symbiotic nitrogen fixation (Jukanti *et al.*, 2012). Chickpea is also a good and cheap source of protein for people in developing countries (Gaur *et al.*, 2012). Globally, chickpea is grown in an area of 13.6 mha; producing 13.1 mt with an average yield of about 0.96-ton ha^{-1} (FAOSTAT, 2013). India is the largest chickpea growing country; with 9.6 mha of chickpea grown area and producing 8.8 mt chickpeas with an average yield of about 0.92ton ha^{-1} (FAOSTAT, 2013). There is growing interest in chickpea consumption and increased global demand but chickpea production has increased slowly at an annual rate of 1.3% in the past 20 years (Rao *et al.*, 2010).

The most intractable impediments to global chickpea production are *Helicoverpa*, aphids, bruchids, weeds, drought, salinity, and low methionine content in the seeds (Acharjee & Sharma, 2013a). Gram pod borer, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) is the most devastating insect pest to chickpea production, which causes severe pod damage and yield failure (ICRISAT, 1992; Yadav *et al.*, 2006). The pod borer is widely distributed throughout the world and has facultative diapauses, which enables them to survive adverse weather conditions.

The larvae feed directly on the pod, causing seed abortion and damage, thereby having the potential to cause major crop losses (Giri *et al*., 1998) (Fig 1). However, there is still no strong resistance which has been identified for *H. armigera* in chickpea cultivars. Therefore, there is urgent need of transgenic chickpea resistant to *H. armigera* to boost production and productivity (Acharjee & Sharma, 2013b). This review will focus on the progress and current status for developing resistance to *H. armigera* in chickpea using Bt toxin genes and the future prospects of using molecular tools like RNAi for plant mediated insect resistance in chickpea.

Conventional approaches for Helicoverpa resistance in chickpea

The conventional breeding approaches and chemical control measures have been useful to create improved chickpea varieties diseases resistance like *Ascochyta* and *Fusarium* but are limited to a certain extent only for insect pests (Acharjee and Sarmah, 2013a). Chemical pesticides are commonly used to control pod borers in chickpea, but unfortunately, extensive and indiscriminate use has resulted in the development of resistance, environmental degradation (Armes *et al.*, 1992). The use of microbial pathogens and biopesticides such as Bt-products have shown some potential to control *H. armigera*, but the high production costs make them uncompetitive compared with the synthetic insecticides (Romeis *et al.,* 2004). Although, the wild relatives, *C. judiacum*, *C. bijugum* and *C. pinnatifidum* have significant levels of resistance to *H. armigera* (Sharma *et al.*, 2005), but these wild relatives are post-zygotic cross incompatible with the cultivated chickpea germplasms (Mallikarjuna, 2001). So, the genetic improvement for insect resistance has been hampered by the limited genomic resources and the narrow genetic diversity in the gene pool of chickpea have hampered breeding for protection (Varshney *et al.*, 2010; Acharjee & Sharma, 2013b). The biotechnological interventions like genetic transformation are likely to improve *H. armigera* resistance in chickpea (Acharjee and Sarmah, 2013a).

Approaches to the generation of transgenic crops using modern genetic transformation technology to incorporate insect resistance have proven suitable for many cultivated crops. *H. armigera* can be effectively controlled by using δ-endotoxin from Bt in transgenic plants, which is very well demonstrated in widely cultivated Bt crops like cotton (James, 2014). The Crystal Insecticidal Protein (CIP) toxins expressed in plants interacts with the mid-gut epithelium receptors and causes an ionic imbalance to break the mid-gut cells and insect death (Schnepf *et al.*,1998; Bravo *et al.*, 2007). The Bt transgenic plants provide a relatively long lasting and seed borne solution for the management of Lepidopteran pests (Tabashnik *et al.*, 2003). Genetic transformation with δ-endotoxin genes from the bacterium *Bacillus thuringiensis* Berliner have been deployed as a means to enhance crop resistance to the insect in several crops for pest management (Sharma *et al*., 2002; James, 2014). The Bt toxins are toxic to lepidopteran pests and non-toxic to humans and animals, which makes Bt crops are one of the most successful plant transgenic technology (BANR, 2000; Cohen *et al.*, 2000). The Bt crops which were commercialized since 1996, have revolutionized the insect pest management strategies and been widely accepted by small and resource-poor farmers and have achieved significant success economically and ecologically in the world (Zhu *et al*., 2012). The area under Bt crops has increased significantly and contributed to more sustainable crop production systems (James, 2014).

Genetic transformation: Bt chickpeas

Genetic improvement by molecular breeding is limited in chickpeas due to their sexually incompatible gene pool of wild relatives (Acharjee and Sarmah, 2013a). Genetic transformation to develop transgenic chickpea expressing toxin genes for various versions of Bt insecticidal genes has been carried out and found to confer resistance to pod borers in the laboratory bioassays (Devi *et al.*, 2011; Acharjee and Sarmah, 2013b). Commercial Bt chickpea lines with resistance to *Helicoverpa* are under development (Sanyal *et al.* 2005; Acharjee *et al.,* 2010; Mehrotra *et al.*, 2011;Asharani *et al.*, 2011; Khatodia *et al*., 2014; Ganguly *et al.,* 2014) which have not yet been released. The first report of successful genetic transformation of chickpea using Bt *cry1Ac* gene came in 1997 (Kar *et al.*, 1997) and thereafter, various research groups initiated genetic transformation of chickpea using *cry1Ac* gene and reported generation of transgenic Bt chickpeas (Sanyal *et al.*, 2005; Indurker *et al.*, 2007; Biradar *et. al.*, 2009) (Table 1). Neelima *et al.*,(2008) presents a non-tissue culture-based *in planta* transformation strategy to generate transgenic plants in chickpea with *cry1AcF* gene using *Agrobacterium*-infected young seedlings. Acharjee *et al.*, (2010) used *cry2Aa* gene to facilitate pyramiding with existing *cry1Ac* chickpea lines. The pyramided transgenic chickpea lines exhibited high levels of *Cry2Aa* and *Cry1Ac* protein and conferred high (98-100%) levels of mortality to *Helicoverpa* larvae in the insect bioassays (Acharjee *et al.*, 2010).

Mehrotra *et al.*, (2011) also generated pyramided *cry1Ac* and *cry1Ab* genes in chickpea. A new synthetic construct *cry1X* was also used forinsect resistance chickpea using *in planta* transformation (Asharani *et al.*, 2011). Ganguly *et al*., (2014) used fused *cry1Ab/Ac* construct to develop different transgenic lines of chickpea expressing constitutively and pod specifically for resistance against *Helicoverpa.* Khatodia *et al*., (2014a & 2014b) developed Bt chickpea plants carrying *cry1Aa3* and *cry1Ac* gene using direct seed *Agrobacterium*-mediated transformation which works without the involvement of any tissue culture procedure and does not require the complex steps for selection of the transgenic events.

Gene pyramiding by incorporating two or more genes may be a more efficient way of enhancing and broadening insect resistance of plants (Li *et al*., 2015). One of the major concerns regarding the development of the transgenic plant is need of expressing high dose of Bt toxin, which can sustain the insect resistance. But the transgenic chickpea lines that showed appreciable levels of expression of Bt toxin were found to exhibit phenotypic abnormalities and these abnormalities ranged from extreme retardation in the growth of the plant to no flowering, and no setting of seeds (Rawat *et al.*, 2010; Acharjee *et al.,* 2010; Khatodia *et al*., 2014). Such observations in chickpea plants have been probably overlooked earlier; however phenotypic and developmental abnormalities with the *cry1Ac* gene have been reported in tobacco (Rocher *et al.*, 1998; Barton *et al.*,1987). A significant reduction in the growth rate and seed production in chickpea lines expressing high levels of Bt toxin when compared to the parental line (Acharjee *et al.*, 2010; Khatodia *et al*., 2014). The high level of Bt toxin protein was causing growth reduction in chickpea. Although, the reasons for this detrimental effect of Bt toxin need to be analyzed.

Field-evolved resistance to Bt crops

The commercialization of transgenic Bt chickpeas containing a single Bt transgene may not give adequate yield advantage, as *H. armigera* is evolved with increased resistance.The widespread use of Bt toxins has prompted concerns that insects might someday become resistant to this important treatment, which can reduce the effectiveness of Bt transgenic crops (Tabashnik *et al*., 2013). Resistance is a genetic change in the insect pest that allows it to avoid harm from Bt toxins. Although the high and consistent levels of toxin production in the Bt plants make them much less favorable for the development of resistance. The laboratory populations of *Cry1A*-resistant Diamond Black Moth have been shown to be able to survive on high levels of Cry1Ac toxin (Tabashnik, 2003). There were no cases of insects developing resistance to Bt transgenic plants in the field initially. The frequency of resistant alleles has increased substantially because of failure to provide adequate refuges of non-Bt cotton and that there is field-evolved *Bt* toxin resistance in bollworm (Tabashnik, 2008). Intensive cultivation of Bt crops has increased field evolved pest resistance to transgenic plants in *H. armigera* in India, China, and Pakistan (Tabashnik *et al*., 2009; Alvi *et al*., 2012; Zhang *et al*., 2013). The field-evolved resistance in *H. armigera* has reduced the efficacy of Bt crops for pest resistance (Tabashnik *et al*., 2013). So, the transgenic crops expressing pyramided two or more Bt toxins to combat the same insect pest have been widely used now to delay the evolution of pest resistance (Carrière *et al*., 2015). But field-evolved resistance and cross-resistance in transgenic plants expressing two different types of Bt toxins has been discovered (Gassmann *et al*., 2014). The insect survival on currently used pyramids is often higher for both susceptible insects and insects resistant to one of the toxins in the pyramid (Carrière *et al*., 2015). The increased resistance to Bt plants suggests that the current approaches for managing Bt resistance should be replaced by new integrated pest management strategies in order to develop the sustainable resistance.

Plant-mediated RNAi for Helicoverpa resistance in chickpea

The insect resistant transgenic Bt plants have been successful to reduce yield loss and pesticide utilization in the past three decades. The potential of using plant-mediated RNAi induced by double-stranded RNAs targeting pest genes came up as a new strategy against coleopteran and lepidopteran pests resistance in crops (Zhu *et al*., 2012). Therefore, down-regulating the crucial physiology-related genes by using specific double-stranded RNAs to induce RNAi in insects, is a key in pest control, which is paving the way for next generation of insect-resistant transgenic crops (Price and Gatehouse, 2008; Huvenne and Smagghe, 2010). The concept of plant-mediated RNAi was first introduced by silencing a cotton bollworm P450 monooxygenase gene, which impairs larval tolerance of gossypol in *H. armigera* (Mao *et al*., 2007). Insect P450 monooxygenase, *CYP6AE14* play a central role in adaptation to plant defense compounds and in developing insecticide resistance (Mao *et al*., 2007). Mao *et al*., (2007) developed transgenic tobacco and Arabidopsis plants expressing double stranded RNA (dsRNA) directed against a detoxification enzyme *CYP6AE14*, which increased the sensitivity to

gossypol leading to mortality. A report of generation and analysis of *CYP6AE14*, dsRNA-expressing cotton plants by Mao *et al*., (2011) showed drastically retarded growth of bollworm larvae and less damage to the transgenic plants. The deleterious effects of RNAi will magnify if multiple genes involved in the P450 complex were targeted (Mao *et al*., 2011). Another target gene for the cotton bollworm RNAi is *CYP6B6*, which is expressed in the fat baby and midgut of the lepidopteran pest, lead to reduced resistance to pyrethroids and other toxic substances (Zhang *et al*., 2013). *HaHR3*, a molt-regulating transcription factor gene, of cotton bollworm has been used as the target gene for plant-mediated RNAi in transgenic tobacco plants resulting in developmental deformity and larval lethality (Xiong *et al*., 2013).

The plant-mediated RNAi technology often results in a mild enhancement of insect resistance (Price and Gatehouse, 2008). Two key steps of plant-mediated insect RNAi are the production of effective forms of dsRNAs in plants and spreading of these silencing molecules into gut cells of insect (Mao *et al.*, 2013). The first barrier to the food components is a midgut peritrophic matrix (PM) layer that prevents large molecules and toxins from entering into midgut cells (Hegedus *et al*., 2009). The plant cysteine proteases could increase the PM permeability and used to improve the plant-mediated RNAi against herbivorous insects (Mao *et al.*, 2013). Expression of dsRNA and protease in the plant provides a better protection as ingestion-mediated RNAi effect against herbivorous insects (Mao *et al.*, 2013).

The nucleotide variations of the dsRNA of target genes in different ecotypes of the target pest, necessitate selection of a highly conserved, off-target, minimized sequence for effective gene silencing using plant-mediated RNAi. The potential insecticidal siRNAs designed *in silico* for *H. armigera* control could be used for crop resistance by synthesizing a plant and delivering a dsRNA (Choudhary and Sahi, 2011). Asokan *et al*., (2012) designed an off-target minimized region for dsRNA synthesis and *in silico* analyzed the nucleotide variationsto design common siRNAs that could be further utilized for downstream applications for *H. armigera*. The effect of diet delivered various concentrations of dsRNA in silencing genes of *H. armigera* revealed that multiple applications of dsRNA resulted in early and persistent silencing of genes (Asokan *et al*., 2013). The chymotrypsin and jhamt were shown to be suitable candidate genes that could be utilized for RNAi-mediated management of *H. armigera* (Asokan *et al*., 2014). Although, the lethal or highly detrimental effects of down-regulating the crucial target genes of *H. armigera* by plant mediated RNAi for resistance in chickpea is yet to be studied. But the accelerated emergence of Bt resistance in *H. armigera* requires plant-mediated RNAi for pod borer resistance in chickpea, which is an alternative tool paving the way for next generation of insect-resistant transgenic crops (Gordon and Waterhouse, 2007).

Future prospects

The sustainability of Bt transgenic crops is already threatened by the accelerated emergence of insect resistance in *Helicoverpa* and Bt chickpea plants have yet to be commercialized. The plant-mediated RNAi have been demonstrated in cotton and tobacco plants using the dsRNA for the target genes (Mao *et al.*, 2007; Xiong *et al*., 2013; Mao *et al.*, 2013; Zhang *et al*., 2013). Therefore, the plant-mediated RNAi using the *in silico* designed siRNA targeting the insect genes may prove to be a very good approach for chickpea plants protection to pod borer. Common high potential insecticidal siRNAs for the target genes of *H. armigera,* designed *in silico* by analyzing the nucleotide variations of the dsRNA of target genes in different populations of the target pest are available for implementation as a pest management strategy in chickpea (Asokan *et al*., 2012). Moreover, the siRNA also reduces the biosafety concerns, being absent in higher eukaryotes, having low off target similarity (Asokan *et al*., 2012). Further, the high expression of Bt toxins in the plants will directly put a great load on the protein production machinery which will ultimately affect the quality and quantity of the Bt crops in terms of growth and development (Rawat *et al.*, 2010; Acharjee *et al.*, 2010; Khatodia *et al*., 2014). Instead, the expression of the dsRNA in the plants to combat the insect will not cause any load on the protein production machinery, which is in particular very important point for chickpea, which is the good and cheap source of proteins with high protein content in seeds.

We propose that the lethal or highly detrimental effect of down-regulating crucial target genes like CytP450 (involved in detoxification of allelo chemicals), *HaHR3* (molt-regulating transcription factor gene) and chymotripsin (involved in digestion of proteins) of *H. armigera* by plant mediated RNAi for resistance in transgenic chickpeas could be studied in future. Transgenic chickpea plants expressing dsRNA will provide the insight of detrimental effects of down-regulating crucial target genes of *H. armigera* by plant mediated RNAi for resistance in chickpea. This strategy could be taken to further advancement for field evaluation and utility in integrated insect pest management.

Conclusions

Genetic improvements of chickpea for *H. armigera* resistance by molecular breeding approaches are limited due to their sexually incompatible gene pool and insufficient to meet up the challenges of the present agricultural state (Varshney *et al*., 2010; Acharjee and Sharma, 2013a). The commercialization of transgenic Bt chickpeas containing a single Bt gene for *H. armigera* resistance may not give adequate yield advantage. This review documents that transgenic chickpeas generated with combinations of suitable genes and approaches like Bt and RNAi is required for protection from *H. armigera* damage in chickpea. The evidence suggests that transgenic plants expressing dsRNA targeting insect-associated genes are able to improve pest resistance. The plant-mediated RNAi approach allows a wide range of potential targets for suppression of gene expression in the insects and holds great promise for future development. So, feeding *H. armigera* with chickpea expressing dsRNA to trigger RNAi could find applications in field control of this insect pest. There is a need for further studies to optimize plant-mediated RNAi for chickpea protection against *H. armigera*. The integrated pest management strategies would require the use of, not only novel Bt transgenics, Plant-mediated RNAi, but also the modern biotechnological tools like targeted CRISPR/Cas-mediated plant genome editing for chickpea protection against the *H. armigera* (Khatodia *et al.,* 2016).

Figure 1. Typical symptoms of *Helicverpa armigera* infestation on chickpea plants. Showing the different stages of larval feeding on leaves and pods, which causes damage and seed abortion respectively, thereby causing major crop losses.

Table 1. The list of the various chickpea transformation made for insect resistance using Bt toxins against *Helicoverpa* pest.

Chickpea Type	Transformation Method	Explants Used	Bt Toxins	Promotors	Selectable Markers	Bt toxin Expression	Stable Integration	Lab Bioassay Mortality	Ref.
ICCV-1, ICCV-6	Biolistic Gene delivery	shoot apex	*Cry1Ac*	CaMV35S	*nptII*	0.004–0.0045%	Yes	72.6% wt reduction	Kar *et al*., 1997
C 235, BG 256, Pusa 362, Pusa 372	*Agrobacterium*	cotyledonary nodes	*Cry1Ac*	CaMV35S	*nptII*	14.5 to 23.5 ng/mg	Yes	>80% mortality	Sanyal *et al*., 2005
ICCC37, PG-12	Biolistic Gene delivery	Epicotyl	*Cry1Ac*	CaMV35S	*nptII*	6 to 20 ng/mg	Yes	13.3-56.6% survival	Indurkar *et al*., 2007
	In planta *Agrobacterium*	apical meristem	*cry1AcF*	CaMV35S	*nptII*	2.06-9.70 µg/g	No	-	Neelima *et al*., 2008
Semsen, ICCV 89314	*Agrobacterium*	embryonic axis	*Cry2Aa*	ats1A	*nptII*	-	Yes	20-98% mortality	Acharjee *et al*., 2010
KAK-2	In planta *Agrobacterium*	embryo axes	*Cry1X*	CaMV35S	*nptII*	0.257-10.77 µg/g	No	49.6% mortality	Asharani *et al*., 2011
P-362	*Agrobacterium*	embryonic axis	*Cry1Ac*	CaMV35S	*nptII*	116 ng/mg	Yes	100% mortality	Mehrotra *et al*., 2011
P-362	*Agrobacterium*	embryonic axis	*Cry1Ab* and *Cry1Ac*	CaMV35S and Pcec	*nptII*	5–40 ng/mg	Yes	86-100% mortality	Mehrotra *et al*., 2011b
C-235	In planta *Agrobacterium*	Seeds	*Cry1Aa3*	CaMV35S	*nptII*	0.091-0.154 µg/g	Yes	55-77% wt reduction	Khatodia *et al*., 2014a & 2014b
C-235, HC-1	In planta *Agrobacterium*	Seeds	*Cry1Ac*	CaMV35S	*nptII*	0.106-0.364 µg/g	Yes	48-75% wt reduction	Khatodia *et al*., 2014 a & 2014b
DCP 92-3	*Agrobacterium*	embryonic axis	Fused *cry1Ab/Ac*	soybean P*msg* and rice *actin1*	*hpt*	4–19 ng/mg	Yes	67-100% mortality	Ganguly *et al*., 2014

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