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Review

Transgenic *Bacillus thuringiensis* (*Bt*) chickpea: India's most wanted genetically modified (GM) pulse crop

Sumita Acharjee and Bidyut Kumar Sarmah*

Department of Agricultural Biotechnology, Assam Agricultural University, Jorhat - 785 013. India.

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Chickpea (*Cicer arietinum*) is grown widely in India because the seeds are rich source of protein for the vegetarian population of country. However, chickpea cultivation is declining over the period of time due to heavy incidences of pests and diseases. *Helicoverpa armigera* is a major pest in the field and non-availability of resistant varieties lead to heavy losses of yield per year. Crop management practices such as application of bio-pesticides, insecticides and integrated pest management are less effective to control this devastating pest. Breeding for development of resistant lines is restricted by lack of resistant sources within the gene pool. Therefore, application of gene technology for chickpea improvement appears to be appropriate approach for development of *Helicoverpa* resistant lines. Genetic transformation of chickpea using various versions of *Bacillus thuringiensis* (*Bt*) insecticidal genes have been carried out and found to confer resistance to pod borers in the laboratory bioassays. The most preferred genetically modified (GM) chickpea for field release is pyramided lines having two or more *Bt* genes with diverse mode of action for effective management of *Helicoverpa*. Here we discuss about the rationale for generation of *Bt* chickpea to enhance production.

Key words: Chickpea, *Bacillus thuringiensis*, genetically modified (GM) pulse crop.

INTRODUCTION

In India, farmers grow many species of grain legumes in an area of 36 million hectare (m ha) with an annual production of 29 million tonnes. Chickpea (*Cicer arietinum* L.) is the most important pulse crop gown in an area of 8.21 m ha and producing 7.48 millon tonnes of the grains (FAOSTAT, 2011). The crop is largely grown by small farmers in rain-fed areas (>70%) which are less fertile and poor in moisture retention capacity. Although, India produces about 75% of the chickpea (Rao, 2010), the production is inadequate to meet the demand of the domestic market. According to 2011 FAOSTAT statistics, India imports about 1, 85,000 metric tons of chickpea valued at US$ 94 millons. The demand for chickpea is projected to be double from 7 to 14 millon tonnes by 2020. In the next 10 years the net import of chickpea will be close to 1.5 millon tonnes to meet the domestic requirements.

During the past two decades, area under chickpea cultivation has declined in India. The factors that discourage farmers to undertake chickpea cultivation are lack of irrigation, high incidences of insect pest (predominantly, *Helicoverpa armigera*) and diseases, lack of supply of quality seeds, non-availability of drought tolerant and short duration varieties. The production constraints have led to increase in the price of pulses in general by two to three folds during the past 10 years in India. These protein rich pulses are now less affordable to average middle class Indian. Recently, the Government

*Corresponding author. E-mail: E-mail: sumita.aus@gmail.com.

Abbreviations: IPM, Integrated pest management; IRM, insect resistance management; ***Bt*,** *Bacillus thuringiensis*; **Vips,** vegetative insecticidal proteins.

of India has initiated various schemes to help pulse growers to improve production by providing subsidies on irrigation, seeds, fertilizers and other farm inputs. However, success of the government schemes depends on development and deployment for varieties resistant to biotic and abiotic stresses.

Chickpea is infected by nearly 60 insect species, of which the major damage is caused by pod borer, *H. armigera* (Hubner). It is a major pest of chickpea in Asia, Africa Australia and the Mediterranean region. Pod borers alone cause 25 to 40% of the crop loss amounting $325 million annually (ICRISAT, 1992; Sharma et al., 2005). *Helicoverpa* females lay eggs on leaves, flowers, and young pods. The larvae feed on the young leaves in chickpea and young seedlings of chickpea may be destroyed completely, particularly under tropical climates in southern India. Larger larvae bore into pods and consume the developing seeds inside the pod. The losses due to *H. armigera* magnify under drought condition. In addition, climate change may aggravate chickpea-*Helicoverpa* interaction. The results of *Helicoverpa* interaction on different crops under elevated CO_2 concentrations (550 to 750 ppm) showed a complex host-pest interaction (Wu et al., 2006; Coll and Hughes, 2008). Therefore, impact of deployment of high-yielding cultivars of chickpea to production and productivity under rain-fed conditions in India would be limited unless varieties are resistant to *H. armigera*.

MEASURES TO CONTROL *H. armigera* INFESTATION IN CHICKPEA

In order to protect the crop from *H. armigera*, various pest management practices have been adopted by Indian farmers. Efforts are being made to develop *H. armigera* resistant varieties both by conventional breeding methods as well as by using modern biotechnological tools to develop transgenic chickpeas resistant to *H armigera*.

Cultural practices, pesticides and IPM strategies

In order to reduce the survival and damage of *H. armigera*, several cultural practices are adopted such as time of sowing, spacing, fertilizer application, intercultural practices and flooding. In order to minimize extent of damage inter-cropping or strip-cropping with marigold, sunflower, linseed, mustard, or coriander is also adopted. These cultural practices are often ineffective because they are dependent on the crop husbandry practices in a particular agro-ecosystem. The chickpea trap crop is planted after the commercial crops to attract *H. armigera* emerging from winter diapause. The trap crops are destroyed before larvae commence pupation. As a result, the overall *H. armigera* pressure on summer crops is reduced, resulting in greater opportunity for adoption of soft control options, reduced insecticide use, and greater activity of the natural enemies.

The importance of biotic and abiotic factors on the seasonal abundance of *H. armigera* is poorly understood. Some parasitic wasps avoid chickpea due to dense layers of trichomes and their acidic exudates. The *Campoletis chlorideae* is an important larval parasitoid of *H. armigera* on chickpea, whereas *Trichogramma* egg parasitoids are rarely present in high numbers in India. The dipteran parasitoids *Carcelia illota*, *Goniophthalmus halli*, and *Palexorista laxa* have been reported to parasitize up to 54% of the larvae on chickpea. Predators such as *Chrysopa* spp*., Chrysoperla* spp*., Nabis* spp*., Geocoris* spp*., Orius* spp*.,* and *Polistes* spp. are common in India. Provision of bird perches or planting of tall crops that serve as resting sites for insectivorous birds such as Myna (*Acridotheris tritis)* and Drongo (*Dicrurus macrocercus)* helps to reduce the numbers of *H. armigera* larvae.

Use of chemical pesticides to control pod borers in chickpea is the most common practice, but indiscriminate use of chemicals lead to resistance development and environmental pollution (Armes et al., 1992). Integrated pest management (IPM) strategies are also being applied in order to reduce the negative effects of chemical pesticides. The IPM strategies include, timely sowing for host avoidance; intercropping with mustard, barley and linseed; use of trap crop such as *Vicia sativa* and African marigold; application of *Helicoverpa* nuclear polyhedrosis virus (HaNPV), or *Bacillus thuringiensis* (*Bt*) formulation; erection of perches; plant (Neem) bioproduct spray or limited application of chemicals like Endosulphan, Monocrotophos, Fenvelarate (Lal, 1990; Jayaraj, 1992). In India, HaNPV has been reported to be a viable option to control *H. armigera* in chickpea. However, the efficiency of the IPM strategies depends on various factors such as pest behavior, diurnal activity, weather condition, crop habitat among others. Besides, the impact of climate change could reduce the effectiveness of present IPM strategies, leading to decrease crop yield.

Wide hybridization

Breeding to transfer gene(s) conferring resistance to *H. armigera* from wild species to the cultivated species was exploited to develop resistant crop varieties. Screening of wild *Cicer* species showed resistance to *H. armigera* such as *Cicer bijugum, Cicer reticulatum, Cicer judaicum, Cicer pinnatifidium, Cicer microphyllum* and *Cicer cuneatum* (Sharma et al., 2005). It was found that the wild relatives, *C. judiacum*, *C. bijugum* and *C. pinnatifidum* have significant levels of resistance to *H. armigera* (Sharma et al., 2005, 2006), but these wild relatives were cross incompatible with the cultivated chickpea germplams. The cross incompatibility between cultivated *Cicer* and other perennial chickpeas are post-

zygotic (Mallikarjuna, 2001; Babb and Muehlbauer, 2005). Thus, cross incompatibility makes the wild relatives under-utilized in plant breeding programme.

Germplasm screening

The crop improvement to increase production and productivity depends on identification and deployment of varieties with resistance/tolerance to pests. Therefore, screening of germplasms maintained at ICRISAT Genebank (>15,000 accessions) and identification of *Helicoverpa*-resistant chickpea lines and performing varietal trails under various agro-climatic conditions is important. Moreover, understanding the molecular basis of resistance to this pest is required to formulate appropriate strategies to manage *Helicoverpa* infestation. So far, screening of the available germplams has led to identification of only moderate levels of resistance to *H. armigera* (Lateef, 1985; Lateef and Pimbert, 1990).

GENETIC MODIFICATION STRATEGIES USING *Bt* GENES

The genome of the *B. thuringiensis* constitutes genes that encode several insecticidal proteins. The insecticidal proteins that accumulate during sporulation are known as crystalline inclusion bodies (Cry and Cty proteins) and those produced during vegetative growth are known as vegetative insecticidal proteins (Vips). Both Cry and Vips proteins are toxic to Lepidoptera, Coleoptera and Diptera insects. These toxins can be expressed in chickpea for resistance to *H. armigera.* The *Bt* proteins have been used as bio-pesticides for the past 40 years and found be species specific and non-toxic to vertebrates. One of the successful applications of recombinant DNA technology to mankind is the development and deployment of transgenic crops expressing *Bt* toxins. In India, transgenic, *Bt* cotton has revolutionized the cotton industry since 2004. The *Bt* cotton has been widely accepted by small and resource poor farmers of India, hence the area under *Bt* cotton has increased significantly from 50,000 ha in year 2004 to 8.4 millon hectare in 2009 (James, 2010). A similar strategy appears to be suitable for generation of *Bt* chickpea for resistance to pod borers.

Selection of insecticidal gene

The choice of Cry toxin for expression in the field crops is critical for pest management. Expression of *Bt* Cry1Ac is most effective against *H. armigera,* however, generation of transgenic chickpea expressing *Cry1Ac* gene alone may not be suitable in terms of insect resistance management (IRM), especially in India. The farmers in India face various challenges in terms of insect management. The marginal and small farmers cannot spare their land for refuge for proper IRM of *Bt* crop. Therefore, pyramiding two or more *Bt* genes with diverse mode of action is essential to avoid resistance buildup within insect population.

The mode of action of Cry proteins has been studied extensively (Aronson et al., 1986; Hofte and Whiteley 1989; Knowles, 1994; Schnepf et al., 1998; De Maagd et al., 2000; 2003 and Bravo et al., 2004; 2007). The *Cry* toxin interacts sequentially to receptors present on the midgut epithelium and insert into membrane forming pores that cause ionic imbalance; break the midgut cells and insect death (Schnepf et al., 1998; De Maagd et al., 2003 and Bravo et al., 2004). Recently, a signal transduction model was proposed where the toxicity is due to activation of an Mg^{2+} dependent signal cascade pathway. The Cry toxin interacts with CAD receptors which lead to activation of G protein. The G protein activates an adenyly-cyclase which results in production of intercellular cyclic adenosine monophosphate (cAMP). The cAMP activates protein kinase A that starts an intercellular pathway resulting in cell death (Zhang et al., 2006).

The first set of transgenic crop commercialized in India is cotton hybrids carrying *Cry1Ac* gene of *Bt* for resistant to cotton bollworm. The cotton industry of India received heavy benefit upon introduction of *Bt* cotton in 2002. Cotton production in India before 2002-2003 was about 2.55 to 2.75 m t, but over the past five years cotton yield has increased by 50%. In the year 2006, five new events, Bollgard II, EventI, GFM Cry1A, BNLA 601 and Event 9124, of *Bt* cotton expressing Cry1Ac, Cry1Ab, Cry2Ab, Cry1C either alone or in combination were approved for release in India (GEAC, 2009). Therefore, applying genetic engineering technologies to develop *Bt* chickpeas using bacterial '*Cry'* genes could be appropriate to protect the crop from *H*. *armigera*.

Pyramiding two or more *Bt* genes such as *Cry1Ac* and *Cry2A* in chickpea could be a preferred option to delay evolution of resistant insects due to different mode of action for these two genes. However, reports suggest that baseline frequency of *Cry2Ab* resistance gene within populations of *H. armigera* (Mahon et al., 2007) is substantially higher than expected. Expressing *Cry1Ac* gene in combination with *Cry1F* gene may be effective to delay insect resistance because *Cry1Ac* in combination with *Cry1F* gives an additive effect against *H. armigera* (Ibargutxi et al., 2008). Moreover, use hybrid Cry proteins such as Cry1Ab- Cry1C also conferred resistance to lepidopteran pest, *Spodoptera exigua* (de Maag et al., 2000). Hybrid *Bt* protein containing domain I and II from Cry1Ba and domain II for Cry1IA was found effective against potato tuber moth and Colorado beetle (Naimov et al., 2003). Development of transgenic plants expressing Vips has been found more effective against many lepidopteran pests, including *H. armigera*. In the

case of maize it was found that Vip3A in combination with Cry1Ab provide complete resistance to *Helicoverpa zea* under field condition (Burkness et al., 2010). Transgenic chickpea stacked with *Bt* genes such as Cry1A along with Vip3A or hybrid *Bt* protein in combination with Vip3A, could be a suitable combination for Indian Agriculture.

The first successful genetic transformation of nuclear genome of chickpea was reported in 1997 using the *cry1Ac* gene (Kar et al., 1997). Subsequently, various research groups within India initiated genetic transformation of chickpea using *Cry1Ac* gene and reported generation of transgenic chickpeas (Sanyal et al., 2005, Indurker et al., 2007; Mehrotra et al., 2011). A second gene, *Cry2Aa*, was also introduced in chickpea to facilitate pyramiding with existing *Cry1Ac* lines (Acharjee et al., 2010). Mehrotra et al. (2011) generated pyramided *Cry1Ac* and *Cry1Ab* gene chickpea; however, pyramiding two or more genes with different mode of action is preferred.

Non *Bt* strategies for *H. armigera* control in chickpea

Exploitation of new genes is essential to avoid reliance on expression of only *Bt* endotoxin in the transgenic plants. A new strategy such as up-regulating secondary metabolites, which are toxic to or repel insects, to escape from insect damage (Gatehouse, 2002) or applying RNAi technology for insect control by silencing endogenous genes of insects could be new strategy to develop GM chickpea. A suitable candidate gene which was found to be effective was cytochrome *P450* gene (*CYP6AE14*) which expresses in the midgut of *H armigera.* Gene silencing in lepidoptera by RNAi technology have been found to be difficult to trigger which may be due to factors absence of RNA dependent RNA Polymerase orthologs (Gordon and Waterhouse, 2007) barrier in uptake of double stranded RNA (dsRNA), improper sorting of dsRNA during endosome trafficking to dsRNA-processing machinery among others. Mao et al. (2007) reported significant growth reduction of *Helicoverpa* larvae reared on transgenic tobacco and Arabidopsis. The efficacy of RNAi silencing can be enhanced by using a tobacco rattle virus vectors (Kumar et al., 2012).

CONCLUSION

Conventional methods of protecting chickpea for insect pest are inadequate to meet the challenges of the present agricultural scenario in India. The limitation of conventional technologies are lack of resistant germplams, enhanced susceptibility of high yielding varieties to pests, barriers to cross cultivated varieties with wild relatives to acquire resistant genes. In order to protect the chickpea yield from losses due to pest infestation resistant gene transfer across the sexual barriers through recombinant DNA technology is mostly preferred. However, selection of suitable gene or combination of genes for genetic modification of chickpea will remain critical to protect from *H. armigera* damage in chickpea.

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