REVIEW

Variation in the Spatio-Temporal Expression of Insecticidal Genes in Cotton

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Abstract: The most significant breakthrough in plant biotechnology is the development of the techniques to transform genes from unrelated sources into commercially important crop plants to develop resistance against targeted insect pests. The spatio-temporal expression of insecticidal genes in transgenic cotton varies with plant age, plant parts and environmental conditions. The understanding of this temporal and spatial variation in efficacy and the resulting mechanisms is essential for cotton protection and production. This review summarizes variations in the efficacy of introduced insecticidal genes in cotton crop. The factors contributing to the variability of endotoxins have also been highlighted. The reduction in Bt protein biosynthesis in late-season cotton tissues could be attributed to the overexpression of the *Bt* gene at earlier stages, which leads to gene regulation at post-transcription levels and consequently results in gene silencing at a later stage. Methylation of the promoter may also play a role in the declined expression of endotoxin proteins. In genetically modified crops several environmental factors have been reported to affect the expression of transgenes. Among environmental factors nitrogen metabolism, inhibition of synthesis, degradation, remobilization and high temperature are attributable to the quantitative reduction in Bt proteins. Applying plant growth regulators or protein enhancers such as Chaperone^{TM} may improve Bt cotton efficacy through enhancing the synthesis of proteins. Also some agronomic practices such as nitrogen fertilization and timely irrigation favour the endotoxin expression. Thus, variations in the efficacy of insecticidal genes in transgenic cotton and the involved mechanisms need to be understood fully so as to plan rational resistance management strategies to retard the rate of resistance development and to control target pests effectively by enhancing the endotoxin expression through genetic or agronomic management.

Keywords: efficacy; spatio-temporal expression; transgenic Bt cotton

Cotton is the most important cash crop and backbone of textile industry of the world. Cotton is susceptible to attack by more than 15 economically important insects, the major lepidopterans being American bollworm (*Heliothis armigera*), pink bollworm (*Pectinophora gossypiella*), spotted bollworm (*Earius insulana*/*vitella*) and army bollworm (*Spodoptera lithura*). Crop protection in agricultural systems in many developing countries relies almost exclusively on the use of broad-spectrum highly toxic agrochemicals, which has led to serious environmental problems and human health concerns, resulting in efforts towards developing its biological control measures (Bakhsh *et al.* 2009).

Cotton breeders have continuously sought to improve cotton through conventional plant breeding

which has introduced numerous improvements in crop yield during the last century. However, the resistance to insect pests and diseases does not exist in available germplasm. This has led to the limited availability of new genetic information in plants and to the creation of plant varieties with novel characters through plant breeding techniques (Hussain 2002). Current approaches to cotton improvements include the use of genetic engineering that has gained momentum in developed as well as developing countries.

One of the goals of plant breeders is to pyramid the genes expressing agriculturally desirable characteristics. This strategy has also been adopted by the biotechnologists. In order to increase the protective efficacy, the spectrum of gene activity and durability of resistance, it is envisaged that packages of different genes will be introduced into the cotton. For this purpose cotton has been genetically engineered by insecticidal genes taken from *Bacillus thuringiensis*, which is considered the most significant breakthrough to develop resistance against insect pests (Lycett & Grierson 1990; Perlak *et al.* 1990; Dhaliwal *et al.* 1998; Zhao *et al.* 2001).

Genes from *B. thuringiensis* encode for crystal proteins which are toxic to larvae of different insects, e.g. *Lepidopteran* (Höfte & Whiteley 1989; Cohen *et al.* 2000), *Coleopteran* (Krieg *et al.* 1983; Herrnstadt *et al.* 1986) and *Dipteran* insects (Andrews *et al.* 1987). Bt cotton is considerably effective in controlling lepidopteran pests, and is highly beneficial to the grower and the environment by reducing chemical insecticide sprays and preserving the population of beneficial arthropods (Gianessi & Carpenter 1999; Tabashnik *et al.* 2002).

The mechanism of endotoxins to kill the targeted insect is actually the action of the Bt Cry proteins involving the solubilisation of crystal protoxins in the insect midgut when ingested by larvae, their conversion to active toxin proteins which then bind to specific receptors in the midgut region. Toxin binding in susceptible insects disrupts the midgut epithelium, thereby causing overall toxic effects and ultimately resulting in the death of the larvae (Kranthi *et al.* 2005).

Transgenic cotton expressing Bt (*Bacillus thuringiensis*) toxins is currently cultivated on a large commercial scale in many countries, but data has shown that it behaves variably in toxin efficacy against target insects under field and greenhouse conditions (Benedict *et al.* 1996; Chen *et al.* 2000; Greenplate *et al.* 2001; Kranthi *et al.* 2005; Olsen *et al.* 2005; Adamczyk *et al.* 2009; Bakhsh *et al.* 2010). The understanding of temporal and spatial variation in efficacy and of resulting mechanisms is essential for cotton protection and production.

Spatio-temporal expression of insecticidal genes

The expression of toxin protein in adequate quantities in appropriate plant parts at the requisite time of the season is important to ensure protection against major target insect pests to contain sustainability. A number of studies conducted in many countries have indicated that the levels of Bt protein in cotton tissues fluctuate during the growing season, and may logically cause variation in the tolerance of Bt cotton to lepidopteran pests (Benedict *et al.* 1996; Chen *et al.* 2000; Greenplate *et al.* 2001; Mahon *et al.* 2002; Kranthi *et al.* 2005). Nearly all transgenic crops around the world utilize the CaMV 35S promoter (Odell *et al.* 1985) (or similar promoters from closely-related viruses) to drive transgenes.

Fitt *et al.* (1998) reported that cotton plants carrying *Cry1Ac* showed a significant decline in efficacy against *Helicoverpa* sp. during the growing season, particularly from flowering onwards (Greenplate *et al.* 1998). It was reported that toxins to *Helicoverpa armigera* in leaves of a commercial Chinese cotton variety GK-12 that contains *Cry1Ac* were significantly decreased as the crop approached maturation (Wu *et al.* 2003), while insecticidal protein levels in GK-19, a transgenic Bt cotton cultivar carrying a *Cry1Ac*/*Cry1Ab* fused gene, were higher during the early stages of cotton growth and significantly declined hereafter, behaving more variably than in GK-12 during the whole period of cotton growth and development (Wan *et al.* 2005).

Season-long differences in the expression of *Cry1Ac* among cultivars can vary as much as twofold throughout the growing season (Adamczyk *et al.* 2001; Adamczyk & Sumerford 2001; Greenplate *et al.* 2001). Resistant power to the targeted bollworm in Bt transgenic hybrid cotton remained only for 110 days, after which the crop was exposed to bollworm attacks. The *Cry1Ac* level declined as the plant grew and was found to drop below its lethal level of 1.9 µg/g within 110 days after sowing (Kranthi *et al.* 2005).

Many researchers reported that it seems to be a common phenomenon that the efficacy is relatively

high in the early growing season, but significantly declines during the late season for most commercialized Bt cotton varieties (Greenplate 1999; Greenplate *et al.* 2000; Xia *et al.* 2005).

Chen *et al.* (2000) reported that Bt protein concentrations also differ in different parts of the plant. The toxin protein content in fully expanded leaves was significantly higher than those in roots, stems and petioles during the seedling stage, while ovaries at anthesis expressed considerably more toxin protein than pistils and stamens at the flowering stage. It was further indicated that in a seven- to nine-leaf stage plant, fully expanded leaves on the main stem were considerably higher than older basal leaves in the toxin protein concentration, while young leaves near the stem terminal expressed the lowest levels of toxin proteins.

Olsen and Daly (2000) concluded that not only there is less Bt protein in older plants but also it appears that the protein is either less available or less toxic to neonates. The concentration of *Cry1Ac* protein, as a proportion of total protein, also declines during the season (Holt 1998).

Guo *et al.* (2001) introgressed transgenic cotton lines containing *Bt* genes among each other as well as with nontransgenic conventional lines. The efficacy of transgenic lines against bollworms was evaluated at different growing stages and it was found out that there was a declining level of efficacy with the plant age as the mortality (%) of *Helicoverpa* and Bt toxin protein level decreased gradually.

Kranthi *et al.* (2005) reported that in Bt transgenic hybrid cotton containing *Cry1Ac*, the leaves were found to have the highest levels of *Cry1Ac* expression followed by squares, bolls and flowers. The toxin expression was the lowest in the ovary of flowers and rinds of green bolls which are the most favoured sites of bollworm attack.

Mahon *et al.* (2002) reported that endotoxin protein concentrations in Bt cotton plants decline markedly after squaring, and molecular analyses pinpointed the change to the production of corresponding mRNA. Finnegan *et al.* (1998) found out that *Cry1Ac* levels decreased consistently throughout the growing season, and concluded that part of the decline in *Cry1Ac* expression was related to a reduction in the levels of mRNA production.

Olsen *et al.* (2005) reported that the developmental decline in bioefficacy in field-grown plants was associated with reduced *Cry1Ac* transcript levels and Bt toxin levels in post-squaring cotton. Changes in the efficacy of Bt toxin were attributed to changes in plant chemistry associated with the maturation of the cotton plant.

Xia *et al.* (2005) investigated the changes of *Bt* gene and its expression at different developmental stages at DNA, mRNA and protein levels in the R_4 generation of GK139-20 insect-resistant transgenic cotton cultivar. It was found out that the expression of Bt toxin gene was in a temporal or spatial manner, and the content of Bt crystal protein in the same tissue decreased along with the growth of the transgenic cotton plants because of a decrease in full-length Bt toxin gene transcripts. The overexpression of Bt toxin gene at earlier stages led to the gene regulation at the post-transcription level and contributed to the consequent gene silencing that was developmentally regulated. The lower expression level of Bt insecticidal gene at late developmental stages correlated with changes in the methylation state of the 35S promoter region.

Manjunatha *et al.* (2009) studied four Bt cotton hybrids (MRC-7201, MRC-6918, RASI XL-708 and SP-11) for *Cry1Ac* protein profiling in fully opened terminal leaf, first terminal pre-candle square and first terminal bolls at 80-90, 110-115, 135-140 and 150-165 days after sowing. The results indicated that the expression of *Cry1Ac* declined over the season independently of the genotype. The expression level was different among the hybrids.

Adamczyk *et al.* (2009) developed a method to determine if differences in the overall level of *Cry1Ac* among Bollgard lines could be correlated with the level of mRNA transcripts using a quantitative real-time polymerase chain reaction (qPCR). The commercial cultivars of Bollgard cotton used in this study differed in the amount of expressed *Cry1Ac* protein. They found out that *Cry1Ac* mRNA transcript differed among Bollgard lines and was correlated with corresponding *Cry1Ac* protein levels. However, they also mentioned that the plant mechanism for which this occurs is still unknown.

Bakhsh *et al.* (2010) studied the spatio-temporal expression of two insecticidal genes (*Cry1Ac* and *Cry2A*) in transgenic cotton. The quantitative levels of both *Cry1Ac* and *Cry2A* genes were found variable among the cotton lines and also varied between different plant parts. The maximum endotoxin expression was found out in leaves of Bt cotton followed by squares, bolls, anthers and petals. The toxin level in fruiting parts was lower compared to other parts showing inconsistency in the toxin level in spite of using the constitutive 35S CaMV promoter.

The commercial cultivars of Bollgard cotton I and II differ in the amount of expressed *Cry1Ac* protein. Many researchers have correlated the variation in Bt protein expression with the increased survival rates of *H. armigera* (Holt 1998; Adamczyk & Gore 2004; Kranthi *et al.* 2005; Olsen *et al.* 2005; Wan *et al.* 2005).

Factors contributing to endotoxin variability

Several factors might be responsible for variation in the gene expression level, e.g. change in the nucleotide sequence of the gene, type of promoter, and the insertion point of the insert/cassette in the DNA of the transgenic variety, transgene copy number, internal cell environment, as well as several external factors in the environment (Hobbs *et al.* 1993; Guo *et al.* 2001; Rao 2005). Therefore, investigations at molecular, genetic and physiological levels should help in understanding the differential expression of transgene and the quantitative changes in insecticidal proteins in Bt cotton plants.

The full expression of transgene(s) in a transgenic cotton variety is crucial to agricultural production, but the expression levels of a gene in the transgenic crop may decrease as the age of the crop advances, vary between young and older parts such as the leaves or between comparable parts in vegetative and reproductive phases. Factors such as soil characteristics, rainfall, severity of pests and diseases, and adequate, appropriate and timely farming management have direct or indirect influences on the performance of the crop and may affect the expression of the transgenes. All these factors, inherent in the varieties and the environment, vary from crop season to season, making the difference between optimal or suboptimal performance of a crop or its failure (Rao 2005).

Regulation can occur at many different stages of gene expression and can be particularly important during transcription. The promoters that drive the transgene expression ensure this control (Buchanan *et al.* 2000). Nearly all transgenic crops around the world utilize the CaMV 35S promoter (Odell *et al.* 1985) (or similar promoters from closely-related viruses) to drive transgenes. It is only now becoming clear that this promoter is not as robust as laboratory and glasshouse studies have suggested and its function is influenced by as yet undefined physiological and perhaps environmental factors (Sunilkumar *et al.* 2002).

Nilsson *et al.* (1992) transformed hybrid aspen with fused bacterial *luxF2* gene under 35S CaMV promoter via *Agrobacterium tumefaciens.* The luciferase expression in all transformants was determined by destructive enzymatic assay as well as by non-destructive image analysis in leaves left attached to intact plants. Variation in the luciferase expression was found out by both measurement techniques. It was found out that the enzymatically assayed luciferase activity in leaves was notably lower in transgenic hybrid aspen plants than in tobacco plants transformed with the same vector. This was not due to a difference in the luciferase enzyme activity between the two species, and therefore it indicated that the 35S promoter is not as active in hybrid aspen as in tobacco.

Wessel *et al.* (2001) reported the variation in expression patterns of three promoters (*Cauliflower Mosaic Virus* (CaMV) 35S, modified CaMV 35S and the promoter of an *Arabidopsis thaliana* Lipid Transfer Protein gene) using a firefly luciferase reporter system. The expression of luciferase gene varied not only among independent transformatants but also between leaves on the same plant and within a leaf. Furthermore, this spatial and temporal expression was also inherited in the next generation.

Sunilkumar *et al.* (2002) studied the expression profiling of 35S promoter using the green fluorescent protein (GFP) gene as a reporter system in cotton during embryo development, and in all the vegetative and floral cell and tissue types. The levels of promoter activity observed in all cell and tissue types in the hypocotyl, cotyledon, stem, leaf, petiole, and roots suggested that the expression of the 35S promoter was developmentally regulated being expressed in most cell and tissue types in cotton albeit at different levels (Pauk *et al.*1995; Haddad *et al.* 2002; Yang & Christou 2005; Bakhsh *et al.* 2010).

We must rely on conventional breeding and selection to solve these problems of variable efficacy of transgenic cotton, but it will be useful, in the longer term, to identify other gene promoters that can drive the strong expression of transgenes throughout the season. It is also important to have such promoters available for the next generation of transgenic cotton so that different traits can be stacked without relying on the same promoter so as to avoid transcriptional gene silencing induced by multiple copies of a single promoter such as the CaMV 35S promoter (Fagard & Vaucheret 2000).

Rochester (2006) evaluated the impact of crop nutrition, plant population density, light intensity,

water management, herbicide application, soil fertility, plant growth regulator application, and cotton cultivars on *Cry1Ac* protein expression in field and glasshouse experiments. The results showed that cultivars are the major source of variation in the leaf *Cry1Ac* protein expression, as suggested earlier (Adamczyk *et al.* 2001). A considerable variation among individual plants of a cultivar was found out. The *Cry1Ac* protein expression was found higher in older leaves as compared to younger ones. These results are in contradiction with the results obtained by Greenplate (1999), Chen *et al.* (2000), Karanthi *et al.* (2005) and Bakhsh *et al.* (2010). They further showed that treatment effects were often more evident in older than in younger leaves. Waterlogging, shading, herbicide application or plant growth regulator application did not significantly affect the leaf *Cry1Ac* protein expression, while severely wilted plants exhibited reduced *Cry1Ac* expression. Severe conditions affecting cotton growth and development or plant survival, such as drought or sodic/saline soil, had a reducing effect on the *Cry1Ac* protein expression.

Thirteen commercial varieties of transgenic *Cry1Ac Bacillus thuringiensis* Berliner (Bt) cotton were examined across two sites known for potential factors that impact endotoxin expression. Two varieties (NuCOTN 33B and DP458B/RR) having the same parental background (DP 5412) were found to express *Cry1Ac* at significantly higher levels compared to the 11 other *Cry1Ac* varieties. The data strongly suggested that the parental background has a stronger impact on the expression of *Cry1Ac* than the environment (Adamczyk *et al.* 2001, 2004; Rochester 2006). These results are in contradiction with the results obtained by Kranthi *et al.* (2005), who tested eight commercial hybrids and concluded that the variability in endotoxins expression was independent of genotypes. Furthermore, Adamczyk and Meredith (2006) showed that cultivars could be selected for the highest overall amount of *Cry1Ac* in addition to desired agronomic traits by using forward breeding (i.e. Bollgard cultivars crossed with Bollgard cultivars).

Olsen *et al.* (2005) reported that a reduction in *Cry1Ac* transcripts was most likely due to the failure of 35S promoter in post-squaring cotton rather than developmentally induced post-transcriptional gene silencing, as two other transgenes under the same promoter also showed a decline in the transcript level post squaring. The expression level of a gene may be influenced by its copy number (Hobbs *et al.* 1993; Agaisse & Lereclus 1995; Guo *et al.* 2001; Rao 2005).

Influence of the environment

In genetically modified crops several environmental factors have been reported to affect the expression of transgenes such as water stress in Bt maize (Traore *et al.* 2000) or nitrogen deficiency (Bruns & Abel 2003), transgenic petunia that contains the gene encoding a dihydroflavonol reductase by high light intensity and temperature (Meyer *et al.* 1992), transgenic tomato that carries a gene encoding polygalacturonase and pectin methylesterase by high temperature (Lurie *et al.* 1996), and transgenic peas containing a seed-specific α-amylase inhibitor by water deficit (Sousa-Majer *et al.* 2004).

Although the molecular mechanism of the differential expression of *Cry* genes in Bt cotton is not fully documented, a number of studies have indicated a close relationship between levels of Bt toxin and the nitrogen as well as carbon metabolism. Nitrogen is an essential nutrient for cotton production, but the traditional rate of nitrogen fertilization for cotton may not have been optimized for the Bt transgenic cotton. It is assumed that the pattern of allocation to defensive compounds depends on the relative availability of carbon and nutrients as well as their relationship with the plant growth rate (Bryant *et al.* 1983). In Bt transgenic cotton, the production of toxin protein was affected by an interaction between CO_2 and nitrogen, and elevated CO_2 decreased N allocation to Bt toxin (Coviella *et al.* 2002). A significant correlation of Bt toxin concentration with whole-plant N concentration in Bt transgenic maize was reported by Bruns and Abel *et al.* (2003).

The increasing levels of available N may increase protein levels in plant tissues, especially in vegetative tissues in most plant species including cotton (Tisdale & Nelson *et al.* 1975). The increased protein is mostly in the form of enzymes and can be used for further growth and development. Therefore, as availability of N to the plant is increased, greater quantities of the endotoxin-synthesizing enzymes and/or mRNA are likely produced, thus greater quantities of the Bt toxin protein will be synthesized (Bruns & Abel 2003).

The leaf tissue with low chlorophyll does not fully express *Cry1A* (Abel & Adamczyk, 2004). It was further suggested that photosynthesis-regulating factors related to mRNA transcription and trans-

lation should have effects on *Cry1A* production and insect control. Pettigrew and Adamczyk (2006) found out that through applying various rates and sources of nitrogen fertilization to three commercial cotton varieties including two transgenic varieties, the rates of nitrogen fertilization did not increase lint yield but produced higher leaf chlorophyll concentrations and increased the expression of the Bt endotoxin protein.

The foliar applications of Chaperone^{TM}, a plant growth regulator, greatly improved late-season endotoxin levels through the enhancement of the protein status in Bt cotton, particularly in the squares, which resulted in increased mortality of neonate bollworms feeding on the treated plants (Oosterhuis & Brown 2003, 2004). It was further suggested that Chaperone^{TM} appears to increase the protein concentration and efficiency of endotoxin expression even at high-temperature stress (Brown & Oosterhuis 2003).

A decline in endotoxin proteins in cotton tissues may also result from degradation of proteins or remobilization (translocation) of total N for further growth and development. It was suggested that a high temperature might result in degradation of total and endotoxin protein in the leaf, and thus it might reduce the amount of the toxin protein and Bt cotton efficacy (Chen *et al.* 2005).

The reduced levels of the toxin protein in early-planted cotton leaves were presumably caused by remobilization of the leaf N to support the larger developing boll load compared with late-planted cotton (Pettigrew & Adamczyk 2006). Rui *et al.* (2005) detected a large amount of endotoxin protein in the xylem sap of Bt cotton plants and leaves of non-Bt cotton plants that were grafted to the Bt plant, respectively, providing strong evidence that the endotoxin protein is transportable. A significant decline in glutamic-pyruvic transaminase (GPT) activity and soluble protein contents was found out suggesting that a high temperature may result in the degradation of soluble protein in the leaf, with a resulting decline in the level of the toxin *Cry1A* (Chen *et al.* 2005).

CONCLUSION

The spatio-temporal expression of insecticidal genes in transgenic cotton against targeted insect pests varies with plant age and plant parts. Results from different groups also suggest that it is considerably affected by environmental stresses, such as high temperature, heavy drought, water logging, elevated CO_2 concentration and nitrogen deficiency. This decline in the efficacy of insecticidal genes has been a major concern for adopting Bt cotton, because it not only increases the costs of pest control, but also it may lead to the development of resistance in pests to the transgenic cotton.

The mechanisms of variation in endotoxin protein content in plant tissues are rather complicated. The reduction in Bt protein content in late-season cotton tissues could be attributed to the overexpression of the *Bt* gene at earlier stages, which leads to the gene regulation at post-transcription levels and consequently results in gene silencing at a later stage. Methylation of the promoter may also play a role in the declined expression of endotoxin proteins. This has triggered research in finding possible new promoters that will induce the more consistent production of insecticidal genes throughout the life of the cotton plant. Therefore, efforts should also focus on evolving new transgenic cotton varieties with tissue-specific promoters to enhance the expression of toxin genes in fruiting parts that are susceptible to attack.

In genetically modified crops several factors have been reported to affect the expression of transgenes. Internal metabolic factors like nitrogen metabolism, inhibition of protein synthesis, degradation and environmental factors such as high temperature, heavy drought, water logging, elevated CO_2 concentration and nitrogen deficiency are attributable to the quantitative reduction in Bt proteins. According to the results shown by researchers, applying plant growth regulators or protein enhancers such as Chaperone^{TM} may improve Bt cotton efficacy through enhancing the synthesis of proteins. Also some agronomic practices such as nitrogen fertilization and timely irrigation favour the endotoxin expression.

Thus, variations in the efficacy of insecticidal genes in transgenic cotton and the involved mechanisms need to be understood fully so as to plan rational resistance management strategies to retard the rate of resistance development and to control target pests effectively by enhancing the endotoxin expression through genetic or agronomic management. It can be concluded that development of new cotton varieties with more powerful resistance, application of certain plant growth regulators and maintenance of general health of the transgenic crop are important in realizing the full transgenic potential in transgenic Bt cotton.

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