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Review

Insect bioassay techniques for screening Bt transgenic cotton plants

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ABSTRACT

Development of efficient insect bioassays is imperative for rapid screening of Bt cotton for insect resistance. The transgenic cotton plants are assessed for insect resistance using part of the plant only ie leaf disc or detached leaf assays. This is the non-destructive method most commonly adopted for screening of transformed plants for insect resistance in early generations. Whereas bioassay using whole plants is the destructive method mainly used for screening further progeny plants since it reflects field condition. However these bioassays are time consuming, often laborious and require a lot of space which make them a hard choice for testing the efficacy of Cry toxins against target pests in greenhouse and large scale field studies. Screening of transgenic cotton plants using efficient bioassay techniques leads to the design of effective tools for controlling insect pests.

Keywords: Seedling bioassay; transgenic cotton; insect resistance

INTRODUCTION

Cotton is an important cash crop playing a key role in the economy of Indian farmers. The use of genetically modified cotton that expresses an insecticidal protein derived from *Bacillus thuringiensis* (Bt) is revolutionizing global agriculture. The introduction of transgenic Bt cotton hybrids and expressing Cry1Ac and Cry2Ab δ-endotoxins has been reported to be highly effective against bollworm complex of cotton. Though Bollgard (BG-I) cotton expressing Cry1Ac is still effective against bollworms; gradually it is being replaced by Bollgard II (BG-II) expressing dual genes (cry1Ac + cry2Ab) for integrated resistance management (IRM) (Hallad et al 2011).

In the process of development of Bt transgenic plants it is imperative to evaluate the efficacy of Cry proteins expressed by transformed plants against target pests using a quick, reliable and sensitive method for the determination of insect resistance (Kranthi and Kranthi 2000) as large number of transformed events need to be screened to identify a single best event.

GM crops and their products can be identified by detecting either the inserted genetic material at DNA level, the mRNA transcribed from the newly introduced gene, the resulting protein, metabolite or phenotype. The screening methods are generally carried out by PCR for detecting the inserted DNA, immunological assays detecting the resulting protein or using bioassays to detect the resultant phenotype. Analytical methods for detection must be sensitive and reliable enough to obtain accurate and precise results (Tripathi 2005). Though these techniques are very effective in identifying transgenic plants ultimately bioassay is the most important screening step that gives the original efficacy of the transformed genes against target pests. Thus the need exists for a sensitive and specific method for detecting the Bt toxin.

Several bioassay techniques (leaf disc method, detached leaf assay, leaf bits, whole plant bioassay, square, flower and fruiting parts bioassay etc) have been successfully employed to study the level of insect resistance in transformed cotton plants (Adamczyk and Gore 2003, Kranthi et al 2005, Rashid et al 2008, Mogali et al 2011). Each of these methods has its own advantages and disadvantages. These techniques typically used either leaf bioassays (detached leaves from young plants) which typically have the highest Bt protein expression or whole plant bioassays under green house conditions. These bioassays help in evaluating efficacy of the plants against target pests.

Bioassays used for screening of transgenic plants

Non-destructive methods

Leaf disc bioassay: In leaf disc bioassay method leaf discs of appropriate diameter excised from young leaves are placed on agar containing antimicrobials in a Petri dish with moistened filter paper (Rashid et al 2008). In leaf disc bioassay desiccation of leaf discs takes place and leaves from the transformed plants need to be replaced with a fresh leaf at least once in two days which would be a constraint for researchers who assay young plants having four to five leaves (Kranthi and Kranthi 2000, Adamczyk et al 2008, Hallad et al 2011). This method is laborious because cutting leaf discs of appropriate size consumes more time. Moreover browning of leaves at cut ends occurs due to accumulation of some secondary metabolites like phenolics and terpenes which may interfere with actual toxicity of Cry proteins and produce inaccurate results. Olsen and Daly (2000) observed that there were many physiological changes in plants that contribute to plant-toxin interactions in cotton. Another drawback of the assay is the degradation of Cry protein (Cry1Ac) after tissues are detached from the plant which produces inaccurate results (Rashid et al 2008).

Detached leaf bioassay: In this method fully expanded leaves are detached from the transgenic plants and washed with distilled water. The leaves are wiped clean of all dirt and other debris and are placed in Petri plates on moist filter papers. Larvae of *H armigera/S litura* are placed on each leaf. Petri plates are covered with lids and sealed with parafilm to avoid escape of larvae. Leaves are replaced with the fresh ones daily. Observations are recorded daily on mortality and growth of the larvae. Screening of large number of plants is laborious and requires lot of laboratory space (Pushpa et al 2013, Ullah et al 2014). For screening cotton plants using this method the plants need to be raised on pots in greenhouse. Hence this method has drawbacks like more input and maintenance cost for potting and maintaining the plants under greenhouse conditions. Moreover there is need to wait until 45 days for testing plants for insect resistance.

Leaf bit bioassay: Mogali et al (2011) standardised a bioassay technique which consists of a leaf bit measuring about 10 mm^2 taken from the transformants placed on agar in a transparent glass vial. The vial is then sealed tightly with sterile cotton to avoid contamination. Though this technique is reliable; obtaining leaf bits and measuring mortality individually is laborious. Leaves are excised from 60 days old transgenic cotton plants. Two ml of 2 per cent agar-agar is poured into small glass vials. Vials are tightly plugged with sterile cotton. A leaf bit measuring about 10 mm^2 is taken from the transformant and is placed on to the agar contained in a transparent glass vial. A second instar larva is placed with the help of a moistened camel hair brush carefully on to the leaf disc. The mouth of the vial is then sealed tightly with sterile cotton plug to avoid contamination which also facilitates aeration. The vials are kept for 14/10 h photoperiod at 28°C temperature with 60 per cent relative humidity. The larval mortality and the damaged leaf in each vial is scored and compared with the control plants after 24, 48 and 72 h.

In-planta/destructive bioassays

Whole plant bioassay: For insect assays with whole plants transgenic greenhouse-grown cotton lines producing Cry proteins are exposed to neonate larvae. This technique was found to give better results because it overcomes the problems associated with detached leaf and leaf disc. Main limitations of this technique are: it takes long time (2-3 months) to evaluate the efficacy of Cry toxins against target pest and requires large area to raise transgenic plants in pots under greenhouse conditions (Rashid et al 2008) which in turn increases the labour requirement and maintenance cost.

Square/boll bioassay: Insect bioassays using leaves of transformed plants are more appropriate where the test insect is a leaf feeder as is the case with the tobacco budworm, *Heliothis virescens* (Fabricius) in the USA. In India where the cotton bollworms are primarily boll feeders and do not prefer feeding on leaves of cotton bioassays using these as test insects often lead to inconsistent results (Kranthi and Kranthi 2000). Hence bioassay on squares and bolls is most reliable and sensitive method. In this method small plastic cups and pet jars are used for squares and bolls. The squares from both BG-I and BG-II cotton hybrids of RCH-2 and Bunny along with non Bt versions are placed in the jar/ cup having 0.5 per cent solidified agar solution at the bottom for maintenance of moisture. The lid of the jar/cup is closed tightly after releasing the larvae at the rate of one per square. The mortality of the larvae at 24 hours interval till three days is recorded. The bioassay is carried out in laboratory at 60, 90 and 120 days after sowing (DAS) by squares feeding method for fourth instar larvae of *E vittella* (Somashekara et al 2011).

Kranthi et al (2005) conducted laboratory bioassays using different plant parts (leaves/squares/flowers/bolls) of Bt and non-Bt cotton hybrids with *H armigera*. *H armigera* larvae were placed on each of the plant parts individually in plastic cups. The plant parts were changed each day for seven days until the end of the bioassay. In order to perform these bioassays the transgenic plants need to be grown in the greenhouse until they reach the ûowering or fruiting stage (3-4 months) and also incur lot of space.

Seedling bioassay: Seedling bioassay technique can be used effectively for rapid and early screening of resistant events from large number of transformants. This method insures and shortens the time needed for screening of Bt plants. This method is very simple, cheap and rapid and requires less space and time (4-15 days) to screen the transgenic plants for insect resistance. Main drawback of this method is that this bioassay can be used only for evaluating segregating generations because the putative transformants cannot be sacrificed. Using the seedling bioassay method the seedlings of transgenic cotton Bollgard II and Widestrike (at two true-leaf stage) were evaluated for resistance to feeding by neonate and late fourth instar saltmarsh caterpillars, *Estigmene acrea* (Drury). The Bollgard II and Widestrike varieties were resistant to neonate saltmarsh caterpillars killing 100 per cent with no visible damage after 3 days of exposure (Kesey and Kerns 2010).

Diet incorporation of Bt transgenic leaves and Bt cotton seed powder

In this method the efficacy of transgenic cotton (Bollgard®, (Bollgard II® and WideStrike™) was evaluated by incorporating lyophilized Bt cotton leaf tissue into a meridic diet. The mortality and developmental parameters of fall armyworms, *Spodoptera frugiperda* (JE

Smith) were observed (Armstrong et al 2011). Similarly in another method reported by Selvi et al (2012) Bt cotton seed powder was incorporated into meridic diet and the efficacy of six Bt cotton hybrids viz NCEH 2R Bt, NCEH 3R Bt, NCEH 6R Bt, NCEH 13 Bt, NCEH 14 Bt, NCEH 21 Bt containing fusion gene cry1Ac-1Ab, Bollgard I (Mallika Bt– cry1Ac), Bollgard II (RCH-2 Bt – cry1Ac + cry2Ab), NCEH 2R non-Bt and NCEH 3R non-Bt was evaluated against *S litura* neonate larvae. This method is useful to screen large number of events within a short period of time and another advantage of this bioassay is that it takes less space. Moreover for testing cotton plants with this bioassay smaller amount of plant material is needed as compared with other bioassays. But the bioassay method may suffer from disadvantages of being need to wait until it reaches certain stage.

CONCLUSION

Leaf level insect feeding bioassays (leaf disc method, detached leaf assay and leaf bit method) are most commonly adopted for screening putative transformants (T_0) with insect resistance genes in early generations. Whereas bioassay using seedlings, whole plants, seedlings, flowers, squares and bolls are mainly used for screening T_1 and other segregating generations.

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