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Böceklere Karşı Dayanıklı cry1Ac ve cry2A Gen Taşıyan Transgenik Pamuğu İle İlgili Çalışmaları

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Özet

Bitki genetik mühendisliği spesifik ihtiyaçlarını karşılamak amacıyla farklılığı yaratmak için yeni yollar açmış olmaktadadır. Bir yerel pamuk çeşidi CIM-482 Agrobacterium tumefaciens'nın LBA4404 hattı kullanarak transform edilmiştir. Agrobacterium hattı recombinant ikili vektör pk2Ac içermekte olup, cry1Ac ve cry2A genleri 35S promoter ile barındarmıştır. 50 mg/l kanammisin Neomisin fosfotransferaz (nptII) genin seleksiyon için kullanılmıştır. Ayrıca, kullanılan iki böcek öldürücü genin (cry1Ac & cry2A) entegrasyon ve ekspresiyon kontrol edilmiştir. Transgenik bitkiler kontrol etmek amacıyla pamuk kozası solucanlara özellikle Amerikan koza solucana (Heliothis armerigera) karşı denemeler 3 yıl sürdererek direnç seviyesini değerlendirilmiştir. Morfolojik ve agronomic olarak yüksek özellikler gösteren ve böceklere karşı yüksek direnç gösteren bitki çeşitler seçilmiştir. Hedef böceklerle muamele sonucunda transgenik bitki hatlarda önemli seviyede direnç görülmüştür. Heliothus larvaların ölüm oranı (%) belirlemek amacıyla transgenik hatların etkinliği incelenmiş olup, laboratuvarda biyotoksisiti denemeler de yapılmıştır. Transgenik hatların çoğununda hedeflenen böceklere karşı %70 ile 100 dirençliği tespit edilmiştir. Sonraki jenerasyonlarda, tüm aktarılmış genlerin stabil olduğu tespit edilmiştir.

Anahtar Kelimeler: Böceklere karşı direnç, pamuk transformasyon, tarla performansı, Bt pamuğu

Insect Resistance Studies of Transgenic Cotton Cultivar Harboring cry1Ac and cry2A

Abstract

Plant genetic engineering has opened new avenues to modify crops and provided new solutions to solve specific needs. A local cotton cultivar CIM-482 was transformed through Agrobacterium tumefaciens LBA4404. The Agrobacterium strain contained the recombinant binary vector pk2Ac harboring cry1Ac and cry2A under 35S promoter. Neomycin phosphotransferase (nptII) gene was used as a selectable marker at a concentration of 50 mg L–1. Furthermore, advance generations of transgenic lines developed out of this transformation event expressing two insecticidal genes (cry1Ac & cry2A) were confirmed for the integration and expression of introduced genes and were evaluated for the resistance against cotton boll worms especially american boll worm (Heliothis armerigera) under field condition for three consecutive years. Homozygous lines showing high insect resistance, morphological and agronomic characteristics were selected. Transgenic lines showed significant resistance levels when subjected to artificial infestation of targeted insect pests. Laboratory biotoxicity assays were also performed to evaluate the efficacy of insecticidal genes against targeted insect pests by calculating the mortality %age of Heliothis larvae. Most of the transgenic lines showed upto 70-100% resistance against targeted insect pests. All the characters were stably inherited in advance generations.

Key Words: Insect resistance, cotton transformation, field performance, Bt Cotton

INTRODUCTION

Cotton is the most important cash crop and backbone of textile industry of the world. Likewise Pakistan is the fifth largest producer of cotton in the world, the third largest exporter of raw cotton, the fourth largest consumer of cotton,
and the largest exporter of cotton yarn [1]. Cotton is the crop being susceptible to attack by more than 15 economically important insects, the major lepidopteron being, american boll worm (*Heliothis armigera*), pink boll worm (*Pectinophora gossypiella*), spotted boll worm (*Earius insulana/vitella*), army bollworm (*Spodoptera lithura*) . Cotton breeders have continuously sought to improve cotton through conventional breeding plant breeding which has introduced numerous improvements in crop yield during past centuries. However, resistance to insect pests and diseases does not exist in available germplasm; this has led to a limit in availability of new genetic information into plants and to create plant varieties with novel characters through plant breeding techniques [2].

Bacillus thuringiensis (*Bt*) is perhaps, the most important source of insect resistant genes. Genes from *B. thuringiensis* encode for crystal proteins, which are toxic against larvae of different insects, *e.g.* Lepidopterans [3, 4, 5] and Dipteran insects [6]. Bt cotton is considerably effective in controlling lepidopterans pests, and is highly beneficial to the grower and the environment by reducing chemical insecticide sprays and preserving population of beneficial arthropods [7, 8]. A variety of issues regarding risk assessment i.e. the effect of transgenic crops on non target insects, horizontal gene flow, vertical gene flow and development of resistance against toxins in targeted insect pests must be considered when developing insect resistant transgenic plants.

It is recommended that lines expressing two insecticidal genes should be released in environment to prolong the resistance development time [9]. Studies have shown that *cry1Ac* and *cry2A* is good combination for lepidopterans insects [10]. In addition to gene pyramiding described above, the possibility of target insect evolving resistance could also be mitigated through the use of planting refugia crop all around the transgenic crop to dilute the insect resistance.

CIM-482, a locally approved cultivar was transformed with two insecticidal genes *cry1Ac* & *cry2A* by *Agrobacterium* mediated transformation [11]. Insect resistance studies of transgenic lines developed out of this transformation event were evaluated for three years 2006, 2007 and 2008 at the campus of National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, Lahore, Pakistan to evaluate the resistance level of these transgenic lines in field conditions.

MATERIALS AND METHODS

Based on yield potential and desired fibre characteristics, cv. CIM-482 was selected for transformation because it had high yield potential and desired fibre characteristics [11]. The *Agrobacterium* strain contained the recombinant binary vector *pk2Ac* harboring *cry1Ac and cry2A* under 35S promoter. *Neomycin phosphotransferase* (*nptII*) gene was used as a selectable marker at a concentration of 50 mg L^{-1}. The seeds of the cotton cultivar were delinted and surface sterilized with Tween-20 for 3 minutes and further subjected to 0.1% $HgCl_2$ and 0.1% SDS solution mixture and further were germinated in the dark at 30°C overnight for the germination. The shoot apices of germinating seedlings were used for *Agrobacterium*-mediated transformation according to the procedure described by Rao et al [12], Maqbool et al [13] and Khan et al [14]. The putative transgenic plants were further shifted to pots containing soil of equal proportion of clay, sand and peat moss (1:1:1). The plants were shifted to greenhouse and were subjected to various molecular analyses. The putative transgenic plants were evaluated using various molecular approaches, self fertilized in green house and seed was obtained to produce further progeny.

Evaluation of Transgenic Plants

Genomic DNA was isolated from transformed plants in subsequent progeny to confirm integration of *cry1Ac* & *cry2A* as described by [15]. PCR was run for the detection of integrated *cry1Ac* & *cry2A* to amplify internal fragments of 565 bp and 600 bp respectively using gene specific primers by a modification of the method by Saiki et al. [16]. DNA extracted from untransformed plants was used as negative control and that of plasmid *pk2Ac* as positive control. Southern Blot Analysis was performed to confirm the integration of 3kb Fragment of *cry1Ac* gene. Genomic DNA was digested with *HindIII* enzyme and rest of the procedure was followed as described by Southern [17]. Gene specific probe of *cry1Ac* was labeled using fermentas biotin decaLabel™ DNA labeling kit (Cat #K0651). Detection procedure was followed as provided in fermentas biotin chromogenic detection kit (Cat# K0661). The expression of the introduced gene was confirmed by western blot assay using the procedure was followed as described by Rao et al [12]. Transgenic progenies were developed out of transgenic plants confirmed by molecular analysis.

Lab Biotoxicity and Artificial Field Infestation

The efficacy of the introduced gene against targeted insect pests, laboratory bioassay and artificial infestation of cotton field with *Heliothis* larvae was conducted each year. Ten leaves from upper, middle and lower portion of each lines were taken in perti plate and 2^{nd} instar larvae of *Heliothis* was fed to them. After 2-3 days mortality rate of *Heliothis* larvae was recorded.

The artificial infestations were conducted twice in August and September (Boll worms activity is optimal at this stage in cotton field) by taking ten 2^{nd} instar larvae of *Heliothis* in a glass vial and then fastening this vial with the plant in the field. Almost 10,000 larvae were released per infestation. Natural infestation was also observed in both years. The plant health condition and number of bolls per plant were recorded before and after each infestation. After 5-6 days of infestation, boll damage %age was calculated.

Agronomic Characteristics of Transgenic Progenies

Along with molecular aspects, agronomic characteristics of the transgenic progenies were also recorded. Different morphological and agronomic charactistics including plant height, number of bolls, number of sympodial and monopodial branches, days to mature and average yield were recorded. Yield of transgenic plants were calculated as a percent increase or decrease relative to control plants.

RESULTS AND DISCUSSIONS

Polymerase chain reaction (PCR) of both genes *cry1Ac* and *cry2A* confirmed the stable inheritance of these genes to subsequent generations. A 565 bp and 600 bp internal fragments for *cry1Ac* and *cry2A* respectively were amplified (Figure-1). No amplification was detected in negative control. Gene integration was detected by gene specific probe after the plasmid *pk2Ac* DNA was digested with *HindIII*. Plant genomic DNA digested with the same restriction enzyme and hybridized with c*ry1Ac* specific probe showed the integration of in Plant genome. Non transformed CIM-482 plant DNA was used as negative control while that of plasmid DNA pk2Ac was used as Positive control (Figure-1). A 68 kDa band of *cry1Ac* protein was observed in transgenic plant samples and this specific protein band was absent in control sample (Figure-1).

Laboratory Biotoxicity assays with 2nd Instar Heliothis larvae showed that expression of both genes is sufficient to kill the targeted insects. In laboratory biotoxicity assay, most of the transgenic lines were showing 70-100 % larval mortality while no any larval mortality was observed in non transformed control plants (Figure-2). The larvae which survived in few cases were too inactive or sluggish to be harmful for the plant. The transgenic lines showing maximum larval mortality were selected to be used in breeding programme. Similarly in artificial infestation assay, the transgenic lines showed appreciable level of resistance against the targeted insects and boll damage varied between 0.09- 4.5% in case of transgenic lines while in control CIM-482, boll damage was calculated to 25%.(Figure-3).

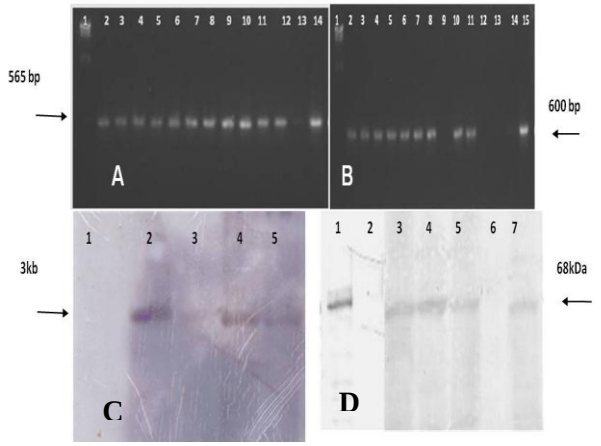

Figure-1: Molecular Evaluation of transgenic progenies using PCR, Southern and Western blot approach A: PCR amplification of c*ry1Ac* (Lane 1: λ/*h* Marker, Lane 2-12: Transgenic plants, Lane13: Negative control (CIM-482), Positive control (plasmid *pk2Ac*), B: PCR amplification of *cry2A* (Lane 1: λ/*h* Marker, Lane 2-13 Transgenic plants, Lane14: Negative control (CIM-482), Lane15: Positive control (plasmid *pk2Ac*), C: Southern blot of Transgenic Plants (Lane 1: Negative control (CIM-482), Lane 2: Positive Control (pk2Ac plasmid), Lane 3-5: Transgenic Lane14: Positive control (plasmid *pk2Ac*), D: Western Blot of Transgenic plants (Lane 1: Positive control (Bt Strain HD-73), Lane 2: Negative control (CIM-482), Lane 3-7: Transgenic Plants

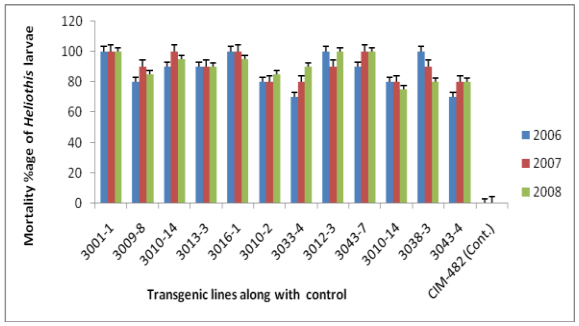

Figure-2: Graph showing mortality rates of *Heliothis* Larvae in different transgenic progenies (2006-08)

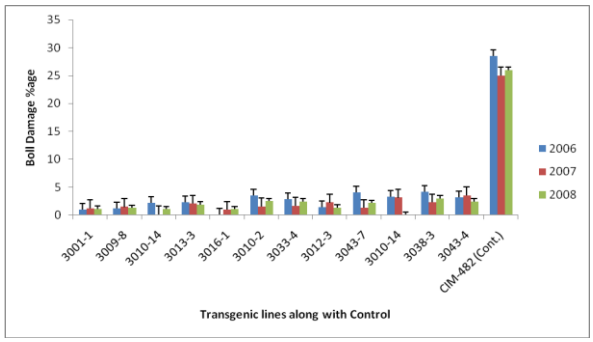

Figure-3: Graph showing boll damage %age in transgenic progenies after the crop was artificial infested with 2nd Instar *Heliothis* larvae. The number of bolls in each transgenic progeny was recorded before and after artificial assay and boll damage percentage was calculated after 5-6 days after infestation.

Difference in resistance level in laboratory biotoxicity and artificial infestation assay can be attributed to the variation in expression of insecticidal gene in transgenic progenies. These results were in agreement with the previous studies conducted by various researchers [18, 19, 20, 21, 22, 23, 24, 25, 26]. who have reported inconsistency in insecticidal gene expression in cotton.

Somaclonal variations seem most likely cause of these changes as it took more time to produce transgenic plants as compared to normal tissue culture procedure and the longer the tissue culture time the higher the frequency of somaclonal variation [9].

The transgenic lines expressing two insecticidal genes *cry1Ac* and *cry2A* provided protection against lepidopterans insects throughout the growth period. These lines provided high resistance against targeted pests till the harvesting and were desirable for agronomic and morphological characteristics. Based on the molecular data obtained from the laboratory and agronomic data recorded from the field it is believed that these transgenic progenies are an excellent source of germplasm to be used in conventional breeding programme.

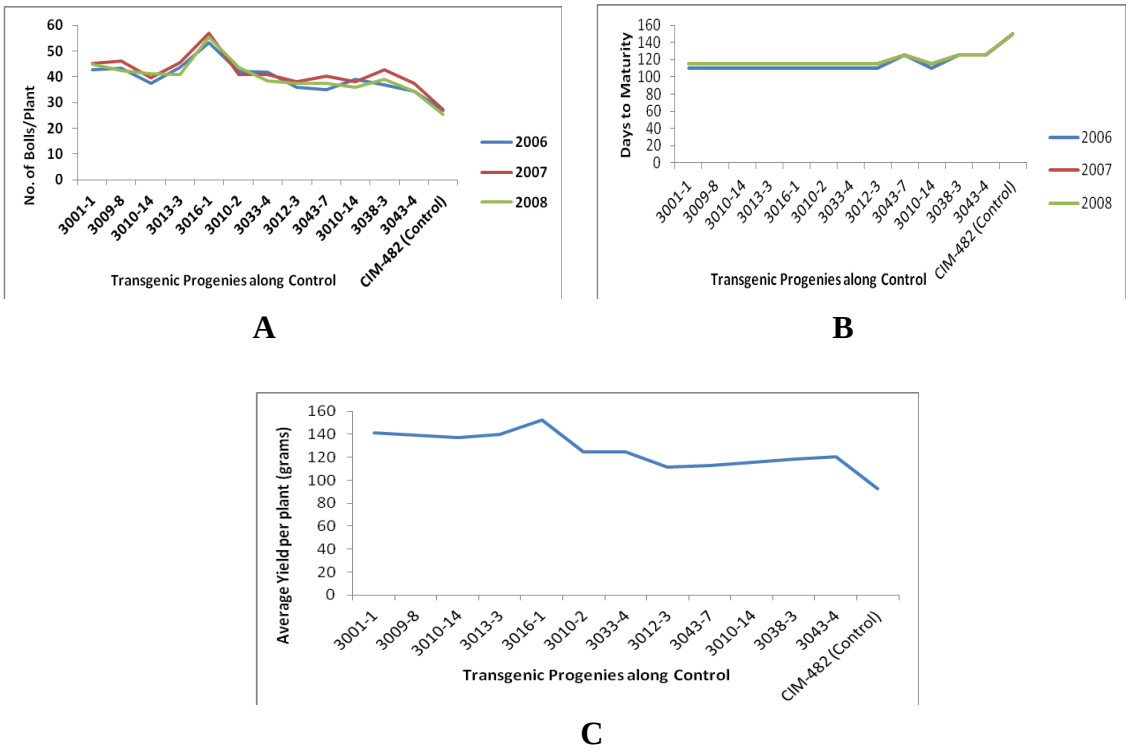

Figure-4: Comparison of Yield contributing factors between transgenic lines and untransformed CIM-482 (2006-08)

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