DEVELOPMENT OF INSECT-RESISTANT COTTON LINES WITH TARGETED EXPRESSION OF INSECTICIDAL GENE

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Abstract: In order to address biosafety concerns regarding the constitutive expression of foreign genes in crops, we applied a strategy aimed at confining foreign gene expression in insect wounding sites of cotton. For this purpose, a plant expression construct was designed by cloning the AoPR1 promoter (pathogenesis-related protein gene isolated from *Asparagus officinalis*) upstream from the insecticidal gene *cry1Ac*. The Turkish cotton cultivar cv. STN-468 was transformed using the *Agrobacterium tumefaciens* strain LBA4404 containing the recombinant binary vector pRD400 harboring *cry1Ac* under a wound-inducible promoter. The neomycin phosphotransferase (*nptII*) gene was used as a selectable marker at a concentration of 100 mg/L. The primary transformants were analyzed for T-DNA integration and expression using standard molecular approaches. The efficacy of insecticidal gene control of the AoPR1 promoter was investigated using leaf bioassays with 2nd instar larvae of *Helicoverpa armigera* and *Spodoptera littoralis*. Positive primary transformants from T_0 progeny were further raised under greenhouse conditions to obtain progeny (T_1). The introduced gene was properly inherited and expressed in T_1 progeny. The mechanical wounding of plants resulted in increased cry1Ac protein levels during 0-48 h of the wounding period. The transgenic lines exhibited appreciable levels of resistance against targeted insect pests in the leaf bioassays. The use of a wound-inducible promoter to drive insecticidal gene expression is a valuable insect resistant management strategy as gene expression will remain limited to the insect biting sites of plant and crop, food and environmental concerns can be minimized.

Key words: cotton modification; cry toxin; confined expression; insecticidal gene

INTRODUCTION

Cotton has a significant contribution in Turkish economy as Turkey is one of the eight largest countries that produce 80% of the cotton in the world. Cotton as a raw material is important to Turkish industries, especially the textile and ready-to-wear clothing sectors. To combat the losses incurred by insect pests, crop protection relies on the use of broad-spectrum highly toxic agrochemicals [1]. No transgenic cotton has been allowed in the country to date for general cultivation.

The area under biotech crops has gradually increased since 1996 and has reached 186.5 million hectares [2]. Cotton is the most extensively planted and marketed crop in world after soybean and maize. The cultivation of insect-resistant cultivars has resulted in increased farm productivity and decreased environmental pollution [3].

With the advancement in genetic engineering technologies, several genes have been incorporated in cotton. Insecticidal (*cry*) genes from *Bacillus thuringiensis* [4-7], cowpea trypsin inhibitor [8] and others provide resistance to insect pests and have been commercialized [9].

In order to confer appreciable protection against targeted insect pests in transgenic cotton expressing insecticidal gene(s), sufficient *Bacillus thuringiensis* (Bt) toxin should be expressed at appropriate times in the cotton growing season. However, the bulk of

literature suggests an inconsistent expression of Bt toxin in cotton at different plant growth stages and plant parts; this has led to the unexpected performance of Bt cotton against lepidopteron pests [10-14,7].
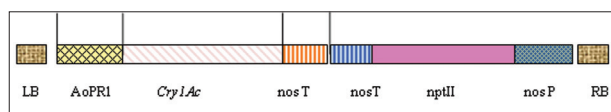
The constitutive expressing 35S promoter isolated from the cauliflower mosaic virus is widely used to drive the expression of foreign gene proteins in transgenic crops, including Bt cotton [15]. The promoter provides robust expression of *Bt* or other related genes in every plant organ and at different growth stages of the crop. The constitutive expression of Bt toxin in transgenic plants can be disadvantageous as it can affect the constant synthesis of foreign gene protein; it can also lead to potential risks of resistance development in targeted insects to the introduced genes [16-18]. In such circumstances, the targeted expression of foreign gene proteins is considered to be very valuable for the next generation of transgenic crops.

Wounding, predator or pathogen invasion induces defense-related proteins in plants. The genes encoding defense-related proteins can be used to targeted foreign gene expression. Paul et al., [19] and Harikrishna et al., [20] reported that mechanically ground fresh suspensions of the mesophyll of asparagus seedlings can be a source of wound-induced messenger RNA, which has led to the isolation of wound-induced cDNA and the corresponding promoter [21,22]. β-glucuronidase (GUS) reporter gene analysis based on expression levels in transgenic tobacco plants demonstrated that the AoPR1 promoter is activated in response to wounding, pathogen invasion and treatment with hydrogen peroxide [23]. The present work was conducted to develop transgenic cotton lines expressing the insecticidal gene *cry1Ac*, which is under the control of the AoPR1 promoter and confined *Bt* expression at insect-biting sites of the plant.

MATERIALS AND METHODS

Construction of plant expression vector

To subclone the AoPR1 promoter (900 bp) into the plasmid pRD400, the plasmid AoPR1 GUS-INT carrying the AoPR1 promoter was excised with BamHI and KpnI. The AoPR1 promoter was subcloned into the plasmid pRD400 predigested with BamHI and KpnI.

Fig. 1. Schematic representation of pAoPR1AcNPT-II plant expression vector containing *cry1Ac* under the control of the wound inducible (AoPR1) promoter isolated from *Asparagus officinalis* and *neomycin phosphotransferase* driven by the nos promoter. The plasmid pAoPR1AcNPT-II contained *nptII* that encodes resistance to kanamycin for plant selection

The resulting plasmid was named pAoPR1. The cry-1Ac gene was excised from its source plasmid, pKUC, by digestion with BamHI and subcloned downstream of the AoPR1 promoter in pAoPR1 predigested with BamHI. The resulting plasmid was named pAoPR1Ac-NPT-II (Fig. 1) and was transferred to *Agrobacterium tumefaciens* strain LBA4404 as reported by Gulbitti-Onarici et al. [24] (Fig. 1). The plasmid contained neomycin phosphotransferase (*nptII*) for plant selection that encodes resistance to kanamycin.

Cotton transformation

Cotton (*Gossypium hirsutum* L.) cv. STN-486 was selected for transformation as it has a good regeneration potential along with other desirable agronomic characteristics. The seeds were delinted and surface-sterilized with Tween-20 for 3 min and further subjected to a 0.1% $HgCl_2$ and 0.1% sodium dodecyl sulphate (SDS) solution mixture; they were thoroughly washed with distilled autoclaved water three times every 5 min. The sterilized seeds were placed in the dark at 30°C overnight for germination. The shoot apices of two-day-old germinating embryos were used for *Agrobacterium*-mediated transformation according to the procedure described earlier by Rao et al. [25], Khan et al. [26] and Bajwa et al. [27]. The inoculated explants were cultured on MS medium [28] containing 100 mM acetosyringone, 10 mM MES and 0.1 mg/L kinetin for 3 days at 28±2°C. After co-cultivation, plantlets were subcultured on selection medium, i.e. MS containing 100 mg/L kanamycin, also supplemented with 0.1 mg/L kinetin, 0.1 mg/L benzylaminopurine (BAP) and 0.1 mg/L α-naphthaleneacetic acid (NAA). Augmentin (amoxicillin and clavulanic acid) was also added (500 mg/L) to inhibit bacterial overgrowth. Subculturing was performed every two weeks. The rooting medium contained half-strength MS medium containing 1.5-2 g/L of activated charcoal, 0.3

mg/L IBA and kanamycin at a concentration of 50 mg/L. The putative transgenic plants were moved to pots containing equal proportions of field soil and peat moss (1:1). Finally, the plants were moved to the greenhouse and subjected to various molecular analyses to confirm gene presence and expression.

Molecular evaluation of putative transformants

Polymerase chain reaction (PCR) was carried out using gene-specific primers to amplify *cry1Ac* from putative transgenic cotton plants. Genomic DNA was extracted and purified from leaves based on the protocol of Li et al. [29]. PCR was performed in a total reaction mixture volume of 20 μL containing 1X reaction buffer, 50 ng of DNA template, 1.5 mM $MgCl_2$, 1 mM of each of the dNTPs, 10 ng of each primer and one unit of *Taq* DNA polymerase. PCR was carried out in a thermal cycler under the following conditions: initial denaturation at 94ºC for 4 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 54ºC for 40 seconds, extension at 72ºC for 40 s, followed by a final extension at 72ºC for 10 min. The plasmid DNA was used as positive control, and the DNA isolated from untransformed plants was used as negative control.

A double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) was used to quantify the accumulated levels of the cry1Ac protein expressed in the leaves of putative transgenic plants using Envirologix kit (Cat# AP051). About 500 mg of fresh terminal leaves were ground in liquid nitrogen, protein extraction buffer was added and a quantification procedure was followed according to the instructions provided in the kit. The optical density (OD) values at 430 nm were used to calculate the amount of cry1Ac protein by comparing it with the standard cry1Ac protein.

Leaf biotoxicity assay

To determine the efficacy of the introduced insecticidal genes (*cry1Ac*) under the wound-inducible promoter against the targeted insect pests, cotton primary transformants in T_0 progeny were subjected to leaf bioassays with second instar larvae of the American bollworm (*Helicoverpa armigera*) and Armyworm (*Spodoptera littoralis*). Five fresh leaves from each plant were taken and placed on wet filter paper in Petri plates, one leaf per plate. A 2nd instar larva, prefasted for 4-6 h, was released onto each plate and allowed to feed on the leaf. The data on insect mortality were recorded on a daily basis up to the third day.
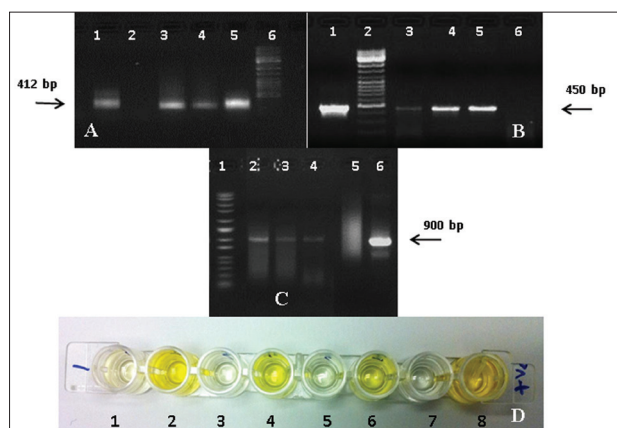
Confirmation of gene integration and expression in T1 progeny

Primary transformant cotton plants positive for the introduced gene (based on PCR, ELISA and leaf bioassay data) were carefully picked and their progenies were grown in the greenhouse and further subjected to various molecular approaches to confirm gene integration and expression using PCR, ELISA and leaf bioassays. PCR was run to amplify the internal fragment of *cry1Ac* (412 bp), AoPR1 (900 bp) and *nptII* (450 bp). ELISA was performed to quantify the accumulated levels of cry1Ac by mechanically wounding the leaves of transgenic plants at 0, 6, 12, 24 and 48 h and data was recorded. Initial screening in T_0 progeny was made using one larva per Petri plate, having in mind the number of larvae available for the experiment. The T_1 progeny was thoroughly screened using five second instar larvae of *Helicoverpa armigera* and *Spodoptera littoralis* per Petri plate, with the transgenic as well as control plant leaves to evaluate the efficacy of cry1Ac protein against these targeted insect pests. Mortality of the larvae was recorded following their incubation.
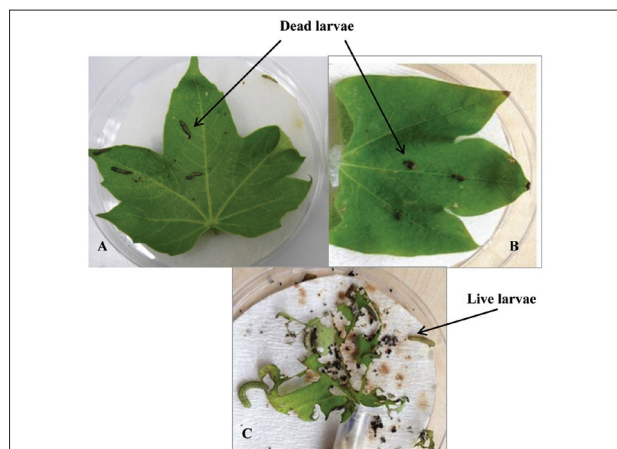
RESULTS

A total of 5000 shoot apical meristems were used for inoculation with the *Agrobacterium* strain LBA4404 harboring the gene of interest. After 8 weeks of selection on 100 mg L^{-1} of kanamycin, nine putative transgenic plants were obtained. The regenerated plants grew well, were allowed to self-pollinate and fertilized normally. The nine primary putative transgenic plants (SP1-SP9) that grew well in soil were subjected to PCR for confirmation of the introduced *cry1Ac* in the cotton genome. Results showed that three (SP2, SP4 and SP6) of the nine plants indicated amplification of the required band of 412 bp of the cry1Ac gene, 450 bp of *nptII* and 900 bp of the AoPR1 promoter (Fig. 2). The primary transformants (SP1-SP9) were also subjected to ELISA for the determination of cry1Ac protein. The

Fig. 2. Molecular evaluation of primary cotton transformants (T$_0$ progeny). **A** – PCR assay showing the amplification of a 412 bp internal fragment of *cry1Ac*. Lane 1: positive control (plasmid DNA); lane 2: negative control (DNA from non-transformed plant), lanes 3-5: Primary transformants of STN-468; lane 6: DNA ladder mix. **B** – PCR assay showing the amplification of required band of *nptII*. Lane 1: positive control (plasmid DNA); lane 2: DNA ladder mix; lanes 3-5: putative transgenic plants; lane 6: negative control (DNA from a non-transformed plant). **C** – PCR assay showing the amplification of 900 bp of the AoPR1 promoter. Lane 1: DNA ladder mix; lanes 2-4: putative transgenic plants of STN-468; lane 5: negative control (DNA from a non-transformed plant); lane 6: positive control (Plasmid DNA). **D** – ELISA confirmed the expression of *cry1Ac* in putative transgenic plants. Positive and negative controls were provided in the kit.

Fig. 3. Leaf biotoxicity assay conducted were on leaves of T$_0$ and T$_1$ progeny plants of cotton. The transgenic plants showed appreciable level of resistance against *H. armigera* and *S. littoralis*. **A** – dead *S. littoralis* larvae after feeding on the transgenic leaf. **B** – dead *H. armigera* larvae after feeding on leaves of transgenic plants. **C** – larvae feeding on the leaf of control plants were alive and healthy

same three plants (P2, P4 and P6) expressed cry1Ac protein (Fig. 2). The quantity of cry1Ac protein recorded as 0.23-0.25 μg/g of fresh tissue weight.

Table 1. Leaf biotoxicity assay in T$_0$ putative transgenic plants. The 2nd instar *H. armigera* and *S. Littoralis* leaves were fed on T$_0$ putative transgenic cotton. P2, P4 and P6 plants showed appreciable levels of resistance against the targeted insect pests. No larval mortality was recorded in non-transformed cotton plants.
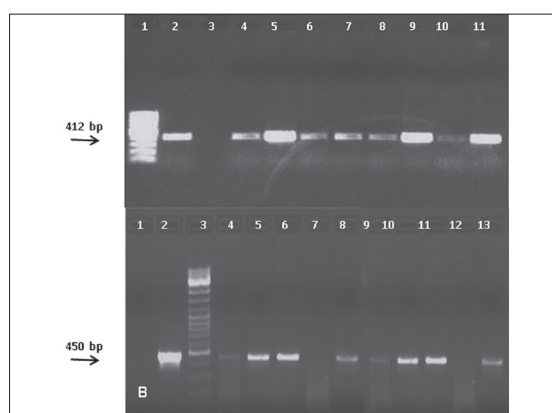
Transgenic lines (T$_0$ progeny)	*H. armigera*	*S. littoralis*
SP1	0.00±0.00	0.00±0.00
SP2	100.00±0.00	100.00±0.00
SP3	40.00±2.49	30.00±1.55
SP4	100.00±0.00	90.00±0.00
SP5	40.00±3.33	30.00±5.77
SP6	100.00±0.00	80.00±0.00
SP7	0.00±0.00	0.00±0.00
SP8	20.00±4.44	20.00±2.55
SP9	0.00±0.00	0.00±0.00
Negative control	0.00±0.00	0.00±0.00

Biotoxicity assays of PCR and ELISA positive T$_0$ plants conducted against larvae of *Helicoverpa armigera* and *Spodoptera littoralis* showed that *cry1Ac* expression was sufficient to kill the targeted insects (Table 1). An appreciable resistance level (80-100%) against *H. armigera* and S. *littoralis* was observed in the SP2, SP4 and SP6 putative transgenic plants; the other plants with less resistance were not considered further. No mortality of larvae was recorded in negative non-transgenic control plants (Fig. 3)

Evaluation of transgene progeny (T$_1$)

Primary cotton transformants positive for the introduced gene (based on PCR, ELISA and leaf bioassay data) were carefully picked and their progenies were raised in the greenhouse. Genomic DNA was isolated and subjected to PCR to amplify the internal fragments of *cry1Ac*, *nptII* and the AoPR1 promoter. A few representative transgenic plants from the progeny of each positive plant were taken for further molecular analysis. The progenies of transgenic plants (SP2, SP4 and SP6) showed the amplified bands of *cry1Ac* (412 bp) and *nptII* (450 bp) (Fig. 4).

The leaves of T$_1$ transgenic plants were mechanically wounded in order to determine the amount of cry1Ac protein expressed with the passage of time. The concentration of cry1Ac protein was recorded after wounding the transgenic leaves. In the T$_1$ progeny of transgenic plants, the levels of cry1Ac increased from 0.090 to 1.875 μg/g of fresh weight between 6
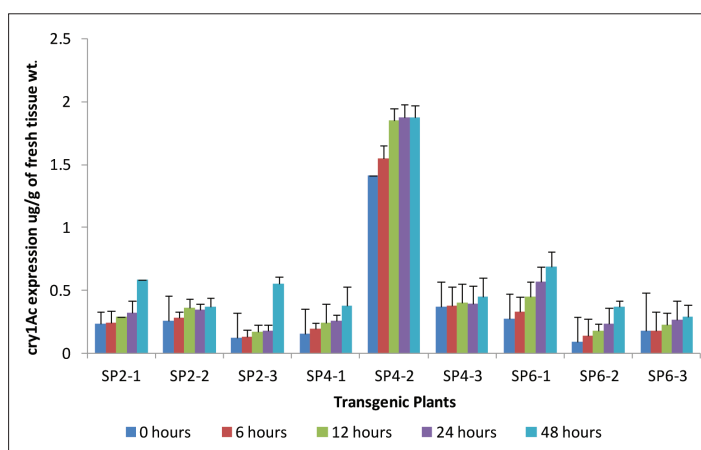
Fig. 4. Molecular evaluation of transgenic cotton plants (T$_1$ Transgenic progeny). **A** – PCR assay showed the amplification of the internal sequence of the 412 bp of *cry1Ac* fragment. Lane 1: DNA ladder 100 bp; lane 2: positive control (plasmid DNA); lane 3: negative control (DNA from non-transformed plant); lanes 4-11: transgenic plants. **B** – PCR showing the amplification of the *nptII* band. Lane 1: negative control (DNA from non-transformed plant); lane 2: positive control (plasmid DNA); lane 3: DNA ladder mix; lanes 4-13: transgenic plants in the T$_1$ progeny

Fig. 5: ELISA conducted to quantify the levels of cry1Ac protein at 0, 6, 12, 24, and 48 h of mechanical wounding. Positive and negative controls were provided in the kit. The results show that cry1Ac protein accumulated after wounding.

Table 2. Leaf biotoxicity assay in T$_1$ transgenic plants. The leaves from T$_1$ putative transgenic cotton were fed to 2nd instar *H. armigera* and *S. littoralis*. Expression of the introduced gene was enough to confer resistance against targeted insect pests. No larval mortality was recorded in non-transformed cotton plant.

Transgenic lines (T$_1$ progeny)	*H. armigera*	*S. littoralis*
SP2-1	100.00±0.00	93.33±6.67
SP2-2	100.00±0.00	100.00±0.00
SP2-3	83.33±3.82	70.00±1.00
SP4-1	90.00±3.00	80.00±2.55
SP4-2	100.00±0.00	90.00±1.80
SP4-3	100.00±0.00	80.00±2.31
SP6-1	90.00±2.55	80.00±1.80
SP6-2	86.67±3.33	40.00±3.33
SP6-3	100.00±0.00	100.00±0.00
Negative control	0.00±0.00	0.00±0.00

and 48 h of the post-wounding period. Fig. 5 depicts increasing levels of insecticidal protein after post-wounding in transformants. The highest levels of cry1Ac protein (1.875 µg/g) were found in SP4-1 after 48 h of post-wounding (Fig. 5) while lower expression levels (0.275 µg/g) were found in SP6-3.

The transgenic plants in T$_1$ progeny were found to be as effective as their T$_0$ parent plants in causing the mortality of *Helicoverpa armigera* and *Spodoptera littoralis* larvae. The bioassay results showed that the wound-inducible expression of *cry1Ac* in transgenic progeny conferred enough protection against targeted insect pests (Table 2). The larval mortality on the transgenic plants was recorded as 70-100% after 24 h of bioassay. The transgenic plants SP2, SP4 and SP6 were highly effective in causing larval mortality in a shorter time. The control non-transgenic plants did not exhibit any larval mortality (Table-2).

DISCUSSION

Most transgenic Bt crops utilize the CaMV 35S promoter [15] that induces a robust foreign gene expression in every cell type and at every plant growth phase. Studies have suggested that the acceptability and adaptability of Bt crops may be reduced as a result of the continuous synthesis of foreign proteins in a plant [30,31]. Additionally, it also increases the risk of resistance evolvement in insects [32,33].

Concerns that the constitutive overexpression of Bt toxins in crop plants will increase resistance development in targeted insects [34] have led the Environmental Protection Agency (EPA) to work out a policy of planting refuges of conventional crops to delay resistance development in insects. In such circumstances, it becomes desirable to use expression-specific promoters that induce foreign gene(s) in specific plant tissues or organs [35,36]. We adopted a similar strategy to avoid the presence of unwanted toxic protein products of insecticidal *cry1Ac* in transgenic Bt cotton

plants at insect wounding sites. The AoPR1 promoter used in the study drives the expression of AoPR1 protein in *Asparagus officinalis*, a class of pathogenesis-related (PR) proteins that are induced in response to different stresses, such as pathogen attacks, wounding and the application of chemicals. Promoters from other PR proteins have previously been shown to drive the expression of *Bt* in transgenic broccoli, cabbage and tobacco [37,38,24].

The *Agrobacterium*-mediated binary vector system is an efficient system of natural exchange of genetic material. Based on our earlier experiments, we used the LBA4404 agrobacterium strain harboring pRD400 plasmid to infect apical meristems in genetic transformation experiments [39]. The transformation efficiency achieved in the present study has been low compared to earlier studies [40-42,25]. This may be attributed to the regeneration capability of cv. STN-468, which was good but comparatively lower than the cultivars transformed by other researchers.

Expression of the insecticidal gene under the control of wound-inducible promoter (AoPR1) in transgenic plants (T_0 and T_1 progeny) was confirmed by standard techniques, including PCR, ELISA and leaf biotoxicity assays. Many researchers have used these approaches to confirm gene integration and expression in transgenes [5,43,44,26,7]. The transgenic cotton plants expressing *cry1Ac* under the control of AoPR1 suggested that the expression of the cry1Ac gene was dependent on wounding of the plant tissues as revealed by the assays. The amount of cry1Ac protein increased enough to cause 80-100% mortality of the larvae tested during 0-48 h of the experiment.

The toxic activities of both progenies of transgenic cotton plants expressing *cry1Ac* under AoPR1 promoter against the larvae of *Helicoverpa armigera* and *Spodoptera littoralis* were examined. The progeny of transgenic cotton plants (T_1) conferred protection against the targeted insect pests. This indicates the wound-specific activity of the AoPR1 promoter to express *cry1Ac* at the wound sites only. The levels of cry1Ac protein accumulated in 48 h were sufficient to cause 80-100% mortality. These results are in agreement with Breitler et al. [33] and Gulbitti-Onarici et al. [24] who obtained insect-resistant rice and tobacco,

respectively, harboring the insecticidal gene driven by wound-inducible promoters.

The previous reports of insect-resistant crops with the 35S promoter [4-6,45,46] indicate a strong and constitutive expression of insecticidal protein, whereas delivering targeted and effective doses of the protein to the insect pest at the threshold level of damage may provide the most effective approach in delaying the build-up of resistance in the target insect population. The ingestion of a small part of transgenic leaf by a targeted insect pest was enough to confer protection against them, offering an efficient insect-resistance management strategy. We conclude that insect control can be achieved by a confined and limited expression of Bt toxin under the control of the wound-inducible AoPR1 promoter.

Author's contributions: A. Bakhsh designed the transformation experiments and conducted initial trials and wrote the manuscript. E. Anayol and S.D. Khabbazi conducted transformation experiments and compiled molecular data. Ö.C. Karakoç reared the larvae and conducted leaf bioassays, and C. Sancak and S. Özcan supervised all the research activities, read the manuscript and critically analyzed the manuscript.

Conflict of interest disclosure: The authors declare no conflict of interest

REFERENCES

1.   Özbek FS and Fidan H. Estimation of pesticides usage in the agricultural sector in Turkey using Artificial Neural Network (ANN). J Anim Plant Sci. 2009;4:373-8.
2.   James C. Global status of commercialized biotech/GM crops, 2014. Ithaca, NY: International Service for the Acquisition of Agri-biotesh Applications; 2014. 259 p. (ISAAA Brief; 49)
3.   Bakhsh A, Khabbazi SD, Baloch FS, Demirel U, Çalişkan ME, Hatipoğlu R, Özcan S, Özkan H. Insect resistant transgenic crops: retrospect and challenges. Turk J Agric For. 2015a;39:531-48.
4.   Perlak FJ, Deaton RW, Armstrong RL, Fuchs RL, Sims SR, Greenplate JT, Fischhoff DA. Insect resistant cotton plants. Bio/Technol. 1990;8:939-43.

5. Cousins YL, Lyon BR, Llewellyn DJ. Transformation of an Australian cotton cultivar: prospects for cotton improvement through genetic engineering. Aus J Plant Physiol. 1991;18:481-94.

6. Jenkins JN, Mccarty JC, Buehler RE, Kiser J, Williams C, Wofford T. Resistance of cotton with endotoxin genes from *Bacillus thuringiensis* var. kurstaki on selected Lepidopteran insects. Agron J. 1997;89:768-80.

7. Bakhsh A, Siddique S, Husnain T. A molecular approach to combat spatio-temporal variation in insecticidal gene (*cry1Ac*) expression in cotton. Euphytica. 2012;183:65-74.

8. Li YE, Chen ZX, Wu X, Li SJ, Jiao GL, Wu JH, Fan XP, Meng JH, Zhu Z, Wang W, Zhu Y, Xu HL, Xiao GF, Li XH. Obtaining transgenic cotton plants with cowpea trypsin inhibitor gene. Acta Gossypii Sinica. 1998;10:237-43.

9. Bakhsh A, Anayol E, Özcan SF, Hussain T, Aasim M, Khawar KM, Özcan S. An insight into cotton genetic engineering (*Gossypium hirsutum* L.): current endeavors and prospects. Acta Physiol Plantarium. 2015b;37:171.

10. Benedict JH, Sachs ES, Altman DW, Deaton WR, Kohel RJ, Berberich SA. Field performance of cottons expressing transgenic *Cry1A* insecticidal proteins for 118 resistances to *Heliothis virescens* and *Helicoverpa zea* (Lepidoptera: Noctuidae). J Econ Entomol. 1996;89:230-8.

11. Chen S, Wu J, Zhou B, Huang J, Zhang R. On the temporal and spatial expression of Bt toxin protein in Bt transgenic cotton. Acta Gossypii Sin. 2000;12:189-93.

12. Greenplate J, Mullins W, Penn SR, Embry K. Cry1Ac Bollgard® varieties as influenced by environment, variety and plant age: 1999 gene equivalency field studies. In: P. Dugger, R. Richter, editors. Proceedings of the Beltwide Cotton Conference. Vol. 2. Memphis, TN: National Cotton Council of America; 2001. p. 790-3.

13. Kranthi K, Naidu S, Dhawad CS, Tatwawadi A, Mate K, Patil E, Bharose AA, Behere GT, Wadaskar RM, Kranthi S. Temporal and intraplant variability of *cry1Ac* expression in Bt-cotton and its influence on the survival of the cotton bollworm, *Helicoverpa armigera*. Curr Sci. 2005;89:291-8.

14. Bakhsh A, Shahzad K, Husnain T. The variation in spatio temporal expression of insecticidal genes in transgenic cotton. Czech J Genet Plant Breed. 2011a;47:1-9.

15. Odell JT, Nagy F, Chua NH. Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature.1985;313:810-2.

16. Kuiper HA, Kleter GA, Noteborn HP, Kok EJ. Assessment of the food safety issues related to genetically modified foods. Plant J. 2011;27:503-28.

17. Shelton AM, Zhao JZ, Zhao RT. Economic, ecological, food safety and social consequences of the development of Bt transgenic plants. Annu Rev Entomol. 2002;47:845-81.

18. Conne A, Glare TR, Nap JP. The release of genetically modified crops into the environment. Part II. Overview of ecological risk assessment. Plant J. 2003;33:19-46.

19. Paul E, Harikrishna K, Fioroni O, Draper J. Dedifferentiation of *Asparagus officinalis* L. mesophyll cells during initiation of cell cultures. Plant Sci. 1989;65:111-7.

20. Harikrishna K, Paul E, Darby R, Draper J. Wound response in mechanically isolated asparagus mesophyll cells: A model monocotyledon system. J Exp Bot. 1991;42:791-9.

21. Warner SA, Scott R, Drape J. Characterization of a wound-induced transcript from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. Plant Mol Biol. 1992;19:555-61.

22. Warner SA, Scott R, Draper J. Isolation of an asparagus intracellular PR gene (AoPR1) wound-responsive promoter by the inverse polymerase chain reaction and its characterization in transgenic tobacco. Plant J. 1993;3:191-201.

23. Özcan S, Firek S, Draper J. Selectable marker genes engineered for specific expression in target cells for plant transformation. Bio/Technology. 1993;11:218-21.

24. Gulbitti-Onarici S, Zaidi MA, Taga I, Ozan S, Altosaar I. Expression of *cry1Ac* in Transgenic Tobacco Plants Under the Control of a Wound-Inducible Promoter (AoPR1) Isolated from Asparagus officinalis to Control *Heliothis virescens* and *Manduca sexta*. Mol Biotechnol. 2009;42:341-9.

25. Rao AQ, Bakhsh A, Nasir IA, Riazuddin S, Husnain T. Phytochrome B mRNA expression enhances biomass yield and physiology of cotton plants. Afr J Biotechnol. 2011;10:1818-26.

26. Khan GA, Bakhsh A, Riazuddin S, Husnain T. Introduction of cry1Ab gene into cotton (Gossypium hirsutum) enhances resistance against lepidopteran pest (Helicoverpa armigera). Spanish J Agr Res. 2011;9:296-300.

27. Bajw KS, Shahid AA, Rao AQ, Kiani MS, Ashraf MA, Dahab AA, Bakhsh A, Latif A, Khan MAU, Puspito AN, Aftab A, Bashir A, Husnain T. Expression of Calotropis procera expansin gene CpEXPA3 enhances cotton fibre strength. Aus J Crop Sci. 2013;7:206-12.

28. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 1962;15:473-97.

29. Li H, Jinhua L, Hemphill JK, Wang JT, Gould J. A rapid and high yielding DNA miniprep for cotton (Gossypium spp.). Plant Mol Biol Rep. 2001;19:1-5.

30. Andow DA, Ives AR. Monitoring and adaptive resistance management. Ecol Appl. 2002; 12:1378-90.

31. Bates SL, Zhao JZ, Roush RT, Shelton AM. Insect resistance management in GM crops: past, present and future. Nature Biotechnol. 2005;23:57-62.

32. Breitler JC, Vassal JM, Catala MDM, Meynard, Marfa V, Mele E, Royer M, Murillo I, San SB, Guiderdoni E, Messeguer J. Bt rice harbouring cry genes controlled by a constitutive or wound-inducible promoter: protection and transgene expression under Mediterranean field conditions. Plant Biotechnol J. 2004;2:417-30.

33. Kim S, Kim C, Li W, Kim T, Li Y, Zaidi MA, Altosar I. Inheritance and field performance of transgenic Korean Bt rice lines resistant to rice yellow stem borer. Euphytica. 2008;164:829-39

34. Huang F, Buschma, Higgins RA, McGaughey WH. Inheritance to Bacillus thuringiensis toxin (Dispel ES) in European corn borer. Science. 1999;284:965-7.

35. Cai M, Wei J, Li KH, Xu CG, Wang SP. A rice promoter containing both novel positive and negative cis- elements for regulation of green tissue-specific gene expression in transgenic plants. Plant Biotechnol J. 2007;5:664-74.

36. Bakhsh A, Rao AQ, Shamim Z, Husnain T. A Mini Review: RuBisCo small subunit as a strong, green tissue-specific promoter. Arch Biol Sci. 2011b;63:299-307.

37. Jin RG, Liu Y, Tabashnik BE, Borthakur D. Development of transgenic cabbage (*Brassica oleracea* var Capitata) for insect resistance by Agrobacterium tumefaciens mediated transformation. In Vitro Cell Dev Biol Plant. 2000;36:231-7.

38. Cao J, Shelton AM, Earle ED. Gene expression and insect resistance in transgenic broccoli containing a *Bacillus thuringiensis* cry1Ab gene with the chemically inducible PR-1a promoter. Mol Breed. 2001;8:207-16.

39. Bakhsh A, Anayol E, Ozcan SF. Comparison of transformation efficiency of five *Agrobacterium tumefaciens* strains in *Nicotiana tabacum* L. Emir J Food Agric. 2014;26:259-64.

40. Smith EF, Towsend CO. A plant tumor of bacterial origin. Science. 1997;25:671-3.

41. Zapata C, Park SH, El-zik KM, R.M. Smith RM. Transformation of a Texas cotton cultivar by using Agrobacterium and the shoot apex. Theor Appl Genet. 1999;98:252-6.

42. Majeed A, Husnain T, Riazuddin S. Transformation of virus-resistant Gossypium hirsutum L. genotype CIM-443 with pesticidal gene. Plant Biotech. 2000;17:105-10.

43. Bashir K, Husnain T, Fatima T, Latif Z, Mehdi SA. Field evaluation and risk assessment of transgenic indica basmati rice. Mol Breed. 2004;13:301-12.

44. Tohidfar M, Ghareyazie B, Mosavi M, Yazdani S, Golab-chian R. Agrobacterium-mediated transformation of cotton (*Gossypium hirsutum*) using a synthetic cry1Ab gene for enhanced resistance against Heliothis armigera. Iranian J Biotechnol. 2008;6:164-73.

45. Ni WC, Zhang ZL, Guo SD. Development of transgenic insect-resistant cotton plants. Sci Agric Sinica. 1998;31:8-13.

46. Rashid B, Saleem Z, Husnain T, Riazuddin S. Transformation and inheritance of Bt genes in Gossypium hirsutum. J Plant Biol. 2008;51:248-54.