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ORIGINAL ARTICLE

Towards better insect management strategy: restriction of insecticidal gene expression to biting sites in transgenic cotton

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Abstract Most of the commercialized Bt crops express *cry* genes under 35S promoter that induces strong gene expression in all plant parts. However, targeted foreign gene expression in plants is esteemed more important as public may be likely to accept 'less intrusive' expression of transgene. We developed plant expression constructs harboring *cry1Ac* gene under control of wound-inducible promoter (AoPR1) to confine *Bt* gene expression in insect wounding parts of the plants in comparison with *cry1Ac* gene under the control of 35S promoter. The constructs were used to transform four Turkish cotton cultivars (GSN-12, STN-468, Ozbek-100 and Ayhan-107) through *Agrobacterium tumefaciens* strains GV2260 containing binary vectors p35SAcBAR.101 and AoPR1AcBAR.101 harboring c*ry1Ac* gene under control of 35S and AoPR1, respectively. Phosphinothricin (PPT) was used at concentration of 5 mg L^{-1} for selection of primary transformants. The primary transformants were analyzed for transgene presence and expression standard molecular techniques. The transformants exhibited appreciable mortality rates against larvae of *Spodoptera exigua* and *S. littoralis*. It was found that mechanical wounding of T_1 transgenic plants was effective in inducing expression of *cry1Ac* protein as accumulated levels of *cry1Ac* protein increased during post-wounding period. We conclude that use of wound-inducible promoter to drive insecticidal gene(s) can be regarded as a valuable insect-resistant management strategy since the promoter activity is limited to insect biting sites of plant. There is no *Bt* toxin accumulation in unwounded plant organs, seed and crop residues, cotton products and by-products, thus minimizing food and environmental concerns.

Keywords Genetic modification · Insect resistance · Confined expression · Insect management

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Introduction

Cotton is the most important cash crop and backbone of textile industry of the world. Cotton is susceptible to insect pests of different orders, thus the insect management in cotton relies on the use of broad spectrum toxic agrochemical that has led to serious environmental problems and human health concerns (Bakhsh et al. 2009). Cotton uses 22.5 % of the world's insecticides and 10 % of all pesticides, on 2.5 % of agricultural land. It is responsible for the release of US$ 2 billion of chemical pesticides each year, within which at least US$ 819 million are considered

toxic enough to be classified as hazardous by the World Health Organization (Olsen 2013; EJF 2007).

Many different foreign gene(s) have been introduced into cotton genome successfully to encode resistance against insect pests (Bakhsh et al. 2015). Among these, *Bt* (Perlak et al. 1991; Cousins et al. 1991; Xie et al. 1991; Jenkins et al. 1997; Ni et al. 1998; Rashid et al. 2008; Khan et al. 2011; Bakhsh et al. 2012; Bajwa et al. 2013), CpTI (Li et al. 1998), API (Thomas et al. 1995) and STKI (Wang et al. 1998) genes provide resistance to insect pests. Coker varieties are responsive to genetic modification; the desirable genes are introduced into Coker cultivars firstly and later on back crossed to other varieties. Recently, improvement of tissue culture techniques to obtain cotton transgenics in genotype-independent manner has been reported successfully (Gould and Magallanes-Cedeno 1998; Bakhsh et al. 2012).

Most of the commercialized transgenic crops around the world express foreign gene (s) under the control of CaMV 35S promoter (Odell et al. 1985) which induces strong gene expression in all tissue types at high levels at different growth stages of the crop. However, such constitutive expression of foreign proteins in transgenic plants may cause adverse effects, such as the metabolic burden imposed on plants for constant synthesis of foreign gene products compared with temporal or spatial expression of the toxin. The constant expression of introduced gene may also increase the potential risk of evolvement of resistance in target insects. We have already started witnessing reports of resistance evolvement in pests against Bt crops (Zhang et al. 2011; Tabashnik et al. 2013). Using CaMV 35S promoter has also raised concerns about the food safety of genetically modified plants (Kuiper et al. 2001; Shelton et al. 2002; Conner et al. 2003).

The different groups of defense-related genes are induced in plants when subjected to wounding, predator or pathogen invasion; resulting in significant changes in mRNA populations and protein synthesis. The freshly isolated suspensions of mesophyll cells generated by the mechanical grinding of asparagus seedlings have been reported as an enriched source of wound-induced messenger RNA (Paul et al. 1989; Harikrishna et al. 1991) that facilitated the isolation of a wound-induced cDNA (AoPR1) and the corresponding promoter from *Asparagus officinalis* (Warner et al. 1992). 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 (Özcan et al. 1993; Warner et al. 1993; Mur et al. 2004). The use of AoPR1 promoter conferred full protection against pests of tobacco; levels of Bt toxin were found increased in insect biting sites (Gulbitti-Onarici et al. 2009). Therefore, targeted expression has become particularly important for the future development of value-added crops as public may be more likely to accept 'less intrusive' expression of the transgene. The purpose of the study was to develop insect-resistant cotton lines with confined insecticidal expression (*cry1Ac*) in insect biting sites under the control of wound-inducible promoter (AoPR1) leading to promising insect management strategy.

Materials and methods

Construction of plant expression vectors

The promoter AoPR1 and *cry1Ac* gene fragments were excised from different source plasmids pAoPR1-GUS-INT (Özcan et al. 1993) and pKUC (Cheng et al. 1998), respectively, using specific restriction enzymes and later on cloned in pTF101 binary vector. The resultant binary plasmids were named p35SAcBAR.101 and pAoPR1AcBAR.101 (Fig. 1) containing *cry1Ac* gene under 35S and AoPR1 promoters. The recombinant plasmids were confirmed using restriction digestion and colony PCR. *Escherichia coli* strain *JM109* cells were used for the cloning and propagation of the recombinant plasmid vector. *Agrobacterium tumefaciens* strain GV2260 was used for transformation. Both plasmids were further transformed in GV2260 strains using Eppendorf Multiporator® device.

Cotton transformation

Cotton (*Gossypium hirsutum* L.) cultivars GSN-12, STN-468, Ozbek-100 and Ayhan 107 were selected for transformation as these cultivars had good regeneration potential through tissue culture and other desirable agronomic characteristics. The sterilized seeds were placed in dark at 30 °C overnight for the germination. The shoot apices of 2-day germinating embryos were used for *Agrobacterium*-mediated transformation using procedure as described by Gould and Magallanes-Cedeno (1998) with some modifications as described by Bakhsh et al. (2012). The inoculated explants were cultured on MS medium (Murashige and Skoog 1962) supplemented with 100 mM acetosyringone, 10 mM MES and 0.1 mg/L kinetin at 28 ± 2 °C. The plantlets were sub-cultured on selection medium containing 5 mg/L phosphinothricin (PPT) for plant selection; 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 to inhibit bacterial overgrowth at rate of 500 mg/L. The rooting medium contained half-strength MS medium supplemented with

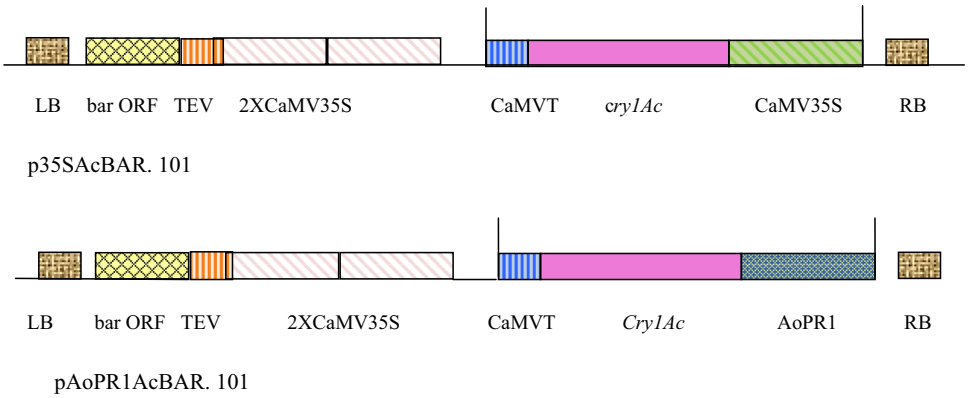


Fig. 1 Schematic representation of plant constructs p35SAcBAR.101 and pAoPR1AcBAR.101 in pTF101.1 containing *cry1Ac* gene under the control of 35S CaMV and AoPR1 promoter, respectively. Phosphinothricin was for the plants selection transformed with plasmids 35SAcBAR.101 and pAoPR1AcBAR.101

1.5–2 g/L of activated charcoal, 0.1 mg/L NAA; PPT was added at this stage to the medium to keep selection pressure for the screening of putative transgenic plantlets. The putative transgenic plants were further shifted to pots containing equal proportion of field soil and peat moss (1:1). Finally, the plants were shifted to greenhouse and were 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 fragment of c*ry1Ac,* AoPR1 and BAR gene from putative transgenic cotton plants. For this purpose, genomic DNA was extracted and purified from leaves based on the protocol of Li et al. (2001). PCR was performed in a total reaction mixture volume of 20 µL containing 1× 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. The primer sequences, annealing temperature and product size are given in Table 1. The plasmid DNA was used as positive control, whereas the DNA isolated from untransformed plants was used as negative control. Amplified DNA fragments were electrophoresed on 1.0 % agarose gel and visualized by ethidium bromide staining under ultraviolet (UV) light.

ELISA

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). The leaves of primary transformants harboring p35SAcBAR.101 and pAoPR1AcBAR.101 were pricked with needle at 0, 12 and 24 h and were collected for protein quantification. This could give us better idea of cry protein accumulation under the control of AoPR1 after wounding. Four PCR-positive plants of each cultivar transformed with different plasmids mentioned above were selected for protein quantification. The protocol for protein quantification was followed as per instruction provided in the kit. The OD values at 430 nm were used to calculate the amount of *cry1Ac* protein by comparing it with the standard *cry1Ac* protein.

Table 1 Primer list and other related information used for amplification of cry1Ac, AoPR1, BAR

Gene	Primer sequence	Annealing temp. (^{0}C)	Product size (bp)
cry1Ac	F-ATGGACAACCCAAACATC	58	412
	R-TCATGTCGTTGAATTGAATACG		
AoPR1 promoter	F-CCGGTACCCTCAGGACTAGACC	64	900
	R-GGCCATGGTTATGTAGCACCGATG		
BAR	F- GCACCATCGTCAACCACTA	60	310
	R-ACAGCGACCACGCTCTTGAA		
cry1Ac (for qPCR)	ATCGGTATCAACAACCAGCA	56	173
	ACCTGTGGGAGAATCCTTG		

qRT-PCR

PCR and ELISA-positive plants were subjected to real-time PCR. Total RNA isolation was performed from leaves of wound-induced plants following protocol as described by Jaakola et al. (2001) and was converted to cDNA. Real-time PCR reactions were carried out with LightCycler® Real-Time PCR Systems (Roche) in 96-well plate using the IQTM SYBR_ Green Super Mix. CDNA from the transgenic cotton plants were used as template. 18S RNA was used as an internal control, cDNA from a known positive plant was used as positive control while cDNA from non-transgenic plant was used as negative control. The reaction conditions were as follows: denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 40 s and final elongation step at 72 °C for 10 min. Melting curve was analyzed by continuous monitoring of fluorescence between 60 and 95 °C with 0.5 °C increments after every 30 s. 18S RNA was used as house-keeping control. Gene-specific primers were designed using BioEdit Software.

Leaf biotoxicity assay

To determine the efficacy of introduced insecticidal gene (*cry1Ac*) under wound-inducible AoPR1 promoter against targeted insect pests, cotton primary transformants in T_0 progeny were subjected to leaf bioassays with third instar larvae of *Spodoptera exigua*. Three fresh leaves along with petiole were detached from each plant and placed on wet filter paper in petri plates. The petiole was wrapped in cotton wool and inserted 1.5 ml Eppendorf tube containing water. Ten third instar larvae, pre-fasted for 4–6 h, were released in each plate and allowed to feed on the leaf. The data on insect mortality were recorded on daily basis up to 5 days.

Confirmation of gene integration and expression in T_1 progeny

Seeds of transgenic cotton plants positive for the introduced gene (based on PCR, ELISA and leaf bioassay data) in T_0 progeny were germinated in vitro on MS medium containing 5 mg/L PPT and grown in the green house and were subjected to various molecular approaches to confirm gene integration and expression. PCR was run to amplify internal fragment of *cry1Ac* (412 bp) and AoPR1 (900 bp). Furthermore, the efficacy of introduced *cry1Ac* gene under different promoter was evaluated using bioassays against *Spodoptera littoralis*. Mortality of the larvae was recorded following their incubation.

Results

Cotton transformation

An optimized procedure by Gould and Magallanes-Cedeno (1998) was followed to transform cotton cultivars with some modifications adopted by Bakhsh et al. (2012). Additionally, we added active charcoal in regeneration medium to suppress phenolic compounds. After 8 weeks of selection on 5 mg/L PPT, putative transgenic plants of cotton cultivars were obtained. The regenerated plants of each cultivar grew well, were allowed to self-pollinate and fertilized normally. Total of 30 plants of GSN-12 (11 plants with p35SAcBAR.101+ 19 plants with pAoPR1AcBAR.101), 9 of Ayhan-107 (3 plants with p35SAcBAR.101+ 6 plants with pAoPR1AcBAR.101), 4 of STN-486 (only with p35SAcBAR.101) and 3 of Ozbek-100 (only with p35SAcBAR.101) were found positive in primary screening. Hence, no primary transformants of STN-486 and Ozbek-100 with pAoPR1AcBAR.101 plasmid could be confirmed. GSN-12 and Ayhan- 107 showed 0.9 and 0.7 % transformation efficiency, respectively (data not provided).

Evaluation of primary transformants

The primary putative transgenic plants that grew well in green house were subjected to PCR for confirmation of presence of introduced *cry1Ac* gene in cotton genome along with promoter and selectable marker gene. Results showed the amplification of required band of 412 bp of *cry1Ac* gene, 900 bp of AoPR1 and 310 bp of BAR in primary transformants that were marked and subjected to further analysis (Fig. 2).

PCR-positive primary transformants with different constructs containing *cry1Ac* under 35S and AoPR1 promoter were subjected to ELISA to quantify expression level of *cry1Ac* after 0, 12 and 24 h of wounding (Fig. 3). The primary transformants with plasmid p35SAcBAR.101 showed varying levels of *cry1Ac* toxin. P16 (STN-486 plant transformed with *cry1Ac* under 35S promoter) had 0.025, 0.030 and 0.20 µg *cry1Ac*/g of fresh tissue weight, respectively. The concentration recorded in P53 (Ayhan-107 plant transformed with *cry1Ac* under 35S promoter) was 0.035, 0.022 and 0.031 µg/g of fresh tissue respectively. Likewise, *cry1Ac* concentration in P15 (GSN-12 plant transformed with *cry1Ac* under 35S promoter) was estimated as 0.042, 0.020 and 0.029 respectively while P27 (Ozbek-100 plant transformed with *cry1Ac* under 35S promoter) had 0.022, 0.030 and 0.036 µg/g of fresh tissue weight respectively after 0, 12 and 24 h of wounding (Fig. 3a).

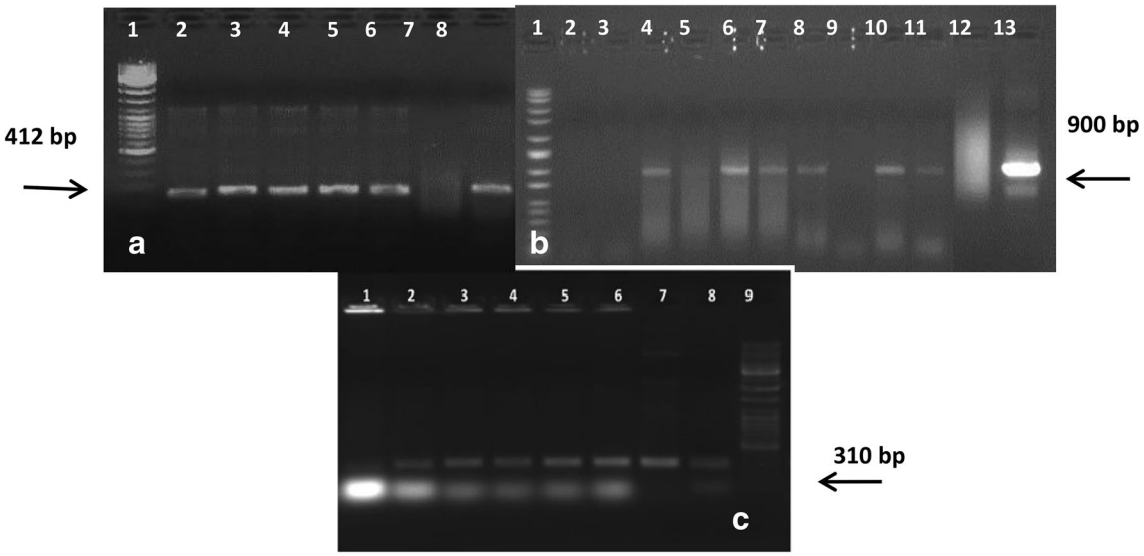


Fig. 2 Molecular evaluation of primary cotton transformants (T_0 progeny). **a** PCR assay showed the amplification of required *cry1Ac* band. *Lane 1* DNA ladder mix; *lanes 2–6* putative transgenic plants P4, P12, P7, P53 and P27; *lane 7* negative control; *lane 8* positive control. **b** PCR assay showed the amplification of AoPR1 promoter fragment, *lane 1* DNA ladder mix; *lanes 2–11* putative transgenic plants P12, P15, P151, P72, P75 and P76; *lane 12* negative control; *lane 13* positive control. **c** PCR confirmation of BAR gene in some of the putative transgenic plants. *Lane 1* negative control; *lanes 2–7* primary transformants P4, P12, P53, P73 and P27, S35-16; *lane 8* positive control; *lane 9* DNA ladder

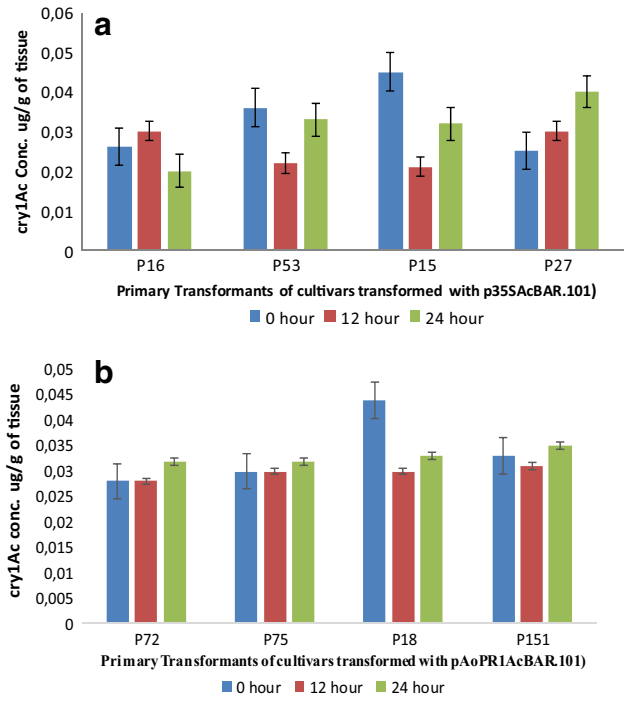


Fig. 3 ELISA assay confirmed the accumulated expression of insecticidal gene (*cry1Ac*) after 0, 12 and 24 h in primary transformants. Positive and negative controls used were provided in kit

Transgenic plants transformed with pAoPR1AcBAR.101 plasmid showed accumulated levels of *cry1Ac* protein. The assay results showed that P72 (Ayhan-107 plant transformed with *cry1Ac* under AoPR1 promoter) had 0.027, 0.027 and 0.032 µg/g of fresh tissue, respectively. There was no difference in protein concentration after 0 and 12 h. The concentration recorded in P75 was 0.031, 0.031 and 0.033 µg/g of fresh tissue, respectively. Protein concentration in P18 (GSN-12 plant transformed with *cry1Ac* under AoPR1 promoter) was estimated as 0.043, 0.03 and 0.033, respectively, while P151 has 0.033, 0.029 and 0.033 µg/g of fresh tissue weight, respectively, after 0, 12 and 24 h of wounding (Fig. 3b).

The PCR- and ELISA-positive primary transformants were selected to quantify *cry1Ac* transcript levels using real-time PCR. The plants exhibited varying expression levels of *cry1Ac* in real-time quantitative PCR analysis. According to the collected data, interestingly maximum expression level of *cry1Ac* (2.85 fold) was found in P15 harboring p35SAcBAR.101 in contrast to the ELISA results; followed by P151 harboring pAoPR1AcBAR.101 with comparatively less expression (0.75-fold). The plants harboring pKGH4 plasmid containing *cry1Ac* under 35S promoter was used as positive control, while non-transformed plant was used as negative control where no amplification of the gene was observed (Fig. 4).

The selected plants from each cultivar were subjected to leaf biotoxicity assays to evaluate the efficacy of *cry1Ac* gene against *Spodoptera exigua* larvae in T_o progeny. The data recorded up to a week of assays showed different toxicity levels against targeted insect pests in the putative transgenic plants obtained from each

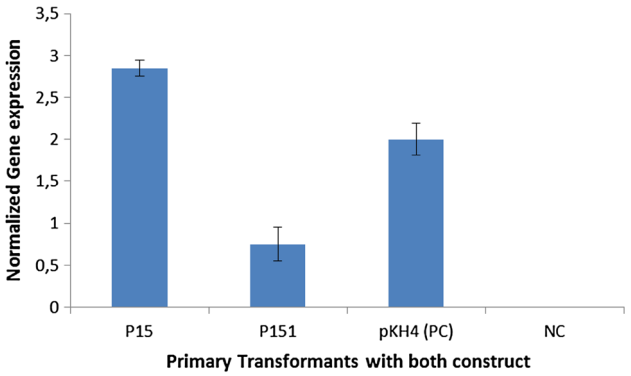


Fig. 4 Expression analysis of insecticidal gene (*cry1Ac*) in primary transformants along with positive and negative control plant by real-time PCR. Data represent means and standard errors of three replications. 18S RNA gene has been used as internal control to normalize data and gene expression has been indicated as a fold-increase relative

cultivar. GSN-12 and Ayhan-107 primary transformants expressing *cry1Ac* gene under AoPR1 showed more larval mortality as compared to transformants with p35SAcBAR.101 (Tables 2, 3). However, the *cry1Ac* gene expression was sufficient to kill the targeted insects in both types of plants. Some of GSN-12, STN-468 and Ozbek-100 putative transgenic plants transformed with p35SAcBAR.101 plasmid did not show encouraging larval mortality results (Tables 2, 3). The transgenic plants with less levels of larval mortality were not considered further. No mortality of larvae was recorded in non-transgenic control plants.

Evaluation of transgene progeny plants (T_1)

The primary cotton transformants positive for the introduced gene of each cultivar (based on PCR, ELISA and leaf bioassay data) were carefully picked and their progenies were raised in the green house. The representative transgenic plants from progeny of each cultivar were taken for further molecular analysis. The progeny results showed the amplification of 412 bp of *cry1Ac*, 310 bp of BAR and 900 bp of promoter fragment (Fig. 5).

The leaf bioassay results showed that T_1 transgenic plants were found to be as effective as their T_0 parent plants in causing mortality of larvae of *Spodoptera littoralis* larvae, although varying mortality rates in each transgenic plant were observed. T_1 progeny results of plants of each cultivar showed that wound-inducible expression of *cry1Ac* in transgenic progeny conferred enough protection against targeted insect pests (Table 4). No significant mortality was observed in larvae fed on non-transgenic cotton leaves (Fig. 6).

Discussion

Most of the transgenic crops around the world contain 35S promoter (Odell et al. 1985) which drives strong gene expression in almost all tissue types at high levels throughout the life cycle of the plant. The constitutive expression may reduce the acceptability and adaptability of *Bt* crops and increase the yield drag that may result from expressing constitutively high levels of foreign gene products in addition to causing target insects to develop resistance (Andow and Ives 2002; Breitler et al. 2004; Bates et al. 2005; Kim et al. 2008). Concerns that constitutive overexpression of *Bacillus thuringiensis* insect toxins in commodity crop plants will increase targeted insect resistance (Huang et al. 1999) have led the Environmental Protection Agency (EPA) to announce rules for resistance management by planting refuges of conventional crops. Therefore, in certain circumstances, it is desirable to use expression-specific promoters which only express the foreign gene in specific plant tissues or organs (Cai et al. 2007; Bakhsh et al. 2011). We have attempted a molecular strategy to minimize unwanted cry toxic protein products in transgenic cotton plants by confining only to the insect wounding sites. For this purpose, wound-inducible promoter isolated from *Asparagus officinalis* was cloned upstream to an insecticidal gene (*cry1Ac*) to construct a binary vector and later on four Turkish cotton cultivars were transformed with *cry1Ac* gene under the control of wound-inducible promoter AoPR1 in comparison with 35S promoter (Fig. 1).

The primary transformants obtained were analyzed for foreign gene integration and expression through conventional PCR, real-time PCR and ELISA. The screening of primary transformants with different constructs and cultivar revealed the successful incorporation (Fig. 2) and expression of insecticidal gene in cotton genome (Fig. 3). The better transformation efficiency recorded in GSN-12 and Ayhan-107 was 0.9 and 0.7 %, respectively. No primary transformants of STN-486 and Ozbek-100 with pAoPR1AcBAR.101 plasmid could be confirmed. Hence not all the primary transformants were positive at this stage; escapes were recorded that probably avoided selection pressure (McCormick et al. 1986). Our results are in line with several research workers who confirmed the integration of foreign DNA in cotton genome through PCR and ELISA (Schrammeijer et al. 1990; Cousins et al. 1991; Tohidfar et al. 2008; Khan et al. 2011; Bakhsh et al. 2012; Hussain et al. 2014).

The insect-resistant cotton plants were developed where the expression of *cry1Ac* gene was under the control of wound-inducible promoter. The results suggested that expression of the *cry1Ac* gene was dependent on wounding

Table 2 Leaf bioassays of first generation individual T_o transgenic plants of different cotton cultivars carrying 35S-Cry1Ac or AoPR1-Cry1Ac genes with third instar larvae of *Spodoptera exigua*

Plasmid	Cultivar	Plant no.	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Mortality (%) ± SE									
GV2260 pTF 101.1 35S-Cry1Ac	Control	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	GSN-12	4	22.15 ± 1.91	29.66 ± 0.70	35.97 ± 2.70	53.35 ± 2.60	70.34 ± 0.70	70.33 ± 0.70	73.80 ± 0.93
	GSN-12	5	20.00 ± 0.00	29.66 ± 0.70	46.65 ± 0.19	56.70 ± 0.19	60.00 ± 0.00	70.33 ± 0.70	76.82 ± 0.26
	GSN-12	6	4.53 ± 1.97	11.62 ± 5.26	19.31 ± 0.96	36.10 ± 1.55	39.37 ± 1.97	39.36 ± 1.97	43.16 ± 0.81
	GSN-12	7	0.00 ± 0.00	8.47 ± 13.84	15.35 ± 10.86	27.96 ± 4.83	38.16 ± 6.24	42.80 ± 3.96	42.80 ± 3.96
	GSN-12	8	1.15 ± 1.97	6.70 ± 3.20	11.62 ± 5.26	29.66 ± 0.70	36.45 ± 0.81	39.86 ± 0.61	39.86 ± 0.61
	GSN-12	9	0.00 ± 0.00	0.00 ± 0.00	8.76 ± 4.73	8.76 ± 4.73	11.62 ± 5.26	28.55 ± 2.50	34.80 ± 4.04
	GSN-12	13	4.53 ± 1.97	6.70 ± 3.20	6.70 ± 3.20	6.70 ± 3.20	25.00 ± 3.20	29.20 ± 1.97	36.10 ± 1.55
	GSN-12	14	32.30 ± 3.37	39.37 ± 2.54	50.00 ± 0.58	53.35 ± 0.78	60.00 ± 0.00	80.00 ± 0.00	90.75 ± 4.04
	GSN-12	15	0.00 ± 0.00	13.95 ± 6.77	27.96 ± 12.56	53.35 ± 2.60	56.84 ± 3.20	60.64 ± 4.32	67.71 ± 3.36
	STN-468	16	13.95 ± 6.77	35.97 ± 2.70	46.65 ± 0.78	60.65 ± 2.54	67.22 ± 1.52	75.00 ± 3.20	78.98 ± 4.04
	STN-468	19	13.01 ± 0.39	26.20 ± 0.93	22.16 ± 1.91	32.78 ± 1.52	36.10 ± 1.55	43.16 ± 1.39	50.00 ± 2.41
	STN-468	23	23.18 ± 0.26	23.18 ± 0.26	26.20 ± 0.93	43.30 ± 0.19	60.00 ± 0.00	60.00 ± 0.00	60.00 ± 0.00
	STN 468	24	1.15 ± 1.97	1.15 ± 1.97	3.69 ± 6.23	11.62 ± 5.26	22.46 ± 1.27	22.46 ± 1.27	25.44 ± 1.99
	Özbek-142	25	0.00 ± 0.00	0.00 ± 0.00	6.70 ± 3.20	13.02 ± 0.39	16.36 ± 0.39	20.00 ± 0.00	26.20 ± 0.93
	Özbek-142	27	26.52 ± 0.26	40.00 ± 0.00	46.65 ± 0.78	50.00 ± 0.58	53.35 ± 0.19	53.35 ± 0.19	60.14 ± 0.61
	Özbek-142	28	1.15 ± 1.97	2.37 ± 4.04	3.69 ± 6.23	19.42 ± 8.56	19.42 ± 8.56	39.36 ± 1.97	39.37 ± 1.97
GV2260 pTF 101.1 AoPR1-Cry1Ac	GSN-12	3	4.53 ± 1.97	22.16 ± 1.91	40.00 ± 0.00	43.30 ± 0.19	70.34 ± 0.70	70.34 ± 0.70	97.63 ± 4.04
	GSN-12	11	18.92 ± 10.61	18.92 ± 10.61	53.35 ± 0.78	53.35 ± 0.78	67.08 ± 0.93	67.08 ± 0.93	73.80 ± 0.93
	GSN-12	12	26.20 ± 0.93	26.20 ± 0.93	26.20 ± 0.93	75.00 ± 11.58	77.84 ± 9.92	77.84 ± 9.92	86.05 ± 6.77
	GSN-12	14	5.12 ± 8.56	22.16 ± 1.91	27.96 ± 4.83	60.00 ± 0.00	60.00 ± 0.00	86.05 ± 6.77	97.63 ± 4.04
	GSN-12	15	39.37 ± 2.54	53.35 ± 0.78	86.05 ± 6.77	86.05 ± 6.77	94.88 ± 8.56	94.88 ± 8.56	94.88 ± 8.56
	GSN-12	18	27.96 ± 4.83	53.35 ± 0.78	67.08 ± 0.93	86.05 ± 6.77	90.75 ± 4.04	97.63 ± 4.04	97.63 ± 4.04
	GSN-12	19	16.36 ± 0.39	39.37 ± 2.54	54.01 ± 3.36	75.00 ± 11.58	80.58 ± 8.56	86.05 ± 6.77	90.75 ± 4.04
	GSN-12	20	36.59 ± 0.21	36.59 ± 0.21	46.65 ± 0.78	60.65 ± 2.54	60.65 ± 2.54	91.53 ± 13.84	93.30 ± 11.08
	GSN-12	23	16.36 ± 0.39	26.20 ± 0.93	26.20 ± 0.93	53.35 ± 0.78	53.35 ± 0.78	80.01 ± 0.00	80.01 ± 0.00

Table 3 Leaf bioassays of second generation individual T_o transgenic plants of different cotton cultivars carrying 35S-Cry1Ac or AoPR1-Cry1Ac genes with third instar larvae of *Spodoptera exigua*

Plasmid	Cultivar	Plant no.	12 h	24 h	48 h	72 h	120 h	144 h
Mortality (%) ± SE								
pTF 101.1 35S-Cry1Ac/BAR	Control	Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Ayhan	7	54.01 ± 3.36	68.97 ± 13.84	68.97 ± 13.84	68.97 ± 13.84	86.05 ± 6.77	97.63 ± 4.04
	Ayhan	53	60.65 ± 2.54	80.58 ± 8.56	86.05 ± 6.77	94.88 ± 8.56	97.63 ± 4.04	97.63 ± 4.04
	Ayhan	59	5.12 ± 8.56	60.65 ± 2.54	60.65 ± 2.54	86.05 ± 6.77	97.63 ± 4.04	100.00 ± 0.00
	GSN	188	25.00 ± 11.58	25.00 ± 11.58	53.35 ± 0.78	67.08 ± 0.93	94.88 ± 8.56	94.88 ± 8.56
	GSN	211	2.37 ± 4.04	38.02 ± 17.65	38.02 ± 17.65	38.02 ± 17.65	45.28 ± 20.54	60.65 ± 7.61
pTF 101.1 AoPR1-Cry1Ac/BAR	Ayhan	71	9.25 ± 4.04	20.01 ± 0.00	20.01 ± 0.00	20.01 ± 0.00	53.35 ± 0.78	60.65 ± 2.54
	Ayhan	72	75.00 ± 11.58	75.00 ± 11.58	75.00 ± 11.58	81.08 ± 10.61	100.00 ± 0.00	100.00 ± 0.00
	Ayhan	73	25.00 ± 11.58	46.65 ± 0.78	46.65 ± 0.78	53.35 ± 0.78	86.05 ± 6.77	97.63 ± 4.04
	Ayhan	75	60.00 ± 0.00	80.58 ± 8.56	80.58 ± 8.56	94.88 ± 8.56	100.00 ± 0.00	100.00 ± 0.00
	Ayhan	76	38.02 ± 17.65	75.00 ± 11.58	81.08 ± 10.61	86.05 ± 6.77	97.63 ± 4.04	100.00 ± 0.00
	Ayhan	257	25.00 ± 16.09	38.02 ± 17.65	38.02 ± 17.65	38.02 ± 17.65	86.05 ± 6.77	94.88 ± 8.56
	GSN	68	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	13.95 ± 6.77	13.95 ± 6.77
	GSN	102	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	25.00 ± 35.81	46.65 ± 28.99
	GSN	106	2.37 ± 4.04	2.37 ± 4.04	2.37 ± 4.04	8.47 ± 13.59	39.37 ± 7.61	75.00 ± 11.58
	GSN	125	0.00 ± 0.00	2.37 ± 4.04	13.95 ± 6.77	13.95 ± 6.77	53.35 ± 5.95	73.80 ± 0.93
	GSN	137	8.47 ± 13.84	8.47 ± 13.84	8.47 ± 13.84	18.06 ± 20.54	75.00 ± 11.58	91.53 ± 13.84
	GSN	141	8.47 ± 13.84	8.47 ± 13.84	8.47 ± 13.84	13.02 ± 20.54	61.98 ± 17.65	81.08 ± 10.61
	GSN	151	53.35 ± 0.78	73.47 ± 0.93	86.05 ± 6.77	90.75 ± 4.04	97.63 ± 4.04	100.00 ± 0.00
	GSN	253	2.37 ± 4.04	5.12 ± 8.56	5.12 ± 8.56	13.02 ± 20.54	31.63 ± 16.22	80.58 ± 8.56
	GSN	254	8.47 ± 13.84	8.47 ± 13.84	8.47 ± 13.84	8.47 ± 13.84	39.37 ± 30.12	53.35 ± 28.99
	GSN	255	18.92 ± 10.61	18.92 ± 10.61	18.92 ± 10.61	46.65 ± 5.95	67.08 ± 0.93	90.75 ± 4.04
	GSN	264	8.47 ± 13.84	8.47 ± 13.84	8.47 ± 13.84	8.47 ± 13.84	46.65 ± 5.95	60.65 ± 7.61
	GSN	269	0.00 ± 0.00	13.95 ± 6.77	39.37 ± 2.54	39.37 ± 2.54	60.65 ± 2.54	60.65 ± 2.54

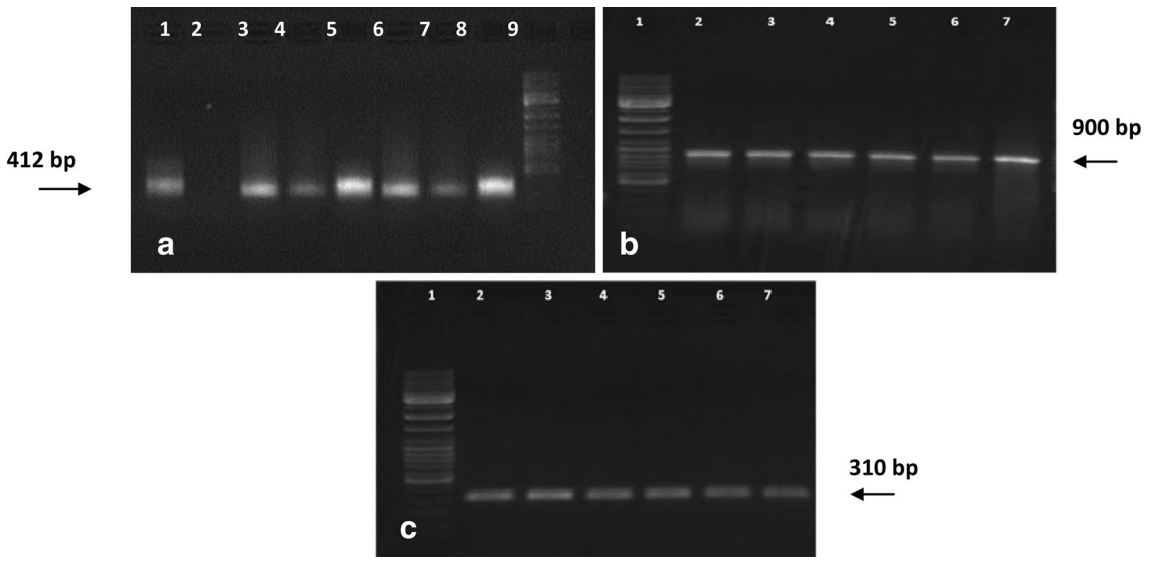


Fig. 5 Molecular analysis of transgenic cotton plants (T_1 progeny). **a** Amplification of *cry1Ac* in transformed plants of cultivar. *Lanes 1–7* P14A, P16, 27A, P15A, P18A, P151; *lane 8* positive control; *lane 9* DNA ladder. **b** PCR assay showed the amplification of AoPR1 promoter fragment. *Lane 1* DNA ladder; *lanes 2–6* transgenic plants P15A, P18A, P18B, P19A and P151; *lane 7* positive control. **c** The amplification of BAR gene fragment, *lane 1* DNA ladder; *lanes 2–7* P14A, P16, P27, P15, P18A and P18B

Table 4 Leaf bioassays of individual T_1 transgenic plants of different cotton cultivars carrying 35S-Cry1Ac or AoPR1-Cry1Ac genes with third instar larvae of *Spodoptera littoralis*

Plasmid	Cultivar	Plant no.	24 h	48 h	72 h	96 h	120 h	144 h	168 h
Mortality (%) ± SE									
pTF 101.1 35S-Cry1Ac/BAR	GSN-12	14A	67.08 ± 0.93	86.05 ± 6.77	86.05 ± 6.77	94.88 ± 8.56	97.63 ± 4.04	97.63 ± 4.04	97.63 ± 4.04
	STN-468	16	13.95 ± 6.77	13.95 ± 6.77	13.95 ± 6.77	46.65 ± 0.78	46.65 ± 0.78	46.65 ± 0.78	60.65 ± 2.54
	Özbek142	27A	18.92 ± 10.61	32.29 ± 3.36	39.37 ± 2.54	54.01 ± 3.36	54.01 ± 3.36	54.01 ± 3.36	60.65 ± 2.54
	Özbek142	27B	53.35 ± 28.99	86.99 ± 20.54	94.88 ± 8.56	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	Özbek142	27C	26.20 ± 0.93	45.99 ± 3.36	60.65 ± 2.54	67.08 ± 0.93	80.01 ± 0.00	80.01 ± 0.00	80.01 ± 0.00
	GSN-12	15A	13.95 ± 6.77	13.95 ± 6.77	26.20 ± 0.93	26.20 ± 0.93	90.75 ± 4.04	100.00 ± 0.00	100.00 ± 0.00
pTF 101.1 AoPR1-Cry1Ac/BAR	GSN-12	15B	39.37 ± 30.12	46.65 ± 28.99	46.65 ± 28.99	46.65 ± 28.99	46.65 ± 28.99	53.35 ± 28.99	100.00 ± 0.00
	GSN-12	15C	26.20 ± 0.93	32.29 ± 3.36	32.29 ± 3.36	39.37 ± 2.54	39.37 ± 2.54	46.65 ± 0.78	81.08 ± 10.61
	GSN-12	18A	25.00 ± 16.09	46.65 ± 28.99	68.97 ± 13.84	75.00 ± 11.58	75.00 ± 11.58	75.00 ± 11.58	86.05 ± 6.77
	GSN-12	18B	20.01 ± 0.00	26.20 ± 0.93	40.00 ± 0.00	60.00 ± 0.00	60.00 ± 0.00	60.00 ± 0.00	60.00 ± 0.00
	GSN-12	18C	5.12 ± 8.56	32.29 ± 3.36	32.29 ± 3.36	80.58 ± 8.56	94.88 ± 8.56	94.88 ± 8.56	100.00 ± 0.00
	GSN-12	18D	39.37 ± 7.61	68.97 ± 13.84	80.58 ± 8.56	80.58 ± 8.56	80.58 ± 8.56	80.58 ± 8.56	80.58 ± 8.56
	GSN-12	19A	5.12 ± 8.56	46.65 ± 28.99	46.65 ± 28.99	46.65 ± 28.99	68.97 ± 13.84	68.97 ± 13.84	68.97 ± 13.84
	GSN-12	19B	39.37 ± 2.54	39.37 ± 2.54	68.37 ± 16.22	75.00 ± 11.58	80.58 ± 8.56	86.05 ± 6.77	100.00 ± 0.00
	GSN-12	19C	68.37 ± 16.22	75.00 ± 11.58	75.00 ± 11.58	80.58 ± 8.56	80.58 ± 8.56	86.05 ± 6.77	100.00 ± 0.00

of the plant tissues. The amount of *cry1Ac* protein increased high enough to cause the mortality of the *Spodoptera exigua* (Fig. 3). Gulbitti-Onarici et al. (2009) also found increased levels of *Bt* gene under control of wound-inducible promoter in tobacco that conferred full protection against *Heliothis virescens* and *Manduca sexta*. The wound-inducible promoter AoPR1 used in the study is a class of pathogenesis-related (PR) proteins that are induced in response to various stresses such as pathogen attacks, wounding and application of chemicals. Promoters from other PR proteins have previously been shown to drive the expression of *Bt* genes in transgenic broccoli and cabbage (Cao et al. 2001; Jin et al. 2000).

Real-time quantitative PCR analysis was used to detect the level of expression of *cry1Ac* in primary transformants of cultivar GSN-12 along with positive and negative controls. The transformed plants showed variable expression level of gene (Fig. 4). Primary transformants with p35SAcBAR.101 interestingly exhibited more level of *cry1Ac* gene compared to the plants with AoPR1AcBAR.101 plasmid. This might be attributed to less wounding time prior to RNA isolation only for 6 h. The plants with 35S promoter may exhibit more mRNA levels in shorter time because of its robust activity. However, it might be fully understood in further studies by inducing wounds in transgenic leaves at different intervals. Despite that, ELISA results showed that expression levels in both constructs are enough to confer protection against targeted insect pests (Tables 2, 3). No amplification of the gene was observed in non-transgenic plant. Many researchers have confirmed the levels of foreign gene expression in cotton plants using real-time quantitative PCR (Maqbool et al. 2010; Rao et al. 2011) who also obtained varying expression levels among transformed plants.

Transgenic cotton plants expressing *cry1Ac* gene under the control of AoPR1 and 35S promoters were evaluated for toxicity against the larvae of *S. exigua* in T_o progeny. Leaf biotoxicity assays exhibited larval mortality at varying levels when data were recorded up to 5 days. The progeny of transgenic cotton plants conferred appreciable protection after 120 h against targeted insect pests. However, primary transformants with *cry* gene under the control of AoPR1 exhibited more larval toxicity (Tables 2, 3) showing strong promoter activity in response. This also indicates the wound-specific activity of AoPR1 promoter which started expressing *cry1Ac* at the wound sites of the wounded leaves. The representative primary transformants from each cultivar were harvested and to confirm gene incorporation and expression in T_1 progeny, the plants were subjected to PCR (Fig. 5) and leaf bioassays using *S. littoralis* (Table 4; Fig. 6) third instar larvae. The levels of *cry1Ac* protein accumulated

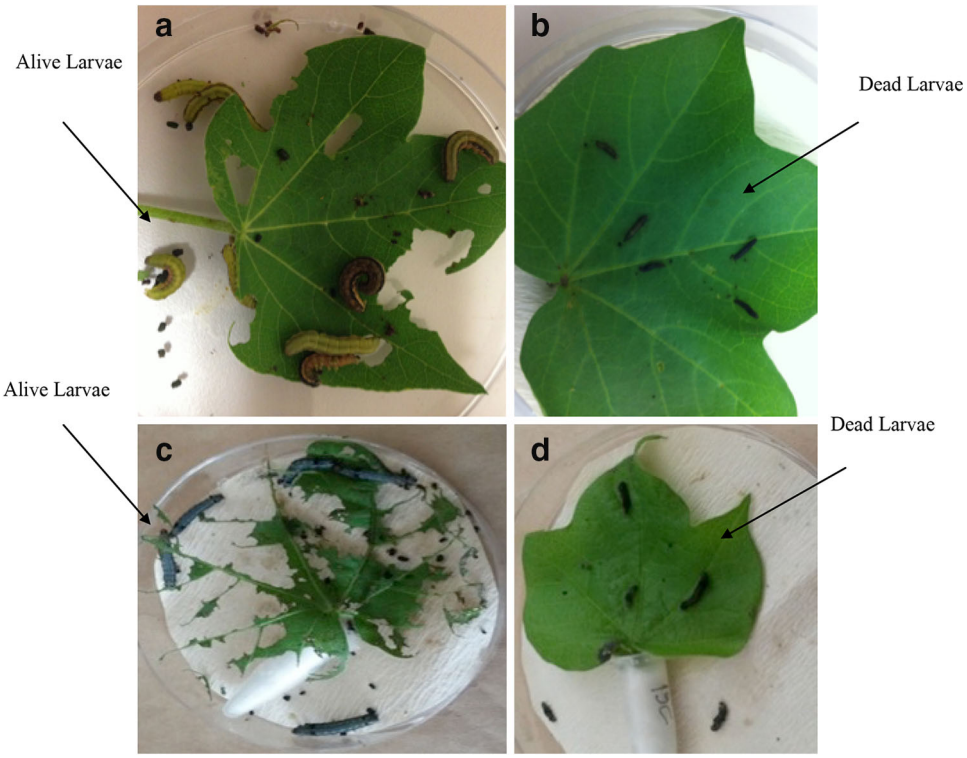


Fig. 6 Leaf biotoxicity assays conducted were on leaves of T_0 and T_1 progeny plants of cotton. The transgenic plants showed appreciable level of resistance against *Spodoptera exigua* in T_0 Progeny (**b**) and *S. littoralis* in T_1 progeny (**d**). The *S. exigua* and *S. littoralis* larvae found dead when fed to transgenic leaf while larvae were noticed alive and chewing leaf of non-transgenic cotton plant (**a**, **c**)

were also sufficient to confer protection against targeted T_1 progenies of each cultivar. These results are in agreement with Breitler et al. (2004), Gulbitti-Onarici et al. (2009) and Kumar who found similar results by using wound-inducible promoter in rice, tobacco and cotton, respectively. The plants showing less mortality in leaf bioassays against targeted pests were rejected at the stage; it can be attributed to low cry toxin production in transgenic plant (Khan et al. 2011).

In the previous studies conducted on Bt crops (especially cotton), researchers used 35S CaMV promoter to drive the expression of insecticidal genes (Perlak et al. 1991; Cousins et al. 1991; Xie et al. 1991; Jenkins et al. 1997; Ni et al. 1998; Rashid et al. 2008; Bakhsh et al. 2012). The levels of insecticidal protein to kill the targeted insects reported earlier are almost the same as we found in our study. However, 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 (Frutos et al. 1999; High et al. 2004). After two decades of intensive cultivation, the sustainability of Bt crops has been questioned as increasing pest resistance to transgenic plants has been frequently reported (Gassmann et al. 2011; van den Berg et al. 2013; Tabashnik et al. 2013). Hence, such strategy to develop insect resistance crops with restricted gene expression seems attractive to ensure biosafety and better insect resistance management strategy.

In the present study, ingestion of small amounts of leaf tissue by targeted insect pests was sufficient to kill the pest proving an efficient insect-resistant management strategy. We conclude that insect control can be achieved by a confined and limited expression of Bt toxin under wound-inducible AoPR1 promoter. Based on the expression of the GUS reporter gene under the control of the AoPR1 promoter by Warner et al. (1992), Özcan et al. (1993) and Firek et al. (1993), it is concluded that Bt toxin will not accumulate in pollen, unwanted plant organs, seed and crop residue, thus minimizing food and environmental concerns; hence, can also lead to the acceptability and marketability of insect resistant crops.

Conclusion

We developed insect-resistant transgenic lines of four Turkish cotton cultivars (GSN-12, STN-468, Ozbek-100 and Ayhan-107) that express insecticidal gene *cry1Ac* expression under the control of a wound-inducible promoter AoPR1 isolated from *Asparagus officinalis;* the prime objective of the study. The presence of BAR gene (that encodes resistance against glufosinate; an active ingredient in several nonselective herbicides such as Basta and Liberty, etc.) in these transgenic lines adds additional herbicide-resistant trait (as we selected transformants on selection pressure of herbicide; further analysis are also in progress). We conclude that transgenic lines are an excellent source of germplasm for cotton breeding program.

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