Development of transgenic tobacco lines with pyramided insect resistant genes

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Abstract: Insect pests are among the major constraints rendering drastic decreases in crop yield. The expression of stacked insecticidal genes in crops can lead to resistance durability and can delay the development of resistance in target insect pests. The present study was designed to introduce an insect resistance trait in locally cultivated Turkish tobacco cultivars (Basma and Nail) with pyramided insecticidal genes. *Agrobacterium* strain LBA4404 harboring plasmid pKGH4 with *cry1Ac* and *cry2A* genes under the control of 35S promoter was used to infect leaf discs of both cultivars; plasmid also contained *uidA* within the T-DNA region for earlier screening of putative transformants. The overall transformation efficiency was calculated as 30.7% and 18.8% in Basma and Nail, respectively. PCR results confirmed the integration of *cry1Ac*, *cry2A*, *uidA*, and *nptII* genes in 40 plants of Basma and 16 plants of Nail. ELISA results showed variation in expression of *cry1Ac* protein among transgenic plants varying from 0.017 to 0.607 µg/g of fresh tissue. Bioassay results with potato tuber moth (*Phthorimea operculella* Zeller) showed significant mortality of the targeted pest on primary transformants. Furthermore, T1 transgenic progeny exhibited the inheritance of T-DNA in Mendelian as well as non-Mendelian fashion. The results revealed that lines can serve as a source of germplasm in tobacco breeding programs.

Key words: Tobacco, genetic transformation, protein expression, insect resistance

1. Introduction

Turkish tobacco, also known as oriental or Grecian tobacco, has slightly lower nicotine contents compared to some other varieties under identical growing conditions, while being smoother and more flavorful. Another tobacco species, *Nicotiana rustica*, or Indian tobacco, is an extremely high nicotine content tobacco used mainly for ceremonial smoking and making poisonous darts. Tobacco is commonly used as a recreational drug and is a valuable cash crop in various countries including Turkey (Villegier et al., 2003). The crop was cultivated on an area of 0.94 million ha with production of 70,000 t (TÜİK, 2016).

Insect pests of tobacco are a serious threat to luscious, leafy, and succulent plants in field conditions, and are considered one of the main factors limiting crop productivity. Insect pests and diseases cause 37% losses of agricultural production around the globe (Gatehouse et al., 1992). Likewise, chemical insecticides are an effective and the predominant type of pest control strategy employed in tobacco fields. However, careless and constant application of insecticides has led to the development of resistance in pests (Zhu et al., 2016). Moreover, insecticides have also been reported to be harmful for nontarget/beneficial

insects and cause potential damage to the natural environment. Insect resistant transgenic plants have emerged as potential alternatives to synthetic insecticides (Sohail et al., 2012).

The modern approaches in biotechnology have revolutionized plant breeding by introducing trait(s) of economic importance in crop crops (Liu et al., 2015; Nadeem et al., 2017). The plantation area of genetically modified (GM) crops has consistently increased each year since the first GM crop was first commercially cultivated in 1996, reaching 185.1 million ha in 2016 (ISAAA, 2016). These transgenic plants are becoming important components of integrated pest management (IMP) worldwide (Kos et al., 2009). *Bacillus thuringiensis* (Bt) is the most important source of insect resistant genes. Genes from *B. thuringiensis* encode for crystal proteins, which are toxic against larvae of different insects, i.e. lepidopterans, coleopterans, and dipterans (reviewed in Bakhsh et al., 2015).

First generation Bt crops effectively controlled some of the target insects but questions have been raised regarding the efficacy of first generation Bt crops. There are many reports that intensive cultivation of Bt crops has led to

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increasing pest resistance against transgenic plants (Bravo et al., 2008; Tabashnik et al., 2008). The evolution of resistance in insect pests threatens the long-term benefits of transgenic Bt crops expressing insecticidal proteins from *Bacillus thuringiensis*. A study by Tabashnik et al. (2013) regarding field monitoring data of pest resistance to Bt crops revealed that 5 out of 13 major pest species had evolved field resistance, whereas this number increased to 16 in 2016, indicating an alarming situation for scientists (Tabashnik and Carrière, 2017). The expression of more than one toxin has been proposed to address the problem of resistance development in crop plants (Sayyed and Wright, 2001). In order to delay the development of resistance in insects, combination of two insecticidal genes with two different binding sites in pest is expected to trigger broad spectrum resistance (Hernandez-Martinez et al., 2008).

The present research was conducted to incorporate two insecticidal genes in Basma (narrowed leaved) and Nail (broad leaves), locally cultivated tobacco cultivars, to develop broad spectrum resistance against targeted insect pests by gene pyramiding strategy.

2. Materials and methods

2.1. Plant material and expression vector

Two tobacco cultivars, Basma and Nail, cultivated in the tobacco growing zone of Turkey were used for the study. The plant expression vector pKGH4 harboring two insecticidal genes, *cry1Ac* and *cry2A*, under the control of 35S promoter individually (Bakhsh et al., 2012) was electroporated in *Agrobacterium tumefaciens* strain LBA4404 (Figure 1). Positive clones were confirmed using PCR and restriction digestion reaction prior to transformation of both cultivars (data not shown). *Escherichia coli* strain Dh5 α cells were used for the cloning and propagation of the recombinant plasmid vector, whereas LBA4404 strain harboring pKGH4 plasmid was used as vector for explant transformation.

2.2. Seed germination and genetic transformation

The seeds of both cultivars were surface-sterilized by immersion in 30% (v/v) sodium hypochlorite solution

with continuous shaking for 15 min. The seeds were rinsed three times with sterile distilled water for 5 min and cultured in petri plates containing MS medium (Murashige and Skoog, 1962) without any growth regulator. The data for germination rates were recorded daily until day 7. Later, plantlets were shifted to magenta boxes for further growth (Duchefa, Cat # S1686).

Leaf discs from young in vitro growing plants of Basma and Nail were subjected to *Agrobacterium*-mediated transformation using the protocol as described by Horsch et al. (1985). After co-cultivation, the leaf discs were moved to regeneration selection medium (MS supplemented with BAP 2 mg L⁻¹, NAA 0.2 mg L⁻¹) along with Duocid 300 mg L⁻¹ (to suppress the growth of agrobacterium) and Kanamycin 100 mg L⁻¹ (for selection of primary transformants). Data for resistant calli, number of shoots per explant, and rooting percentage were recorded. The shoots reaching a length of 2 to 3 cm were excised and transferred to large magenta box containing the same medium as mentioned above. Later, plantlets with well-developed roots were transferred into pots with peat and perlite (3:1) and were allowed to set seeds. The transgenic plants grew normally and were self-fertilized.

2.3. GUS histochemical assay

As pKGH4 contained the *uidA* gene in the T-DNA region encoding β -glucuronidase enzyme, it was relatively easy to screen for putative transgene at an earlier stage. The kanamycin resistant leaf discs from putative transformants were subjected to GUS histochemical assay. The GUS solution was prepared containing 10 mg/L X-gluc, 10 mM EDTA, 100 mM NaHPO₄, 0.1% Triton X-100, and 50% methanol (pH was adjusted to 8.0). The resistant leaf discs were dipped in solution and incubated at 37 °C overnight. The staining solution was removed and washed with 70% ethanol until the tissue became cleared.

2.4. Evaluation of putative transgenic tobacco plants

DNA extractions were carried out from primary transformants using a Nucleo Spin Plant II Kit (Macherey-Nagel GmbH & Co). Polymerase chain reaction (PCR) was carried out using gene specific primer to amplify internal

Figure 1. Schematic representation of the T-DNA region of pKGH-4. Both insecticidal genes (*cry1Ac* and *cry2A*) were under the control of 35S promoters individually. The construct contained the *uidA* gene interrupted by an intronic sequence facilitating earlier screening of primary transformants. Kanamycin was used at concentration of 100 mg/L for plant selection.

fragments of *cry1Ac*, *cry2A*, *uidA*, and *nptII* genes from putative transgenic plants. PCR was performed in a total reaction mixture volume of 20 µL containing 1X reaction buffer, 10 ng of DNA template, 1.5 mM MgCl₂, 0.1 mM of dNTPs mix, 0.5 µM of each primer, and one unit of Taq DNA polymerase (Thermo Scientific Cat. No. EP042). PCR was carried out in a thermal cycler using initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 to 60 °C for 30 s (temp. varied with the type of primers used), and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 10 min. In all PCR reactions, the plasmid DNA was used as a positive control, whereas the DNA isolated from untransformed plants was used as a negative control. Table 1 provides information about primer sequences, product sizes, and annealing temperatures.

Agrobacterium infection of a plant host is dependent on activation of chromosomal virulence *chvA* genes along with Vir genes. The *chvA* gene has a role in the attachment of the bacteria to the host cell (Nester, 2015). Therefore, primary transformants can be confirmed for the presence or absence of agrobacterium contamination using gene specific primers of the above-mentioned genes. The transformed tobacco lines developed were subjected to PCR to evaluate the presence of any agrobacterium.

PCR positive plants were subjected to real-time PCR to determine the relative expression of the *cry1Ac* gene. Total RNA isolation was performed from leaves of selected transgenic plants using E.Z.N.A. Total RNA Kit I (Cat. No. R6834); 2 µg of RNA was reverse transcribed to cDNA using a Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Cat No. K1622). Real-time PCR

reactions were carried out with LightCycler Real-Time PCR Systems (Roche) in a 96-well plate using the IQTM SYBR Green Supermix. 18S RNA primers were used as an internal 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 58 °C for 30 s, and extension at 72 °C for 40 s, and a final elongation step at 72 °C for 10 min. The melting curve was analyzed by continuous monitoring of fluorescence between 60 °C and 95 °C with 0.5 °C increments every 30 s.

The primary transformants were subjected to double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) to quantify the accumulated levels of the *cry1Ac* protein expressed in the leaves of putative transgenic plants using an Envirologix kit (Cat# AP051). Approximately 100 mg fresh terminal leaves was ground in liquid nitrogen and extraction buffer (provided in the kit) was added and the 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.

To determine the efficacy of the introduced insecticidal gene (*cry1Ac*), tobacco primary transformants in T0 progeny were subjected to leaf bioassays with third instar larvae of potato tuber moth (PTM), which has been a minor pest of tobacco for more than 100 years, although it has emerged as a problem in tobacco plantings over the last 5 years (Vaneva-Gancheva and Dimitrov, 2013). The fresh leaves from each plant were taken and placed on wet filter paper in petri plates accommodating one leaf per plate. The five larvae were prefasted for 4–6 h, and allowed

Table 1. Primer used in PCR studies to detect introduced gene(s) in primary transformants of lines developed out of Basma and Nail cultivars.

Primer	Sequence (5'-3')	Product size (bp)	Annealing temp. (°C)
<i>npt-II</i>	TTGCTCCTGCCGAGAAAG GAAGGCGATAGAAGG CGA	780	58
<i>cry1Ac</i>	ATGGACAACCCAAACATC TCATGTCGTTGAATTGAATACG	690	58
<i>cry2A</i>	AGATTACCCAGTTCCAGAT GTTCCCGAAGGACTTTCTAT	600	62
<i>uidA</i>	CCCTTACGCTGAAGAGATGC GAGCGTCGCAGAACATTACA	350	55
<i>cry1Ac(RT)</i>	ATCGGTATCAACAACCAGCA AACCTGTGGGAGAATCCTTG	173	58
<i>chvA</i>	CGAAACGCTGTTTCGGCCTGTGG GTTACAGCAGGCCGCGCATCCTGG	890	65

to feed on the transgenic and control leaf. Mortality data of the larvae were recorded for 48 h.

2.5. Transgene confirmation in subsequent progeny (T1)

The primary transgenic plants developed out of both tobacco cultivars were self-fertilized and were subjected to different assays to confirm the gene integration and expression in subsequent progeny. The chi-square test was applied to determine the segregation pattern of introduced T-DNA in transgenic plants of Basma and Nail. The seeds of 20 PCR-positive plants of Nail and Basma were allowed to germinate on MS medium supplemented with kanamycin at a concentration of 100 mg/L and 50 sterilized seeds harvested from each plant were incubated on MS media in three replicates. The petri plates were cultured in a growth chamber using standard culture conditions. Kanamycin-resistant shoots were subjected to GUS, PCR, and ELISA.

3. Results

As the seeds of Basma and Nail were collected from open field conditions, seed sterilization with sodium hypochlorite solution resulted in containment of pathogens in MS media, while appreciable germination results were recorded. Germination rates of Nail and Basma remained

90% and 70%, respectively (data not provided). Sodium hypochlorite is a commonly used surface sterilizing agent in in vitro culture experiments of many plants (Day et al., 2016). A total of 2400 leaf discs (1200 from each cultivar) were excised and inoculated with *Agrobacterium* strain LBA4404 harboring recombinant plasmid. The calli initiation started a week after incubating explants on RSM, whereas shoots started emerging 4 weeks later. The resistant shoots continued to grow on RSM, and later were excised and transferred to magenta boxes (107 × 94 × 96 mm). The plantlets developed roots on rooting medium in the presence of selectable marker (Figure 2).

As plasmid contained the *uidA* gene with the T-DNA region, the kanamycin-resistant tobacco plantlets were subjected to GUS histochemical assays facilitating earlier screening of putative transgenic shoots (Figure 3). The well rooted putative transgenic shoots grew well and were allowed to self-fertilize normally to set seed. Based on the GUS assay, molecular analysis, and protein expression, the transformation efficiency was recorded as 30.7% and 18.8% in transgenic plants of Basma and Nail, respectively (Table 2).

Figure 2. Different steps of genetic transformation of tobacco. Infection with *agrobacterium* harboring plasmid (a), co-cultivation (b), induction of resistant calli/shoots from leaf discs of Basma on RSM (c), resistant calli with shoots (d), a rooted putative plant (e), putative transgenic plants in soil (f).

Figure 3. GUS histochemical assay of primary transformants. Control and putative transgenic leaf after co-cultivation (A), transgenic shoots on RSM in comparison with control (B), newly regenerated shoot and plantlets expressing GUS at later stage (C and D).

Table 2. Transformation efficiency of Basma and Nail cultivars.

Cultivar	No. of explants used	No. of resistant calli (%)	No. of shoots/explant	Rooting efficiency (%)	No. of rooted plants in soil	No. of PCR positive plants	Transformation efficiency (%)
Basma	1200	75.6	12.2	90	130	40	30.7
Nail	1200	55.5	8.5	90	85	16	18.8

3.1. Molecular evaluation of transgenes (T0)

PCR assays of the primary transformants revealed the amplification of required bands of 690 bp of the *cry1Ac* gene, 600 bp of *cry2A*, 365 bp of *uidA*, and 450 bp of *nptII* genes in primary transformants (Figures 4A–4D). The primary transformants were also evaluated to confirm the presence of any agrobacterium contamination, and results revealed no agro contamination in transgenic plants (Figure 4E).

The real-time quantitative data of PCR positive plants exhibited accumulated transcript levels of *cry1Ac* though plants exhibited varied expression levels of *cry1Ac*. According to the collected data, the maximum expression level of *cry1Ac* (3.75-fold) was found in plant B9, followed by B13 (3.0-fold), N25 (2.7-fold), and N11 (2.48-fold). A positive cotton plant harboring pKGH4 plasmid containing *cry1Ac* under 35S promoter was used as a positive control (C1), while nontransformed tobacco (C2) was used as a negative control (Figure-5).

Figure 4. PCR analysis of putative transgenic plants of Basma and Nail (A) amplification of *cry1Ac* in primary transformants of Basma and Nail. Lane 1: DNA ladder Mix, Lanes 2–9: Basma plants, Lanes 10–16: Nail plants, Lane 17: –ve control, Lane 18: +ve control (B) amplification of *nptII*. Lanes 1–7: Basma plants, Lanes 8–12: Nail plants, Lane 13: +ve control, Lane 14: –ve control, Lane 15: 1 kb plus ladder (C) amplification of *cry2A* gene. Lanes 3–8: plants of Basma and Nail, Lane 2: +ve control, Lane 1: 100 bp plus ladder (D) amplification of *uidA* plants of Basma and Nail (2–9), Lane 1: 100 bp plus ladder, Lane 10: +ve control, (E) amplification of *chvA*. Lane 1: 1 kb plus ladder, Lane 2: Agrobacterium colony harboring pKHG4, Lanes 3–8: plants of Basma and Nail.

Figure 5. Expression analysis of *cry1Ac* in primary transformants by real-time PCR. Data represent means and standard errors of three replications. 18S rRNA gene was used as internal control to normalize data and gene expression is indicated as a fold-increase relative.

PCR-positive plants were subjected to ELISA to determine the concentration of cry1Ac protein in primary transformants. The results showed the maximum expression of cry1Ac protein (0.58 µg/g of fresh wt.) in plant B9, followed by B40 (0.42), B5 (0.25), B2 (0.13), and B25 (0.13). N22 showed the maximum cry1Ac expression (0.54), followed by N11 (0.27), N33 (0.17), N2 (0.14), and N3 (0.03). No cry1Ac protein was detected in nontransformed plants (Figure 6).

The leaf bioassays with third instar larvae of the potato tuber moth showed that *cry1Ac* expression in transgenic plants conferred protection against PTM (Figure 7A). The mortality rates of PTM varied with different transgenic plants (Figure 7B), reaching between 65% and 100%. Larvae were either dead or sluggish to harm the transgenic leaves. No mortality of larvae was recorded in negative nontransgenic control plants.

3.2. Evaluation of transgenic progeny (T1)

The seeds collected from PCR positive self-fertilized T0 transgenic plants were allowed to germinate on MS media supplemented with kanamycin (100 mg/L) and the chi-square goodness of fit test was applied. The germination rates on selective media showed variation, mainly exhibiting distorted Mendelian ratios of resistant and susceptible plants. Only six lines (N33, B10, B19, B29, B32, and B35) showed an expected 3:1 Mendelian ratio (Table 3). The germinated plants subjected to GUS histochemical, PCR, and ELISA assays also confirmed the stable inheritance of introduced genes in T1 progeny. GUS histochemical analysis revealed the functionality of the *uidA* gene in T1 progeny (Figure 8). PCR assays also showed the proper integration of transgenes in subsequent

progeny (Figure 9). Appreciable levels of cry1Ac protein were recorded in subsequent progeny sufficient to confer protection against targeted pests as revealed by our biotoxicity assays (Figure 10).

4. Discussion

The agrobacterium-mediated binary vector system has been an efficient system of transfer of gene(s) encoding traits of economic importance. Agrobacterium strains are responsible not only for infectivity but also for the efficiency of gene transfer (Chetty et al., 2013; Bakhsh et al., 2014). *Agrobacterium tumefaciens* strain LB4404 was found to be infective with respect to transformation and also was more efficient than other strains (Bakhsh et al., 2014). The transformation efficiency in the present study was 30.7% and 18.8% in Basma and Nail, respectively (Table 1). Various factors affect *Agrobacterium*-mediated transformation in different crops and within cultivars of one species (Rao et al., 2009). Varying transformation rates have been reported in tobacco as well (Sohail et al., 2012).

The primary transformants developed out of both cultivars were analyzed for the integration and expression of gene(s) within the T-DNA region. Out of 130 putative transgenic plants of Basma and 85 plants of Nail transferred in the greenhouse, 40 plants of Basma and 16 plants of Nail were positive based on GUS and PCR analysis of the *cry1Ac*, *cry2A*, *uidA*, and *nptII* genes (Figure 3). The transgenic plants grew well while no abnormal phenotypes were observed. It has been established that use of kanamycin constitutes a good selection system for regeneration of transgenic tobacco plants (Sohail et al., 2012; Bakhsh et al., 2014); however, few escapes in

Figure 6. Enzyme-linked immunosorbent assay of primary transformants of Basma and Nail plants. The concentration of cry1Ac protein varied between 0.1 and 0.6 µg/g of fresh tissue weight of transgenic plants.

Figure 7. Leaf biotoxicity assay conducted using leaves of primary transformants against third instar larvae of *Phthorimaea operculella*. (A) Representative plants of Nail (11N) and Basma (9B) are shown. The transgenic plants showed appreciable level of resistance against targeted pest in comparison with control plants. (B) Mortality rates of PTM when fed to transgenic leaves.

our study (data not shown) were also noted. Likewise, in earlier studies, escapes have been reported (Horsch et al., 1985; McCormick et al., 1986). The improved selection efficiency for obtaining primary transformants on in vitro culture medium is critical for the development of a satisfactory transformation system. The sensitivities of the explants, nonembryogenic callus, embryogenic callus,

and somatic embryos to kanamycin have been observed to be different (Zhang et al., 2001). The instability of the T-DNA, genomic rearrangements, nonrigorous selections, and chimeras might be the possible reasons for the escapes (McHugen and Jordan, 1989). In our study, only plants positive for introduced gene(s) were marked and subjected to further studies.

Table 3. Chi-square test to evaluate goodness of fit test of *nptII* gene in T1 transgene.

T1 Transgenics (50 seed of each line per replication)	χ^2 Value = $\sum (O - E)^2/E$
N2	7.7
N3	16.66
N11	6.00
N22	11.76
N33	0.24
B4	16.66
B5	44.82
B9	3.22
B13	11.76
B15	49.3
B16	29.06
B18	16.66
B19	0.66
B21	14.1
B23	7.7
B28	16.66
B29	1.3
B32	0.24
B33	16.66
B35	2.38

Tabulated chi-square value ($df = 1$) at 5% level of significance probability level is 3.84

Real-time quantitative PCR has emerged as a standard molecular technique to analyze relative expression of targeted genes (Maqbool et al., 2010; Rao et al., 2011; Anayol et al., 2016). In the present study, GUS and PCR positive transformants of Basma and Nail were subjected to qPCR and we observed that transgenic plants had increased expression of *cry1Ac*. Many researchers have found varied levels of foreign gene expression among transgenic lines when subjected to qPCR (Maqbool et al., 2010; Rao et al., 2011) (Figure 5).

The expression level of *cry1Ac* protein varied among different transgenic plants of both cultivars (Figure 6). The variability in *cry1Ac* expression in transgenic crops has previously been reported (Olsen et al., 2005; Puspito et al., 2015; Anayol et al., 2016) and has been attributed to the regulatory nature of 35S promoter (Sunilkumar et

al., 2012). The cry toxin amount varied among transgenic plants in T0 and T1 generations (Figures 6 and 10). However, the expressed amount was enough to confer protection against PTM as revealed in leaf bioassays.

Earlier reports of the variation in insecticidal gene expression in different crops have been reported by different researchers. The spatio-temporal expression of Bt protein has been documented in cotton (Adamczyk et al., 2009; Bakhsh et al., 2012), rice (Ansari et al., 2014), and tobacco (Sohail et al., 2012). The various factors that can contribute variation in gene expression are gene sequence, type of promoter, gene integration site in plant genome, transgene copy number, and internal cell environment and several external factors have been reported (Hobbs et al., 1993; Guo et al., 2001; Manjunatha et al., 2009; Sunilkumar et al., 2012).

Figure 8. GUS histochemical assay of Basma and Nail transgenic plants in T1 progeny in comparison with control plants. A and B: Basma, C and D: Nail.

Figure 9. PCR analysis of *cry1Ac* and *uidA* genes in transgenic plants of Basma and Nail. (A) *cry1Ac* Lane 1: -ve control, Lane 2: +ve control, Lane 3: 100 bp plus ladder, Lanes 4–8: T1 progeny plants of Basma, Lanes 9–12: T1 progeny plants of Nail (B) *uidA* gene, Lane 1: 100 bp plus ladder, Lane 2: -ve control, Lane 3: +ve control, Lanes 4–13 transgenic plants of Basma and Nail lines.

The chi-square goodness of fit test revealed that only six lines out of 20 showed an expected 3:1 Mendelian ratio while the other lines exhibited distorted Mendelian ratios (Table 3). Mendelian inheritance of the foreign genes in

transgenic plants has been observed in alfalfa (Micallef et al., 1995), rice (Duan et al., 1996), maize (Fearing et al., 1997), cotton (Tang et al., 2000; Xia et al., 2007; Rashid et al., 2008; Daud et al., 2009), and cow pea (Ivo et al.,

Figure 10. ELISA assay to determine cry1Ac protein in transgenic lines of Basma and Nail. The amount of cry1Ac protein expressed was sufficient to confer protection against targeted pests.

2008). However, in many cases, the foreign gene displayed complicated segregation ratio profiles (Altman et al., 1996; Wu et al., 2002; Rashid et al., 2008., Bakhsh 2010). In the present study, the distorted Mendelian ratio can be attributed to the presence of transgene in multiple loci belonging to different segregating homologues (Hashmi et al., 2011).

The transgenic lines in subsequent progeny showed proper integration and expression of introduced genes as revealed by GUS, PCR, and ELISA assays (Figures 8–10). The development of resistance in the targeted insect pests against single Bt toxin led to an imperious need to stack

more than one insecticidal gene in crops (reviewed in Lombardo et al. (2016)). The tobacco plants with stacked insecticidal genes developed in the present work can be efficiently used in a tobacco breeding program and can be considered a promising tool for integration into insect pest management.

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