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GENETIC TRANSFORMATION AND EXPRESSION DETECTION OF TOBACCO BY USING A MULTI-GENE PLANT TRANSFORMATION VECTOR

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ABSTRACT

Tobacco was transformed by employing the plant transformation vector p209-*Cry1Ac-Cry3A-BADH* by using *Agrobacterium*-mediated method to obtain completely regenerated plants screened by kanamycin sulfate. PCR detection indicated out of the nine lines in which *NPT II* gene was detected, *Cry1Ac*, *Cry3A*, and *BADH* were detected in seven lines; *Cry1Ac* and *Cry3A* were detected in one line; and *Cry1Ac* was detected in one line. Fluorescence quantitative PCR detection indicated in all target gene lines, three target genes were differentially expressed at the transcriptional level in four lines. *BADH* expression was absent in three lines. ELISA analysis revealed Cry1Ac and Cry3A toxin expression were detected in all target gene lines. The content of Cry3A toxin (up to 13,749.30 $ng \cdot g^{-1}$) was significantly higher than that of Cry1Ac toxin (up to 290.70 $ng \cdot g^{-1}$). The indoor insect-resistance test showed each transgenic line with insect-resistant gene inhibited the survival, growth, and development of *Prodenia litura* (*Fabricius*) larvae to varying degrees. Average corrected mortality of five lines was significantly higher than that of control, reaching up to 70.6%. Two lines were selected for further salt-tolerance research, and results showed transgenic lines had an individual salt tolerance compared with control.

Keywords: Multi-gene, plant transformation vector, tobacco, genetic transformation, expression detection.

INTRODUCTION

Transgene, as one of the principal methods employed in modern molecular breeding, serves an important function in directional improved varieties. Great progress has been made in the research on transgenic plants. In particular, progress has been achieved in terms of the *Bt* insect-resistant gene, *betaine aldehyde dehydrogenase* (*BADH*) salt-tolerant gene, and other stress-resistant genes (Jia *et al*., 2002; Sunkar *et al*., 2003; Baumgarte and Tebbe, 2005; Yu and Wei, 2008; Jouzani *et al*., 2008; Cao *et al*., 2008; Zhou *et al*., 2008). The *Bt* gene is considered to be the most promising insect-resistant gene. Numerous *Bt* genes have been successfully transferred to tobacco, corn, cotton, and other plants. Such transfer has established a large number of transgenic plant varieties with good insect-resistance to realize commercial production with significant economic benefits (Fromm *et al*., 1990; Koziel *et al*., 1993; Stewart *et al*., 2001; Zhang *et al*., 2007; Wang *et al*., 2008; George *et al*., 2012; Krishna and Qaim, 2012).

With the continuous progress in genetic engineering research, the limitations of single-gene transformation have gradually been recognized. Thus, multi-gene transformation has recently become the main direction of genetic engineering research. Considerable research has been conducted on transgenic plants characterized by a multi-gene complex (Zhu *et al*., 2007; Azadi *et al*., 2010; Rao *et al*., 2011; Sun *et al*., 2012; Qgawa *et al*., 2014). Through genetic engineering, the insect-resistant, drought-resistant, and salt-tolerant genes are transformed into plants simultaneously. These can expand the insect-resistant spectrum, effectively delay the onset of pest resistance to insecticide, and improve the comprehensive resistance capability of plants (Lian *et al*., 2008; Rao *et al*., 2011; Sun *et al*., 2012). In 2010, 41% of transgenic crops with complex character were planted in the US. These plants comprised 78% of transgenic corn and 67% of transgenic cotton. These transgenic plants with complex character have been obtained by using hybrid polymerization, repeated transformation, gene gun, and other methods. The main drawback of these methods is that a higher number of transformed genes require a longer test cycle. Moreover, the separation of transgenic progeny is complicated and has little stability. Thus, transgenic pure lines that contain multiple target genes and exhibit good comprehensive character are difficult to obtain.

With the development of Gateway, Cre/LoxP recombinant, and other technologies, the construction technology for multi-gene plant transformation vector continues to improve, and it has been gradually applied (Earley *et al*., 2006; Tanaka *et al*., 2011). The multi-gene vector transformation method constructs multiple genes and their regulatory elements in the same plant transformation vector. This method can be used to transform multiple genes into plant genomes once to obtain transgenic plants in which multivalent genes are simultaneously expressed. The keys to successful

transformation by the multi-gene vector are the stability of exogenous genes in the transformant and the effectiveness of expression. That is, whether all multiple exogenous genes are expressed and whether mutual influence exists among the gene expressions. These issues have to be verified by evaluating the character of the transgenic plant.

Two *Bt* genes (*Cry1Ac* and *Cry3A*) were combined with *BADH* to construct a multi-gene plant transformation vector. Tobacco (wisconsin35) was transformed by using the *Agrobacterium*-mediated method. Molecular detection and biological detection of the target character were conducted for transgenic plants to determine whether three exogenous genes can be normally expressed in these plants. The findings will lay a foundation for vector transformation in other plants.

MATERIALS AND METHODS

Strains and vectors: Strains: *Agrobacterium tumefaciens* strain EHA105. Plant transformation vector: p209-*Cry1Ac-Cry3A-BADH*, carrying *NPT II*, two *Bt* genes *Cry1Ac* (GenBank accession: AF148644.1) and *Cry3A* (GenBank accession: M84650.1), as well as *BADH* (GenBank accession: DQ497233.1). *NPT II* was induced by the NOS promoter, whereas the three target genes were induced by the CaMV35S promoter. The plasmid vector was stored in *Agrobacterium tumefaciens* strain EHA105 and constructed by the Key Laboratory of Germplasm Resources of Forest Trees and Forests Protection of the Agricultural University of Hebei. The structure is illustrated in Fig. 1.

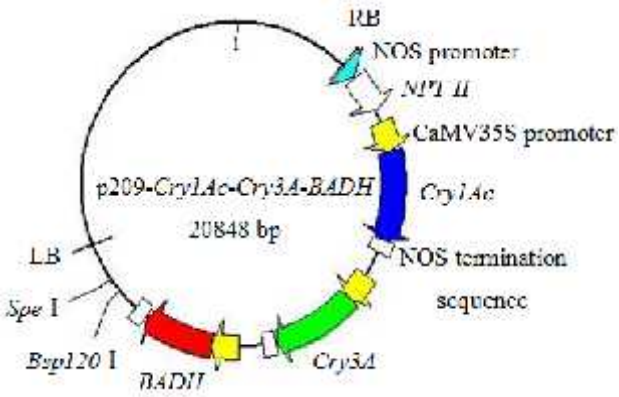

Fig. 1: Structure of p209-*Cry1Ac-Cry3A-BADH*
*Each target gene has a respective promoter and termination sequence in the vector, and these genes connect between the left and right borders in the vector.

Plant material and tested insects: Plant material: tobacco, varieties: wisconsin35. Tested insects: *Prodenia litura* (*Fabricius*), Insecta Lepidoptera Noctuidae, purchased from Henan Province, Jiyuan Baiyun Industrial Co., Ltd.

Primers and their sequences used in the experiment: Detection primers of *NPT II*: 103F:5'-ATCTCCTGTCATCTCACCTTGCTCCT-3'; 103R: 5'-TCAGAAGAACTCGTCAAGAAG-3'. Detection primers of *Cry1Ac*: *Bt1*-F1: 5'-ATGGATAACAATCCGAACATCA-3'; *Bt1*-R1: 5'-CCACCTTTGTCCAAACACTGAA-3'. Detection primers of *Cry3A*: *Bt3*-F1: 5'-CACTGTTCCCACTGTACGATGT-3'; *Bt3*-R1:5'-ATGTTGAAGAAGTCCACGCTCT-3'. Detection primers of *BADH*: *BADH*-F1:5'-TGGTGCTCATCGTGCTAAAT-3'; *BADH*-R1: 5'-CTCCCAGTAAATGCTACCTTGT-3'.

Forward primer designed according to the terminal end sequence of *Cry1Ac*: 133-1F: 5'-TCCAGTTACTGCAACACTCGAGTAG-3'. Reverse primer designed according to the front end sequence of *Cry3A*: 133-2R: 5'-TCCGTGTTGTTATCAGCAGTCAT-3'. Forward primer designed according to the terminal end sequence of *Cry3A*: 133-3F: 5'-CATAGACAAGATTGAGTTCATTCCAGT-3'. Reverse primer designed according to the front end sequence of *BADH*: 133-4R: 5'-GAAGAGTTGACGAGCAGGAATTG-3'. Fluorescence quantitative PCR primers of *Cry1Ac*: *Bt1*-F2: 5'-GAATTTTTGGTCCCTCTCAAT-3'; *Bt1*-R2:5'-AGGATCTGCTTCCCACTCTCT-3'. Fluorescence quantitative PCR primers of *Cry3A*: *Bt3*-F2: 5'-TGGGGATACGAGAAGGAGGAT-3'; *Bt3*-R2:5'-AGTGGGAACAGTGCGATGAGA-3'. Fluorescence quantitative PCR primers of *BADH*: *BADH*-F2: 5'-CCCAATTCCTGCTCGTCAACTCT-3'; *BADH*-R2:5'-CACTGCAACCTCCACATCCTCTG-3'.

Transformation of tobacco by *Agrobacterium*-mediated leaf disc method: *Agrobacterium tumefaciens* strain EHA105 containing transformation vector was extracted and then cultured on YEB solid medium containing 25 mg·L^{-1} of rifampicin (Rif) and 50 mg·L^{-1} of kanamycin sulfate (Kan). The single colony that grew was placed in liquid YEB medium containing Kan and Rif (concentration ibidem) at 28 °C. The bacterial liquid was shaken into turbidity at 150 rpm. One-tenth of the liquid was placed in the new YEB liquid medium containing Rif and Kan. The mixture was shaken until OD_{600} was 0.4–0.6, which indicates fluid infection.

Sterile, robust, fresh, and green tobacco leaves were chosen and then cut into leaf discs of 0.5–1 cm^2 along the leaf veins. These leaves were infected with the bacterial liquid that was diluted with 5% sucrose solution (1:1) for 8 min. The leaf discs were removed and dried by sterile blotting paper and then inoculated in the co-culture medium (MS+2.0 mg·L^{-1} 6-BA+0.1 mg·L^{-1} IBA). After dark culture for 2 d, the leaves were transferred to the screening culture medium (MS+2.0 mg·L^{-1} 6-BA+0.1

mg·L^{-1} IBA+50 mg·L^{-1} Kan+400 mg·L^{-1} Cef). The medium was replaced every two weeks. The resistant buds, after growing to approximately 2 cm, were transferred to the rooting medium (MS+75 mg·L^{-1} Kan+400 mg·L^{-1} Cef) for screening. The samples placed in a chamber were to be cultured at a temperature of 25 ± 2 °C, light intensity of 1,500–2,000 Lx and light/dark cycle of 14/10 h. The rooting plants in the rooting culture medium were propagated and domesticated, after which they were transplanted into the field.

PCR detection of transgenic lines: In May 2012, field seedling leaves of each line screened by Kan and non-transgenic tobacco (control) were collected, and DNA was extracted. The detection primers were designed according to the sequence information on *NPT II* and the three target genes from NCBI. The extracted DNA was amplified by PCR for the preliminary determination of whether the target genes were integrated into the tobacco genome.

The reaction system of *NPT II*, *Cry1Ac*, *Cry3A*, and *BADH* genes detected by PCR was as follows: ddH_2O, 15.4 µL; 10 × PCR Buffer, 2 µL; dNTP (10 mM), 0.4 µL; forward primer F (20 µM), 0.4 µL; reverse primer R (20 µM), 0.4 µL; rTaq DNA polymerase (5 U·µL $^{-1}$), 0.2 µL; and DNA, 1.2 µL. The reaction program of PCR detection of *NPT II* gene was as follows: First was pre-denaturation for 5 min at 95 °C, followed by denaturation for 45 sec at 94 °C, renaturation for 50 sec at 50 °C, and extension for 50 sec at 72 °C for 35 cycles. The final extension was for 7 min at 72 °C and for 1 min at 25 °C. The reaction program of PCR detection of *Cry1Ac*, *Cry3A*, and *BADH* genes was as follows: First was pre-denaturation for 5 min at 95 °C, followed by denaturation for 50 sec at 95 °C, renaturation for 1 min at 50 °C (*Cry1Ac* and *Cry3A*) or 52 °C (*BADH*), and extension for 1 min at 72 °C for 30 cycles. The final extension was for 7 min at 72 °C and for 1 min at 25 °C.

The test results showed that a few lines only transformed part of the target genes. The lines that transformed part of the target genes were selected for further verification. The forward primers 133-1F and 133-3F were designed according to the terminal end sequence of *Cry1Ac* and *Cry3A*. The reverse primers 133-2R and 133-4R were designed according to the front end sequence of *Cry3A* and *BADH*. The forward and reverse primers were designed to test whether untransformed genes were detected in the lines that only transformed part of the genes. The lines that only transformed *Cry1Ac* gene were amplified by using 133-1F and 133-2R, as well as the 133-3F and 133-4R primer pair. The lines that transformed both *Cry1Ac* and *Cry3A* were amplified by using the 133-3F and 133-4R primer pair to verify the transgene situation further. The reaction system and procedure employed were the same as those detailed above with an annealing temperature of 55 °C.

Fluorescence quantitative PCR detection: In July 2012, field seedling leaves were collected from each transgenic line and the control. The total RNA in tobacco was extracted by using Beijing ComWin Biotech Co., Ltd. ultrapure RNA kit. The reverse transcription of the first chain of cDNA was performed by using a TUREscript 1st Strand cDNA Synthesis Kit from Aidlab Biotechnologies Co., Ltd.. The steps were proceeded according to manufacturer's instructions.

Fluorescence quantitative PCR primers were designed according to the full sequence information of target gene lookup from NCBI. Fluorescence quantitative PCR was employed by using 2 × Sybr Green qPCR Mix. In a total volume of 50 µL reaction system, it contained 2 × SYBR qPCR Mix 25 µL, DNA template 2 µL, forward and reverse primer (10µM) 1 µL respectively, and ddH_2O 21 µL. The reaction process was as follows: First was pre-denaturation for 3 min at 94 °C, followed by denaturation for 20 sec at 94 °C, renaturation for 20 sec at 55 °C–60 °C, and extension for 30 sec at 72 °C to read the board for 40 cycles. The final extension was for 5 min at 72 °C. The melting curve of the samples was analyzed. The annealing temperature of *Cry1Ac* and *Cry3A* PCR reactions was 55 °C. The annealing temperature of *BADH* PCR was 60 °C. The samples were placed sequentially into the fluorescence quantitative PCR instrument connected to a computer. The program was then set up to perform the PCR. The data were collected after the reaction.

ELISA for detection of Bt toxin: In July 2012, the top-down third leaf of the field seedlings of each transgenic line and control was collected (the process for each line was repeated thrice). The Bt toxin of each line was detected by using Bt-Cry1Ab/1Ac and Bt-Cry3A ELISA kits from Agdia, Inc. The positive control was provided, whereas the non-transgenic tobacco was used for the negative control. The detection process was performed according manufacturer's instructions. The data were detected by using a BioRad 550 microplate reader. The concentration of toxin was calculated per gram of fresh leaves containing toxin in nanograms (ng).

Insect-resistance test: In July 2012, field seedling leaves of each line were collected for the insect feeding test of *Prodenia litura* (*Fabricius*) larvae. Newly hatched instar larvae were introduced to feed on the seedling leaf. The test for each line was repeated thrice, and 30 insects were used in each test. The tobacco leaves were changed daily. The instar number and mortality of insect larvae were recorded. The insect feeding test stopped when the number remained stable and no further death occurred. The length and the weight of surviving larvae were measured. The data were recorded, and the average corrected mortality was calculated [average corrected mortality = (transgenic lines mortality - control mortality) / (1 - control mortality)].

Salt-tolerance test: Cultured tissue seedlings of two transgenic lines transferred to the salt-tolerant gene and control seedlings were chosen for the salt-tolerance test. The concentration gradient of NaCl was set to 0, 50, and 100 mM. Robust leaf blades were selected from each line. The blades were cut into 1 cm^2 pieces along the main vein (four edges were wound). The samples were inoculated into differentiated medium with different concentrations of NaCl. Five leaf discs were inoculated in each Petri dish, and the process was repeated thrice for each line and each gradient. After 25 d, the status of the grown callus of each line in the Petri dishes was observed. The weight of each leaf disc was determined. Three leaf discs were randomly chosen, and each was randomly cut out into 1 cm^2 blocks. The number of buds per square centimeter was counted.

Data analysis: The data were processed by using Microsoft Excel 2003 graphics and DPS v7.05 statistical analysis software. The significant differences among indexes were compared by using Duncan's method.

RESULTS AND ANALYSIS

Obtaining transgenic lines: In the screening differentiation medium, the leaf discs of transgenic tobacco can differentiate the resistant shoots, but the control cannot. The resistant shoots were transferred to the screening rooting medium. Nine lines exhibited rooting, whereas the control did not. The rooting lines were transferred to the screening differentiation medium and were well differentiated. The re-differentiated resistant shoots were transferred to the rooting medium and then transplanted to the pot. After domestication, the rooting shoots were transplanted to the field. Nine transgenic lines were eventually obtained by Kan screening (Fig. 2).

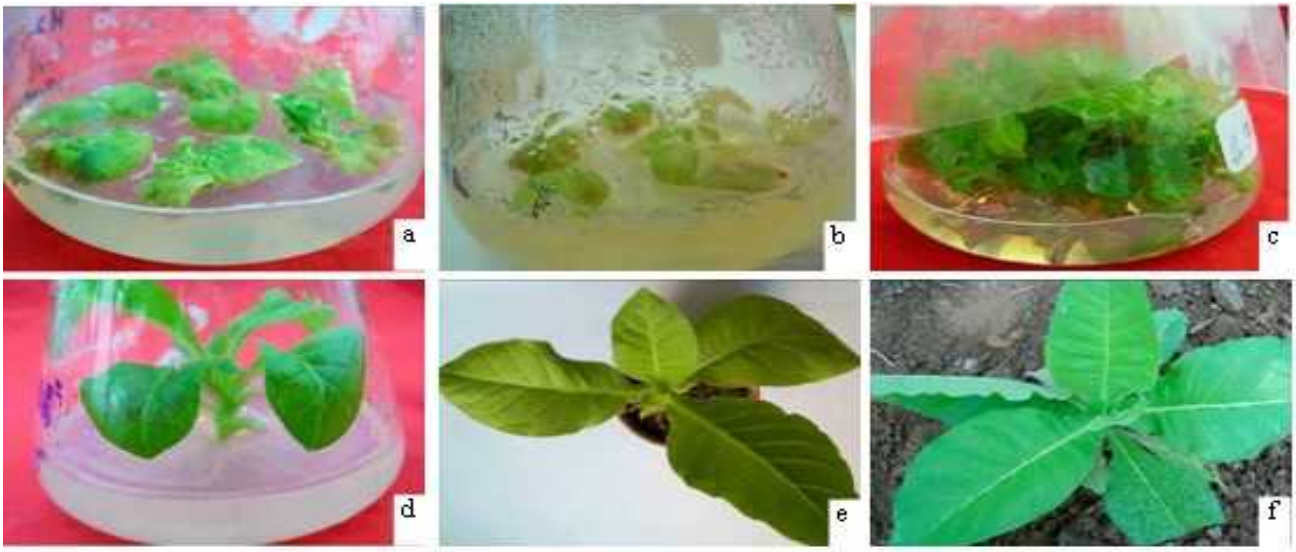

Fig. 2 Obtaining the transgenic lines

a: Leaves grew resistant shoots after agrobacterium infection; b: Untransformed leaves turned yellow in the selective medium; c: Proliferation of resistant shoots; d: Rooting of resistant shoots; e: Rooting plants were transplanted to small pots; f: Rooting plants were transplanted to field

PCR detection of transgenic lines: The DNA of nine transgenic lines and control leaves was extracted to amplify exogenous genes. The results are shown in Table 1. The results of *NPT II* gene amplification by PCR proved that the target fragment of 473 bp was amplified from all nine lines and positive control plasmid. The results of *Cry1Ac* gene amplification by PCR proved that the target fragment of 546 bp was amplified from all nine lines and positive control. The results of *Cry3A* gene amplification by PCR showed that the target fragment of 667 bp was amplified from eight transgenic plants. The results of *BADH* gene amplification by PCR demonstrated that the target fragment of 507 bp was amplified from seven transgenic plants. Finally, seven transgenic lines were found to possess the transgenic *Cry1Ac*, *Cry3A*, and *BADH* genes through PCR detection. Only the *Cry1Ac* gene was detected in line 9. By using the primer pairs 133-1F and 133-2R, as well as 133-3F and 133-4R, the fragment of 1.5 kb was again verified to be amplified from other lines. However, the fragment cannot be amplified from line 9. The results indicated that the line was only transferred to the *Cry1Ac* gene. The *BADH* gene was not detected in line 8. Through the primer pair 133-3F and 133-4R, the fragment of 1.5kb was again verified to be amplified from other lines.

However, the fragment was not obtained from line 8. The results further proved that the line only transferred to *Cry1Ac* and *Cry3A* genes.

Table 1. Result of PCR detection of each target gene

Number of lines	Amplified result of *NPT II*	Amplified result of *Cry1Ac*	Amplified result of *Cry3A*	Amplified result of *BADH*
1	+	+	+	+
2	+	+	+	+
3	+	+	+	+
4	+	+	+	+
5	+	+	+	+
6	+	+	+	+
7	+	+	+	+
8	+	+	+	-
9	+	+	-	-
plasmid vector	+	+	+	+
Non-transgenic tobacco	-	-	-	-

*"+" means amplify the target fragment, "-" means not amplify the target fragment.

Fluorescence quantitative PCR detection: The transcription abundance of each target gene was detected by fluorescence quantitative PCR (Table 2). The detection results of *Cry1Ac* gene by fluorescence quantitative PCR showed that in all detected lines, the transcription abundance of *Cry1Ac* gene was 2.89E+04–1.45E+05, and that of line 8 in which the double *Bt* gene was detected was 1.72E+05. Only *Cry1Ac* was detected in line 9, which also exhibited transcription that was not detected in the control. The testing results of the *Cry3A* gene showed that the transcription abundance of *Cry3A* in the lines detected for all genes was 2.37E+04–1.86E+06. Compared with the transcription abundance of the *Cry1Ac* gene, the transcription abundance of the *Cry3A* gene was higher by an order of magnitude, whereas that of line 8 in which the double *Bt* gene was detected was 1.45E+05. The *Cry3A* gene was not detected in the remaining lines and control. The testing results of the *BADH* gene showed that among the lines in which all genes were detected, the transcription of the *BADH* gene was detected in four lines. The transcription abundance was 5.29E+04–3.24E+05. The *BADH* gene was not detected in the remaining lines and control.

Table 2. Transcription abundance of target genes detected by fluorescence quantitative PCR

Transgenic type	Number of lines	Transcription abundance of *Cry1Ac*	Transcription abundance of *Cry3A*	Transcription abundance of *BADH*
Cry1Ac+Cry3A+BADH	1	2.91E+04	5.87E+05	1.39E+05
	2	1.22E+05	7.38E+04	-
	3	8.72E+04	3.54E+05	3.24E+05
	4	8.46E+04	1.31E+05	5.29E+04
	5	2.89E+04	2.37E+04	-
	6	1.45E+05	1.86E+06	6.15E+04
	7	1.33E+05	4.37E+04	-
Cry1Ac+Cry3A	8	1.72E+05	1.45E+05	-
Cry1Ac	9	7.52E+04	-	-
untransformed	CK	-	-	-

ELISA detection of the expression of Bt toxin: The detection results of Cry1Ac toxin (Table 3) showed that the toxin was detected in nine lines. The toxin content of seven lines in all genes were detected was 116.44–290.70 $ng \cdot g^{-1}$, and that of line 8 in which the double *Bt* genes were detected was 248.59 $ng \cdot g^{-1}$. The toxin content of line 9 in which only the *Cry1Ac* gene was detected was very little. The detection results of Cry3A toxin (Table 3) showed that the toxin content was detected in eight lines, whereas the content of all lines detected was 19.53–13,749.30 $ng \cdot g^{-1}$. The toxin content of line 8 in which double *Bt* gene was detected was 6.46 $ng \cdot g^{-1}$, and the toxin in the remaining lines and control were not detected. The Cry3A toxin content in most of the lines was significantly higher than that of Cry1Ac toxin based on a comprehensive analysis.

Insect-resistance test: In the insect-resistance test, each transgenic line had a different effect on the survival and growth of larvae. Insect test results after feeding for 9 d are shown in Table 4. The average body length and average weight of surviving larvae showed that compared with the control, the growth of the surviving larvae in each transgenic line was inhibited to different degrees. The insect instars of most living larvae were 4–6. For the average corrected mortality, the values for lines 2, 3, 6, 7, and 8 were significantly higher than those of the control with a maximum of 70.6%. Other lines exhibited no significant difference from the control. Most insect test results showed a positive correlation with Cry1Ac toxin content.

Salt-tolerance test: Two lines (1 and 3) with high transcription abundance and the control were chosen for the salt-tolerance test. In the differentiation medium containing NaCl, leaf disc differentiation was inhibited to different degrees. The results are shown in Table 5. The callus rate of line 1 was similar to that of the control in every gradient, whereas that of line 3 at the gradient of 100 mM was slightly higher than that of the control. From the average weight of leaf discs, when the concentration of NaCl was 0, line 1, line 3, and the control exhibited no significant difference. When the concentration of NaCl was 50 mM, the value for line 3 was significantly higher than that of the control, whereas those of line 1 and the control exhibited no significant difference. When the concentration of NaCl was 100 mM the values for lines 1 and 3 were significantly higher than that of the control. From the average number of shoots per square centimeter, the low concentrations of the two lines were not apparently different from that of the control. The value for line 1 was significantly higher than that of the control, only when the concentration was 100 mM. The results showed that when the concentration was high, a significant difference was observed between the transgenic lines and the control, but no obvious difference was observed when the concentration was low. The transgenic lines had an individual salt tolerance.

Table 3. Bt toxin content of various lines

Transgenic type	Number of lines	Content of Cry1Ac toxin (ng·g^{-1})	Content of Cry3A toxin (ng·g^{-1})
Cry1Ac+Cry3A+BADH	1	146.95±20.56 cd	13,749.30±651.61 a
	2	215.66±14.95 b	3,262.86±84.77 c
	3	204.48±63.64 bc	7,428.38±615.72 b
	4	116.44±10.66 d	5,449.42±471.64 b
	5	290.70±79.22 a	19.53±2.26 d
	6	243.08±24.82 ab	7,259.40±467.63 b
	7	132.15±29.19 d	721.14±62.44 d
Cry1Ac+Cry3A	8	248.59±15.11 ab	6.46±0.58 d
Cry1Ac	9	7.84±0 e	0±0 d
untransformed	CK	0±0 e	0±0 d

*Within each column, means with the same letter are not significantly different (P = 0.05); ANOVA FISHER's LSD test.

Table 4. Results of insect-resistance test of various lines

Transgenic type	Number of lines	Average body length (mm)	Average weight of larvae (g)	Average corrected mortality (%)
Cry1Ac+Cry3A+BADH	1	17.3±2.7 b	0.093±0.016 b	7.0±7.6 def
	2	13.8±2.1 c	0.077±0.016 c	70.6±14.4 a
	3	12.7±2.2 cd	0.056±0.009 d	48.7±14.5 abc
	4	12.0±2.2 d	0.046±0.008 d	27.5±24.3 cde
	5	18.0±1.7 b	0.102±0.013 b	18.1±14.9 cdef
	6	16.8±3.0 b	0.102±0.036 b	42.9±29.1 abc
	7	13.9±2.1 c	0.079±0.022 c	31.6±13.4 bcd
Cry1Ac+Cry3A	8	10.1±1.5 e	0.025±0.008 e	60.8±18.7 ab
Cry1Ac	9	17.5±2.1 b	0.125±0.032 a	18.1±15.9 cdef
Untransformed	CK	20.0±4.2 a	0.131±0.049 a	0±0 ef

*Within each column, means with the same letter are not significantly different (P = 0.05); ANOVA FISHER's LSD test.

Table 5. Results of salt-tolerance tests of line 1, line 3, and control

Concentration of NaCl (mM)	Number of lines	Callus rate (%)	Average weight of blisks (g)	Average number of buds (piece)
0	1	100	0.765±0.019 a	20.7±3.8 a
	3	100	0.942±0.326 a	25.0±4.8 a
	CK	100	0.932±0.302 a	21.2±6.7 a
50	1	100	0.629±0.060 b	20.0±3.3 a
	3	100	0.861±0.326 a	20.2±6.2 a
	CK	100	0.560±0.057 b	19.6±6.6 a
100	1	80	0.904±0.163 a	18.1±4.9 a
	3	86.7	0.913±0.117 a	13.8±3.0 ab
	CK	80	0.702±0.172 b	13.0±5.3 b

*Within each column, means with the same letter are not significantly different (P = 0.05); ANOVA FISHER's LSD test.

DISCUSSION

Constructing multi-gene single plant transformation vector to transform multiple target genes into plant genome and to be expressed in plants is a highly efficient method of multi-gene transformation; thus, this method become a major research hot spot (Goderis *et al*., 2002; Lin *et al*., 2003; Fitzgerald *et al*., 2006; Underhill *et al*., 2007; Lian *et al*., 2008; Hu *et al*., 2010; Zeevi *et al*., 2012). Two insect-resistant *Bt* genes *Cry1Ac* and *Cry3A*, as well as salt-tolerant gene *BADH*, were constructed in a plant transformation vector. These genes were used to achieve seven tobacco lines carrying three exogenous genes by employing the *Agrobacterium*-mediated method. Significant differences were observed in the transcription abundance, Bt toxin expression, and insect resistance between different lines of exogenous genes. These results proved that significantly different expressions of exogenous genes from various lines were caused by the insertion position. Another noteworthy phenomenon was that the expression of three genes was very distinct. The transcription abundance and toxin expression of *Cry3A* gene were significantly higher than those of the *Cry1Ac* gene. The difference between the expressions of three exogenous genes may be related to the sequence and direction of exogenous genes in the vector, species of exogenous genes, promoter type, and so on. Thus, further study is necessary.

Conclusion: Tobacco was transformed by using plant transformation vector p209-*Cry1Ac*-*Cry3A*-*BADH* through the *Agrobacterium*-mediated method. Nine rooting lines were achieved by Kan and Cef screening at a suitable concentration. Through PCR detection, seven lines were detected to have *Cry1Ac*, *Cry3A*, and *BADH* genes; one line was detected to have double *Bt* genes; one line was detected to have only the *Cry1Ac* gene. Through fluorescence quantitative PCR detection, among the lines in which all target genes were detected, the expression of target gene transcription was detected in four lines, whereas the *BADH* transcription was not detected in three lines. Furthermore, only part of the target genes was detected in the other two lines, and the transcription of target gene was also detected. ELISA detection of toxin indicated that Cry1Ac toxin expression was detected in nine transgenic lines, whereas Cry3A toxin expression was detected in eight lines transforming into *Cry3A*. A certain difference was observed between lines. The results showed that the target genes were expressed at the level of translation. The feeding insect test showed that the average corrected mortality of lines 2, 3, 6, 7, and 8 on *Prodenia litura* (*Fabricius*) larvae was higher than that of the control. The highest was 70.6%. The insect-resistant effect of other lines was weak. Salt-tolerance test results showed that compared with the control, two transgenic lines exhibited certain salt tolerance.

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