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Genetic transformation and expression of transgenic lines of *Populus* x *euramericana* with insect-resistance and salt-tolerance genes

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ABSTRACT. We characterized new transgenic varieties of poplar with multiple insect-resistant and salt stress tolerant genes. Two insect-resistant *Bacillus thuringiensis* (*Bt*) genes, *Cry1Ac* and *Cry3A*, and a salt-tolerant gene, *Betaine aldehyde dehydrogenase* (*BADH*), were inserted into a vector, p209-*Cry1Ac*-*Cry3A*-*BADH*. The clone of *Populus* x *euramericana* was transformed by the vector using the *Agrobacterium*-mediated method. Three transgenic lines were assessed using genetic detection and resistance expression analysis. PCR revealed that exogenous genes *Cry1Ac*, *Cry3A*, *BADH* and selective marker gene *NPTII* were present in three transgenic lines. Quantitative real-time PCR (qPCR) showed significant differences in the transcriptional abundance of three exogenous genes in different lines. Results of assays

for Bt toxic proteins showed that the Cry1Ac and Cry3A toxic protein content of each line was 12.83-26.32 and 2108.91-2724.79 ng/g, respectively. The Cry1Ac toxic protein content of different lines was significantly different; the Cry3A toxic protein content was about 100 times higher than that of the Cry1Ac toxic protein. The insect-resistance test revealed the mortality rate of transgenic lines to *Hyphantria cunea* L1 larvae varied by 42.2-66.7%, which was significantly higher than non-transgenic lines. The mortality rate of L1 and L2 *Plagiodera versicolora* larvae was 100%. The insecticidal effect of transgenic lines to *P. versicolora* larvae was higher than that to *H. cunea* larvae. NaCl stress tolerance of three transgenic lines under 3-6% NaCl concentration was significantly higher than that of non-transgenic lines.

Key words: Transgenic poplar; Multiple transgenes; *Populus* x *euramericana*; Insect-resistance; Salt tolerance

INTRODUCTION

Recently, plant genetic engineering has developed rapidly in the cultivation of new plant varieties with good qualities, such as insect, disease, and stress resistance, and high yields. With the development of transgenic plants, the expression of a single gene cannot meet the needs of social development. Transgenic plants with multiple gene transformations have become an important focus of transgenic research (Bates et al., 2005; Christou et al., 2006). At present, advanced genetic transformation technology is widely used in plant breeding, and many plants with resistance to multiple stresses have been developed (Feng et al., 2001; Krens et al., 2011; Zhou et al., 2012).

The preferred method for multi-gene transformation of plants is the construction of multiple gene expression cassettes in a vector, and multiple gene insertion has been completed by a single transformation. This method can effectively overcome the time-consuming, low efficiency problems of co-transformation, and the separation of genes in the progeny, etc. (Taverniers et al., 2008).

The poplar is an important economic tree species globally, and it is a model species in forest genetic engineering research (Hawkins et al., 2003; Li et al., 2008). Significant progress has been made in the research of genetic transformation of using the *Agrobacterium*-mediated method to obtain transgenic poplars (Parsons et al., 1986) after nearly 30 years of development. Transgenic plants with herbicide, insect, disease, and drought resistance, and salt tolerance were obtained. However, most of the reports introduced only a single gene or a certain class of genes (Fladung et al., 2013; Klocko et al., 2014; Movahedi et al., 2015), and different agronomic traits, such as salt tolerance and insect resistance, are usually controlled by different genes. Therefore, multiple gene transformation is a feasible way to obtain a new poplar variety with multiple resistances (Yang et al., 2003; Wang et al., 2007a; Su et al., 2011).

Multiple insect resistant *Bt* genes, such as *Cry1Ac* and *Cry3A*, and salt tolerance genes, such as *BADH*, were inserted into a vector to transform poplars and develop new varieties. These varieties are resistant to Lepidoptera and Coleoptera pests, and have a certain degree of salt tolerance. This could improve the broad-spectrum insect resistance, and expand the planting range of poplars, which has important practical significance for forestry development.

MATERIAL AND METHODS

Strains and vectors

Strains: *Agrobacterium tumefaciens* strain EHA105

Plant transformation vector: p209-*Cry1Ac*-*Cry3A*-*BADH*, carrying the neomycin phosphotransferase gene (*NPT II*), *Bacillus thuringiensis* (*Bt*) crystal protein genes *Cry1Ac* (GenBank accession No.: AF148644.1), *Cry3A* (GenBank accession No.: M84650.1), and the betaine aldehyde dehydrogenase gene (*BADH*) (GenBank accession No.: DQ497233.1). *NPT II* was induced by the NOS promoter, and the three target genes were induced by the CaMV35S promoter. All the above genes were terminated by the NOS terminator. The multi-gene transformation vector system was constructed using the existing plasmid, and adding a two isocaudamer system, which were *Not*I/*Bsp*120I and *Spe*I/*Xba*I/*Nhe*I. The system comprised a transformation vector containing the restriction enzyme site *Bsp*120I and *Xba*, and a cloning vector containing the enzyme cutting site *Not*I, *Bsp*120I, *Spe*I, and *Nhe*I in sequence. The original restriction enzyme site disappeared after connecting to the isocaudamer, so the open reading frame of the new target genes could be connected to the transformation vector. The plasmid vector was stored in *A. tumefaciens* strain EHA105, and constructed by the Hebei Key Laboratory for Tree Genetic Resources and Forest Protection. The structure is illustrated in Figure 1.

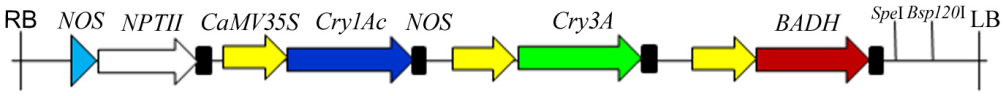

Figure 1. Schematic representation of construct p209-*Cry1Ac*-*Cry3A*-*BADH* used in transformation experiments. Blue triangle: promoter of the nopaline synthase gene; White arrows: neomycin phosphotransferase gene (*NPT II*); Black rectangles: terminator of nopaline synthase; Yellow arrow: 35S promoter from CaMV (CaMV35S); Blue arrow: *Bacillus thuringiensis* crystal protein genes *Cry1Ac*; Green arrow: *B. thuringiensis* crystal protein genes *Cry3A*; Red arrow: betaine aldehyde dehydrogenase gene (*BADH*); RB and LB: right border and left border of T-DNA.

Tested insects

In this study, we tested the insects *Hyphantria cunea* (Lepidoptera, Arctiidae) and *Plagiodera versicolora* (Coleoptera, Chrysomelidae).

Transformation of the *Populus* clone by *Agrobacterium*-mediated method

The expression vector p209-*Cry1Ac*-*Cry3A*-*BADH* was transformed into the *Populus* clone. Fully expanded, dark green, sterile, young poplar leaves were chosen and cut with 3-5 incisions perpendicular to the main vein. These leaves were infected with the *Agrobacterium* liquid for 10 min. Then they were removed and dried using sterile blotting paper, and inoculated on the co-culture medium (MS + 0.5 mg/L 6-BA + 0.15 mg/L IAA), and cultured in the dark for 3 days at 28°C.

The non-infected leaves were used as the control, and the rest of the experiment was the same as the above steps. After co-culture for 3 days, the leaves were transferred to the differentiation and screening culture medium (MS + 0.5 mg/L 6-BA + 0.15 mg/L IAA + 30 mg/L Kan + 400 mg/L Cef). The medium was replaced about every two weeks. After growing

to approximately 2 cm, the resistant buds were transferred to the rooting medium (1/2MS + 0.3 mg/L IAA + 50 mg/L Kan + 400 mg/L Cef) to induce the rooting. The rooting plants were transplanted to small pots, and propagated and domesticated. Then, they were transplanted into the specialized nursery. The full names of the abbreviations in this section are shown in **Table S1**.

DNA extraction and PCR

Annual field seedling leaves of each transgenic line, No.1, No.2, No.3, and the non-transgenic line (ck^-) were collected, and genomic DNA was extracted by the improved CTAB (hexadecyl trimethyl ammonium bromide) method. The recombinant plasmid, *p209-BtCry1Ac-BtCry3A-BADH*, was used as the positive control, and genomic DNA of the non-transgenic line as the negative control for the exogenous genes. PCR amplification was performed with detection primers of *NPT II*, *Cry1Ac*, *Cry3A*, and *BADH* genes (Table 1) to preliminarily determine whether the target gene was integrated into the poplar genome.

The reaction system of *NPT II*, *Cry1Ac*, *Cry3A*, and *BADH* genes detected by PCR was: 15.1 μL ddH_2O, 2 μL10X PCR buffer, 0.4 μL dNTP (10 mM), 0.4 μL forward primer F (20 μM), 0.4 μL reverse primer R (20 μM), 0.2 μL rTaq DNA polymerase (5 U/μL), and 1.5 μL template DNA. The reaction program of PCR detection of *NPT II*, *Cry1Ac*, *Cry3A*, and *BADH* genes was: pre-degeneration for 5 min at 93°C; degeneration for 45 s at 94°C, renaturation for 55 s at 50°C (*NPT II*), 54°C (*Cry1Ac*), 57°C (*Cry3A*) or 60°C (*BADH*), and extension for 1 min at 72°C, 35 cycles; and extension for 10 min at 72°C. The theoretical sizes of the amplified fragment of the *NPTII*, *Cry1Ac*, *Cry3A*, and *BADH* genes were 473, 546, 667, and 507 bp, respectively.

RNA extraction and qPCR

In early August 2014, the newly expanded leaves were collected from the top of the transgenic lines and the control. The total RNA in the *Populus* clone was extracted using EASYEx PLUS Plant RNA Kit from SaiLe Biotech Co., Ltd., and the reverse transcription of the first chain of cDNA was performed using a TUREscript 1st Strand cDNA Synthesis Kit from Aidlab Biotech Co., Ltd., according to manufacturer instructions.

According to the full sequence information of the target genes from NCBI, qPCR primers (Table 1) were designed. qPCR was carried out using 2X Sybr Green qPCR Mix. The reaction system (50 μL) was: 25 μL 2X SYBR qPCR Mix, 2 μL DNA template, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), and 21 μL ddH_2O. The reaction process was as follows: pre-degeneration for 5 min at 94°C; degeneration for 45 s at 94°C, renaturation for 50 s at 55°-60°C, and extension for 60 s at 72°C, 35 cycles; and extension for 10 min at 72°C. The annealing temperatures of 3 target gene PCRs were 55°, 58°, and 60°C, respectively. The samples were placed sequentially into the qPCR instrument. The data were collected and analyzed after the reaction.

ELISA detection of the *Bt* toxic protein

In early August 2014, the newly expanded leaves were collected from the top of the transgenic lines and the control. The *Bt* toxic protein of each line was detected using Bt-

Cry1Ab/1Ac and Bt-Cry3A ELISA kits from Agdia, Inc. A kit for the positive control was provided, whereas non-transgenic poplar was used for the negative control. The detection process was performed according to the manufacturer instructions. Data were printed using a BioRad 550 microplate reader in ng/g.

Table 1. Primers used in the experiment and their sequences.

Primer name	Sequences of the primers
Detection primers of *NPT II*	F: 5'-ATCTCCTGTCATCTCACCTTGCTCCT-3'
	R: 5'-TCAGAAGAACTCGTCAAGAAG-3'
Detection primers of *Cry1Ac*	F: 5'-ATGGATAACAATCCGAACATCA-3'
	R: 5'-CCACCTTTGTCCAAACACTGAA-3'
Detection primers of *Cry3A*	F: 5'-CACTGTTCCCACTGTACGATGT-3'
	R: 5'-ATGTTGAAGAAGTCCACGCTCT-3'
Detection primers of *BADH*	F: 5'-TGGTGCTCATCGTGCTAAAT-3'
	R: 5'-CTCCCAGTAAATGCTACCTTGT-3'
qPCR primers of *Cry1Ac*	F: 5'-GAATTTTTGGTCCCTCTCAAT-3'
	R: 5'-AGGATCTGCTTCCCACTCTCT-3'
qPCR primers of *Cry3A*	F: 5'-TGGGGATACGAGAAGGAGGAT-3'
	R: 5'-AGTGGGAACAGTGCGATGAGA-3'
qPCR primers of *BADH*	F: 5'-CCCAATTCCTGCTCGTCAACTCT-3'
	R: 5'-CACTGCAACCTCCACATCCTCTG-3'

Insect-resistance test

The insect resistance of the Cry1Ac toxin protein was detected by *H. cunea* L1 larvae. The eggs were collected from the nursery of the Agricultural University of Hebei, and hatched into L1 larvae, which were used in feeding experiments. The insect resistance of the Cry3A toxin protein was detected by *P. versicolora* L1 larvae. The imagoes of *P. versicolora* were collected from the nursery of the Agricultural University of Hebei and fed with fresh, non-transgenic line leaves at about 25°C. The larvae hatched from collected eggs were cultured with non-transgenic leaves. Three days later, L1 larvae were used for testing.

Leaves of the same size in each line were selected as a treatment. We cleaned the surface of the leaves, cut them into oblique sections of about 1-cm lengths of the petiole, and inserted them into wet mud to keep them fresh. Then, with a soft brush, the test insects were placed on the leaves, and into the culture bottles of about 7cm. The bottles were tied with wet gauze to maintain the humidity and permeability. Thirty insects were used in each test, and the test for each line was repeated three times. The leaves were changed daily. The instar number and mortality of insect larvae were recorded. The insect feeding test stopped when the number of larvae remained stable. The cumulative mortality was analyzed and calculated. Cumulative mortality on the Nth day = mortality on the Nth day/initial feeding numbers x 100%.

Salt-tolerance test

One-year-old branches of each plant were used as cuttings, raising seedlings by cuttage, and treatment with salt stress. Branches with consistent growth (thickness, length) of each line were selected as a treatment, about 1 cm thick and 15 cm long, cuttings (with one bud) were clipped and transplanted into flowerpots, and 1cm at the top of the cuttings was retained for a robust bud. Four seedlings were planted per pot, which were Control, No.1, No.2, and No.3. The concentration gradient of NaCl was set to 0, 3, and 6‰, and each treatment was repeated five times. When the cutting seedlings showed stable growth, they were watered with salt water

once every 7 days using 2 L per bucket to better simulate a realistic saline environment. The growth of each seedling line was observed and recorded. Salt stress tests were performed in May and June 2014.

At the end of the experiment, the growth indexes, such as seedling height and ground diameter, were determined. The photochemical efficiency of each seedling was measured with an LI-6400 XT-40 portable photosynthesis analyzer. The analyzer was opened to preheat, and the test leaves were shaded for about 20 min with a dark-adapted clip. Then, the fluorescence parameters were set, and the leaves were placed into the leaf chamber. Finally, the data were measured and saved.

At the end of the test, each seedling was washed and wiped. The roots, stems, and leaves were clipped and placed into paper bags. The samples were dried in a drying cabinet at 105°C for 15 min, and 85°C to a constant weight. The dry weights of samples were measured after cooling.

RESULTS

Obtaining transgenic plants

After a week of culture on differentiation and screening culture medium, most of the leaves began to lose the green, dark coloration at the cut, but some developed green calluses in the main vein or petiole base of the cut. The other parts of the leaves and leaves without differentiated buds gradually turned yellow or brown. The resistant shoots from calluses were transferred to a new differentiation and screening medium, and most of them were well differentiated. When the newly differentiated resistant shoots grew to 2 cm, they were transferred to rooting medium. After 5-7days, most of the resistant shoots grew out of the root system. Twelve transgenic lines with trivalent genes were obtained by Kan screening, showing 3% transformed seedlings. After acclimatization, the rooting shoots were transplanted to the nursery (Figure 2). Three healthy transgenic lines, Nos. 1, 2, and 3, were selected for genetic and resistance detection.

Figure 2. The transgenic plants. **A.** Leaves grew resistant shoots after *Agrobacterium* infection. **B.** Proliferation of resistant shoots. **C.** Resistant shoots (right), and control (left) on the screening rooting medium. **D.** Rooting plants were transplanted to small pots.

PCR detection of transgenic lines

The target genes of *NPTII*, *Cry1Ac*, *Cry3Ac*, and *BADH* were amplified using specific PCR primers. The target gene fragments of 473, 546, 667, and 507 bp were amplified in each of the transgenic lines tested and in the positive control plasmid, but not in the control line ck$^-$. The results are shown in Figure 3. The results reported the presence of the exogenous genes in the poplar lines.

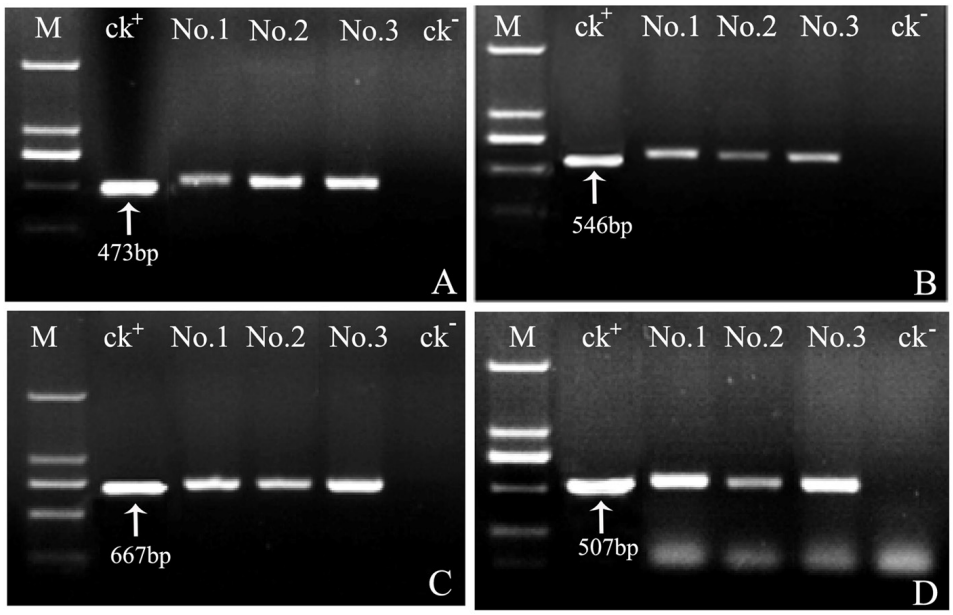

Figure 3. PCR detection of exogenous genes in three transgenic poplar lines. *Lane M* = DL2000 DNA molecular weight markers (From top to bottom, these are 2000, 1000, 750, 500, 250, and 100 bp); ck$^+$: p209-*Cry1Ac*-*Cry3A*-*BADH* plasmid; Nos. 1-3: The transgenic lines; ck$^-$: non-transformed line. **A.** *NPTII* gene. **B.** *Cry1Ac* gene. **C.** *Cry3Ac* gene. **D.** *BADH* gene.

qPCR detection

For qPCR detection of the *Cry1Ac* gene, the standard curve equation was y = -3.978 x + 47.95, $r^2 = 0.996$, where y was the CT value, x was the logarithm of the exogenous gene start copy, r^2 was the linear correlation coefficient; for detection of the *Cry3Ac* gene, the standard curve equation was y = -3.606 x + 44.72, $r^2 = 0.999$; for detection of the *BADH* gene, the standard curve equation was y = -3.180 x + 37.29, $r^2 = 0.997$.The transcriptional abundance results of the three target genes is shown in Table 2. From Table 2, there were differences in transcriptional abundance of different exogenous genes in the same plant receptors. For the three genes in each line, the transcriptional abundance of *Cry3A* was greater than that of *Cry1A* and *BADH*. There were significant differences in the transcript abundance of target genes in the three transgenic lines ($P < 0.05$). Among them, the transcriptional abundance of the three target genes of No.1 were the highest, and that of No.3 were the lowest.

Table 2. Transcriptional abundance of *Cry1Ac*, *Cry3A*, and *BADH* detected by qPCR.

Number of lines	Transcriptional abundance		
	Cry1Ac	*Cry3A*	*BADH*
ck-	0[d]	0[d]	0[d]
No. 1	2.652E+5[a]	4.985E+005[a]	1.010E+004[a]
No. 2	7.258E+4[b]	3.650E+005[b]	8.161E+003[b]
No. 3	2.775E+4[c]	2.916E+005[b]	1.910E+003[c]

Column data marked with different letters are significantly different ($P < 0.05$); Fisher LSD method.

ELISA detection for *Bt* toxic protein expression

The detection results of Cry1Ac and Cry3Ac toxic proteins (Table 3) showed the toxic protein was detected in three lines, and there was no color reaction of ck-.The Cry1Actoxic protein content of each line was 12.83-26.32 ng/g, and the Cry3Ac toxic protein content of each line was 2108.91-2724.79 ng/g. The contents of the Cry3A toxin protein in the threelines were about 100 times higher than those of the Cry1Ac toxin protein. There were differences between different lines in toxin protein contents. The two kinds of toxic protein contents of No.1 were the highest, and those of No.3 were the lowest.

Table 3. *Bt* toxic protein content of various lines.

Lines	Content of *Cry1Ac* toxic protein (ng/g)	Content of *Cry3A* toxic protein (ng/g)
No. 1	26.32 ± 21.29[a]	2724.79 ± 699.57[a]
No. 2	18.16 ± 7.83[b]	2244.77 ± 557.01[a]
No. 3	12.83 ± 2.23[b]	2108.91 ± 89.91[a]
ck	0.00 ± 0.00[c]	0.00 ± 0.00[b]

Column data marked with different letters are significantly different ($P < 0.05$); Fisher LSD method.

Insect-resistance detection

The mortality of the pest larvae, *H. cunea* (Lepidoptera) and *P. versicolora* (Coleoptera), fed leaves of the transgenic lines are shown in Table 4, and the conditions of larvae feeding on the leaves are shown in Figures 4 and 5. After five days, the cumulative mortality rate of L1 *H. cunea* larvae gradually stabilized at 66.7% for No.1, 45.5% for No.2, and 42.2% for No.3, respectively. One day later, the cumulative mortality rate of L1 *P. versicolora* larvae of the three lines reached 100%, and that of L2 *P. versicolora* larvae of the three lines were 88.9, 82.2, and 44.4%, respectively. Two days later, the cumulative mortality rate of L2 *P. versicolora* larvae of the three lines reached 100%.

Table 4. Mortality of *Hyphantria cunea* and *Plagiodera versicolora*.

Insect instar	Feeding time	Mortality (%)			
		ck-	No.1	No.2	No.3
L1 *H. cunea*	1st day	0	57.7	37.7	38.8
	2nd day	1.1	62.2	40.0	40
	3rd day	3.3	66.7	45.5	42.2
	4th day	3.3	66.7	45.5	42.2
	5th day	3.3	66.7	45.5	42.2
L1 *P. versicolora*	1st day	0	100	100	100
L2 *P. versicolora*	1st day	1.1	88.9	82.2	44.4
	2nd day	1.1	100	100	100

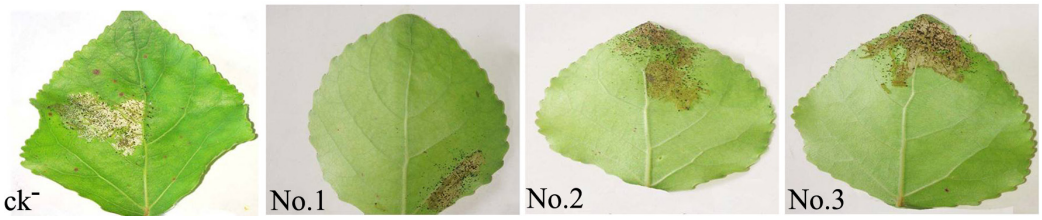

Figure 4. *Hyphantria cunea* larvae feeding on the leaves. ck-: Control; Nos.1-3: transgenic lines.

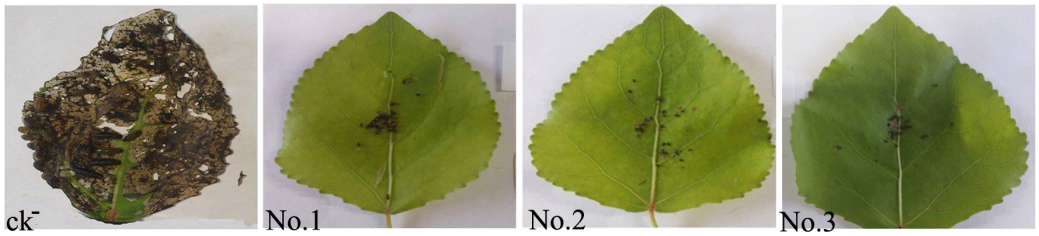

Figure 5. *Plagiodera versicolora* larvae feeding on the leaves. ck-: Control; Nos.1-3: transgenic lines.

Salt-tolerance detection

Differences in seedling height and ground diameter of each transgenic line under salt stress

Plant height and ground diameter are indicators for plant growth, and are important for evaluating the salt tolerance of plants. Under the same salt stress concentrations, the weaker the inhibition of seedling height and ground diameter, the stronger the salt tolerance. Seedling height and ground diameter of transgenic lines and the control after 30 days of NaCl stress are shown in Table 5. With an increase of NaCl concentration, the height increment of each line was progressively reduced. When the NaCl concentration was 0 and 3‰, the differences of seedling height and ground diameter of each line were not significant. When the NaCl concentration was 6‰, there were significant differences of seedling height and ground diameter between transgenic lines and control ($P < 0.05$). Under 6% NaCl stress, the three transgenic lines differed - the growth of No. 2 was faster and that of No.3 was slower.

Table 5. The transgenic plant heights and diameters at different NaCl concentrations after 30 days of treatment (mm).

Character	Number of lines	Concentration of NaCl		
		0‰	3‰	6‰
Height	ck-	35.67^{a}	23.33^{a}	10.50^{c}
	No. 1	30.50^{a}	22.83^{a}	21.67^{ab}
	No. 2	27.67^{a}	23.35^{a}	23.50^{a}
	No. 3	27.67^{a}	22.50^{a}	17.83^{b}
	Mean ± SD	30.38 ± 3.77^{a}	23 ± 0.41^{ab}	18.38 ± 5.76^{b}
Diameter	ck-	4.68^{a}	3.85^{a}	2.53^{b}
	No. 1	4.36^{a}	3.89^{a}	3.83^{a}
	No. 2	4.28^{a}	3.79^{a}	4.03^{a}
	No. 3	4.08^{a}	3.49^{a}	3.35^{ab}
	Mean ± SD	4.35 ± 0.25^{a}	3.76 ± 0.18^{ab}	3.44 ± 0.67^{b}

Within each column, means with different letters are significantly different of the same characters or different salt stress concentrations of different lines ($P < 0.05$); Fisher LSD method.

Chlorophyll fluorescence under salt stress

The *Fv*/*Fm* value reflects the PSII primary photochemical transformation efficiency. Under salt stress, with the increase of NaCl concentration, the reduction of *Fv*/*Fm* value increased, photosynthetic apparatus was damaged, and the photosynthetic capacity of plants decreased. After 30 days of salt treatment, the results of the maximal photochemical efficiency of *Fv*/*Fm* value are shown in Table 6. With increased NaCl concentration, the *Fv/Fm* value of each line decreased. Compared with ck$^-$, No.2 was tolerant to salt at both the concentrations used, and No.3 was tolerant to 3‰ NaCl concentrations.

Table 6. *Fv/Fm* values of transgenic plants under different NaCl concentrations.

Number of lines	Concentration of NaCl		
	0‰	3‰	6‰
ck$^-$	0.55^a	0.36^b	0.40^b
No.1	0.71^a	0.52^{ab}	0.45^b
No.2	0.68^a	0.67^a	0.72^a
No.3	0.59^a	0.69^a	0.43^b
Mean ± SD	0.64 ± 0.06^a	0.56 ± 0.15^a	0.50 ± 0.15^a

*Column data marked with different letters mean significant difference ($P < 0.05$); Fisher LSD method.

Differences in biomass of each line under salt stress

Soil salinity can reduce plant growth. The biomass of salt-intolerant plants decreased with increased NaCl concentrations. As shown in Table 7, under different concentrations of NaCl, the average relative biomass of the three transgenic lines was much higher than that of ck$^-$. The average relative biomass of No.2 (0.724) was the highest.

Table 7. Biomass and relative biomass change of transgenic plants under different NaCl concentrations.

Number of lines	Concentration of NaCl (‰)	Biomass (g)	Relative biomass	Average Relative biomass
CK$^-$	0	4.848 ± 2.128	**(a)**	**(b)**
	3	2.943 ± 1.121	0.607	0.394
	6	0.881 ± 0.827	0.182	
No.1	0	4.049 ± 0.396	**(c)**	**(d)**
	3	3.062 ± 0.898	0.756	0.690
	6	2.526 ± 0.228	0.624	
No.2	0	3.981 ± 1.572	**(e)**	**(f)**
	3	3.144 ± 0.285	0.790	0.724
	6	2.621 ± 1.104	0.658	
No.3	0	4.355 ± 1.542	**(g)**	**(h)**
	3	2.832 ± 0.856	0.650	0.564
	6	2.080 ± 0.181	0.478	

DISCUSSION

Characterizations and technical difficulties of multiple gene transformation

With the development of transgenic technology, the limitation of single gene transformation has gradually been recognized, and the generation of transgenic plants with multiple good traits has become a focus of current research. There are many methods

to express multiple exogenous genes in plants, such as crossing plants containing single transgenes, repeated transformation, multi-gene vector transformation, and so on (Goderis et al., 2002; Lin et al., 2003; Que et al., 2010). The multi-gene vector transformation method comprises the multi vector co-transformation and multi-gene single vector transformation methods. The multi-gene single vector transformation method constructs multiple target genes into a single expression vector, carrying multiple expression cassettes and each gene has its own independent promoter. This method, which is efficient for multi-gene transformation, can be used to transform multiple genes into plant genomes once to obtain transgenic plants, and multivalent genes are simultaneously expressed (Fitzgerald et al., 2006; Underhill et al., 2007). The advantages of multi-gene single vector transformation method are as followings: 1) multiple exogenous genes can be transferred into plants by a single transformation, which can simplify the operation steps, improve the integration rate, and reduce the damage to plants; 2) transgenic plants include a variety of genetically modified traits, expand the function of transgenic plants, and meet the needs of plants in different conditions (Halpin, 2005).

However, the construction and transformation of multi-gene plant expression vectors has several technical difficulties, and the limitation of the capacity of the vector restricts the construction of multi-gene vector. In addition, the losses of exogenous genes, gene silencing, and efficient expression are the key problems to be solved in genetic engineering (Halpin et al., 2001; Untergasser et al., 2012). In particular, when the exogenous gene has some homology, it may produce a certain disturbance. For example, the transformation of multiple *Bt* genes into a vector, which can improve the resistance of transgenic plants, may affect the expression of genes because of the influence of gene interference.

In the present study, two insect-resistant *Bt* genes, *Cry1Ac* and *Cry3A*, and one salt-tolerant gene, *BADH* were inserted into a plant expression vector. The clone of *P.* x *euramericana* was transformed by the vector with the use of the *Agrobacterium*-mediated method. Transgenic clones of *P.* x *euramericana* with trivalent genes were screened with kanamycin, and were determined to be stable in resistance. The transcriptional level of different target genes was different, and there were significant differences in transcriptional abundance of the three exogenous genes in different lines. There are many reasons for the difference in transcriptional abundance between the different lines and the different exogenous genes, such as the differences of the gene itself, insertion site of exogenous gene, order of the exogenous gene, and interference between different genes (Fan et al., 2002).

Bt gene expression

The *Bt* gene is widely used globally. The *Cry1Ac* gene is widely used for the toxic control of Lepidoptera, and the *Cry3A* gene is used to control Coleoptera in poplar at present. Studies have indicated that poplar with two kinds of *Bt* genes have selective resistance to insects and high mortality rates of target insects. Simultaneously, there were significant differences in *Bt* gene expression (Yu et al., 2009). For example, Wang et al. (2012) carried out ELISA tests on 8 lines of 741 poplar transferred with the double *Bt* genes, *Cry3A* and *Cry1Ac*, and the results showed Cry3A protein expression was significantly higher than that of the Cry1Ac toxin protein expression by more than 10 times. In the present study, the *Cry1Ac* and *Cry3A* gene were simultaneously expressed in the clone of *P.* x *euramericana*, and the expression of each *Bt* gene was different. The Cry3A toxic protein content was about 100 times higher than that of Cry1Ac toxic protein in the three lines.

In the insect-resistance test, the cumulative mortality of the two larvae reached a high level. The results showed that the combined use of the two insect resistance genes expanded the insect resistant spectrum and improved the insect resistance of transgenic clone of *P.* x *euramericana*.

The results for the *Bt* toxic protein and insect resistance in the three transgenic plants were consistent with previous reports (Wang et al., 2008; Dong et al., 2015). There were significant differences in the expression of different *Bt* gene, and it was speculated that the two exogenous genes may have been be inserted into the same site, which promoted *Cry3A* gene expression, and could be related to the modification of the*Cry3A* gene, which is closer to the plant's DNA (G+C)mol%. The specific mechanism still needs to be further studied.

Salt tolerant gene expression

Betaine is a non-toxic osmotic adjustment compound, which is rapidly accumulated in the cell during salt and drought stress. Under osmotic stress, betaine accumulation can make many important metabolic enzymes remain active. Betaine aldehyde dehydrogenase is a key enzyme in the synthesis of betaine, which is distributed in the chloroplast stroma, and its activity is induced by salt and drought. The *BADH* gene is the best drought resistant and salt tolerant gene for plants, and is widely used in plant breeding (Liu et al., 2011; Fan et al., 2012). Many studies have shown the *BADH* gene can be introduced into plants to improve salt tolerance. Sun et al. (2011) transformed the vector containing the *BADH* gene into tobacco, and successfully obtained transgenic tobacco. The growth of the control was restrained and that of the transgenic plant was normal at 300 mM NaCl. In the salt tolerance test, at concentrations of 3 and 6‰ NaCl, the growth of transgenic and control plants were inhibited to some extent. However, seedling height, ground diameter, and biomass of the transgenic plants was significantly better than those of the control plants under the same conditions because of the expression of the *BADH* gene. The results were consistent with that of *P.* x *euramericana* 'Neva' (Wang et al., 2007b) and corn (Wang et al., 2014) transferred with the *BADH* gene. In addition, the present study showed the transcriptional abundance of the *BADH* gene was the highest in line No.1, but the salt tolerance test results of line No.2 were superior to those of line No.1. It also showed the salt tolerance of plants is a complex trait that is determined by multiple genes and signal pathways (Parida and Das, 2005), involving physiological, biochemical, and cellular aspects. The introduction of a single gene can improve the salt tolerance of plants to a certain extent; however, further research on the interaction between the various factors and the specific molecular mechanism is needed.

Problems and suggestions for further improvement

In the present study, three functional genes were successfully transferred into the genome of the clone of *P.* x *euramericana* by a multi-gene single vector transformation method. The three target genes were transcribed and expressed, which effectively improved the insect resistance and salt tolerance of transgenic lines. However, there were significant differences in the expression of two *Bt* genes, and the expression of the Cry1Ac protein was low. The transcriptional abundance of salt tolerant *BADH* gene was low, and the salt tolerance of different transgenic lines also showed some differences. It is possible that the expression of each gene was influenced by the other genes in the multi-gene transformation; therefore, the *BADH* gene

could not be expressed efficiently. In addition, the physiological metabolism and biological signal transduction of woody plants was more complex than that of herbaceous plants, which made the plant show the different characteristics. In future studies, to improve the expression of multiple exogenous genes in transgenic plants, the following aspects should be considered: First, when multiple gene vectors are constructed, we focus on the coordination between the exogenous gene and the promoter to develop multiple exogenous genes that are constructed on the vector for efficient expression. Combinations of different promoters, enhanced sequences, and different types of exogenous genes were developed to study the interaction between them, and improve the gene expression. Second, to eliminate the position effect, the plant expression vector containing the MAR-gene-MAR structure could be constructed by adding the matrix associated region MAR to both sides of the exogenous gene, which can be used for genetic transformation. In this way, the expression level of the target gene could be increased, and the difference of the gene expression level between different transgenic plants could be decreased, thus, reducing the location effect. Third, by not changing the amino acid sequence, the target gene, such as *Cry1Ac*, was modified and the plant preference codon was used. It is more suitable for plant expression patterns to increase its target gene expression.

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Supplementary material

Table S1. Abbreviation list.