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ORIGINAL ARTICLE

Genetic transformation and expression of *Cry1Ac–Cry3A–NTHK1* genes in *Populus × euramericana* "Neva"

Dongyan Liu[1,2] · Jun Zhang[1,2] · Yan Dong[1,2] · Xu Zhang[1,2] · Minsheng Yang[1,2] ·
Baojia Gao[1,2]

Abstract To realize the genetic transformation of *Cry1Ac, Cry3A,* and *NTHK1* genes in *Populus × euramericana* "Neva" and obtain transgenic plants, the plant transformation vector p09687199–*Cry1Ac–Cry3A–NTHK1* was transformed in *Populus × euramericana* "Neva" through *Agrobacterium*-mediated transformation. The complete regeneration of the transformed plant was screened with kanamycin. Resistant roots of the transformed plants were detected through PCR, fluorescence quantitative PCR, and ELISA analysis of the toxic protein. The insect resistance and salt tolerance of transgenic plants were determined based on the findings of these methods. PCR detection showed that among six lines, five lines indicated the presence of three target genes, *Cry1Ac* and *Cry3A* genes were detected in one line. Fluorescence quantitative PCR detection showed that the transcript abundance of the *Cry1Ac* gene was within 1.13E+3 to 3.17E+4, that of *Cry3A* was within 4.40E+6 to 1.97E+7, and that of *NTHK1* was within 2.48E+3 to 8.17E+3. Bt toxic protein detection showed that the content of the *Cry1Ac* gene was from 1.08 to 22.99 ng·g^{-1}, and that of *Cry3A* gene was from 146.12 to 17027.96 ng·g^{-1}. The expression of the *Cry3A* gene was significantly higher than that of the *Cry1Ac* gene. The insect resistance test demonstrated that the mortality of transgenic plants on *Hyphantria cunea* first instar larva reached 68.89 %. The mortality on *Plagiodera versicolora* first and second instar larvae reached 100 %, whereas that on the third instar larva was as high as 81.71 %. Four lines were selected to detect potting salt tolerance, and only the No. 2 line showed strong salt tolerance.

Keywords *Populus × euramericana* "Neva" · *Bt* gene · *NTHK1* gene · *Agrobacterium* · Genetic transformation

✉ Minsheng Yang
yangms100@126.com

Baojia Gao
baojiagao@163.com

1 Institute of Forest Biotechnology, Forestry College, Agricultural University of Hebei, 071000 Baoding, People's Republic of China

2 Hebei Key Laboratory for Tree Genetic Resources and Forest Protection, 071000 Baoding, People's Republic of China

Introduction

In recent years, transgenic technology exhibited much progress in the directional improvement of plant traits and has become one of the most important approaches in contemporary molecular breeding. Many studies have examined transgenosis, especially insect-resistant transgenosis, of various *Bt* and salt tolerance genes in the poplar genome (Génissel et al. 2003; Fladung and Ewald 2006; Yang et al. 2008; Hu et al. 2010).

However, research on transgenic poplar mainly focused on 1–2 gene transformations to change traits via genetic engineering technology. Despite the continuous development of transgenic technology, changing a single gene of plant traits has not been realized. The transformation of two or more resistant genes in the same plant and the improvement of multiple plant traits have become popular research topics (Bates et al. 2005; Li and Zhu 2005; Christou et al. 2006; Wang et al. 2010). Multi-gene transformation can alter insect resistance, salt tolerance,

and other qualities of genes in the same plant genome, change the multiple traits of plant variety, and improve the comprehensiveness of plant stress tolerance (Sun et al. 2011; Lian et al. 2008; Gao et al. 2012). The multi-gene transformation methods of plants include *agrobacterium*-mediated method and particle bombardment induction. Methods applied on tobacco (Komari et al. 1996), rice (Feng et al. 2001), corn (Zhu et al. 2008), and other plants are reported to be successful.

With the constant improvement of multi-gene vector construction technology, the multi-gene vector co-transformation method has been extensively implemented in gene engineering. Multi-gene vector transformation aims to transform the expression vector containing multiple genes and their regulatory elements in the plant to obtain a multi-gene transformation plant co-expressed with the multivalent gene via screening. This method can overcome the substitute of the selectable marker gene in the single-gene repeated transformation method and reduce the workload of genetic transformation, screening, and identification. As for the high-efficiency expression of multiple exogenous genes in a plant and the interaction of gene expressions, transgenic plant traits still need to be studied. In this study, two insect-resistant genes, namely *Cry1Ac* and *Cry3A*, and *NTHK1* salt tolerance gene were transformed into *Populus × euramericana* "Neva." Molecular and biological detections of the transgenic plants were performed, and the expressions of the three exogenous genes were analyzed to provide a fundamental basis for multi-gene transformation and expression.

Materials and methods

Agrobacterium strains

Agrobacterium tumefaciens was GV3101. The plasmid vector p09687199 in *Agrobacterium tumefaciens* carries the *Bt* gene (containing *Cry1Ac* and *Cry3A*) and the ethylene receptor gene *NTHK1*. The selectable marker gene was kanamycin resistance gene *NPT II* (Fig. 1). The *Cry1Ac* gene is resistant only to Lepidoptera pests, whereas the *Cry3A* gene is resistant to coleoptera pests. The *NTHK1* gene can improve plant salt tolerance (Wang et al. 2008, 2011).

Receptor materials

The transgenic receptor material was *Populus × euramericana* "Neva", a hybrid of *Populus deltoides* and *Populus nigra* L.; it is a new hybrid variety from Italy introduced by the Chinese Academy of Forestry. The control materials were non-transgenic *Populus × euramericana* "Neva."

Test insects

Hyphantria cunea belongs to Insecta Lepidoptera Arctiidae. Larvae egg masses were collected from Baoding. *Plagiodera versicolora* Laicharting belongs to Insecta Coleoptera Chrysomelidae. Adult worm and egg masses were also collected from Baoding.

Genetic transformation of *Populus × euramericana* "Neva"

Revival of Agrobacterium tumefaciens

Agrobacterium tumefaciens GV3101 containing plasmid p09687199 preserved at low temperature was utilized for lineation on a YEB solid medium through the three-level dilution method. It was cultured at 28 °C in a thermostat incubator for 16–24 h. After the bacterial colonies grew, a single colony was selected, dissolved in YEB liquid medium, placed on a shaker at 160 rpm (away from light), and cultured to the logarithmic growth phase (OD600 = 0.4–0.6) for explant dip dyeing.

Genetic transformation mediated by Agrobacterium tumefaciens

Under sterile conditions, fully extended and dark green tender leaves of *Populus × euramericana* "Neva" tissue culture seedling were selected and transected into 3–5 vertical incisions deep into the main vein. The leaves were immersed for 10 min with *Agrobacterium*. The bacterial suspension was gently shaken to allow the leaves to fully touch the *Agrobacterium*. The leaves were then removed. The excess bacterial suspension was emptied using sterile filter paper, and the leaves were placed in a co-culture medium   (MS + 2.0 mg·L^{-1}6-BA + 0.5 mg·L^{-1}NAA +
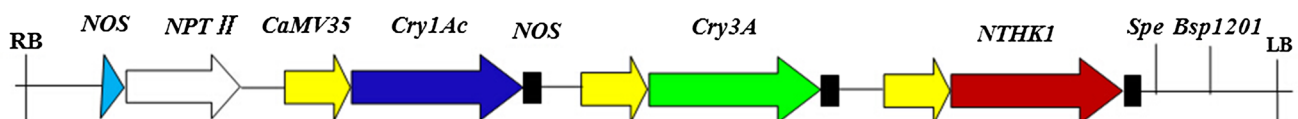

Fig. 1 Structure of multi-gene plasmid vector p09687199–*Cry1Ac*–*Cry3A*–*NTHK1*. Each target gene has a respective promoter and termination sequence in the vector, and these genes connect between the *left* and *right* borders in the vector

10 mg·L^{-1} acetosyringone) and co-cultured for 3 days at 28 °C in dark conditions. The sample was then transferred to a differentiation screening medium (MS + 2.0 mg·L^{-1}6-BA + 0.5 mg·L^{-1}NAA + 30 mg·L^{-1}Kan + 400 mg·L^{-1} Cefotaxime Sodium) every 15 d to 20 days. When the resistance buds grew to 1 cm, they were cut off and transferred into a rooting screening medium (1/2MS + IBA0.3 mg·L^{-1} + 0.5 mg·L^{-1}NAA + 30 mg·L^{-1}Kan + 400 mg·L^{-1} Cefotaxime Sodium). The adventitious roots grew within a week and regenerated into a complete plant. Their stem tips were removed and cultured in a differentiation screening culture. When the adventitious buds grew to approximately 2 cm, they were transferred to a rooting screening medium. The rooting seedlings were transplanted into small pots and placed in an isolated garden after domestication. The 30 mg·L^{-1}Kan + 400 mg·L^{-1} Cefotaxime Sodium were selected for screening referring Yang's research (Yang 2012).

PCR detection of transgenic plants

Transgenic seedling leaves were collected from the garden in the same year. The genomic DNA of transgenic *Populus × euramericana* "Neva" and control plants were extracted with improved CTAB method (Wang et al. 2002). The forward and reverse primers of *Cry1Ac, Cry3A, NTHK1*, and *NPTII* genes were utilized in PCR detection with genomic DNA as the template (Table 1). A 20 μL reaction system was prepared (ddH$_2$0: 7 μl, 2 × Mix: 10 μl, primer R (10 pM): 1 μl, primer F (10 pM): 1 μl, and DNA: 1 μl). The plasmid DNA was considered the positive control, and the non-transgenic *Populus × euramericana* "Neva" was regarded as the negative control. PCR was conducted as follows: pre-degeneration at 95 °C for 5 min, degeneration at 94 °C for 45 s, renaturation at 50 °C for 60 s, extension at 72 °C for 50 s (30 cycles), extension for 7 min at 72 °C, and preservation for 1 min at 25 °C.

Fluorescence quantitative PCR detection of transgenic plants

The transgenic seedlings' annual top leaves that had just fully expanded were acquired at the end of July 2014.

About 0.1 g of leaves was placed into a mortar. Liquid nitrogen was added, and the leaves were rapidly ground into powder. Total RNA was extracted with EASYEx PLUS Plant RNA Kit (Cyrus, China). Reverse transcription of the first cDNA chain was performed with a reverse transcription kit (Kangwei, China). Fluorescence quantitative PCR primers were designed according to the sequence information of the target genes. cDNA synthesized by reverse transcription was utilized as the template, and 2* Sybr Green qPCR Mix was used for fluorescence quantitative PCR.

The fluorescence quantitative PCR primer is shown below.

Cry1Ac gene F: 5′-GAATTTTTGGTCCCTCTCAAT-3′
R: 5′-AGGATCTGCTTCCCACTCTCT-3′
Cry3A gene F: 5′-TGGGGATACGAGAAGGAGGAT-3′
R: 5′-AGTGGGAACAGTGCGATGAGA-3′
NTHK1 gene F: 5′-CCCTTATCACCCTCTTTCCTAT-3′
R: 5′-AGTGTCTTTGCTAACTCCACCA-3′

The reaction system (20 μL) comprised 10 μL 2* SYBR qPCR Mix, 0.5 μL forward primer (10 μM), 0.5 μL reverse primer (10 μM), 1 μL template, and 8 μL ddH2O. The reaction procedure was as follows: pre-degeneration at 95 °C for 5 min, degeneration at 95 °C for 10 s, and renaturation at 55 °C for 30 s. The amplified products of the known fragment were considered the standard and diluted 10 times for use in real-time fluorescence quantitative PCR. The standard curve was then obtained. According to the cycle threshold (Ct) that indicates whether the fluorescence signal reached the setting threshold in each PCR tube and the standard curve, the transcript abundances of *Cry1Ac, Cry3A*, and *NTHK1* genes in cDNA were calculated with mRNA as the transcript.

Cry1Ac and Cry3A detection by ELISA

The transgenic and control seedlings' top annual shoots with fully extended leaves acquired at the end of July 2014 were used for *Bt* toxic protein detection. The *Cry1Ac* and *Cry3A* toxic proteins of all lines were detected with *Bt-Cry1Ab/1Ac* and *Bt-Cry3A* ELISA kits (Agdia, USA). The detection methods were in accordance with the instructions provided by the manufacturers. The positive control was

Table 1 Primers used for PCR detection

Genes amplified by primers	Primer F	Primer R	Size of the amplified fragment (bp)
NPTII	5′-ATCTCCTgTCATCTCACCTTGCTCCT-3′	5′-TCAGAAGAACTCGTCAAGAAG-3′	473
Cry1Ac	5′-ATGGATAACAATCCGAACATCA-3′	5′-CCACCTTTGTCCAAACACTGAA-3′	546
Cry3A	5′-CACTGTTCCCACTGTACGATGT-3′	5′-ATGTTGAAGAAGTCCACGCTCT-3′	667
NTHK1	5′-TGAGCGTATCTTAGAGTGCC-3′	5′-TGTCTTTGCTAACTCCACCA-3′	478

the appendant of the kit, and the negative control was the non-transgenic *Populus × euramericana* "Neva". The results were determined with a BioRad 550-type microplate reader. Protein concentration was calculated by the number of toxic protein nanogram (ng) in each gram of fresh leaves.

Insect resistance detection

Hyphantria cunea first instar larvae were used to detect the insect resistance of the Cry1Ac toxin protein. *Hyphantria cunea* larvae were obtained from egg masses in the nursery of Agricultural University of Hebei in July 2014. The first instar larvae that hatched in the greenhouse were directly utilized for the insect feeding test. The insects used in the Cry3A toxic protein insect resistance test were the first, second, and third instar larvae of *Plagiodera versicolora*. Adult *Plagiodera versicolora* was acquired from the nursery of Agricultural University of Hebei at the end of April 2014 and cultured at room temperature. Fresh and tender non-transgenic *Populus × euramericana* "Neva" was replaced every day. The eggs were collected and cultured daily. Non-transgenic *Populus × euramericana* "Neva" was utilized to feed the larvae. *Plagiodera versicolora* first instar larvae were obtained in 3 days and cultured with the second and third instar larvae for testing.

All transgenic lines and non-transgenic control seedling fresh leaves on annual shoots were collected. The petioles were inserted in humid flower mud to maintain the fresh leaves. The test insects were gently and evenly placed onto the leaves. The leaves were then placed in a small beaker with 6.5 cm diameter and 8 cm height. The rim of the cup was covered with double-layer moist gauze and tied up. The small beaker was placed in a plastic bag and bound gently to maintain humidity and ventilation. Each treatment was repeated thrice. One bottle underwent one repetition of the treatment, and 30 larvae were placed in a bottle. The fresh leaves were changed every day. The mortality of the larvae was recorded. The test continued until all larvae stabilized or died.

The corrected mortality equation is as follows:

Corrected mortality = (mortality rate of transgenic lines − mortality of control)/(1 − mortality of control).

Salt tolerance test

Four transgenic lines of *Populus × euramericana* "Neva" containing ethylene receptor gene *NTHK1* were regarded as the materials, and the non-transgenic *Populus × euramericana* "Neva" was considered the control. Branches that were approximately 15 cm in size, a year old, and with constant diameter were cut and placed in a pot with a depth of 40 cm and a diameter of 30 cm during spring. Each pot

had five seedlings, including one line of non-transgenic *Populus × euramericana* "Neva" and four lines of transgenic seedlings. Fifteen pots of seedlings were set for each line. The water container was covered with a plastic film to preserve moisture. The film was gradually removed as the branch sprouted. The salt tolerance treatment was performed when the seedlings reached a height of approximately 15 cm.

NaCl solution concentration gradients of 0, 3, and 6 ‰ were set. Stress treatment was performed on the transgenic lines and control cutting seedling. Five potted plants were set for each treatment, that is, five treatments were repeated for each line. The saline solution was watered each week at 1 L/pot.

Measurement of seedling height The growth of each line and control plant was observed regularly while under salt stress. Seedling height was measured every two weeks.

Biomass measurement The soil of the cutting seedling root was washed with water after subjecting the plant to salt stress for 40 days. The entire seedling was placed inside an envelope and returned to the laboratory. The seedling was baked at 80 °C until the weight was constant after de-enzyming at 105 °C for 10 min. The dry weight was then measured.

Measurement of photosynthetic rate The photosynthetic rate of each line was determined by Li-6400XT photosynthesis in a sunny morning after subjecting the plant to salt stress for 40 days. The first mature leaf of each line was selected. The dust on the surface of the leaves was removed; the main vein was avoided as the leaves were clamped in the leaf chamber. Data were continuously recorded until the numbers stabilized.

Data analysis

Figures were drawn in Microsoft Excel 2007, and the data were processed with DPS v7.05 statistical analysis software. Multiple comparisons were performed on significant differences with Duncan's new multiple range method.

Results and analysis

Acquisition of transgenic plants

The green buds were differentiated from the transgenic *Populus × euramericana* "Neva" in the screening medium. The resistant buds grew in the culture medium containing a certain concentration of kanamycin because of neomycin phosphotransferase *NPT II* gene. The non-transgenic *Populus × euramericana* "Neva" etiolated and died. The differentiated buds were transferred to the rooting medium. Six lines rooted, but the control did not root.
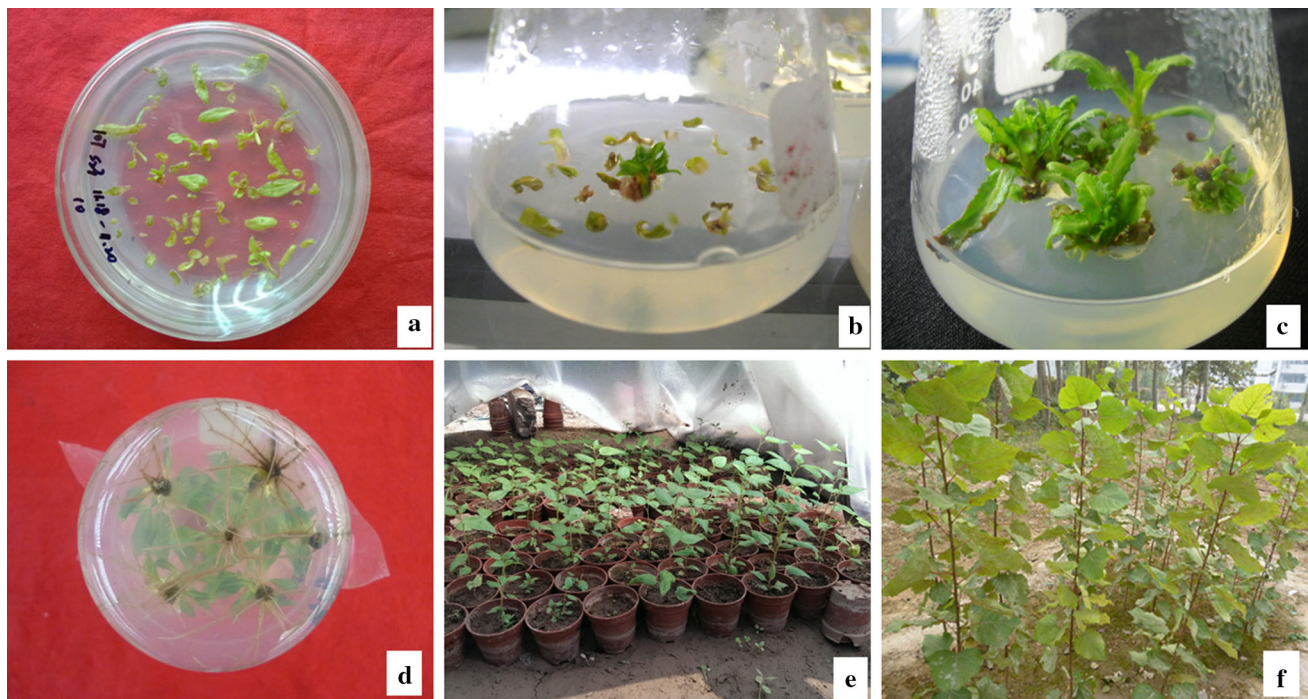
Fig. 2 Acquisition of transgenic lines. **a** *Agrobacterium* infection after co-culture stage. **b** Leaves with resistant shoots after *agrobacterium* infection. **c** Proliferation of resistant shoots. **d** Rooting of resistant shoots. **e** Rooting plants transplanted to small pots. **f** Rooting plants transplanted to the field

Six resistant lines were obtained after repeated subculture and selection. When the root length was approximately 2 cm in the rooting medium, it was transplanted to a small pot and placed in the field. The transgenic lines were obtained by kanamycin preliminary screening, as shown in Fig. 2.

PCR detection of transgenic lines

PCR amplification of six pre-acquired transgenic plants was performed with the two *Bt* genes and *NTHK1*-specific primers. The p09687199 plasmid and non-transgenic *Populus × euramericana* "Neva" were considered positive and negative controls. Multiple PCR amplification results showed that *NPTII* (Fig. 3a), *Cry1Ac* (Fig. 3b), and *Cry3A* (Fig. 3c) genes were detected in the six pre-acquired transgenic plants. *NTHK1* gene was only detected in five lines (Fig. 3d).

Fluorescence quantitative PCR detection of transgenic lines

The cDNA obtained from mRNA reverse transcription was detected by fluorescence quantitative PCR, that is, exogenous gene expression was detected on the transcriptional level. Real-time monitoring of the entire PCR process by fluorescence signal integration showed that the expression of the *Cry1Ac* fluorescence signal was detected in six lines and those of *Cry3A* and *NTHK1* were detected in five lines. The transcript abundance of the *Cry1Ac* gene was within 1.13E+3 to 3.17E+4, that of the *Cry3A* gene was within 4.40E+6 to 1.97E+7, and that of *NTHK1* was within 2.48E+3 to 8.17E+3. The transcript abundance of the *Cry3A* gene was high and that of *Cry1Ac* and *NTHK1* was low. The expressions of the three genes showed no significant difference (Table 2, Fig. 4).

Detection of Bt toxin protein in transgenic lines

The two *Bt* toxic proteins detected by ELISA are shown in Table 3. The expression of *Cry1Ac* toxic protein reached as low as 1.08–22.99 ng·g^{-1} in the transgenic lines. A significant difference was observed among the transgenic lines. The contents of the toxin protein were high in No. 3 and No. 6 lines. The content of *Cry3A* toxin protein was as high as 17,027.96 ng·g^{-1}, and a significant difference was evident among the transgenic lines. The contents of No. 3 and No. 4 lines were the highest. The toxic protein was not detected in the control. Comprehensive analysis showed that the content of *Cry3A* toxin protein in transgenic plants was significantly higher than that of *Cry1Ac*.

Fig. 3 PCR amplification of the exogenous genes of various transgenic lines. **a** *NPTII* gene PCR amplification. **b** *Cry1Ac* gene PCR amplification. **c** *Cry3A* gene PCR amplification. **d** *NTHK1* gene PCR amplification. *CK*[+] plasmid, *CK*[−] control, *M* DL5000 DNA marker (from top to bottom: 5000, 3000, 2000, 1000, 750, 500, 250, and 100 bp)
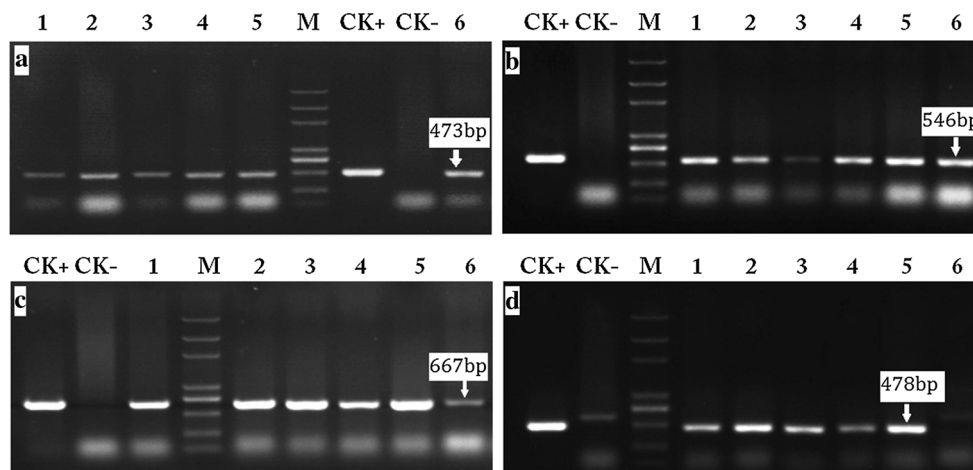

Table 2 Transcript abundance of target genes detected by fluorescent quantification PCR

Various transformed lines	Transcript abundance of *Cry1Ac* gene	Transcript abundance of *Cry3A* gene	Transcript abundance of *NTHK1* gene
1	1.45E+4	1.85E+7	3.23E+3
2	5.61E+3	5.24E+6	3.97E+3
3	3.17E+4	1.35E+7	2.48E+3
4	1.07E+4	1.97E+7	8.17E+3
5	1.13E+3	4.40E+6	2.57E+3
6	1.02E+4	–	–

Insect resistance detection of transgenic lines

Lethal effect of transgenic lines on Lepidoptera Hyphantria cunea

The transgenic and control lines were utilized to feed the *Hyphantria cunea* larvae. The insect resistance effect significantly differed among various transgenic lines. Only No. 3 and No. 6 lines exhibited a highly obvious lethal effect on the larvae and a certain inhibitory effect on the growth of the larvae (Fig. 5). The lethal rate and larvae growth of the other lines did not significantly differ from those of the control lines. Correlation analysis between the lethal rate of *Hyphantria cunea* larvae and *Cry1Ac* toxic protein expression was performed. The correlation coefficient was $r = 0.9832$, which indicates significant correlation at $a = 0.01$ ($r_a = 0.8745$, $n = 7$) level, thereby suggesting that the indoor insect feeding result is consistent with the ELISA kit test results. The higher the expression of Cry1Ac toxic protein, the higher the lethal rate of *Hyphantria cunea* larvae was. In contrast, the lethal rate of *Hyphantria cunea* larvae was low (Table 4).

Lethal effect of transgenic lines on Coleoptera Plagiodera versicolora

As shown in Table 5, except in the No. 6 line, the insect resistance of transgenic *Cry3A* gene lines to *Plagiodera versicolora* larvae was high. The lethal rate of the first instar larvae reached 100 % and that of the second instar larvae reached more than 90 %. However, the lethal rate of the third instar larvae decreased. The insect resistance of the No. 3 line on *Plagiodera versicolora* larvae was the best. Correlation analysis of the lethal rate of *Plagiodera versicolora* larvae and the expression of Cry3A toxin protein produced a correlation coefficient of $r = 0.8451$. Therefore, both are significantly correlated at $a = 0.01$($r_a = 0.7545$, $n = 7$)level, suggesting that the insect test result of *Plagiodera versicolora* is basically consistent with the ELISA kit detection result. The No. 6 line did not show insect resistance, similar to the control.

Salt tolerance test of transgenic lines

Effects of salt stress on seedling height and biomass

Seedling heights of 3 and 6 ‰ were compared with 0 ‰ seedling height. The proportion of 3 and 6 ‰ seedling heights accounting for 0 ‰ was calculated to determine the extent of decrease in different line seedling heights under salt stress. As shown in Fig. 6, the seedling heights of different lines decreased with the increase in salt concentration. Treated by 3 ‰ saline, the monthly growth heights of the control, No. 1, No. 3, and No. 4 lines decreased to approximately 60–70 %; the No. 2 line decreased to only

Fig. 4 Fluorescence quantitative PCR detection of transgenic plants. **a** Dissociation curve of *Cry1Ac*. **b** Amplification curve of *Cry1Ac*. **c** Dissociation curve of *Cry3A*. **d** Amplification curve of *Cry3A*. **e** Dissociation curve of *NTHK1*. **f** Amplification curve of *NTHK1*
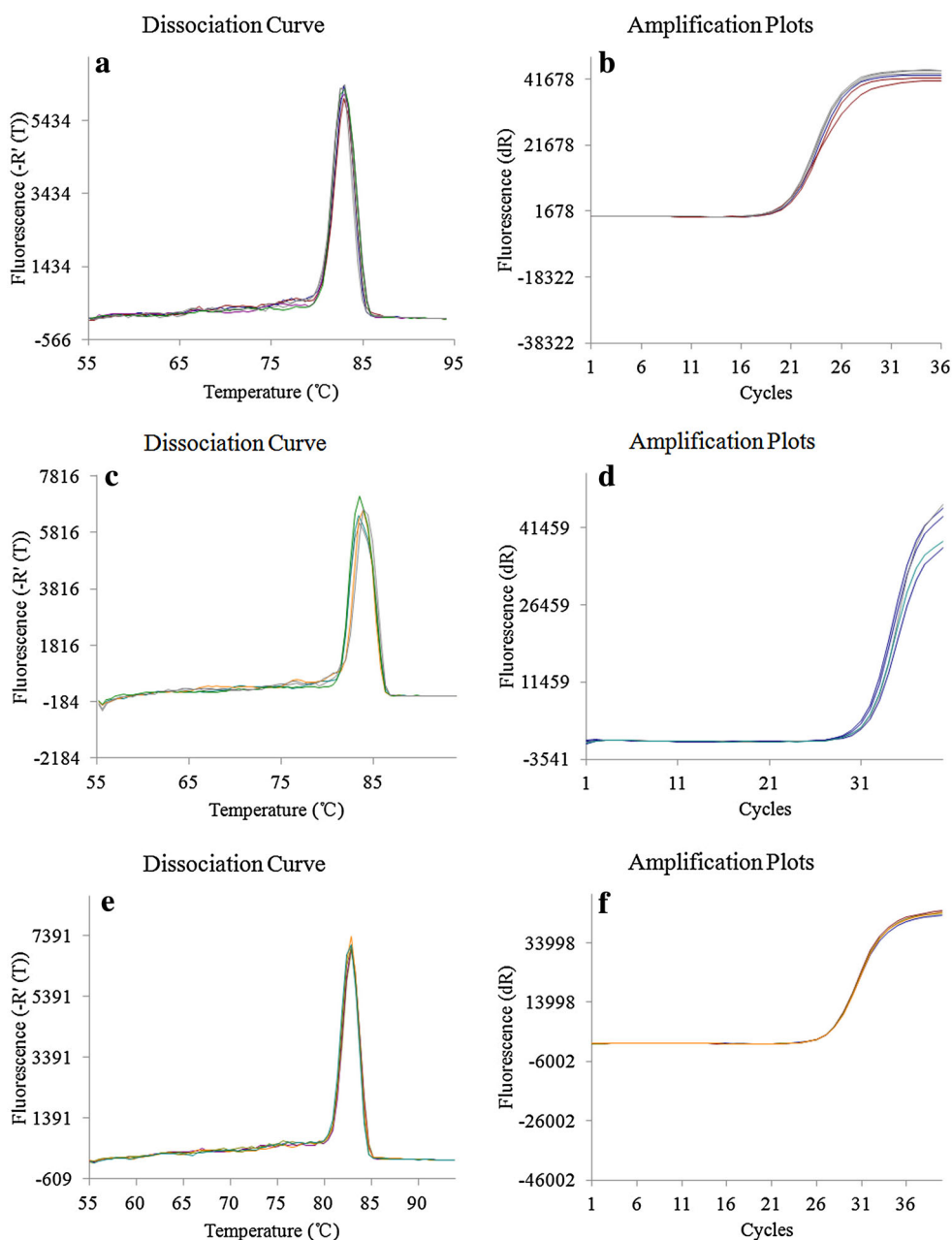

Table 3 Bt toxic protein content of various transformed lines

Various transformed lines	Content of *Cry1Ac* toxic protein/ng·g^{-1}	Content of *Cry3A* toxic protein/ng·g^{-1}
1	5.35 ± 1.66[b]	8912.23 ± 759.04[c]
2	2.11 ± 1.07[bc]	4358.15 ± 365.79[d]
3	22.05 ± 2.99[a]	17027.96 ± 3137.36[a]
4	1.08 ± 0.58[c]	13712.85 ± 2770.94[ab]
5	2.91 ± 1.83[bc]	10994.24 ± 3126.47[bc]
6	22.99 ± 3.79[a]	146.12 ± 35.74[e]
CK	0.00 ± 0.00[c]	0.00 ± 0.00[e]

Within each column, means with the same letter are not significantly different ($P < 0.05$)

90 %. Treated with 6 % saline, the monthly height growth of the control, No. 1, No. 3, and No. 4 lines decreased to about 50–60 %. The height growth of the No. 2 line decreased to 78 %. The increase in seedling height showed no significant difference in No. 1, No. 3, No. 4, and control lines. The extent of the decrease in seedling growth height was large under salt stress. Compared with that of the control line, the extent of the decrease in the No. 2 line was smaller under salt stress, suggesting that the No. 2 line can still maintain high growth and has a level of certain salt tolerance.

Seedling total biomass amounts of 3 and 6 ‰ were compared with 0 ‰ biomass. The percentages of 3 and
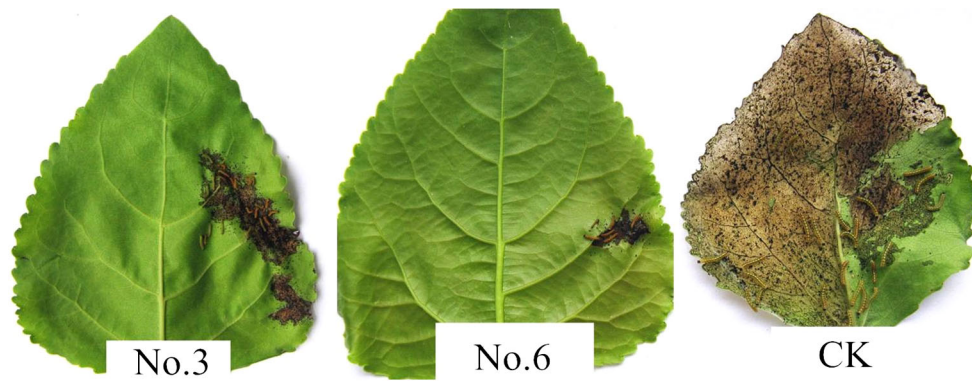
Fig. 5 Larval development and result of leaf eating by L_1 *H. cunea* on the fourth day

Table 4 Mortality of L_1 *H. cunea* fed with various transgenic line leaves

Various transformed lines	Corrected mortality(%)
1	10.00 ± 6.67^b
2	16.67 ± 3.33^b
3	62.22 ± 21.17^a
4	0.00 ± 0.00^b
5	4.44 ± 3.85^b
6	68.89 ± 13.47^a
CK	0.00 ± 0.00^b

Within each column, means with the same letter are not significantly different ($P < 0.05$)

6 ‰ total biomass accounting for 0 ‰ were calculated to compare the extent of decrease. The total biomass of each line decreased with the increase in salt concentration (Fig. 7). The decrease in CK (Control check) was most significant when treated by 3 ‰ saline. The total biomass of No. 1, No. 3, and No. 4 lines decreased to about 80–90 %. In contrast, the total biomass of the No. 2 line slightly increased. Treated by 6 ‰ saline, the total biomass of the control and No. 4 lines decreased to 40–50 %. The total biomass of No. 2 and No. 3 lines were 82 and 77 %, respectively, reflecting a small extent of decrease. Overall, compared with the control, the total biomass measures of No. 1, No. 3, and No. 4 lines were not significantly different because of the main effect of salt stress. The decrease in total biomass in the No. 2 line was smaller than that in the control.

Effects of salt stress on net photosynthetic rate

The net photosynthetic rates of the control and transgenic lines decreased after treatment with salt solution (Fig. 8), indicating that salt stress inhibited the photosynthesis of poplar. Treated by 3 ‰ saline solution, the net photosynthetic rates of the control, No. 1, and No. 4 lines decreased by 50–60 % compared with 0 ‰ treatment. The No. 3 line decreased by 40 %, and the No. 2 line declined by only 21 %. Treated by 6 ‰ saline solution, the net photosynthetic rates of the control, No. 1, and No. 4 lines decreased by 60–70 % compared with that treated by 0 ‰ saline solution. The No. 3 line decreased by 52 %, and the No. 2 line declined by only 19 %. The net photosynthetic rate of each line showed a downward trend. No significant difference was observed in the extent of decrease between No. 1 and No. 4 lines. The decrease in the No. 3 line was smaller than that in the control line. The extent of decrease in the No. 2 line was the smallest and indicated the least effect of salt stress.

Table 5 Mortality rates of different instar larvae of *P. versicolora* fed with various transgenic line leaves (%)

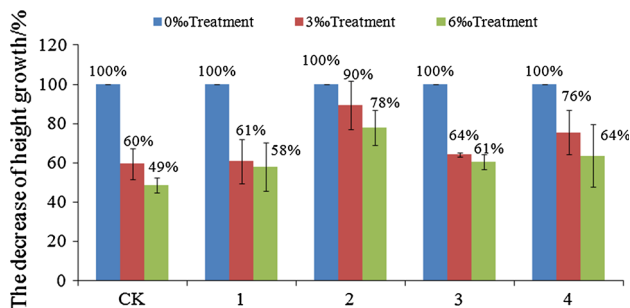| Various transformed lines	Average corrected mortality of first instar larvae	Average corrected mortality of second instar larvae	Average corrected mortality of third instar larvae
1	100.00	98.00	68.18
2	100.00	100.00	73.66
3	100.00	100.00	81.71
4	100.00	97.60	75.62
5	100.00	95.20	78.05
6	0.00	0.00	0.00
CK	0.00	0.00	0.00

Fig. 6 Effects of different concentrations of salt stress on height growth of various transgenic lines

Discussion

Bt gene is a kind of insect-resistant genes derived from microorganisms, and it can express the insecticidal crystal proteins (ICPs). Insecticidal crystal proteins are hydrolyzed by protease in the intestine after ingested by insect, and they turn into toxic peptide which has resistance on protease. The toxic peptide can bind to the insect midgut epithelial cell-specific binding sites, forming perforation and causing cell expansion and cracking, and finally leading to the death of the insect (Li et al. 2010). Different types of crystal proteins have insecticidal specificity. The *Bt* gene is an insect-resistant gene obtained from microbes. Current studies on poplar focus on the *Cry1* gene having a toxic effect on Lepidoptera and the *Cry3* gene having a toxic effect on Coleopteran; various transgenic lines were also observed to exhibit insect resistance (Yang et al. 2008; Zhang et al. 2005; Hjältén et al. 2012; Axelsson et al. 2012). Many studies have proven that transgenic *Cry1Ac* gene poplar has high insect resistance on *Hyphantria cunea*, *Clostera anachoreta*, and *Porthetria dispar*, and so on. Transgenic *Cry3A* gene poplar has a lethal effect on Coleoptera *Plagiodera versicolora* (Yang et al. 2003; Wang et al. 2008; Niu et al. 2011). Yanping Wang et al. (2008) fed *Plagiodera versicolora* and *Clostera anachoreta* with seven lines of transgenic *Cry3A* hybrid poplar 741 and two lines of

transgenic *Cry1Ac* gene hybrid poplar 741. The result showed that transgenic *Cry3A* gene lines have a high lethal rate on *Plagiodera versicolora*. No significant difference was observed in the lethal rate between Lepidoptera *Clostera anachoreta* larvae and the control. Transgenic *Cry1Ac* gene lines showed a high lethal rate on *Clostera anachoreta*, and no significant difference was shown in the lethal rate between Coleopteran *Plagiodera versicolora* larvae and the control. These results suggest that with different transgenic *Bt* genes, poplars exhibit selective insect resistance. Guiying Wang et al. (2012) compared different transgenic *Bt* gene lines and lines containing double *Bt* genes obtained by the twice conversion method. The results showed that with different transgenic *Bt* genes, poplars exhibit selective insect resistance. The effect on the target insects was significant. No toxicity was inflicted on non-target insects. The transgenic double *Bt* genes had digenic resistance. ELISA detection showed that the expression of *Cry3A* toxic protein was more than ten times higher than that of *Cry1Ac* toxic protein.

In the current study, six transgenic lines were obtained with *Agrobacterium*-mediated method. The expressions of two *Bt* genes differed. The transcript abundance of *Cry3A* was 10^3–10^4 times higher than that of *Cry1Ac* at the transcription level. The expression of *Cry3A* toxic protein was 10^2–10^3 higher than that of *Cry1Ac* toxic protein at the translation level. In the insect resistance test, among the six lines, the lethal rates of five lines to Coleoptera *Plagiodera versicolora* first instar larvae reached 100 % and that of third instar larvae reached 68.18–81.75 %. However, the lethal effect on Lepidoptera *Hyphantria cunea* was weak. Only the lethal rates of two lines reached more than 60 %. Compared with the transgenic single *Bt* poplar line, the expression of the *Cry3A* gene was maintained at a higher level, and the lethal effect on *Plagiodera versicolora* larvae was significant. However, the expression of the *Cry1Ac* gene decreased. Generally, the lethal effect of transgenic single *Cry1Ac* gene line on Lepidoptera *Hyphantria cunea* is 80–100 % (Yang et al. 2003; Wang et al. 2008; Tian
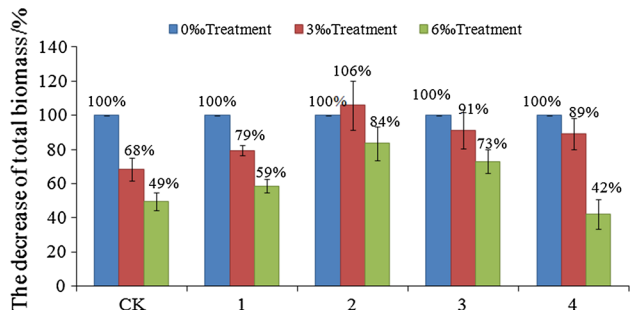

Fig. 7 Effects of different concentrations of salt stress on the total biomass of various transgenic lines
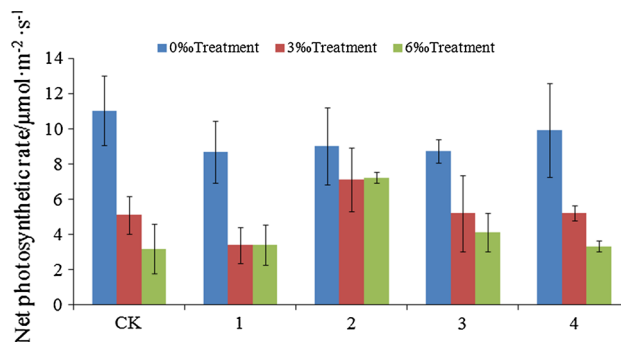

Fig. 8 Effects of different concentrations of salt stress on the net photosynthetic rate of various transgenic lines

et al. 2000). This phenomenon may be caused by gene interaction. The two *Bt* genes were constructed on the same vector and inserted into the plant genome. These genes were closely adjacent and had high homology, resulting in potential interference, namely one gene inhibiting another gene. In addition, the sequence of the target gene may also affect gene expression. Related studies on tobacco showed that when these two *Bt* genes are constructed on one transformation vector, regardless of whether they are *Cry3A* and *Cry1Ac* genes, the toxin protein expression of the *Bt* gene in the upstream decreases. This observation requires further study and verification.

NTHK1 is an ethylene receptor gene cloned from tobacco; it can be induced by mechanical injury, NaCl, and PEG. Thus, *NTHK1* may be involved in the signal transduction process of salt and drought (Zhang et al. 1999, 2001). Tao Feng (2008) injected *NTHK1* into soybean through *Agrobacterium*-mediated method. The result showed that *NTHK1* can be effectively expressed and can enhance salt tolerance and drought resistance. Shasha Du et al. (2014) cultured tobacco lines with transgenic *NTHK1* gene placed in media containing different NaCl concentrations. The adventitious bud differentiation and callus growth of the explant were observed. The result showed that transgenic *NTHK1* tobacco plant growth is superior to that of wild control plants, proving that the *NTHK1* gene can improve the salt tolerance of tobacco to a certain degree. In the present study, four transgenic pot seedlings were subjected to a series of NaCl stress. The No. 2 line was proven resistant to salt tolerance. Under NaCl stress, seedling height, the biomass of transgenic lines, and the extent of the decrease in net photosynthetic rate were lower than those of non-transgenic controls. No significant difference in salt tolerance was observed between the three other lines and the control line. However, the salt tolerance test involving short-term pot experiments was insufficient. Comparative tests of the experimental forest should be conducted on salty and alkaline soil to accurately detect the salt tolerance of transgenic lines.

In the six detected transgenic lines, *Cry1Ac* gene was detected in one line only, and the other two genes were lost. The loss of exogenous gene in transgenic plants had been reported (Jin et al. 2005; Byebier et al. 1987). In general, this phenomenon may be due to the loss in T-DNA integrated into the plant genome or before the integration process (Hua and Huang 1999). Because more repeated sequences existed on the vector, part of target genes was sheared in the T-DNA transformation and integration process, resulting in gene loss. In the study, the missing genes were located near the left border of T-DNA, which was in accordance with the literature reported that T-DNA transformation can cause the loss of the left edge sometimes (Wang et al. 2002; Byebier et al. 1987). But the molecular mechanism of exogenous gene loss is not clear.

The expressions of the three target genes showed a significant difference in this study. In the mRNA transcription, Bt toxic protein expression and biological resistance detection showed significant differences. However, no obvious difference was observed in the mRNA transcript abundance of different transgenic lines. Bt toxic protein expression and biological resistance showed a significant difference between different transgenic lines, suggesting that lines with a low expression of exogenous gene demonstrate posttranscriptional gene silencing. The expressions of various genes showed no correlation in the six transgenic lines. The expressions of the *Cry3A* and *NTHK1* genes were not ideal. The *Cry1Ac* gene was expressed well in the No. 6 line. The expression of the *Cry3A* gene in the No. 2 line was generally acceptable. The expression of the *Cry1Ac* gene was not ideal, whereas the *NTHK1* gene was expressed well. The expressions of *Cry1Ac* and *Cry3A* toxic proteins were relatively high in the No. 3 line probably because three exogenous genes had their own independent promoter and reading frame. When the exogenous gene was inserted into the plant genome, many patterns and mechanisms about its integration patterns emerged.

Due to the different T-DNA insertion sites in the genome of poplar, the gene sequences around the exogenous gene insertion sites in host genome may affect the exogenous gene expression, resulting in different expression conditions of exogenous genes in different lines. On the multi-gene vector, three exogenous genes have independent reading frame, but the same promoter and terminator and more repeated sequences of two *Bt* genes may also lead to genes mutual inhibition at the transcriptional level, resulting in different expressions of different genes. The plant genome may also affect the expression of a certain gene. For example, after the methylation balance of the plant genome was destroyed, the defense occurring on the plant genome allowed different degrees of methylation to occur on the integrated exogenous gene, thereby causing gene silencing (Nan et al. 1998). Therefore, to improve the expression effect, the exogenous gene expression and interaction mechanism must be further studied in polygenic transformation.

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