J. For. Res. (2016) 27(6):1249–1256
DOI 10.1007/s11676-016-0254-x

ORIGINAL PAPER

# Temporal and spatial changes in Bt toxin expression in Bt-transgenic poplar and insect resistance in field tests

**Yiwen Zhang[1,2] · Jun Zhang[1,2] · Jinping Lan[3] · Jinmao Wang[1,2] · Junxia Liu[1,2] · Minsheng Yang[1,2]**

Received: 28 November 2014 / Accepted: 8 April 2015 / Published online: 6 May 2016


**Abstract** Extensive planting of *Bacillus thuringiensis* (Bt)-transgenic plants economically benefits society; however, the potential risk they pose is receiving increasing attention. This study used enzyme-linked immunosorbent assay and fluorescence quantitative PCR (RT-PCR) to monitor the temporal and spatial dynamics of the expression of Bt toxic protein in a forest of 6- to 8-year-old trees of transgenic insect-resistant poplar 741 for three consecutive years. The enrichment, distribution, and degradation of Bt toxic protein and the influence of transgenic poplars on the targeted insect population, *Hyphantria cunea*, were investigated. The content of Cry1Ac toxic protein dynamically changed in transgenic poplar. During the annual growth cycle, the content initially increased, then decreased in the long and the short branches of the crown and in the root system, peaking in August. During the study, the protein did not accumulate overtime. The mRNA transcription of gene *Cry1Ac* was almost consistent with the level of the protein, but transcription peaked in July. In the transgenic and control forestland, microscale levels of the Cry1Ac toxic protein were detected from the soil, but increased accumulation was not observed with the planting year of transgenic poplar. Meanwhile, Bt was isolated and detected molecularly from the soil in the experimental forestland. A systematic investigation of the density of *H. cunea* in the experimental transgenic poplar forest indicated that transgenic Pb29 poplar could resist insects to a certain degree. At peak occurrence of the targeted insects, the density of *H. cunea* in the experimental forest was significantly lower than in the nontransgenic poplar forest.

**Keywords** Biosecurity · Bt toxic protein · Targeted insect · Transgenic insect-resistance poplar

Yiwen Zhang and Jun Zhang have contributed equally to this work.

Project funding: This research was supported by the National High Technology Research and Development Program of China (863 Program) (Grant No. 2013AA102703).

The online version is available at http://www.springerlink.com

Corresponding editor: Chai Ruihai

✉ Minsheng Yang
yangms100@126.com

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

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

[3] Life Science Research Center, Hebei North University, Zhangjiakou 075000, People's Republic of China

## Introduction

Poplar is a widely distributed, essential species of deciduous tree with a long planting history and numerous varieties. It is the predominant fast-growing economic tree variety in timber forests, protection forests, and green belts. To date, forest areas artificially planted with poplar in China exceeds 700,0000 ha, accounting for 19 % of the total artificial forest area (Lu 2008). However, *Populus* are severely attacked by insects, causing great economic losses every year (Zhang and Liu 2006). Therefore, breeding insect-resistant poplar varieties is urgently needed. A number of studies have thus focused on developing transgenic insect-resistant poplars, mostly using *Bacillus thuringiensis* (Bt). At present, transgenic *BtCry1Ac Populus nigra* and transgenic (*Cry1Ac* + *API*) poplar 741

[*Populus alba* × (*Populus davidiana* + *Populus simonii*) × *Populus tomentosa*] developed in China are commercially planted in a controlled region, and China is the first country to genetically engineer forest trees for insect resistance (Hu et al. 2001; Zheng et al. 2000).

Forest trees have a long growth cycle, regenerate slowly and are vital for several. Consequently, for genetically transformed trees, in-depth studies are required to evaluate the sustained expression of the transgene after its insertion into the genome; the possible presence of silencing, drifting, and the influences on other species and microorganisms. Leaves of poplar transformed with a Bt gene are toxic to young larvae, can continuously delay the growth and development of the next generation larvae, and affect the growth and development of other insect species (Gao et al. 2004; Yang et al. 2005). In field experiments during 3 years on European *P. nigra* with the *Cry1AC* gene, the percentage leaf damage and number of pupae in the soil were significantly lower than for the control. The transgenic trees had 10 % damaged leaves while the control trees reached 80–90 %; the average number of pupae per $m^2$ of soil at 20 cm depth in transgenic poplar plantation was 18 which was only 20 % of that found in the non-transgenic control field. Leaf damage and number of pupae in the soil were significantly lower than for the control trees (Hu et al. 2001). In addition, *H. cunea* larvae that are artificially fed on leaves of transgenic insect-resistant hybrid poplar 741 have up to a 100 % mortality rate (Yang et al. 2003). Transgenic *Cry3A* aspen exhibits high resistance in the indoors greenhouse against the leaf beetles *Crysomella tremula* and *Phratora vitellinae* (Génissel et al. 2003; Hjältén et al. 2012). In transgenic *Cry3A* poplar (BGA-5) leaves used to feed targeted insects *Plagiodera versicolora* and nontargeted insects *Clostera anachoreta* in the laboratory, BtCry3A protein was detected; it effectively inhibits coleopteran insects belonging to *Chrysomelidae*, but it has no effect on nontargeted insects and other arthropods (Zhang et al. 2011a, b).The transgenic insect-resistant hybrid BOGA-5 poplar (Bt-Cry3A and oryzacystatin I) has a lethal rate against coleopteran larvae up to 76.7 % in the laboratory and had significant insect resistance in a field experiment (Zhang et al. 2011a, b).

Biosecurity has thus received increasing attention, and risk evaluation and safe management of transgenic trees have been important focuses of transgenic tree investigations (Hu et al. 2010). Transgenic plants are planted at a large scale, and their branches and leaves, root secretions, and dispersed pollen unceasingly enter the soil and accumulate, thereby possibly affecting the microbial activity process and mutually acting with soil microorganism to influence the soil ecological system. Nevertheless, whether transgenic poplars significantly influence conditions around the root system and the populations and species diversity of soil microorganisms remains unknown (Danielsen et al. 2012; Hou et al. 2009).

To take advantage of the insect resistance of Bt poplar and evaluate its ecological risk to the environment, we selected the commercial transgenic *Btcry1Ac* poplar 741 with high insect resistance as the plant material for an experimental transgenic poplar forest. Enzyme-linked immunosorbent assay (ELISA) and fluorescent quantitative PCR method were used to detect and analyze the temporal and spatial dynamics of the Bt toxic protein content of adult trees in the experimental forest and the influence of transgenic poplar on targeted insect *H. cunea* for three consecutive years. We aimed to evaluate the ecological safety of the transgenic poplar system, introduce a safe and reasonable planting mode, and provide a scientific basis for developing a safety evaluation system and guidelines for transgenic trees.

## Materials and methods

### Experimental land and planting material

In March 2005, experimental transgenic poplars were planted in 3.5 ha of experimental land in a field release base approved by the Biology Genetic Engineering Security Council of the State Forestry Administration. Forty-five transgenic line Pb29 and control trees were planted in 10 rows in three replicates with 3 × 4 m between each 5-year-old poplar seeding developed in our laboratory. Both the transgenic and control plants were poplar 741, a high-quality hybrid poplar clone. The clone combination is [*P. alba* L. × (*P. davidiana* Dode. + *P. simonii* Carr.)] × *P. tomentosa* Carr. The transgenic poplar 741 was acquired by simultaneous transcription of the Bt insecticidal protein gene (*BtCry1Ac*) and arrowhead protease inhibition gene (*API*) into the high-quality hybrid poplar 741 clone with an *Agrobacterium*-mediation method (Zheng et al. 2000). The Pb29 line selected in this experiment is a transgenic insect-resistant hybrid poplar 741, which is a highly insect-resistant line. Pb29 was granted a commercialization license by the Biology Genetic Engineering Security Council of State Forestry Administration and has been planted in controlled regions.

### Research method

#### *Material collection*

On June 10, 2009, Pb29 lines and control poplar 741 lines were randomly selected from the experimental forest. Current-year young leaves in the middle of the plants were collected, placed in an ice box, and stored in a laboratory

refrigerator at −80 °C for DNA extraction and PCR detection of the transgenes. Every June to October from 2009 to 2011, one strain of transgenic Pb29 and one strain of control poplar 741 were randomly selected from each replicate area; a total of six strains were obtained. Long-branch leaves, short branch leaves, and current-year new root samples were regularly collected every month from the middle of the crown of the sample plants. Rhizosphere soil for the corresponding poplar samples was collected in each area; that is, the current-year new root was dug out at 15 cm below the soil surface and vertically below the peripheral crown to collect soil 2 mm or less surrounding the root. The collected samples were brought to the laboratory in an ice box and then stored in a refrigerator at −80 °C.

*DNA extraction of the poplar genome and detection of Cry1Ac gene*

The CTAB method (An et al. 2001) was performed to extract the DNA of transgenic poplar Pb29 and poplar 741 genomes. Primers were designed based on the *Cry1Ac* gene sequence in transgenic Pb29 and synthesized by Shanghai Technical Service Industry and Biological Engineering Co., Ltd. The primer sequence was AF: 5′-CAA CCC GAA CAT CAA CGA AT-3′, AR: 5′-GCC AAT AAG CCT AGT TAA ATC A-3′. PCR reaction was carried out in a Biometra T1 Thermocycler (Germany). The PCR amplification procedure was as follows: 4 min of pre-denaturation at 94 °C; 30 cycles of 50 s at 94 °C, 60 s at 55 °C, 60 s at 72 °C; and a final extension of 10 min at 72 °C.

*Extraction and detection of Cry1Ac protein*

Phosphate buffer solution with Tween-20 (8 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, 10 mM KCl, 140 mM NaCl, 0.05 % Tween-20, pH 7.4) was used to extract Cry1Ac toxic protein from the long branch leaves, short branch leaves, root system, and rhizosphere soil of transgenic poplar. The Cry1Ac protein was quantified with BtCry1Ab/1Ac ELISA kit of Agdia, including one 96-well plate with antibody embedded and supplementary reagents. Quantitative ELISA consisted of protein extraction and assay using the BtCry1Ab/1Ac ELISA kit and its instructions (Applied Agdia, USA).

*Transcription analysis of transgenic poplar gene Cry1Ac*

From June to October of 2011, one transgenic poplar was randomly and regularly selected in each replicate region of the experimental forest every month; three poplars were obtained. The long-branch leaves from the mid crown were collected for transcription analysis of *Cry1Ac*. The RNA extraction kit from Shanghai Technical Service Industry and Biological Engineering Co., Ltd. was used for RNA extraction, and Quantscript RT kit from TAKARA Biotechnology Co., Ltd. was used for reverse transcription. A fluorescence dye method was employed for real-time fluorescent quantitative expression analysis as follows. According to the full sequence information of gene *Cry1Ac*, fluorescent quantitative PCR primers were designed as follows: Bt1-F2: 5′-GAATTTTTGGTCCC TCTCAAT-3′ and Bt1-R2: 5′-AGGATCTGCTTCCCACT CTCT-3′. The reaction system (50 μL) comprised 2 × SYBR qPCR Mix (25 μL), DNA template (2 μL), 10 μM forward primer (1 μL), 10 μM reverse primer (1 μL), and $ddH_2O$ (21 μL). The reactions were done in the CHROM4 fluorescent quantitative PCR device (BioRad) using the cycling conditions described earlier. The data were obtained with Sequence Detector Version 1.3.1 and input to Excel for analysis.

## Identification of Bt in soil

In June 2010, a five-point sampling method was used in each region of the experimental forest to collect the surface soil samples from around transgenic line Pb29 and control poplar 741. In the laboratory under sterile conditions, 1 g of the respective soil sample was suspended 9 mL of sterile distilled water, them 1 μL of the supernatant was transferred to a centrifuge tube; sterilized water was added to 1000 μL, and the tube held in a water bath at 80 °C for 20 min. After the solution had cooled to room temperature, the dilution streak plate method was used to separate colonies on LB agar plates. After 24 h at 37 °C, A single colony was selected for a subsequent smear, fuchsin staining, and microscopic examination. Single colonies selected from the strains were used for colony PCR amplification with 16S universal primer. The 16S universal primer was 8F: 5′-AGA GTT TGA TCC TGG CTC AG-3′, 1492R: 5′-GGT TAC CTT GTT ACG ACT T-3′. The PCR amplification cycling were done as described earlier using the Biometra T1 Thermocycler (Germany). The PCR products were sequenced by Biogenro Biotechnology Co., Ltd.

## Influence of experimental transgenic poplar forest on *H. cunea*

From July to September of 2010, *H. cunea* were systematically counted by region random sampling method from five trees of transgenic line Pb29 and five of the control poplar randomly selected from each region of the experimental forests. Individuals of *H. cunea* were counted on 50-cm long branches selected in the eastern, southern, western and northern of the four orientations, upper and

lower of the two levels. Individuals of *H. cunea* on stems 5 m or less from the soil surface were also counted.

## Results and analysis

### PCR detection of gene *Cry1Ac* in transgenic poplar

The PCR using genomic DNA from individual young leaves of transgenic line Pb29 and control poplar 741 yielded a distinct amplification band between 500 and 750 bp for transgenic poplar line Pb29, consistent with the fragment size of *Cry1Ac* in positive control amplification (Fig. 1). No band was present in the PCR amplification of the control. This result indicates that the planted poplar was transgenic *Cry1Ac* Pb29, consistent with the result of Tian et al. (2000). Therefore, it further verifies that gene *Cry1Ac* stably existed in the experimental transgenic poplar forest.

### Distribution of the Cry1Ac toxic protein in transgenic poplar

Figure 2 shows the temporal changes in theCry1Ac toxic protein content in long-branch leaves, short branch leaves, and root system of transgenic line Pb29 in the experimental forest during the growing seasons from 2009 to 2011. Protein content changed consistently in long-branch leaves, short branch leaves, and root system of transgenic poplar, initially increasing and then decreasing during the entire growing season in 1 year (Fig. 2a). Every June to July, the content of *Cry1Ac* toxic protein in all three tissues was lower than 50 ng $g^{-1}$. The content peaked in August, then decreased. The content of Cry1Ac toxic protein from highest to lowest in the three tissues was root system >short branch leaves >long-branch leaves. Figure 2b shows the three-year statistical data grouped by tissue type. The toxic protein content of *Cry1Ac* in the long-branch leaves, short branch leaves, and root system decreased as the years progressed. This finding demonstrates that the toxic did not accumulate in the long-branch leaves, short branch leaves, or root system of poplar each year.

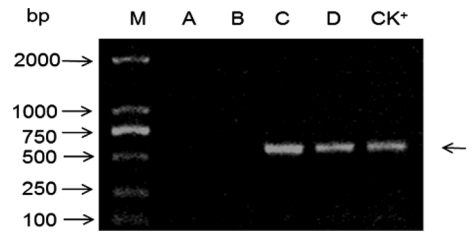


**Fig. 1** PCR detection of *Cry1Ac* in transgenic poplar. **a**, **b** Nontransgenic poplar 741; **c**, **d** *Cry1Ac*-transgenic Pb29; $CK^{+}$*Cry1Ac* vector. The *arrow* indicates the amplified fragment

### Detection of Cry1Ac toxic protein in rhizosphere soil of experimental forestland

Figure 3 shows the level of Cry1Ac toxic protein detected in the rhizosphere soil collected from around the transgenic and nontransgenic poplars. In 2009, toxic protein was detected only in the rhizosphere soil from transgenic poplars, not in the nontransgenic poplar plots (Fig. 3a). In July and September in 2010 and 2011, a small amount was detected in the nontransgenic poplar plots. The toxic protein level in nontransgenic poplar soil was slightly higher than in the transgenic poplar soil, especially in July and September of 2010. This higher content may be attributed to the amount of Cry1Ac toxic protein in the soil, the residues and falling leaves of transgenic poplars washed by rain or blown by wind into nontransgenic poplar lands, or experimental error. Figure 3b shows the changes in Cry1Ac toxic protein content in nontransgenic poplar rhizosphere soil as the years progressed. Toxic protein was not detected in the rhizosphere soil of nontransgenic poplar soil in June 2010 and June 2011. The three-year consecutive detection results indicated that the amount of Cry1Ac toxic protein in transgenic poplar rhizosphere soil was extremely small compared with that in the different tissues of transgenic poplar. Moreover, toxic protein did not accumulate as years progressed, and no special pattern encompassing the changes of Cry1Ac toxic protein in different years and months was found.

### Transcription analysis of gene *Cry1Ac* in long-branch leaves of transgenic poplar

The long-branch leaves of transgenic poplar were collected from June to October 2011 for RNA extraction and reverse transcription and RT-PCR. In 1 year, the transcription abundance of gene *Cry1Ac* initially increased then decreased, similar to the expression pattern of Cry1Ac toxic protein (Fig. 4). Transcript levels peaked in July, then gradually decreased and were almost no detectable by October. Expression of Cry1Ac was highest in August, possibly because of fluorescence quantitation, which reflects the RNA level, or the rapid transcription from DNA to RNA. ELISA reflects the protein level. Consequently, the expression of *Cry1Ac* in transgenic poplar long-branch leaves peaked early.

### Identification of Bt in experimental land soil

Colonies that were detected from the surface soil around transgenic line Pb29 and control poplar 741 looked like a pale yellow, opaque, dripping wax with irregular margins that had jagged, small protrusions. Viewed with an oil immersion lens, the cells were long, rod-shaped, and had

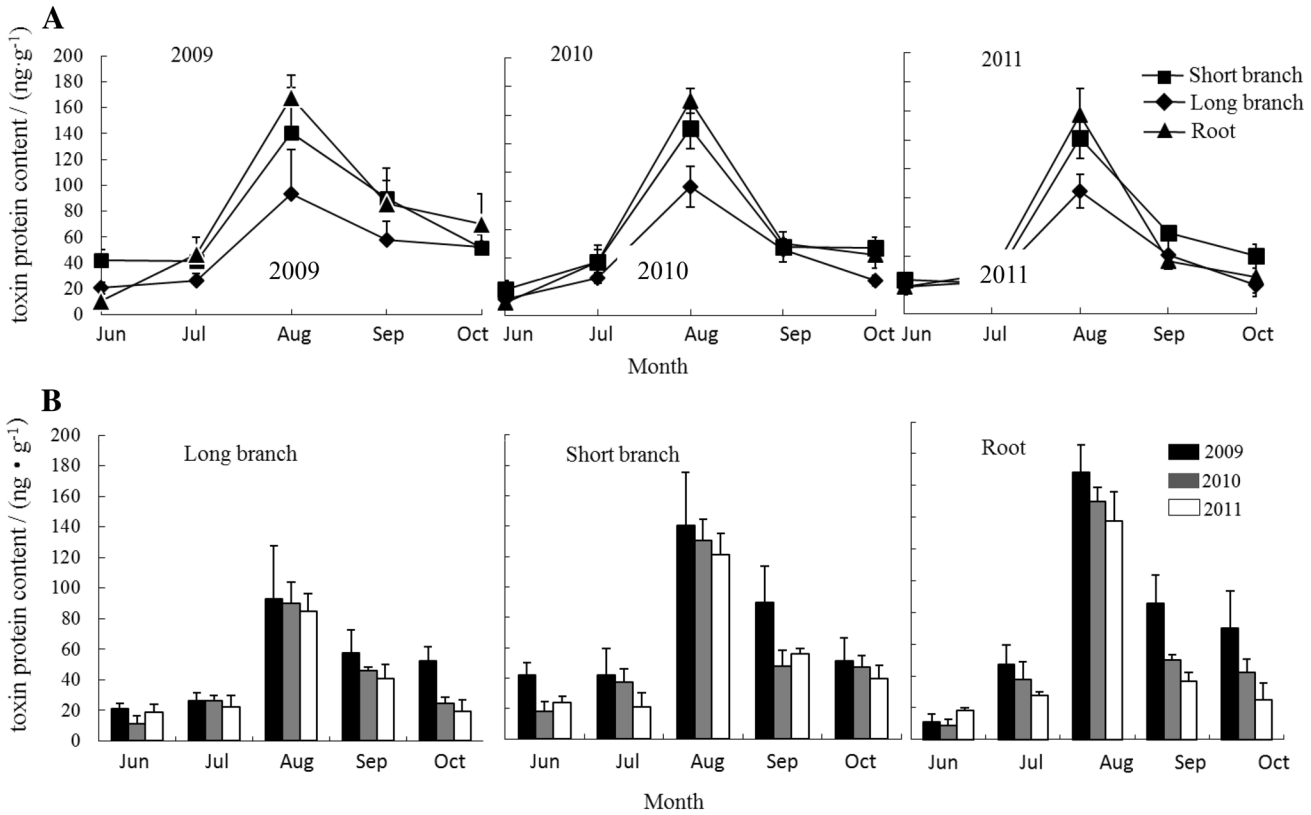


**Fig. 2** Temporal changes in Cry1Ac toxic protein content in different tissues of transgenic line Pb29 from 2009 to 2011. Content shown for the three tissues at each sampling (**a**) and for each tissue grouped by month (**b**) for the 3 years. Protein levels were quantified with ELISA

various sizes. The crystals were rhomboid or rhombic, and the microorganisms were short rod shaped and transparent (Fig. 5a) and were potentially Bt. Subsequent colony PCR using the 16S full universal primer indicated that the amplification efficiency of colony PCR products was high (Fig. 5b) and could therefore be used for product sequencing. A comparison of the PCR product sequences with the NCBI database (http://www.ncbi.nlm.nih.gov) indicated that the microorganisms were Bt. Bt was found in soils from the transgenic and the control forests.

### Influence of the experimental transgenic poplar forest on *H. cunea* population

In 2010, the *H. cunea* populations in the transgenic and nontransgenic planting areas were dynamically consistent (Fig. 6). During the year, the population was low in July, gradually decreased in August and peaked in early September, The level of the Cry1Ac toxic protein in the transgenic plant tissues was highest in August when the population of *H. cunea*in transgenic Pb29 poplar planting area was lowest and also significantly lower than that of nontransgenic control 741.Thus, the population levels of *H. cunea* in transgenic Pb29 poplar gradually decreases as the Cry1Ac toxic protein increased, illustrating that the Cry1Ac toxic protein inhibits or kills *H. cunea* and that transgenic Pb29 poplar is insect-resistant compared with the control poplar 741.

## Discussion

The expression of *Cry1Ac* after integration into the poplar genome is influenced by genetic structure, gene insertion site, receptor gene epistasis, environment, and other factors. Our data on its expression during three consecutive years (Fig. 2) showed that *Cry1Ac* is stably expressed and that transcript content changes almost in parallel with regular changes in the growth period. This result indicates that the protein content is high and increases with vigorous physiological activity in poplar. In the later part of the growing season, the toxic protein content also decreased, in the different tissues in parallel with developmental stage and *Cry1Ac* expression. Transgenic poplar Pb29 stably expressed Cry1Ac toxic protein and significantly decreased the population density of the targeted insect *H. cunea* (Fig. 6).The present study also showed that transgenic Pb29 poplar significantly decreased population of on *H.*

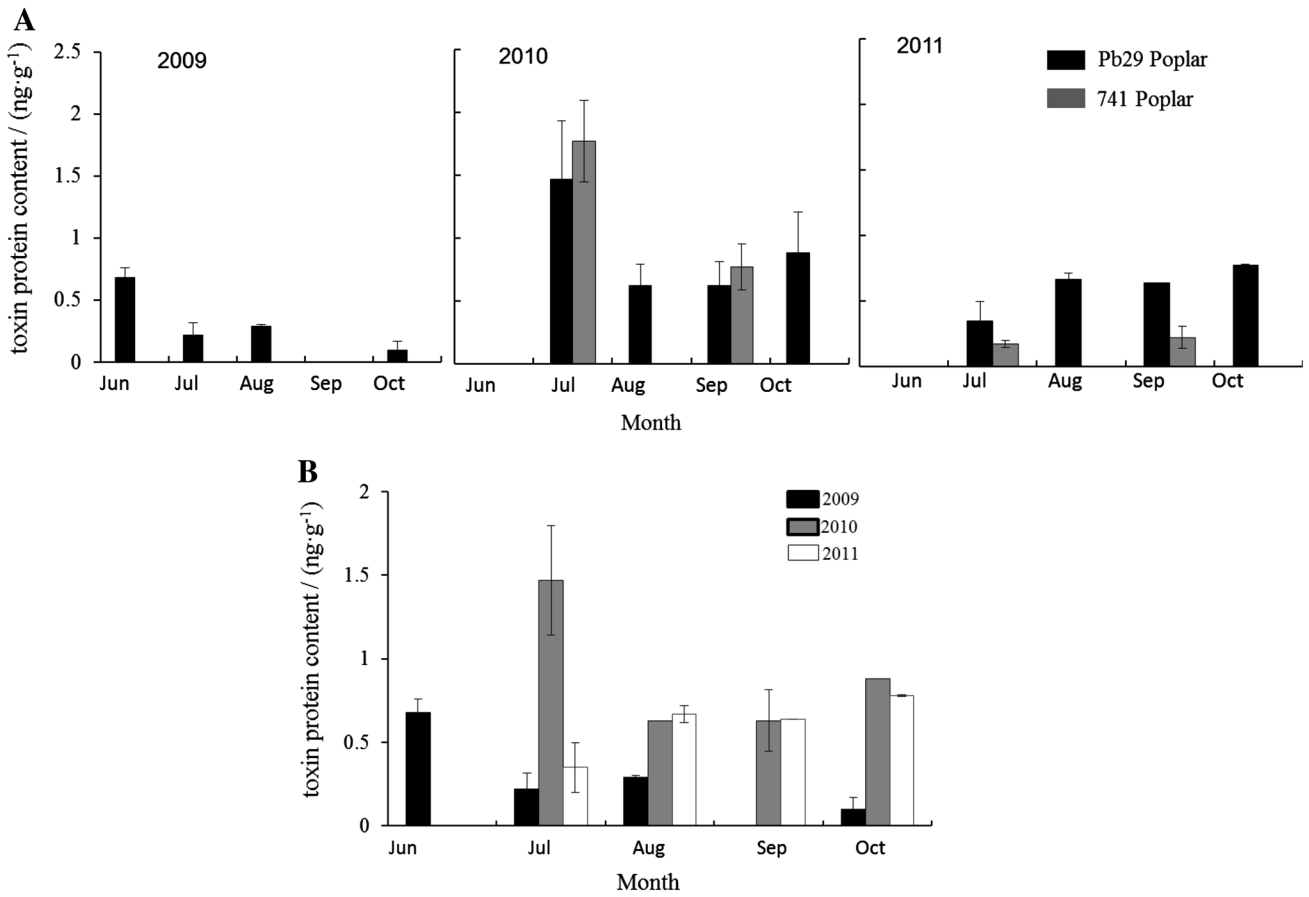


**Fig. 3** Content of Cry1Ac toxic protein in rhizosphere soil from transgenic (Pb29) and nontransgenic (741) poplars from 2009 to 2011 as quantified by ELISA

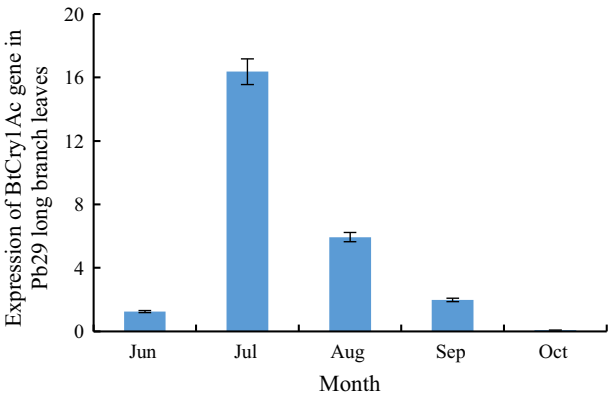


**Fig. 4** Fluorescent quantitative PCR analysis of gene *Cry1Ac* in long-branch leaves of transgenic poplar

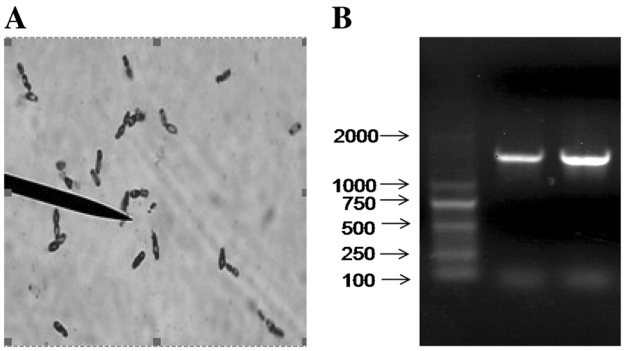


**Fig. 5** Microscopic examination of colony and colony PCR in soil. **a** Cells separated from soil and viewed with an oil immersion lens; **b** PCR detection in soil isolates

*cunea*in the experimental forest, consistent with lethality up to 100 % of the first and second instar larvae fed on transgenic Pb29 poplars (Gao et al. 2004).

The Cry1Ac toxic protein content in transgenic Pb29 poplars initially increased and then decreased during different stages in the annual growth cycle, as did its mRNA transcript levels (Figs. 2, 4). However, the transcript levels were highest in July, and the content of Bt toxic protein peaked in August. Thus, mRNA and protein levels differed in the same period, and the level of the Bt toxic protein lagged behind the transcript levels, demonstrating that the regulation of post-transcriptional level or translation level affected the protein level. The inconsistent changes in the

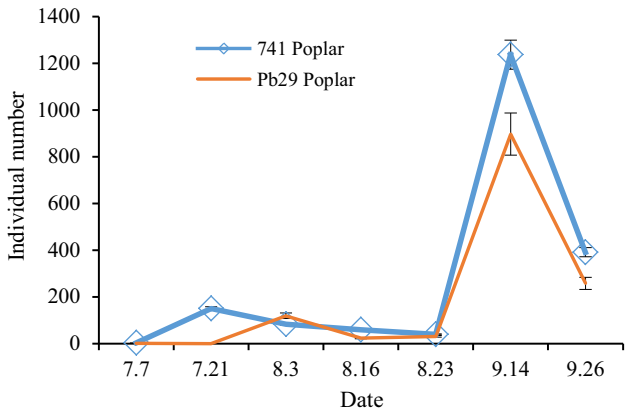


**Fig. 6** *Hyphantria cunea* population dynamics in experimental transgenic poplar forest from July through September 2010

transcription and translation signals suggests that related protein expression and gene transcription must be given attention. Reports showing inconsistent transcription and translation are common (Hiwasa-Tanase et al. 2011; Li and Xie 2011; Perlak et al. 1991). Our experiment indicated that the changes in Bt toxic protein, mRNA level, and insect resistance followed the same trends, but changes over time were inconsistent. Although mRNA transcript levels were highest in July, Bt toxic protein expression gradually increased and peaked in August when the population of *H. cunea* was gradually increasing and the transgenic poplar had the best resistance. In September, RNA transcription and expression of *Cry1Ac* gene were lower, and the *H. cunea* population was subsequently at its highest. Axelsson et al. (2012) also indicated that the extent of target pest damage to the blade in high-expression transgenic *Btcry3Aa P. davidiana* lines is significantly lower than that of the low-expression lines. Therefore, the higher the expression quantity of Bt toxic protein, the better its insect resistance.

Given the large-scale cultivation of transgenic plants, their branches, leaves, and other residues, root system secretions, and pollen enter the soil continuously and can accumulate. They may interact with soil microorganisms, influencing the activity of the microorganisms and the soil ecological system (Axelsson et al. 2010). Studies have shown that the Bt protein is held in plant residues and root secretions in the soil. It does not begin to degrade until it has remained in the soil for several weeks, even in specific soil environments, and it can maintain its activity in the soil for more than 200 days (Head et al. 2002; Zwahlen et al. 2003). Zhen et al. (2011) found that Bt toxic protein surrounds the root system of transgenic plants in the experimental poplar 741 forest, but its concentration decreases tenfold in the following order: root tissue, root surface soil, rhizosphere soil and earth surface soil. The distribution of Bt toxic protein is not significantly correlated to changes in the number of soil microorganisms. The annual average content of Cry1Ac toxic protein in the rhizosphere soil of transgenic planting area was highest in the second year of investigation and gradually decreased in the third year (Fig. 3).The level of the Cry1A*c* toxic protein did not increase over time. Cry1Ac toxic protein detected in soil may be due to existing Bt in the soil (Fig. 5), rain wash, transgenic poplar residues and leaves blown by wind, or experimental error. Wang et al. (2012) investigated the mode of migration of the Bt-Cry1Ac protein within poplar grafts and found Cry1Ac protein in the phloem, xylem, pith and leaves of the grafted poplar and that it was transported between rootstock and scion mainly through the phloem. Migration of Bt-Cry1Ac protein in the grafted union was also evidenced in that the leaves of the control graft did have a lethal effect on *C. anachoreta* larvae in laboratory feeding experiments. However, whether Cry1Ac toxic protein can lead to changes in soil environment requires further investigation.

Molecular detection and biological assays are important methods to study transgenic insect resistance. Positive molecular detection suggests no resistance to insects. Transgenic Pb29 poplar can stably express Cry1Ac toxic protein and decrease the number of targeted insect *H. cunea* (Fig. 6). Similar to transgenic Bt crops such as cotton, corn, and rice (Dively 2005; Head et al. 2005; Liu et al. 2004), transgenic Pb29 poplar can significantly decrease the population density of targeted insects. Nevertheless, this field experiment was limited by many factors, including the relatively short experimental time, small test area, and movement of targeted insects between transgenic and nontransgenic poplars; thus, experimental errors may have occurred (Fladung and Ewald 2006). The resistance of transgenic poplar to *H. cunea* is evidenced by a lethal effect and largely by the deferred development of *H. cunea* larva. The forestland investigation also indicates that the population of *H. cunea* in transgenic poplar forest is small and has a long developmental period. Thus, this preliminary analysis shows that the amount of experimental forestland did affect the population and development of *H. cunea* to some degree, but the system needs an in-depth investigation and research on the variables, including nontargeted insects, the population structure and amount of natural enemies, species diversity index, uniformity, and ecological dominance.

Information on the long-term and stable existence of an exogenous gene in transgenic trees is the basis of breeding and safety evaluation. The transgene *Cry1Ac* stably existed in the transgenic poplar genome. However, given the unknown integration sites, whether the exogenous gene can stably remain in the genome requires necessary and in-depth investigations to provide reference for further evaluation of its safety. Given its low frequency, this gene is

difficult to detect using the current techniques. We stress that the data in this research are only preliminary. Multiple lines of research and studies are necessary to obtain more data for precise valuation of the biological safety of transgenic poplar.

**Acknowledgments** This research was supported by the National High Technology Research and Development Program of China (863 Program) (Grant No. 2013AA102703).

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

## References

An XL, Dong JG, Han JM (2001) RAPD analysis of *Exserohilum turcicum*I. CTAB method of isolating DNA from *Exserohilum turcicum* for RAPD. J Agric Univ Hebei 24(1):38–41 **(in chinese)**

Axelsson EP, Hjältén J, LeRoy CJ, Julkunen-Tiitto R, WennstrÖm A, Pilate G (2010) Can leaf litter from genetically modified trees affect aquatic ecosystems? Ecosystems 13(7):1049–1059

Axelsson EP, Hjältén J, LeRoy CJ (2012) Performance of insect-resistant *Bacillus thuringiensis* (Bt)-expressing aspens under semi-natural field conditions including natural herbivory in Sweden. For Ecol Manag 264:167–171

Danielsen L, Thurmer A, Meinicke P, Buee M, Morin E, Martin F, Pilate G, Daniel R, Polle A, Reich M (2012) Fungal soil communities in a young transgenic poplar plantation form a rich reservoir for fungal root communities. Ecol Evol 2(8):1935–1948

Dively GP (2005) Impact of transgenic VIP3A × Cry1Ab lepidopteran-resistant field corn on the nontarget arthropod community. Environ Entomol 34:1267–1291

Fladung M, Ewald D (2006) Tree transgenesis: recent developments. Springer, Berlin-Heidelberg

Gao BJ, Wang YF, Wang JM, Liang HY, Yang MS, Zhang JH (2004) Researches on the insect-resistant effect of transgenic hybrid poplar 741 with two insect-resistant genes. Acta Ecol Sin 02:297–301 **(in chinese)**

Génissel A, Leplé JC, Millet N, Augustin S, Jouanin L, Pilate G (2003) High tolerance against *Chrysomela tremulae* of transgenic poplar plants expressing a synthetic *cry3Aa* gene from *Bacillus thuringiensis* ssp tenebrionis. Mol Breed 11(2):103–110

Head G, Surber JB, Watson JA, Martin JW, Duan JJ (2002) No detection of Cry1Ac protein in soil after multiple years of transgenic Bt cotton (Bollgard) use. Environ Entomol 31(1):30–36

Head G, Moar W, Eubanks M, Freeman B, Ruberson J, Hagerty A, Turnipseed S (2005) A multiyear, large-scale comparison of arthropod populations on commercially managed Bt and non-Bt cotton fields. Environ Entomol 34(5):1257–1266

Hiwasa-Tanase K, Nyarubona M, Hirai T, Kato K, Ichikawa T, Ezura H (2011) High-level accumulation of recombinant miraculin protein in transgenic tomatoes expressing a synthetic miraculin gene with optimized codon usage terminated by the native miraculin terminator. Plant Cell Rep 30(1):113–124

Hjältén J, Axelsson EP, Whitham TG, LeRoy CJ, Julkunen-Tiitto R, Wennstoem A, Pilate G (2012) Increased resistance of Bt aspens to *Phratora vitellina*e (Coleoptera) leads to increased plant growth under experimental conditions. PLoS One 7(1):e30640

Hou YJ, Su XH, Jiao RZ, Huang QJ, Chu YF (2009) Effects of transgenic *Populus alba* × *P. glandulosa* on soil microorganism. Sci Silvae Sin 45(5):148–152 **(in chinese)**

Hu JJ, Tian YC, Han YF, Li L, Zhang BE (2001) Field evaluation of insect-resistant transgenic *Populus nigra* trees. Euphytica 121(2):123–127

Hu JJ, Yang MS, Lu MZ (2010) Advances in biosafety studies on transgenic insect-resistant poplars in China. Biodivers Sci 18(4):336–345

Li GW, Xie XS (2011) Central dogma at the single-molecule level in living cells. Nature 475(7356):308–315

Liu Z, Ye G, Hu C (2004) Effects of *Bacillus thuringiensis* transgenic rice and chemical insecticides on arthropod communities in paddy-fields. Chin J Appl Ecol 15(12):2309–2314

Lu MZ (2008) Advance in transgenic research and its biosafety of forest trees in China. In: Proceeding of the 8th symposium in agricultural biochemistry and molecular biology of the Chinese Society of Biochemistry and Molecular Biology, September 2008, Guiyang, China, pp 1–2

Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci USA 88(8):3324–3328

Tian YC, Zheng JB, LI CQ (2000) Studies of transgenic hybrid poplar 741 carring tow insect-resistant genes. Acta Bot Sin 42(3):263–268

Wang LR, Yang MS, Akinnagbe A, Liang HY, Wang JM, Ewald D (2012) *Bacillus thuringiensis* protein transfer between rootstock and scion of grafted poplar. Plant Biol 14:745–750

Yang MS, Lang HY, Gao BJ, Wang JM, Zheng JB (2003) Insecticidal activity and transgene expression stability of transgenic hybrid poplar clone 741 carrying two insect-resistant genes. Silvae Genet 52(5–6):197–201

Yang MS, Gao BJ, Wang YF, Wang JM, Du GJ (2005) Analysis of main characteristic of white poplar hybrid 741 transformed two insect-resistant genes. Sci Silvae Sin 01(1):91–97 **(in chinese)**

Zhang HC, Liu ZS (2006) Agricultural statistics of China 2006. China Agricultural Press, Beijing, pp 230–257

Zhang BY, Chen M, Zhang XF, Luan HH, Diao S, Tian YH, Su XH (2011a) Laboratory and field evaluation of the transgenic *Populus alba* × *Populus glandulosa* expressing double coleopteran-resistance genes. Tree Physiol 31(5):567–573

Zhang BY, Chen M, Zhang XF, Luan HH, Tian YH, Su XH (2011b) Expression of Bt-Cry3A in transgenic *Populus alba* × *P. glandulosa* and its effects on target and non-target pests and the arthropod community. Transgenic Res 20(3):523–532

Zhen ZX, Wang JM, Yang MS (2011) Effects of transgenic insect-resistance hybrid poplar 741 groves on soil microorganisms. J Agric Univ HeBei 34(1):78–81 **(in chinese)**

Zheng JB, Liang HY, Gao BJ, Wang YF, Tian YC (2000) Selection and insect resistance of transgenic hybrid poplar 741 carrying two insect-resistant genes. Sci Silvae Sin 36(2):13–19

Zwahlen C, Hilbeck A, Gugerli P, Nentwig W (2003) Degradation of the Cry1Ab protein within transgenic *Bacillus thuringiensis* corn tissue in the field. Mol Ecol 12(3):765–775