Empirical assessment of transgene flow from transgenic poplar plantation

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To assess the possible impact of transgenic poplar plantations on the ecosystem, we analyzed the frequency and distance of gene flow from a mature male transgenic Populus nigra carried Bacillus thuringiensis toxin gene (Bt-OE poplar) plantation, and the survival of Bt-OE poplar seeds as well. The resultant Bt-OE poplar seeds occurred at a frequency from ~0.15% at 0 m to ~0.02% at 500 m far away from the Bt-OE poplar. The Bt-OE poplar seeds weaken and even lost germinated activation within 3 weeks in the field (from 68% to 0%), compared to the 48% germination rate after 3 weeks in 4°C. The survival rate of seedlings in the field is 0% without any treatments, but increased to 1.7% under four treatments (Clean and trim, watering, weeding, and cover with plastic to keep moisture) together after seeded in the field for eight weeks. Results of this study indicate that gene flow originated from Bt-OE poplar plantation through pollen and seed could happen at a very low rate and in a limited range in natural conditions. This study provide the first-hand field data on the extent of transgene flow in poplar plantations, and offer guidance for risk assessment of transgenic poplar plantations.
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Abstract

To assess the possible impact of transgenic poplar plantations on the ecosystem, we analyzed the frequency and distance of gene flow from a mature male transgenic *Populus nigra* carried *Bacillus thuringiensis* toxin gene (Bt-OE poplar) plantation, and the survival of Bt-OE poplar seeds as well. The resultant Bt-OE poplar seeds occurred at a frequency from ~0.15% at 0 m to ~0.02% at 500 m far away from the Bt-OE poplar. The Bt-OE poplar seeds weaken and even lost germinated activation within 3 weeks in the field (from 68% to 0%), compared to the 48% germination rate after 3 weeks in 4°C. The survival rate of seedlings in the field is 0% without any treatments, but increased to 1.7% under four treatments (Clean and trim, watering, weeding, and cover with plastic to keep moisture) together after seeded in the field for eight weeks. Results of this study indicate that gene flow originated from Bt-OE poplar plantation through pollen and seed could happen at a very low rate and in a limited range in natural conditions. This study provide the first-hand field data on the extent of transgene flow in poplar plantations, and offer guidance for risk assessment of transgenic poplar plantations.

Keywords: Biosafety; Bt gene; Gene flow; Pollen dispersal; Transgenic poplar
Introduction

A major concern on the commercialization of transgenic forest trees is their gene flow, through which the transgenes may spread from the transgenic trees to nature forests. Although gene flow is a very common natural phenomenon and an important process of evolution, the movement of transgenes from genetic modified plants to populations of wild or relatives may be considered undesirable and lead to undesirable environmental consequences, such as more aggressive weeds, loss of germplasm, or increased non-target organisms and biodiversity losses.

Trees are long-lived, and their exposure to the environment occurs over a much longer time than annual crops (Neale and Ingvarsson, 2008; Niinemets, 2010). Poplar, as a fast-growing tree with 8-15 years in rotation, has been used for genetic transformation both for research and breeding (Bradshaw et al., 2000; Jansson and Douglas, 2007). Cross-pollination of poplars in plantation with their wild relatives is of major concern due to the broad compatibility within and among species even belonging to different sections of *Populus* (Eckenwalder, 1996). Several modeling and simulation methods have been proposed to measure the gene flow between poplar stands (Niggemann et al., 2012; Slavov et al., 2009) and co-dominant markers have been developed for monitoring gene flow among species even belonging to different sections (Khasa et al., 2005). However, no empirical data on transgenic poplar plantation is available to address this issue.

Poplar plays an important role in afforestation and timber supply worldwide. In China, for instance, about 7 million hm² poplar plantation has been established for shelterbelt and timber production (Fang, 2008). However, insects cause severe damages to the plantation and millions of US dollars loss is estimated annually. The infected trees are usually sprayed with pesticide or cut down in order to control the infestation, thus resulting in serious economic and ecological consequences (Chen et al., 2009). Genetic engineering provides a promising tool to breed superior poplar clones with improved tolerance to insects, drought, and wood properties. For example, insect-tolerance transgenic *Populus nigra* with *Bacillus thuringiensis* toxin (*Bt*) gene was planted for field test in 1994, and proved to be effective to avoidance of the leaf-insects damage (Hu et al.,...
To date, transgenic poplars are not only released in field trials but are planted as a commercial crop in plantation forestry (Hu et al., 2014). In order to develop poplars that were more tolerant to insect attack, *Populus nigra* were transformed with cry1Aa in 1993 and field-tested since 1994. The *Bt* poplars were first commercialized in 2001 and occupied 490 ha in China up to 2014. The transgenic poplar plantations have effectively inhibited the fast-spread of target insect pests and have significantly reduced the number of insecticide applications required (Hu et al., 2014).

In China, transgenic poplars are mainly planted in the northern areas, where the climate is dry or semidry with average precipitation from 100 to 600 mm concentrated in July and August. The poplar seed dispersal occurs during May to June thus misses the rain season favorable seed germination. Indeed, the successful establishment of a poplar plantation in these areas is much dependent on the quality of seedlings planted and the availability of irrigation. In addition, no natural regenerated poplar stands have been found and no natural regeneration of poplar plants has been observed in and around plantations. Taken together, it is perceived that transgenic poplar plantation will have a little chance to spread transgene into the nature forestry and this gives the rational to the National Forestry Administration to grant the permission for the commercialization of transgenic poplar clones in six provinces in China far away from natural poplar stands in 2002. However, so far no empirical data has been collected to test and support this hypothesis.

In this study, we used the mature *Bt*-OE poplar plantation in Manas Plain Forest Station, Xinjiang Uygur Autonomous Region, to access the possible gene flow from transgenic poplar plantation through pollens and seeds. We would like to address the following questions: (1) How far can the *Bt*-OE poplar pollen travel and successfully fertilize other poplar trees under the local climate? (2) In a population of the mixed non-transgenic and transgenic male poplars, are the seeds with *Bt* gene produced according to the ratio of the two types of males? (3) What is the probability of *Bt*-OE poplar seeds germinate and establish under different conditions? The answers to these questions would provide the basis for accessing the safety issue of the application of transgenic trees.
Results

Identification of Bt transgene by PCR amplification of Bt fragments from transgenic leaves, pollens and hybrid seeds

Seeds collected from non-transgenic CK3 (P. nigra) in the transgenic poplar plantation (TPP, Fig. 1) and P. nigra cv. ‘Pioneer’ trees (Fig. 1 and Fig. 2A) at four sites (No.4, No.10, No.13 and NE01, Fig. 1) were used for DNA extraction and amplification of Bt fragment with gene-specific primers. The high cross compatibility between Pioneer and Bt-OE poplars was revealed by pollinating female flowers (Fig. 2B) with pollens from transgenic male flowers (Fig. 2C) to produce fruits (Fig. 2D) and seeds (Fig. 2E). The PCR products of Bt fragments amplified from leaves of female Pioneer trees, the leaves and pollens of transgenic male tree and the leaves of their progenies are shown in Fig. 3A. The success in amplification of the Bt fragments from transgenic clones and their progenies indicated that the Bt gene still existed in the genome of transgenic poplars and could be transferred to its offspring by crossing. The amplified products of seeds collected from the Pioneer trees at the four sites were also performed to measure the probability of the transgene flow and some of them at No. 4 were provided in Fig. 3B.

Bt seeds produced with pollens from Bt poplar plantation

The collected seeds with the number from 3,045 to 6,190 for each female trees at the four sites (No. 4, No. 10, No. 13 and NE01, Fig. 1) in two adjacent years were used to amplify Bt fragments (Fig. 3B) and the frequency of Bt seeds occurred in the collected seeds were listed in Table 1. Bt seeds occurred at the rate of 0.16%/0.15% (in two years respectively) collected at the site No. 0 in the transgenic poplar plantation, but only 0.05%/0.07% at site No. 4, and 0.03%/0.02% at site No. 10. Although more collected seeds were tested, no Bt gene was detected in the seeds at sites No. 13 and NE01. No. 13 located at 794 m south-east TPP, while NE01 located at 368 m north-east. It indicated that the number of Bt-containing seeds detected was not only related to the distance from but also to the direction to the TPP.

Paternal assessment of the seeds collected from the sampled trees
To further analyze the potential pollens other than \textit{Bt-OE} poplars contributed to the seeds collected from the plantation, 77, 115 and 116 seeds from the sampled trees at sites No.1, No.4 and No.10 had been collected respectively for paternity analysis (Fig. 1). Seeds from the female tree at No.1, No.4, and No.10 dominantly pollinated by \textit{P. nigra} cv. ‘Russkii’ male trees with 92.21%, 57.39%, and 71.05%, respectively. This might be explained by that there were about 72 ‘Russkii’ trees distributed in the entire poplar plantation, accounting for 51% of the total male trees. In addition, 4.35% and 7.89% seeds from No.4 and No.10 were produced by unknown poplar males. These two sites were more open to the outside of the plantation, which could be pollinated by male poplar trees other than that in the plantation, while the female tree at site No.1 was a bit inside thus not easy to receive outside pollens.

**Germination ability and seedling survival of \textit{Bt-OE} poplar seeds under different conditions**

The germination rates of seeds after storage at 4ºC, room temperature and under field conditions were summarized in Table 2. After three weeks storage, the germination rate of \textit{Bt} seeds stored at room temperature and in the field decreased to 7% and 0%, respectively, while 48% of the germination rate was obtained for the seeds stored in 4ºC even after four weeks. It was determined that poplar seeds remain viable for only two weeks in nature (Imbert and Lefèvre, 2003). Therefore, there was no significant change of the viability of \textit{Bt-OE} poplar seeds. The higher germination rate of seeds stored at room temperature than that stored under field condition indicates that besides temperature, other factors such as exposure to sun light and air moisture may also affect the germination ability of seeds.

\textit{Bt-OE} poplar seeds were sowed in field conditions under different treatments (see materials and methods). No seedlings were found in the two plots without any treatments. For the six cleaned/trimmed, watered and plastic-film covered plots at the two sites, the rate of seedling establishment varied from 0.3 and 11.3% after one week but decreased to 0.2%~3.5% after two months (Table 3). There was only 0.3% of the germination rate of \textit{Bt-OE} poplar seeds at one plot due to water flow formed by raining two days after the seeding.
Discussion

The concern about transgene escape via pollen and seeds of transgenic trees is its possible negative effect on the natural forest ecological system, which has hurdled the application of transgenic trees (Haggman et al., 2013). It is thus important to access the pollen dispersal, the seed production and seedling survival of transgenic trees. Previous studies have used established stands and simulation models to explore the consequences of introducing new genes into the environment. In this study, we take the advantage of the commercialized insect-resistance transgenic poplar (Ewald et al., 2006) and for the first time, we have provided the empirical data to evaluate the possibility of gene flow through pollen and seeds.

In natural population, the possibility of introgression is especially high in poplars because reproductive barriers between species are weak. Meirmans et al. studied the rate of spontaneous hybridization from two poplar plantations into adjacent natural population of *P. deltoides* and *P. balsamifera*. The significantly high rate of hybridization in the natural population suggests that small peripheral populations carry a higher risk of introgression (Meirmans et al., 2010). Generally, biases in relative abundance can influence the direction of gene flow. Relative abundance may contribute to asymmetric introgression of *P. nigra* genes, as this exotic species is much less abundant and expected to contribute less to the pollen cloud than the native *P. balsamifera* in North America (Lepais et al., 2009; Thompson et al., 2010). These results indicate the access of gene flow is very important and influenced by many factors, which may lead to different genetic consequences of populations. Therefore, the transgene flow using transgenic trees in the field conditions needs to be studied.

Without commercialized transgenic tree plantations, it is not practical to assess transgene escape to conventional populations. The alternative would be to develop and exploit simulation models such as STEVE, AMELIE to gain insight into the gene flow in forest trees. Several models of pollen and seed dispersal have been proposed for forest trees (DiFazio, 2002; DiFazio et al., 2004; Kuparinen and Schurr, 2007; Nathan et al., 2008). For example, Kuparinen and Schurr (Kuparinen and Schurr, 2007) investigated the rate of transgenic escape in cases where the modified organism carries...
mitigation genes, and DiFazio (2002) used his model to predict transgenic escape assuming long
distance dispersal as a common phenomenon. Many results indicate that long-distance dispersal is
extensive and pollen dispersal curves are similar in populations with very different ecological and
demographic characteristics (Slavov et al., 2009). Indeed, Roe et al. compared and contrasted exotic
hybridization and introgression to the same processes occurring among native poplars in eastern
Canada. In this native hybrid poplar zone, gene flow was asymmetric, with exotic alleles
predominantly introgressing into *P. balsamifera*, while hybrid formation among natives occurs
primarily with the female *P. deltoides*. They also found that the majority of gene flow was
intraspecific (over 98%) (Roe et al., 2014a; Roe et al., 2014b). DiFazio et al. (2004) reported the mean
pollination distances for *P. trichocarpa* between 140-1,100 m, with a strong dependency on the
area sampled. Pospísková & Sálková (2006) found that the effective pollination distances were 10-
230 m within a *P. nigra* population along the Morava River. The study of Rathmacher et al. (2009)
showed that only a minor part of gene flow took place at distances beyond 1 km, and poplar seeds
generally had shorter dispersal distances with a maximum distance of 500 m. Bialozyt (2012)
reported that most of the effective pollinations (75%) occurred within a distance < 1,000 m
between native black poplar trees (*P. nigra*) and its commercial hybrid (*P. × canadensis*), and only
a very limited proportion of effective pollinations occurred at distances > 2 km in central Germany.
Therefore, there was a wide range in the distance of pollen dispersal accessed in poplar plantations
based on population studies and models. In our study, the results indicated that the pollen dispersal
of *Bt* poplars occurred within 500 m in distance, which is close to the minimum limit of the
predicted based on models or population analysis. Dispersal of *Bt* pollens is also affected by the
wind direction during flowering period in spring. Due to the prevailing wind direction from
northwest to south-east in the study site, it is reasonable that no *Bt* pollen pollinated seeds has been
detected in the north-east site (NE01), though the separation of about 200 meter poplar plantation
in wide between the TPP and the site NE01 may also limit the *Bt* pollens dispersal.

The formation of a hybrid plant does not mean a wild population will be established (Chandler
and Dunwell, 2008). Hypocotyls develop within 6–8 h after moisture has reached the seed and the
Pappus has degraded (Zsuffa, 1974), and the seedling dies if conditions are not favorable for further development. Germination occurs exclusively on bare soil (Barsoum and Hughes, 1998). The results present in this study reveal that the transgenic poplar seeds lose germination ability under field conditions after 3 weeks, but retained the ability almost unaffected at 4°C in long storage. Therefore, it is a crucial period of 3 weeks for germination of transgenic seeds in the field. When sowed at test sites without watering, no transgenic poplar seeds were germinated in the bare field but 3.5% of seedling survival rate was obtained with watering, weeding and tillage measures. According to the study by Guilloy-Froget et al. (2002), successful germination of *P. nigra* seeds depends on a change to hydrated conditions. Therefore, soil, water, weed could all affect the germination and survival of seeds, and water seems to be the most important limiting factor. The significance of seed dispersal could also be highly dependent on plantation size (DiFazio et al., 2012). In this study, the number of male transgenic poplar trees (23) was small, which might affect the fertilization of these pollens under the competition with non-transgenic pollens from 141 males. As a result, there were only 0.16% and 0.15% Bt seeds from the control (No. CK3) were detected in the transgenic poplar plantation in two years, respectively. The paternity analysis also support this suggestion that the non-transgenic male trees dominated the pollen pool would produce more non-Bt seeds thus the transgenic seeds would not be readily produced in such a situation.

In summary, our study provides evidence that the pollen from transgenic trees travels only at limited distance, and under the presence of large number of non-transgenic males, the probability for the Bt pollen to successfully produce Bt progeny is quite rare. In addition, the transgenic seeds could not easily germinate and the seedlings not easily survive in dry areas in northern China. Therefore, it could be concluded that the transgene may not easily flow to the local poplar forestry based on this empirical data.
Materials and methods

Plant materials and seeds collection

The experimental site is located in Manas Plain Forest Station (N44°15' 56", E86°19' 60"), Xinjiang Uygur Autonomous Region. The transgenic P. nigra plantation (TPP) was planted with 2-year cuttings in an agriculture land about 0.68 ha (Fig. 1) in 1994. The adjacent poplar plantations included P. alba plantation in the north (0.74 ha), P. × canadensis (a female clone) in the east (2.3 ha) and P. nigra cv. ‘Pioneer’ (P. nigra cv. ‘Italica’ × P. nigra, female) in the southeast (11.3 ha) and northeast (1.2 ha). The field in the south of the transgenic poplar plantation was used in agriculture. There were 23 Bt flowering transgenic male poplar trees, and 63 non-transgenic male trees (39 and 24 clonal trees of non-transgenic P. nigra and P. × euramericana cv. ‘Robusta’ respectively in TPP), while 72 and 6 clonal trees of P. nigra cv. ‘Russkii’ (P. nigra cv. ‘Italica’ × P. nigra) and P. × euramericana cv. ‘Leipzig’ respectively distributed in southeast Pioneer plantation, no other male trees were planted in the experimental site. The detail of each plantation was shown in Table S1.

High cross-ability between transgenic P. nigra and P. nigra cv. ‘Pioneer’ was observed in our pilot experiment (Fig. 2) and the flower-time of these two clones was also compatible in the experimental site. The sampling trees in Pioneer plantation for seed collection were located in both southern east (No.1, No.4, No.10 and No.13) and northern east (NE01). One female P. nigra tree control (CK3) in the TPP was also sampled. The plantation of P. × canadensis (female) was established in the same year (1994) with TPP. The sampling sites were chosen in the southern east plantation were due to the wind direction favored the pollen dispersal in this direction in northern China. The period of pollination of the male trees is 5-6 days starting from April 8, at the temperature ranging from -5 ~ 23°C, with the prevailing winds (0.2 ~ 10.7 m/s wind speed) from north-west.

The Seeds were collected from single trees at the above sites when the start of dehiscing capsules grouped in catkins in the spring of 2006 and 2007. Seeds were dried at room temperature
and the wool was removed by hand. Seeds were put inside envelopes in sealed plastic bag filled
with adequate silicon and then stored in coldness. Germination rates were tested in triplicates with
100 seeds each after storage for 1, 2, 3 and 4 weeks. Seeds were sowed on wet filter paper in Petri
dishes. Germination rates were determined at the fourth day after sowed. A seed was considered
to have germinated with the appearance of two healthy cotyledons. To access the germination of
seeds and the survival of the seedlings in natural fields, we sowed 1,000 seeds in total 16 plots
treated with or without cleaning/trimming, weeding, watering or plastic film covering at two sites
with 8 plots each, respectively.

Detection of *Bt* fragments by PCR

For detecting *Bt* seeds, the collected seeds were put on a wet filter paper in Petri dish and
germinated at room temperature. After 3 days germination, each 5 seedlings were pooled as one
sample for DNA extraction to reduce the lab work (An extremely low *Bt* detection rate were based
on our pre-experiments) using CTAB method. DNA concentration was estimated by comparing
the brightness of the band with standard markers. About 50 ng DNA were used to amplify the *Bt*
DNA fragment using gene-specific primer pairs (5′-GAA TTC GCT AGG AAC CAA GCC ATT-
3′ and 5′-AAG TAT ATC CAT CAA ATG TGG ACT-3′) and PCR was performed as described
previously (Wang et al., 1996). After 5 min at 94°C, the PCR reaction was carried out for 30 cycles
at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min. After a final incubation at 72°C for 5 min,
5μl PCR products were analyzed by electrophoresis in 1.5% agarose gel and visualized under UV
light after staining with EtBr solution. *Bt* seeds were used as controls to check the fidelity of PCR
amplification.

Paternity analysis

Four candidate paternal poplar clones (*P. nigra*, *P. × euramericana* cv. ‘Robusta’, *P. nigra*
cv. ‘Russkii’, *P. × euramericana* cv. ‘Leipzig’) were identified in the plantations (Fig. 1). To test
the paternal source of seeds of the open pollinated *P. nigra* cv. ‘Pioneer’, total 308 seeds (from
tree No. 1, 4 and 10) were analyzed at four SSR loci (wpms04, wpms14, wpms18 and wpms20),
which were selected for distinguish the 4 candidate males (Fig. S1). Primer sequences and PCR-profiles were same as described by van der Schoot et al. (2000) and Smulders et al. (2001). Fragment analyses were performed on QIAxcel System (QIAGEN, Germany). The Biocalculator software program was used to present the results as both simulated bands in gel images and peaks in electrophoregrams. The potential paternal parent of each seed was conferred by the appearance of the SSR patterns for each loci.

Acknowledgment

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Competing financial interests

The authors declare no competing financial interests.
Table 1. Summary of the proportion of seeds with *Bt* from sampled sites

<table>
<thead>
<tr>
<th>Sites</th>
<th>Distance to TPP</th>
<th>Numbers of tested seedlings</th>
<th>DNA samples</th>
<th>Bt seedling (%)</th>
<th>Numbers of tested seedlings</th>
<th>DNA samples</th>
<th>Bt seedling (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.0</td>
<td>0 m</td>
<td>3045</td>
<td>609</td>
<td>0.16%</td>
<td>6190</td>
<td>1238</td>
<td>0.15%</td>
</tr>
<tr>
<td>No.4</td>
<td>210 m</td>
<td>6180</td>
<td>1236</td>
<td>0.05%</td>
<td>4355</td>
<td>871</td>
<td>0.07%</td>
</tr>
<tr>
<td>No.10</td>
<td>500 m</td>
<td>6055</td>
<td>1211</td>
<td>0.03%</td>
<td>4315</td>
<td>863</td>
<td>0.02%</td>
</tr>
<tr>
<td>No.13</td>
<td>794 m</td>
<td>6060</td>
<td>1212</td>
<td>0%</td>
<td>4375</td>
<td>875</td>
<td>0%</td>
</tr>
<tr>
<td>NE01</td>
<td>368 m</td>
<td>6040</td>
<td>1208</td>
<td>0%</td>
<td>4735</td>
<td>947</td>
<td>0%</td>
</tr>
</tbody>
</table>

TPP, transgenic poplar plantation. After 3 days germination, each 5 seedlings were pooled as one sample for DNA extraction. The data was collected from two continues years (2006-2007).
Table 2. The average germination rate of Bt-OE poplar seeds after storage

<table>
<thead>
<tr>
<th>Condition</th>
<th>1 W</th>
<th>2 W</th>
<th>3 W</th>
<th>4 W</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C</td>
<td>68±1.58 a</td>
<td>67±1.34 a</td>
<td>60±1.22 a</td>
<td>48±1.06 a</td>
</tr>
<tr>
<td>Room temperature</td>
<td>65±1.69 a</td>
<td>30±0.87 b</td>
<td>7±0.18 b</td>
<td>0</td>
</tr>
<tr>
<td>Field</td>
<td>12±0.32 b</td>
<td>3±0.21 c</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

W: week; SD: Standard deviation. Different letter: significant at 0.05 among the three conditions in each week.
The average germination rate is 68±1.53 before treatment.
Table 3. Seedlings survival rate during 8 weeks in the field after sowing

<table>
<thead>
<tr>
<th>Group</th>
<th>Condition a</th>
<th>Survival rate b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 W</td>
</tr>
<tr>
<td>A</td>
<td>No action</td>
<td>0%</td>
</tr>
<tr>
<td>B</td>
<td>C&amp;T, Wa</td>
<td>3.0%</td>
</tr>
<tr>
<td>C</td>
<td>C&amp;T, Wa, We</td>
<td>6.6%</td>
</tr>
<tr>
<td>D</td>
<td>C&amp;T, Wa, We, C</td>
<td>8.8%</td>
</tr>
</tbody>
</table>

a: Four conditions (Group A-D) were differentially combined with four treatments: clean and trim (C&T) watering (Wa) weeding (We) and cover with plastic to keep moisture (C).

b: For each condition, total of 2,000 seeds were used for evaluate the survival rate during 8 weeks (W) after sowed in two independent area.
Figure Legends:

Fig. 1 Sketched map of the sites of transgenic poplar plantation (TPP) and the surrounding non-transgenic plantations.
There were 23 Bt flowering transgenic male poplar trees, and 63 non-transgenic male trees (39 non-transgenic *P. nigra* clonal trees, 24 *P. × euramericana* cv. ‘Robusta’ clonal trees) in TPP, while 72 *P. nigra* cv. ‘Russkii’ (*P. nigra* cv. ‘Italica’× *P. nigra*) clonal trees, 6 *P. × euramericana* cv. ‘Leipzig’ clonal trees distributed in southeast Pioneer plantation. The sampling trees in Pioneer plantation for seed collection were located in both southern east (No.0, No.4, No.10 and No.13) and northern east (NE01). The distances from sampling trees to TPP were shown in Table 1. Pies show the proportion of the candidate paternity for the collected seeds at three sites.

![Female, male flowers, hybrid fruits and seeds of *P. nigra* cv. ‘Pioneer’ and Bt *P. nigra.*](https://doi.org/10.7287/peerj.preprints.2335v1)

- (a) Pioneer Plantation.
- (b) Female flowers of Pioneer tree.
- (c) Male flowers of transgenic *P. nigra* (Cl. 222).
- (d) Fruits produced from Pioneer female branch crossed with pollens from Bt poplar.
- (e) Seeds of Pioneer pollinated with Bt poplar.
Fig. 3  Agarose gel electrophoresis of products amplified from non-transgenic, transgenic poplars (a) and partial seeds collected at No.4 site (b).

(a) Line 1, *Bt* plasmid as a positive control; line 2, leaves of *Bt* transgenic poplar; line 3, pollens of *Bt* poplar; line 4, leaves of non-transgenic poplar; lines 5-32, partial results of *Bt* detection of offsprings from controlled crosses between Pioneer poplar and *Bt* transgenic poplar. (b) Lines 24-47 and lines 72-94, partial results of *Bt* seed detection on site No.4; line 95 leaves of non-transgenic poplar; line 96, *Bt* plasmid as a positive control. Lines 35 and 91 are DNA samples showing positive *Bt* (arrow) in detection.
Supporting information

Additional Supporting information may be found in the online version of this article:

Fig. S1  The microsatellite patterns of female parent and four possible male parents (‘Russkii’, ‘Leipzig’, ‘Robusta’, or nigra, Fig. 1) using four SSRs (wpms04, wpms14, wpms18, and wpms20). The red asterisks indicate the corresponding amplicon.

Table S1  Basic information of each plantation. (docx)
References


