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# Insecticidal Activity and Transgene Expression Stability of Transgenic Hybrid Poplar Clone 741 Carrying two Insect-Resistant Genes

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**Abstract**

The insecticidal activity and the influence of transgenic hybrid poplar clone 741 carrying a Bacillus thuringensis gene (Bt*Cry1Ac*) and the arrowhead proteinase inhibitor gene (*API*) on the growth and development of different defoliating insects were tested during two years. The transgene expression stability during this period was documented.

Bioassays of transgenic poplar clone 741 on the larvae of Gypsy moth [*Lymantria dispar* (Linnaeus)], Scarce Chocolate-tip [*Clostera anachoreta* (Fabricius)] and other defoliating insects were carried out annually. The results showed that three transgenic subclones tested had a high resistance against these insects, but the insecticidal activity was different between years and with different insect species. Transgenic expression was generally stable during the 4 years. The insecticidal activity on the first larvae stage was obvious and decreased gradually with the development of the larvae (instar stage). Growth and development of the surviving larvae was seriously inhibited and delayed and in some cases could not even complete their development. Nevertheless feeding of transgenic poplar leaves could not kill the pests entirely. Proper planting strategies are necessary in order to prolong and optimize their resistance against the pests.

*Key words:* transgenic hybrid poplar clone 741, Bt*Cry1Ac* and *API* genes, insecticidal activity, transgenic expression stability, insect resistance.

## Introduction

Inserting foreign DNA and into plants expressing insect-resistance is a new way to accelerate plant breeding. The development of transgenic insect-resistant plants provides a quick and safe approach to pest management. Some articles reported that the transformation of insect-resistant genes into poplars (*Populus nigra, P. euramericana, P. tomentosa P. tremula* x *P. tremuloides* and *P. alba* x *P. grandidentata*), larch (*Larix gmelinii*), spruce (*Picea asperata*) and Formosan sweetgum (*Liquidambar formosana*) (YU, 2000; XIE, 1999; ZHU, 1997; WU *et al.,* 1999). MCCOWAN *et al.* (1991) obtained regenerated plants (*Populus alba* x *Populus grandidentata*) with resistance to Gypsy moth (*L. dispar* (L)) and Tent caterpillar (*Malacosoma neustria testacea* [Motschulsky]). TIAN *et al.* (1993) transferred the 35S-Ω-Bt-Nos gene into black poplar (*Populus nigra*) with the binary vector Agrobacterium tumefaciens LBA4404. WANG *et al.* (1997) transferred the 35S-Ω-Bt-Nos gene into hybrid poplar (*Populus euramericana*) mediated by a binary vector

and the *Agrobacterium tumefaciens* transfer system. There are many reports on transgenic poplar, such as KLOPFENSTEIN *et al.* (1991, 1993), KARL and DAVID (1995), HOWE and GOLDFARB (1994), LEPLE and BRASILERIO (1992), CONFALONIERI *et al.* (1998), HAO *et al.* (1999, 2000) and RAO *et al.* (2000). Regenerated plants with insect-resistance were obtained in all of these studies. The effectiveness of insect-resistance in transgenic plants is related to the side effects of gene transfer methods (site of gene insertion, copy number, gene silencing etc.). Thus molecular biological analysis of transgenic plants by PCR, Southern blotting and Western blotting has to be supported by long-term bioassays. This is a very important precondition for a possible commercialization of transgenic plants. In most reports of insect-resistant plants, bioassays confirmed the biological activity and the toxicity after transfer of plants into the soil for only a short time (TIAN *et al.,* 1993; WANG et al., 1997; KLOPFENSTEIN *et al.,* 1991). Using transgenic trees it is now possible to extend these observations over a long period of time (several years, or even half of the rotation length).

In the past 10 years, the transformation vector of modified *Bacillus thuringensis* (Bt) and arrowhead proteinase inhibitor (*API*) genes [*API* gene came from the tuber of the arrowhead (*Sagittaria sagittifolia var.sinensis*) which is one kind of vegetable planted in the southern China. The *API* gene can restrain the insect growth and finally kill the insects, provided that it restrained some assimilation enzyme in the insect intestine and induced an anorexia response.] A plasmid was constructed containing the two genes, and successfully transferred into hybrid poplar clone 741 in our laboratory. Over 50 subclones of transgenic plants were regenerated and three subclones were demonstrated to be highly resistant to the insects tested (subclones No.s 29, 3 and 11). This paper reports the results of bioassay studies of transgenic hybrid poplar clone 741 subclones carrying two insect-resistant genes over four years. These results provide additional proof for the possible application of transgenic poplar.

## Materials and Methods

*Plant material*

The poplar clone 741 [*P. alba* x (*P. davidiana* + *P. simonii*)] x *P. tomentosa*] was used for transformation receptor, because of its elite characteristics and the inability to form viable seeds (JIANG *et al.,* 1991). Different transgenic subclones were used for the tests (subclone No. 1, 3, 11, 12, 17 and 29) which were chosen according to their effectiveness in the early tests from a set of at least 50 transgenic subclones. Non-transformed plants of poplar clone 741 were used as a control.

The transgenic plants were regenerated and planted in a nursery trial in spacing 50 cm x 30 cm in 1997. Plants were cut back every autumn because the area for each plant was restricted. The leaves from freshly sprouted shoots were harvested every year.

*Partial modification of Bt Cry1Ac gene and subcloning of API gene*

According to the restriction sites in Cry1Ac gene, *Bam*H *I-EcoR I* fragment containing coding region 1-273 bp was subcloned into the same sites of pBluescript SK+ and modified by PCR method. Nine modification primers were used to modify the region of 1632-1836 bp of *Cry1Ac* gene as described previously (TIAN *et al.,* 1995). The modified DNA sequence was verified by sequence analysis and reassembled into the original Bt*Cry1Ac* gene to form the recombinant plasmid pSKBtpm. Conventional recombinant DNA techniques were used for PCR amplification and subcloning of *API* gene (XU *et al.,* 1993).

*Construction of expression vector containing two insect-resistant genes*

Restriction enzyme digestion of Bt*Cry1Ac* and *API* gene fragments, ligation with binary vector, transforming of *E. coli* and selection of the recombinant plasmid were performed as described by AUSUBEL (1995).

*Transformation of hybrid poplar clone 741 leaves*

Leaves from test-tube grown poplar plantlets were used as explants and transformation was carried out using *Agrobacterium tumefaciens* harboring the expressing vector pBtiA containing the genes Bt*Cry1Ac* and *API*. The MS medium used for shoot induction contains 1.0 mg/L 6-BA, 0.1 mg/L NAA and 50 mg/L kanamycin, while the root induction medium contains 50 mg/L kanamycin, 1.0 mg/L IBA and 1/2 MS salts. The tubes grown plantlets were cultivated at 25°C temperature, 1500-2000 lx intensity of illumination and 12h light /dark photo period.

*Molecular biological analysis of transformed poplar plants*

Transgenic plants were selected by regeneration on a kanamycin-containing medium. PCR detection and DNA Southern bloting analysis were performed. Twenty ug of poplar genomic DNA was digested overnight with *Hind*III and used for Southern bloting analysis. $^{32}$P-labled Bt*Cry1Ac* gene fragment (1.8kb) and *API* gene (0.6kb) fragment were used as probes respectively.

The results of the transformation and molecular biological analysis of transformed hybrid poplar clone 741 have been described by TIAN *et al.* (2000) and ZHENG *et al.* (2000).

*Bioassays in vitro (laboratory test of insect feeding): Insects tested*

The following insects were used to test the insect-resistance of transgenic poplar clone 741:

a) insect species with large larvae
Gypsy moth (*Lymantria dispar* (L)),
Scarce Chocolate-tip (*Clostera anachoreta* (Fabricius)),
b) insect species with small larvae
Fall webworm (*Hyphantria cunea* (Drury)),
*Micromelalopha troglodyta* (Graeser),
Vapourer moth (*Orgyia antiqua* (L)).

Fresh egg blocks of *C. anachoreta* were collected from the experimental field of the forestry college, Agriculture University of Hebei and artificially incubated in lab; those of *Hyphantria cunea* and *L. dispar* were from Qin Huangdao city and the larvae of *Micromelaopha troglodyta* and *Orgyia antiqua* were from the nursery of Shenzhou city.

The egg blocks were cultivated on fresh leaves of untransformed control plants of poplar clone 741 at room temperature in the laboratory. Larvae (caterpillars) in different developmental stages (instar larvae) were collected and used for testing.

The different instar insects were characterized as follows:

Instar larvae I: larvae were normally used 20 hours after appearing from the egg block depending on the species. Other larval stages (II-V) were depending on the insect species and characterized by their stages of moulting.

*Test method*

Jars, 12 cm high with a bunghole of 6 cm wide, were used for testing. The strong first-instar larvae (incubated less than 20h on leaves of control plants) were selected and transferred slightly into the jars. Up to 50 insects per leaf, depending on the size of instar larvae I, were used in the beginning of the

experiment. Later, the larger instar larvae were separated depending on their size. Detached leaves of different transgenic clones and control plants were tested. Every repeated trial had at least 10 (maximum of 30) larvae for insect species with larger larvae (Gypsy moth and Scarce Chocolate-tip) and every experiment was repeated three times. The jars were sealed with white cloth to provide enough air and proper humidity for the growth of larvae and to prevent the larvae from escaping. Fresh leaves were provided every two days. The jars were incubated at 23 °C. The larvae mortality rate was determined every day until the full development of the insects. The overall larval mortality rate was recorded at the end of experiment. In some cases weight of larvae was measured at the end of experiment.

## Results

*Insecticidal activity of transgenic hybrid poplar 741 on different defoliating insects*

The average larval mortality of some main defoliating insects on the leaves of transgenic poplar was compared with leaves of the non-transformed control over three years (*Table 1*). The results showed that all the transgenic subclones exhibited a high resistance against the tested insects. The mortality of *M. troglodyta* on transgenic plants was the highest. After 6 days, nearly all the larvae of *M. troglodyta*, an insect with smaller larvae, were dead, while the mortality of *L. dispar*, characterized by larger instar larvae, was lower. There was a significant difference among the effects of different subclones. Subclones Nos. 3, 11 and 29 showed higher insecticidal activity to all tested insects and the larvae mortality was over 70%. While subclone Nos. 1, 12 and 17 showed higher insecticidal activities against certain insects, such as *M. troglodyta* with a larval mortality of 100%. Under identical conditions the mortality of insects fed with the non-transformed plant leaves was very low.

*The transgene expression stability of transgenic hybrid poplar clone 741*

The results of a four-year test with the insects characterized by large larvae, (*C. anachoreta* and *L. dispar*), and the most resistant transgenic subclones (Nos. 3, 11 and 29) were listed in *Table 2*. The insecticidal effect of these subclones to these insects exhibited different results. The average mortality of *C. anachoreta* varied from 76.7 to 99.9% and the average mortality of *L. dispar* varied from 60.0 to 94.5%. The insecticidal effect of transgenic subclones No. 29 and 11 to *C. anachoreta* was lower in 1999, while the effects of subclone No. 29 to *L. dispar* was lower in 1999 and 2000. Nevertheless the insecticidal effect of each subclone showed no tendency of a decrease in insect resistance in general.

*The insecticidal effect of the transgenic leaves on insect larvae in different instar stages*

The influence of transgenic leaves on the development of insect larvae was tested in two ways. One way was the use of instar larvae I, following the development either until the end of the insect development or the death of the larvae (*Table 3*). The other way which was tested used equal numbers of different instar larvae (stage I–V) from control leaves and their transfer to transgenic leaves thereafter. The results in *Table 3* show that the insecticidal effect was more obvious in the earlier larval stages. The insecticidal effects to *C. anachoreta* and *L.*

*Table 1.* – Average total mortality (%) with their standard errors of different insect species after feeding on detached leaves (*in vitro*) of transgenic subclones of hybrid poplar clone 741 over a period of 3 years (1998-2000).

| Transgenic subclone | *C. anachoreta* (Fabricius) | *L. dispar* (Linnaeus) | *H. cunea* (Drury) | *M. troglodyta* (Graeser) | *O. antiqua* (Linnaeus) |
|---|---|---|---|---|---|
| Control | 3.7±2.2$^{c}$ | 4.7±4.2$^{d}$ | 14.5±4.3$^{c}$ | 8.4±8.4$^{b}$ | 0 |
| No.1 | 75.0±8.3$^{b}$ | 61.7±11.7$^{bc}$ | --- | 100±0$^{a}$ | --- |
| No.3 | 89.1±3.2$^{a}$ | 92.1±0.4$^{a}$ | --- | --- | --- |
| No.11 | 88.1±9.9$^{a}$ | 78.2±7.7$^{b}$ | 92.3±4.2$^{a}$ | 100±0$^{a}$ | 100±0 |
| No.12 | 75.0±21.7$^{b}$ | 50.0±23.1$^{c}$ | 71.7±18.4$^{b}$ | --- | --- |
| No.17 | 70.0±0$^{b}$ | 67.6±6.6$^{bc}$ | 28.1±6.2$^{c}$ | 100±0$^{a}$ | --- |
| No.29 | 91.6±7.9$^{a}$ | 69.8±12.8$^{b}$ | 100±0$^{a}$ | 99.5±0.6$^{a}$ | ---- |

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

*Table 2.* – Variation in insect-resistance of three transgenic subclones expressed as average mortality (%) with their standard errors in different years.

| Insect Species | test date | Transgenic Subclone | | | |
|---|---|---|---|---|---|
| | | No.3 | No.11 | No.29 | Control |
| *C. anachoreta* | Aug.1-Aug.20,1998 | 89.6±5.2$^{ns}$ | 94.3 ± 5.8$^{a}$ | 90.8 ± 2.3$^{b}$ | 1.7 ± 1.7$^{ns}$ |
| | Jul.25-Aug.25,1999 | 92.0±4.8$^{ns}$ | 76.7 ± 13.4$^{b}$ | 84.2 ± 3.0$^{c}$ | 3.3 ± 2.7$^{ns}$ |
| | Aug.15-Aug.25,2000 | 85.7 ± 5.0$^{ns}$ | 93.4 ± 6.7$^{a}$ | 99.9 ± 0.2$^{a}$ | 6.1 ± 5.6$^{ns}$ |
| | Aug.1-Aug20,2001 | 98.8±3.2$^{ns}$ | 88.7 ±5.0$^{ab}$ | 99.6±4.2$^{a}$ | 0.0±0.0$^{ns}$ |
| *L. dispar* | May.10-Jun.20,1998 | 91.8±11.7$^{ns}$ | 77.8±5.9$^{ab}$ | 84.4 ± 1.6$^{b}$ | 8.1 ± 11.5$^{ns}$ |
| | Apr.30-May.30,1999 | 92.5±5.0$^{ns}$ | 86.0 ± 4.1$^{a}$ | 60.6 ± 7.6$^{c}$ | 0.0 ± 0.0$^{ns}$ |
| | Apr.30-May.30,2000 | 92.0 ± 8.1$^{ns}$ | 70.7 ± 7.4$^{b}$ | 64.4 ± 5.6$^{c}$ | 6.0 ± 6.0$^{ns}$ |
| | Apr.10-May.30,2002 | 94.0 ± 0$^{ns}$ | 85.5 ± 2.5$^{a}$ | 94.5 ± 0.5$^{a}$ | 2.7 ± 0.6$^{ns}$ |

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

*Table 3.* – The insecticidal effect of three transgenic poplar subclone leaves on the development of the different instar larvae of *C. anachoreta* and *L. dispar* (beginning with instar larvae I) in different years.

| Insect Species | Transgenic subclone | Tested year | Instar of insect (% mortality) | | | | |
|---|---|---|---|---|---|---|---|
| | | | I | II | III | IV | Uncertain instar |
| *C. anachoreta* | No.3 | 2000 | 75.0 | 14.5 | 0.8 | - | - |
| | | 2001 | 98.0 | 1.6 | 0.2 | - | - |
| | No.11 | 2000 | 72.9 | 13.3 | 2.1 | - | - |
| | | 2001 | 69.0 | 7.8 | 1.0 | -- | - |
| | No.29 | 2000 | 70.5 | 15.4 | 0.8 | - | - |
| | | 2001 | 95.0 | 4.3 | 0.3 | - | - |
| *L. dispar* | No.3 | 2002 | 52.5 | 10.5 | 12.5 | 5.5 | 12.5 |
| | No.11 | 2000 | 52.4 | 27.6 | 8.0 | 0.5 | - |
| | | 2002 | 60.0 | 8.5 | 6.5 | 0.5 | 10.0 |
| | No.29 | 2000 | 71.2 | 7.2 | -- | - | - |
| | | 2002 | 56.0 | 11.5 | 10.5 | 6.5 | 9.0 |
| Control | | 2002 | 2.73 | 0 | 0 | 0 | - |

*Table 4.* – The average mortality (%) with their standard errors of different instar larvae of *C. anachoreta* fed on two different transgenic poplar subclone leaves (equal numbers of instar larvae were used of every stage [I – V]).

| Instar | Control | Subclone No.29 | Subclone No.3 |
|---|---|---|---|
| I | 0.0 | 95.0±3.0$^{a}$ | 98.5±1.0$^{a}$ |
| II | 0.0 | 90.5±4.8$^{a}$ | 86.2±4.5$^{b}$ |
| III | 0.0 | 64.3±2.2$^{b}$ | 65.3±5.2$^{c}$ |
| IV | 0.0 | 35.2±6.5$^{c}$ | 35.4±2.0$^{d}$ |
| V | 0.0 | 21.6±3.5$^{d}$ | 18.5±2.5$^{e}$ |

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

*dispar* were significant in first-instar (stage I) larvae. Different subclones had the same tendency in different years. *Table 4* shows the results of insecticidal effect of transgenic poplar leaves on larvae at different instar stages which were originally fed on normal leaves. Results show that as the insect larvae *C. anachoreta* grew older, the effect of the toxoprotein of the transferred genes became less. The mortality decreased in the third-instar and was only about 20% in the 5th instar insect. Thus the sensitivity of insect larvae is directly related to the development of the larvae.

*The effect of transgenic poplar on the speed of larvae development*

The growth of surviving larvae was seriously inhibited fed on the transgenic poplar (*Table 5*, *Figures 1* and *2*). The duration of development of larvae was prolonged compared with the control. Larvae of *C. anachoreta* needed over 20 days to finish the whole development on the transgenic plant leaves while the larvae needed only 13 days on the control plant leaves to complete development. The development of larvae (*L. dispar*) lasted 47.6 days on average on leaves of subclone No. 29 and 46.6 days on leaves of subclone No. 11, while the development of the larvae on control leaves required 35 days.

The feeding of transgenic poplar leaves had obvious impacts on larval weight compared with that of control plant. The weight of one individual of *C. anachoreta* fed on transgenic plant leaves was 162 to 185 mg, which was nearly 1/3 of the control (468 mg) at 14th day. The difference between the weight of one individual of L. dispar fed on transgenic plant leaves and control was not significant during the first 25 days. After day 25 the weight of control insects showed an exponential growth. At day 36 the weight of control was 579 mg while the weight of insects fed on transgenic plant leaves was 43 to 102 mg. There were no significant differences among the transgenic subclones tested.

*Table 5.* – The duration of development of every instar of *C. anachoreta* fed on the leaves of three different transgenic subclones or non-transformed plants (in days).

| Development duration | 2000 | | | 2001 | | | |
|---|---|---|---|---|---|---|---|
| | No.29 | No.3 | Control | No.29 | No.11 | No.3 | Control |
| I-II | 8.0 | 9.0 | 3.0 | 4.9 | 5.5 | 5.8 | 2.8 |
| II-III | 6.0 | 5.0 | 4.0 | 5.1 | 4.7 | 7.2 | 3.1 |
| III-IV | 4.0 | 4.0 | 3.0 | 5.6 | 4.8 | 4.6 | 2.7 |
| IV-V | 3.0 | 4.0 | 3.0 | 5.4 | 4.5 | 5.2 | 3.7 |
| average (in total) | 21.0 | 22.0 | 13.0 | 21.0 | 19.5 | 23.0 | 12.3 |

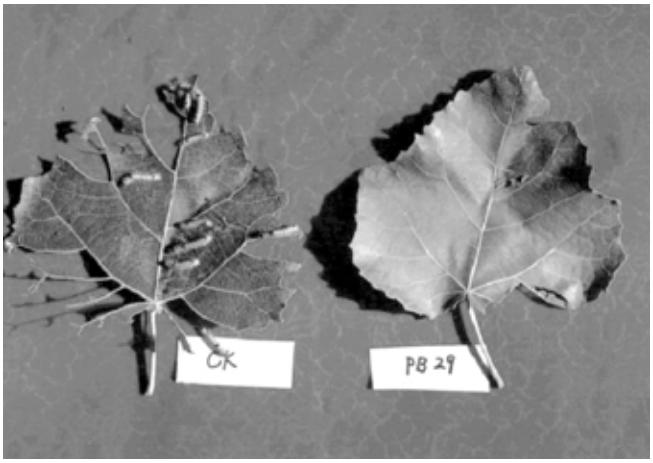


*Figure 1.* – Damages on a single leaf of feeding *C. anachoreta* larvae after 1 day (left untransformed control, right transgenic subclone No. 29).

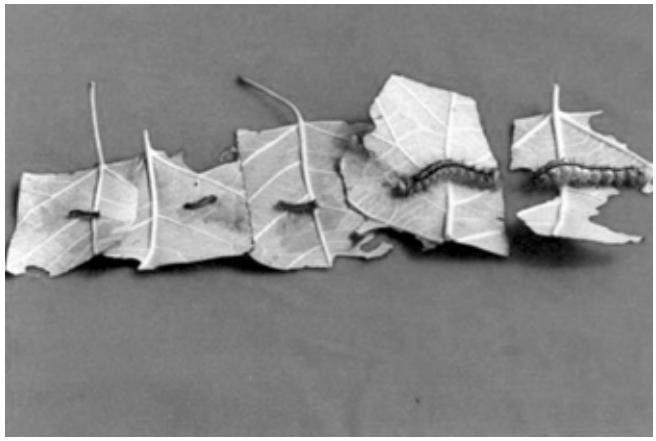

*Figure 2.* – The size of living larvae of *L. dispar* feeding 42 days (from left to right, transgenic subclones Nos. 3, 11, 29 and 15 [not very efficient and therefore not mentioned in the paper], and untransformed control).

## Discussion

Great progress has been made in genetic transformation of poplar. At the beginning of these experiments only a single gene could be transferred. The regenerated plants expressed various degrees of insect-resistance. For example LEPLE and BRASILERIO (1992) transferred the genes of serine protease inhibitor, sulfhedryl protease inhibitor and Bt *Cry III(A)* into two poplar clones and the genes resulted in increased insect-resistance. In 1995 the cysteine gene (OCI) was transferred into *Populus tremula* x *P. tremuloides* and after a 25-day insect feeding bioassay, the mortality of the larvae of *Chrysomela tremulae* was 43.5% while the larval mortality on the control leaves was only 4.5% (LEPLE *et al.,* 1995). TIAN et al. (1993) transferred the 35S-Bt-Nos gene mediated by the binary vector *Agrobacterium tumefaciens* LBA4404 into *Populus nigra* and the mortality of the *Apocheima cinerarius* Ersch. was 80 to 96% within 5 to 9 days. WU and FAN (1991) transferred the Bt toxin gene into *Populus nigra* and the insert of the foreign gene was proved by the Southern blotting. CONFALONIERI *et al.* (1998) reported that they transferred the proteinase inhibitor gene of soybean into *Populus nigra* but the insecticidal effect was not very high. We constructed a vector containing Bt*CryIAc* and *API* genes and transferred this vector into the poplar hybrid clone 741. The regenerated plants were determined by PCR, Southern blotting and an insect bioassay. Different subclones had different insect-resistance levels. Subclones Nos. 3, 11 and 29 showed higher resistance against all the tested insects with an average mortality of over 70%, while Nos. 1, 12 and 17 showed higher resistance against a certain specific insects. Therefore it can be assumed that both the insertion sites of foreign genes in different transgenic subclones and the expression quantity of the toxic protein were different. In the insect feeding selection, only 5 to 10% of transgenic clones had shown insecticidal effect.

There are two main questions in transgenic application: one is transgenic integrations and gene regulation; the other is gene silencing (WANG *et al.,* 2000; SU *et al.,* 1999). Using the poplar genetic transformation system HAN (1997) studied the factors that influence the foreign gene expression and discovered that it would improve the expression efficiency when both sides of the foreign genes have matrix attachment regions

(MAR). It proved that the insertions with the MAR has a greater effect on the foreign gene expression. The insecticidal effect of the transgenic hybrid poplar clone 741 with insect-resistance observed in our laboratory was different in the different years (*Table 2*), which might be affected by different climatic conditions, different laboratory environmental conditions, different times and nutritional state of the insects in the different years. In 4 years an obvious decreasing trend (e.g. inactivation of expression – gene silencing effects) was not observed. It suggested that the insecticidal effect was based on the expression of both genes was stable and the insects did not show any adaptation to the transgenic poplar within four years. One possible reason is that the transgenic poplar carried two genes (Bt*Cry1Ac* and *API*). Many transgenic plants reported in earlier studies were carrying only one gene, which can be overcome after a short term. Transgenic plants carrying two or more different genes can improve the insecticidal effect and prevent the insects from developing resistance against the transgenic plants because the receptors of different insecticidal proteins and the insecticidal mechanism are different.

The transgenic plants had many effects on the growth of the insects, such as the reduction of larvae weight, delay of insect development and the inability to complete development and pupate. According to some publications, Bt gene has strong impacts on the development, reproduction and nutrition of some insects. The results of other authors showed also that development of larvae was prolonged, the weight of larvae and pupae decreased, moulting rate, pupating rate, number of egg decreased and many of the larvae were dead (YU, 2000). The proteinase inhibitor can also inhibit the growth of larvae obviously and delay pupation and moulting (YU, 2000; WANG and QIN, 1999). The transgenic hybrid poplar clone 741 carrying both Bt*Cry1Ac* and *API* gene showed not only an insecticidal effect but also the inhibition of the insect growth and development. The application of transgenic plants can result in insecticidal effects but a certain variation is also obvious. Besides environment safety problems of transgenic poplar, another important question is how the transgenic poplar can prevent the pest from developing a resistance to it. Many scholars believed that the pests' resistance evolution should be controlled. In order to reduce the resistance evolution, we should develop some strategies in deployment to overcome the problem, such as 1) the mixing of various kinds of transgenic plants of different species, 2) the mixing of transgenic plants with different genes, and 3) the mixing of transgenic plants and ordinary plants (LIU *et al.,* 1998). The success of such strategies in China has to be investigated in the future according to the existing biosafety regulations (JIA and PENG, 2002).

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