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RESEARCH ARTICLE

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Insect-resistant mechanism of transgenic triploid of Chinese white poplar

Abstract The activities of antidotal enzymes and digestive enzymes of *Clostera anachoreta* (Fabricius) instar larvae, feeding on leaves of three kinds of insect-resistant clones of transgenic triploid of Chinese white poplar, after 4, 12, 24, 48, 72 and 96 h, were investigated. The results showed that, feeding on clone 7, the activity of esterase, carboxylesterase, and mixed-function oxidases in the midgut of the larvae was very much decreased. Feeding on clone 10, those results were less than those of clone 7 and there were few changes on the larvae, which fed on clone 26. The changes of the amylase in the midgut of larvae were the same as those described above. However, the activities of glutathione S-transferase and proteinase were complex, increased markedly after 24 h feeding on clone 7, and then declined rapidly. The same changes were taking place on the larvae feeding on clone 10. There were many slight changes in glutathione S-transferase of the larvae, feeding on clone 26; no changes occurred in the proteinases of the midgut. Thus, the antidotal enzymes and digestive enzymes in the midgut of the larvae were inhibited. This may be the main mechanism of the transgenic triploid of Chinese white poplar.

Keywords transgenic triploid of Chinese white poplar, *Clostera anachoreta* (Fabricius), antidotal enzymes, digestive enzymes

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1 Introduction

In the 1990s, transgenic anti-insect plants were obtained by introducing the *Bt* (*Bacillus thuringiensis*) gene into abele × large-toothed aspen (first reported by Mc.Coun). This initiated the research of transgenic poplar in the world. Since then, many studies have been conducted on the European white berry with the *Bt* gene, American black poplar, European poplar, and the transgenic 741 poplar, and others (Tian et al., 1993; Chen et al., 1995; Chen et al., 1995). Recently, the *BtCry1ac* gene and the *API* gene were transferred into the triploid Chinese white poplar and a series of transgenic anti-insect plants were obtained in the Laboratory of the Bio-technical College of Forestry, Agricultural University of Hebei (AUH). Studies on the insect toxicology indicated that enzyme activity in the insect, especially the enzyme related to detoxification, changed after the insecticide had been absorbed. Detoxification enzymes in the insect include esterase, carboxylesterase, mixed-function oxidase (MFO), and glutathione S-transferase (GSTs). These enzymes can affect the outer-substance directly. No corresponding research on the anti-insect mechanism of the eaten transgenic plant leaves has been reported yet. Our research focused on enzyme activity variation within the insect caused by the transgenic poplar, with the aim to explain the anti-insect mechanism of the transgenic plant and to find a base for suitable application.

2 Materials and methods

2.1 Materials

2.1.1 Preparation of insect

Clostera anachoreta (Fabricius) instar larvae were caught in Raoyang County, Hengshui City and fed in wide-mouthed preserve jars. The temperature was between 26 and 28°C, relative humidity between 75% and 80% and illumination was 14 h/day. Before taken out for testing, the larvae were

fed on the transgenic triploid of Chinese white poplar for 4, 12, 24, 48, 72 and 96 h, separately.

2.1.2 Preparation of materials

The leaves with double transgenic insect-resistant genes (*Populus tomentosa* × *P. bolleana*) × *P. tomentosa* (transgenic poplar for short in following text) were provided by the Laboratory of Forestry Bio-technical, College of Forestry, Agricultural University of Hebei. The ordinary triploid of Chinese white poplar is regarded as CK. Clones 7, 10, and 26 were, respectively, highly, moderately and low insect-resistant transgenic poplar clones.

2.2 Methods

2.2.1 Insect midgut antidotal enzyme determination

1) Esterase

Carboxylesterase activity determination refers to the Asperen (1962) and Chen (1978) methods. Ten of the fourth to fifth instar larvae of *C. anachoreta* were taken with their head and the rump cut off. The bodies of larvae were homogenized at 4°C in a 0.04 mol/L phosphate (pH=7.0) buffer and the homogenate was centrifuged at 10,000 r/min for 10 min. The supernatant fluid was stored for the experiments. The assay used 0.1 mL of supernatant fluid (diluted 10 times), 0.5 mL of 0.04 mol/L pH=7.0 buffer, and 5.0 mL of 0.003 mol/L α-naphthyl acetate (contained 10^{-6} mol/L physostigmine when assay carboxylesterase). After incubation for 30 min at 30℃, we added 1.0 mL of Coomassie brilliant blue to the reaction; the solution was placed for 30 min before measuring absorbency at $OD_{600\ nm}$. The specification curve was made by α-naphthol, the amount of supernatant fluid was indicated on the X-axis, and the number of absorbency on the Y-axis. We then counted the α-naphthol of supernatant fluid per mL and measured carboxylesterase activity. A buffer replaced the supernatant fluid CK.

2) Glutathione s-transferase (GSTs) assay

Referred to as Oppenoorth's (1979) method, the reaction liquid was homogenized at 4℃ in a 0.06 mol/L phosphate (pH=7.0) buffer (contained 2 mmol/L EDTA). Other specifications were as described above. The assay used 3 mL of diluted supernatant fluid, 20 μL of 0.2 mol/L CDNB and 100 μL of 0.4 mol/L GSH. After incubation for 20 min at 25℃, a spectrum absorbency test was made at $OD_{340\ nm}$.

3) Mixed-function oxidase

Referred to as Hansan and Hodgson's method (Hansan and Hodgson, 1971), the reaction liquid was homogenized at 4℃ in a 0.2 mol/L phosphate (pH=7.8) buffer (contained 2 mmol/L EDTA). Again, the other specifications are as mentioned above. The assay used 1.0 mL of diluted supernatant fluid, 2.5 mg of NADPH, and 10 μL of a 0.1mol/L NTAN buffer (incubated 5 min). After shaking for 30 min at 34℃, the reaction was stopped by the addition of 1.0 mL of 0.1 mol/L NaOH. The solution of this reaction was extracted by 5 mL of aether and then the solution of aether was surged by 3 mL of 0.5 mol/L NaOH before measuring the absorbency NaOH solution at $OD_{410\ nm}$. CK did not contain NADPH.

2.2.2 Insect midgut digestive enzymes measurement

Twenty larvae of *C. anachoreta* were collected and dissected. The midguts and their contents were homogenized at 4℃ in 15 mL of 0.15 mol/L NaOH and the homogenate was centrifuged at 11,200 r/min for 15 min. The supernatant fluid was stored for the assay (Zhou et al., 2001).

1) Proteinase measurement. Referred to as Folin-phenol's method (Guo and Zheng, 1996), the assay used 1 mL of supernatant fluid (warmed up to 40°C) and 1 mL of 2% warmed casein. After incubation for 10 min at 40°C, the reaction was stopped by adding 2 mL of 10% trichloroacetic (TDA). The solution was placed for 10 min before centrifuging at 4℃ 11,200 r/min for 15 min. One mL of supernatant fluid, 5 mL of 0.4 mol/L Na_2CO_3, and 1 mL of Folin-phenol were added. The solution was placed on 40°C for 15 min before measuring absorbency at $OD_{680\ nm}$. When CK was assayed, terichloroacetic was added first, followed by zymolyte and supernatant fluid. The specification curve was made by tyrosine.

2) Amylase determination. Referred to as the dinitrosalicylic method (Northwest Agricultural University, 1986; Guo and Zheng, 1996), the assay used 1 mL of supernatant fluid and 1 mL of citric acid buffer (pH=5.6). After incubation for 15 min at 40℃, 2 mL of a 1% warmed starch solution was added and incubated for 5 min at 40°C. Then 4 mL of 0.4 mol/L NaOH was added (CK was added before warming up). Then, 2 mL of solution was taken out, 2 mL of 3,5-dinitrosalicylic acid was added, foiled boiled for 5 min, and diluted with distilled water in a final volume of 25 mL before measuring absorbency at $OD_{520\ nm}$. The specification curve was made by maltose.

2.2.3 Determination of contents of insect's enzyme

Referred to as Coomassie brilliant blue G-250 method, bovine serum albumin (BSA) was used as the standard protein. The activity of protein was expressed per gram of protein (Chen, 1993; Wang, 1998).

3 Results and analysis

3.1 Esterase, carboxylesterase activity variation in the midgut

3.1.1 Activity variation of the esterase

Table 1 shows that after 96 h, in comparison with CK, the specific activities of the esterase in the larva fed on highly, moderately, and low-resistant plants were 36.5%, 59.7%, and 78.9% respectively. The activity decreased over time.

3.1.2 Activity variation of the carboxylesterase

Table 2 shows that the carboxylesterase-specific activity also decreased correspondingly to 47.2%, 59.6%, and 80.0% of the CK fed on highly, moderately and low-resistant plants respectively.

Table 1 Effect of insect-resistant clones of transgenic triploid of Chinese white poplar on the activity of esterase in the midgut of *C. anachoreta* instar larvae (mmol·mg^{-1}·min^{-1})

Time /h	Transgenic poplar			
	CK	Clone 7	Clone 10	Clone 26
4	0.155,2	0.150,3	0.151,9	0.154,6
12	0.158,7	0.142,3	0.143,5	0.152,3
24	0.164,2	0.131,2	0.139,1	0.149,7
48	0.165,7	0.110,5	0.120,6	0.142,1
72	0.176,8	0.089,1	0.110,6	0.142,3
96	0.179,8	0.065,6	0.107,3	0.141,8

Each value is the average of three replicates

Table 2 Effect of insect-resistant clones of transgenic triploid of Chinese white poplar on the activity of carboxylesterase in the midgut of *C. anachoreta* instar larvae (mmol·mg^{-1}·min^{-1})

Time /h	Transgenic poplar			
	CK	Clone 7	Clone 10	Clone 26
4	0.110,5	0.105,2	0.108,1	0.109,3
12	0.100,9	0.090,2	0.089,1	0.096,7
24	0.112,9	0.091,4	0.095,6	0.102,3
48	0.118,1	0.081,7	0.093,3	0.103,5
72	0.123,5	0.072,6	0.086,2	0.104,6
96	0.138,3	0.065,3	0.082,4	0.110,6

3.2 Activity variation of the glutathione S-transferase

Measurements of the activity of the glutathione S-transferase reached its lowest level taken after 12 h, accounting for 65.2%, 71.6%, and 86.9% of the CK activity, respectively. Then it increased to 1.13 times, 1.09 times, and 1.01 times of the CK activity, 24 h after feeding on the leaves. But it decreased again to 54.2%, 63.7%, and 85.8% of CK after 96 h.

3.3 Activity variation of the MFO

The activity of the mixed-function oxydases MFO in the larva's midgut decreased gradually. It accounted for 32.6%, 52.1%, and 83.3% of the CK activity after 96 h fed on highly, moderately and low-resistant plants, in which it decreased the most when fed on highly resistant plants.

Table 3 Effect of insect-resistant clones of transgenic triploid of Chinese white poplar on the activity of glutathione S-transferase in the midgut of *C. anachoreta* instar larvae (*OD* / mg protein • min^{-1})

Time /h	Transgenic poplar			
	CK	Clone 7	Clone 10	Clone 26
4	0.259,4	0.226,3	0.231,6	0.245,0
12	0.295,1	0.192,5	0.211,2	0.256,3
24	0.350,3	0.394,7	0.381,6	0.353,7
48	0.396,5	0.334,6	0.349,5	0.333,8
72	0.436,9	0.285,6	0.344,1	0.410,1
96	0.472,5	0.256,1	0.301,2	0.405,3

Table 4 Effect of insect-resistant clones of transgenic triploid of Chinese white poplar on the activity of mixed-function oxidases in the midgut of *C. anachoreta* instar larvae (mmol·mg^{-1}·min^{-1})

Time /h	Transgenic poplar			
	CK	Clone 7	Clone 10	Clone 26
4	0.673,5	0.651,1	0.658,9	0.660,3
12	0.697,3	0.586,3	0.611,5	0.652,1
24	0.735,1	0.512,1	0.566,1	0.660,2
48	0.790,8	0.400,3	0.561,2	0.687,9
72	0.854,2	0.421,2	0.550,1	0.724,3
96	0.924,7	0.301,7	0.481,6	0.770,5

3.4 Digestive ferment activity variation in the mid-intestines

3.4.1 Activity variation of the protease

The activity of the protease in the larva's midgut reached its trough after 12 h and then increased to a peak, with 1.13, 1.07, and 1.01 times of the CK activity, 24 h after feeding on the leaves. The protease activity decreased when fed on the highly and moderately resistant plants after 96 h and accounted for 69.7%, 78.7% of the CK activity. There was no obvious variation when feeding on the low resistant plants.

Table 5 Effect of insect-resistant clones of transgenic triploid of Chinese white poplar on the activity of proteinases in the midgut of *C. anachoreta* instar larvae (mg • mg protein • min^{-1})

Time /h	Transgenic poplar			
	CK	Clone 7	Clone 10	Clone 26
4	15.96	14.51	15.03	15.73
12	16.23	12.52	14.21	15.67
24	16.59	18.89	17.78	16.87
48	16.97	14.21	14.86	16.82
72	17.55	12.98	14.23	17.65
96	18.03	12.56	14.19	17.99

3.4.2 Activity variation of the amylase

The activity of the amylase in the larva's midgut decreased gradually to 80.0% and 85.8% of that of CK, 96 h after feeding on highly and moderately resistant plants and to 98.9% of the CK feeding on low-resistant plants.

Table 6 Effect of insect-resistant clones of transgenic triploid of Chinese white poplar on the activity of amylase in the midgut of *C. anachoreta* instar larvae (mg • mg protein • min^{-1})

Time /h	Transgenic poplar			
	CK	Clone 7	Clone 10	Clone 26
4	1.245	1.202	1.231	1.242
12	1.381	1.235	1.325	1.376
24	1.402	1.203	1.302	1.393
48	1.472	1.213	1.331	1.465
72	1.485	1.201	1.311	1.476
96	1.582	1.265	1.357	1.565

4 Discussions

The control of transgenic poplar to the leaf-eating insect is the result of interaction between plants and insects. The reaction was mainly controlled by the expression and activity of the insect-killing protein in the transgenic poplar and the insect's growth stage and the corresponding feeding behavior. Many studies indicated that highly anti-insect and moderately anti-insect clones of transgenic poplar had a high fatality rate over a short period of time to 1–2 instar of the larva of *C. anachoreta*. This decreased significantly with the growth of the larva. Transgenic clones may disturb the normal physiological metabolism of the insect by inhibiting the activity of detoxification enzymes. With the decrease of the activity of detoxification enzymes, the resistance would decrease correspondingly. Thus, to facilitate the intrusion of the outer-substance may be one of the important mechanisms of insect killing for the transgenic poplar.

As for the activity of the glutathione S-transferase (GSTs) in the insect, it decreased at the beginning of feeding and increased after 24 h, and then decreased again. This may be related to the enzyme itself. GSTs can detoxify by combining with strong activity compounds. That is, the GSTs can combine with the poisoned electrophile and thus protect the other nucleophilic centers (protein, nucleotide, etc.). After the leaves were eaten, the GSTs of the larva's midgut could compete with the poisoned protein and the sensitivity at the functional loci decreased. Therefore, the specific activity increased quickly. With time, the poisoned protein accumulated and the activity of the enzyme decreased correspondingly.

The esterase, carboxylesterase, and mixed-function oxidases (MFO) are the important detoxification enzymes in the insect; and they play an important role in decomposing the outer poison and maintaining the normal metabolism. Our study concentrated on the activity variation of the enzyme of the larva of *C. anachoreta* caused by various resistance levels. Leaves with high and moderate resistance of transgenic plants have significant levels of resistance to inhibit the activity of these three enzymes and the inhibition was getting stronger as time passed by. The highly resistant plants caused the maximum decrease, while the low-resistant plants had the least decrease. There was no obvious variation of the specific activity of the protease in the larva midgut fed on the low-resistant plants and CK. But it decreased at the beginning when fed on highly and moderately resistant plants and reached its lowest activity after 12 h and then began to increase after 24 h. Furthermore, the activity of the protease was higher than the activity when fed on CK. The decrease in decomposing of the protease inhibitor compound and the protease may be the cause. So the larva of *C. anachoreta* will excrete much protease suitable for digestion, thus leading to an increase in specific activity. The protease accumulated and regulation became stable in the body of the insect. The specific activity of the protease increased gradually.

The activity of starch enzyme decreases gradually when fed on the highly and moderately resistant plants compared with CK. But it made little difference when fed on

low-resistant plants. The protease inhibitor can suppress the activity of the protease in the midgut. This disorder of the insect's metabolism made the insect excrete massive amounts of protease, which consumed much energy on the part of the insect, so that the insect finally died.

The midgut is the important place for synthesis and excretion of digestive enzymes. The *Bt* protein can act on the cells of the intestinal wall and destroy it partially, or the cavumlization can interrupt the normal synthesis and excretion of the starch enzyme and the protease in the midgut. The protein inhibitors acted on the protease, had an impact on the digestion, and absorbed the protein. It is a mechanism for the killing of insects.

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