Breeding Science 62: 288–291 (2012)
doi:10.1270/jsbbs.62.288

Note

Overexpression of *poplar* wounding-inducible genes in *Arabidopsis* caused improved resistance against *Helicoverpa armigera* (Hübner) larvae

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Four highly inducible genes of poplar trees, *PtdKTI5*, *PtdWIN4*, *PtdPOP3* from hybrid poplar (*Populus trichocarpa* × *P. deltoides*) and *PtKTI2* from trembling aspen (*Populus tremuloides* Michx.) have been individually transformed into *Arabidopsis thaliana* for overexpression. High transcriptional level of each transgene in transgenic *Arabidopsis* lines was confirmed by RT-PCR analysis. The development, body weight and survivorship of cotton bollworm (*Helicoverpa armigera*) fed on four types of transgenic *Arabidopis* plants were evaluated in the laboratory. Our data indicated that these four *Populus* defense-related genes exhibited various degree of insectital activity on larval and postlarval development of cotton bollworm and may be utilized for herbivore resistance improvement in plant genetic engineering.

Key Words: wounding-inducible gene, *Populus*, transgenic *Arabidopsis*, *Helicoverpa armigera*, anti-herbivore.

Introduction

Plants have developed sophisticated protective and defensive adaptations to different stresses including herbivore and pathogen attack. Such adaptations include stress-induced enzymes and proteins as well as morphological and physiological changes. An advantage of inducible, as opposed to constitutive defenses, is that they are only produced when needed, and are therefore potentially less costly, especially when herbivory is variable (Karban *et al*. 1997). Poplar is a long-lived woody model system and its genome has a large suite of highly inducible genes associated with insect resistance. For example, Kunitz trypsin inhibitors (KTIs) feature prominently in poplar defense responses against insects (Bradshaw *et al*. 1990, Christopher *et al*. 2004, Haruta *et al*. 2001, Saarikoski *et al*. 1996). The expression profiles of *Populus* KTI gene family following herbivore attack or mechanical wounding have been characterized by microarray and real-time PCR analyses and different KTI genes exhibited differential constitutive and insect-induced gene expression patterns (Ma *et al*. 2011, Philippe *et al*. 2009). Among them, PtdKTI5 has been shown to be the most stable KTI and could retain its activity at high levels of DTT and high temperature (Major and Constabel 2008). Thus, PtdKTI5 may be inherently more stable and resistant to proteolysis within the guts of lepidopteran and perhaps other herbivores (Major and Constabel 2008). Another member of *Populus* KTIs, PtKTI2 has been shown to be induced by wounding and herbivory in *Populus tremuloides* and its *in vitro* activity to inhibit bovine trypsin has been confirmed (Haruta *et al*. 2001). POP3, also named as stable protein 1 (SP1) was previously isolated from *Populus tremula* (Pelah *et al*. 1995, 1997a, 1997b). POP3 is a boiling stable, stress-responsive protein with no significant sequence homology to other stress-related proteins (Dure 1993, Ingram and Bartels 1996, Thomashow 1999). The poplar genome appears to contain several clustered *win4* genes which are systemically wound-inducible in leaves and stems (Davis *et al*. 1993), however, their physiological functions still remained unknown. The common feature of the four above-mentioned genes is to be induced systemically upon wounding and herbivore infestation and this fact attracted us to test their potential anti-herbivore effects in transgenic plants. Our results may help in targeting useful genes for improving herbivore tolerance in economically important crop plants.

Communicated by A. Watanabe
Received October 28, 2011. Accepted May 16, 2012.
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Materials and Methods

Plant materials

Poplar hybrid H11-11 (*Populus trichocarpa* × *P. deltoides*) and trembling aspen (*Populus tremuloides*) were propagated and grown as previously described (Constabel *et al*. 2000). For mechanical wounding, leaf margins were crushed three times at 2 h intervals with pliers at LPI 12–14 (leaf plastochron index) and leaf samples were harvested at 24 h after wounding (Constabel *et al*. 2000). Total RNA was extracted from 100 mg crushed leaves using the RNeasy Mini Kit (Qiagen) and the first strand of cDNA was synthesized using

Table 1. List of primer used in cloning and gene expression level analysis

Target genes	Forward Primers (5′→3′)	Reverse Primers (5′→3′)
	Cloning Primers	
PtdKTI5	atgttactacccctctccttcc	ttatacaacagcttttaatccatca
PtdWIN4	atgtcgagcgttaacttggtag	tcattcacaagccaaacgtg
PtdPOP3	atggcaaccagaactccaaa	tagtagagaaagtagtctatcacaagacgc
PtKTI2	atgaagatcactaaatttctagggc	tcattctgacaccattttatcacc
	Genomic PCR Primers	
PtdKTI5	ttctcctggcttccacattga	tgggcacgtaccgtttatgtc
PtdWIN4	tcacaggagcttggaattgga	aaccagaaaacatcctgcggt
PtdPOP3	acatggcaaccagaactccaa	cgtgccccaattgaaactctt
PtKTI2	cggcctcccagtaacattttc	aaagggatcggactttggaca

Superscriptase II (Invitrogene). Nucleotide primers designed to amplify the coding regions of target genes were listed in Table 1.

Analysis of transgenic plants

In order to heterogeneously express these four genes, *PtdKTI5* (GenBank Aceesion number AAQ84218), *PtKTI2* (AAK32690), *PtdPOP3* (XP_002314976) and *PtdWIN4* (AAA16342) in *Arabidopsis thaliana*, their cDNA sequences were positioned between the *CaMV35S* promoter and the *NOS* terminator of the binary pCGN1548 vector. The resultant plasmid was used to transform *Arabidopsis thaliana* (Col-0) plants by the *Agrobacterium*-mediated floral dip method (Clough and Bent 1998). Homozygous T_2 seeds were identified and used for further bioassay studies. The expression level of the target gene in each transgenic *Arabidopsis* line was analyzed by the semi-quantitative RT-PCR using gene-specific primers (Fig. 1).

Insect bioassays

The entomocidal activity of the overexpressed *populus* protein in the leaf tissue of the transformed *Arabidopsis* was assayed through a no-choice detached leaf feeding bioassay. Briefly, newly emerged nymphs of cotton bollworm were brushed carefully in plastic Petri dishes containing fresh leaves on moist filter paper and the larvae were reared individually to prevent cannibalism. The *Arabidopsis* leaves were changed daily and the leaf consumption was recorded. Mature caterpillars were allowed to pupate in moist soil substratum.

Statistical analysis

Analysis of variance (ANOVA) was used to compare growth rates, larval fresh weights and leaf consumption among lines. Differences were considered to be significant for $P < 0.05$.

Results and Discussion

Analysis of transgenic plants

After transformation by *Agrobacterium*, the expressional levels of each transgene in the kanamycin-resistant

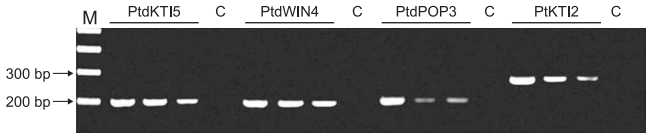

Fig. 1. Expression level of target genes was quantified by semi-quantitative PCR using cDNA from putative transgenic *Arabidopsis* plants. Lane M: DNA molecular marker; lane 1–12: independent transgenic *Arabidopsis* plants; C: control plants transformed with empty vector.

Arabidopsis lines were analyzed by semi-quantitative RT-PCR (Fig. 1). We chose three independent transgenic lines with the highest expression levels for each target gene in order to obtain the appropriate expression level higher than the pest's sensitivity threshold and thus induce deleterious effects on the larvae.

Insect resistance bioassays

The cotton bollworm *Helicoverpa armigera* (Hübner) is one of the most serious insect pests in China and every year the larvae of this species cause substantial economical losses to cotton, corn, tomato, legumes and other vegetable crops (Liu *et al*. 2004). No-choice bioassays were performed in triplicate and at the end of larval stadium, no significant difference in survivorship was found among larvae reared on different types of *Arabidopsis* plants (Table 2). However, an obvious reduction of the mean body weight compared to the control group was observed for *PtdPOP3* and *PtdKTI5* transgenic groups (Table 2). The feeding behavior of larvae fed on four types of transgenic plants seemed to be normal and the larvae did not consume higher or lower amount of foliage when compared to the larvae fed on control leaves (Table 2), which indicated that the larvae fed on transgenic *Arabidopsis* did not overcome the possible negative effect of the digested foliage by increasing or decreasing their food intake. In the end of bioassay, about 43% of the larvae fed on control plants entered pupation and nearly all of them emerged from chrysalis as adults (Table 2). In comparison, although the larvae fed on transgenic *Arabidopsis* entered pupation stage at about the same time point and no morphological deformation was observed, the percentage of them entering the pupation stage was significantly reduced (Table 2). This indicated a more pronounced deleterious and accumulative effect of the transgenic plants happened at this pupation stage. Individual insects were then checked twice daily for molting by observation of the head capsule. For each group of pupa, the molting process finished within 6 or 7 days and no obvious difference was found between transgenic and control groups. In the case of PtKTI2, the adult emergence was affected seriously and the percentages of mature adults decreased sharply, however; only moderately decline was observed for other three transgenic groups. Considering the larval body weight and the percentage of pupation and adult emergence, the four types of transgenic plants can be arranged in a descending order according to

Table 2. Ratio of survival, larval growth, leaf consumption, pupation and adult proportion of cotton bollworm larvae reared on control or the transgenic *Arabidopsis* lines

	Survival (%)	Larval weight (mg)	Consumed leaf weight (g/larvae)	Pupation (%)	Adults (%)
Control	57.1 ± 5.8	229.1 ± 7.8	2.85 ± 0.12	42.9 ± 5.7	40.9 ± 3.6
PtdPOP3-1	48.6 ± 4.2	206.2 ± 12.3*	3.01 ± 0.21	14.6 ± 3.7**	12.2 ± 1.5**
PtdPOP3-2	50.7 ± 6.2	185.6 ± 13.8*	2.97 ± 0.18	19.2 ± 3.1**	18.2 ± 1.6**
PtdPOP3-3	57.1 ± 4.7	203.9 ± 10.6*	2.88 ± 0.20	18.5 ± 4.3**	15.7 ± 1.2**
PtKTI2-1	62.9 ± 6.8	231.4 ± 10.7	2.91 ± 0.19	23.2 ± 3.6**	8.6 ± 0.8**
PtKTI2-2	54.3 ± 7.0	219.9 ± 11.2	2.84 ± 0.16	25.1 ± 4.8**	11.2 ± 0.7**
PtKTI2-3	58.6 ± 4.9	222.2 ± 14.5	2.87 ± 0.21	21.6 ± 3.9**	8.8 ± 0.6**
PtdKTI5-1	48.6 ± 5.3	187.9 ± 10.6**	2.85 ± 0.17	31.4 ± 4.2*	30.1 ± 2.6**
PtdKTI5-2	45.7 ± 5.7*	173.3 ± 19.2**	2.94 ± 0.20	30.6 ± 3.9*	28.3 ± 1.9**
PtdKTI5-3	60.8 ± 4.4	191.0 ± 17.4**	3.03 ± 0.26	35.2 ± 2.2*	32.2 ± 2.4*
PtdWIN4-1	48.8 ± 6.8	203.4 ± 25.6	2.89 ± 0.13	18.0 ± 2.7**	12.4 ± 1.1**
PtdWIN4-2	52.3 ± 7.3	226.3 ± 12.5	3.05 ± 0.25	19.3 ± 4.1**	15.2 ± 1.5**
PtdWIN4-3	56.6 ± 5.6	218.2 ± 17.2	2.82 ± 0.19	20.1 ± 4.9**	18.9 ± 1.2**

* Significant differences ($P < 0.05$) versus the vector-only (control) line. ** Significant differences ($P < 0.001$) versus the control line.

their anti-herbivore activity:PtdKTI2 > PtdPOP3 = PtdWIN4 > PtKTI5. Due to the low adult numbers, we did not investigate the adult longevity and reproduction and future work is required to investigate the effect of the transgenic lines on the survival and fertility of the subsequent adults.

In literature, plant defensive proteins have been extensively reported for their anti-herbivore properties. In a suite of systemically induced genes by wounding or herbivore identified in *Populus* genome, the four genes under investigation in this study were found among the most abundant ESTs in the leaf transcriptome, suggesting they may be functionally important for hybrid poplar defense (Christopher *et al*. 2004, Davis *et al*. 1993, Haruta *et al*. 2001). The data in this study demonstrated that these four *Populus* defense-related genes could interfere with the performance and growth of this agronomically important insect, which make them attracting candidates to be used in genetic engineering. Protease inhibitors expressed in transgenic plants have been reported to provide enhanced resistance to important pest species since that they could bind to the digestive proteases of insects, thus block the digestion of proteins, leading to developmental delays and increased mortality (Abdeen *et al*. 2005, Amirhusin *et al*. 2007, Dunse *et al*. 2010). In this study, two *Populus* KTI genes varied in their anti-herbivore effects. At the larval stage, both KTIs did not decrease the survival rate of the cotton bollworm larvae and did not induce excessive foliage consumption. However, PtdKTI5 could significantly decrease the average larval weight, while PtKTI2 could not. However, the PtKTI2 seemed to exert more pronounced negative impact on the larval development at the following pupation stage. Much fewer larvae raised on PtKTI2 transgenic *Arabidopsis* entered pupation and turned into adults when compared with those raised on PtdKTI5 transgenic plants, indicating their different working mechanisms. *PtdWIN4* and *PtdPOP3* are two other wounding-inducible genes without defined functions, and their anti-herbivore effects were reported for the first time in this work. Both *PtdWIN4* and *PtdPOP3* transgenic *Arabidopsis* showed a high inhibitory activity on the development of the cotton bollworm and the highest inhibitory activity appeared at pupation stage for both of them. It has been suggested that gene pyramiding by expression of different defense genes could be a better plant protection strategy, thus these four genes identified in this study may constitute good candidates for such a combination in order to achieve a synergistically enhanced anti-herbivore activity.

Acknowledgments

The authors wish to thank C. Peter Constabel for supplying the plasmid constructs. This study was funded by National High-tech R&D Program of China (2011AA100201), National Natural Science Foundation of China (31100493), Tianjin Research Program of Application Foundation and Advanced Technology (10JCYBJC08600) and Tianjin Science & Technology Pillar Program (11ZCGYNC01400).

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