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*Full Length Research Paper*

# Effects of *Lymantria dispar* feeding and mechanical wounding on defense-related enzymes in *Populus simonii* × *Populus nigra*

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**In plants, lipoxygenase (LOX) and polyphenol oxidase (PPO) have been related to defense mechanisms against insect feeding and wounding and these roles were investigated in poplar (*Populus simonii* × *Populus nigra*) regarding induction by third-instar *Lymantria dispar* larvae feeding and punching mechanical wounding. The results showed that *L. dispar* feeding and mechanical wounding caused different responses of protein and transcript levels of LOX and PPO. PPO was obviously induced by *L. dispar* feeding during a 72 h period, while LOX was not significantly induced by *L. dispar* feeding. For mechanical wounding, activity and mRNA expression level of LOX and PPO were not significantly induced but were mostly inhibited. Therefore, LOX was not involved in defensive mechanisms of *P. simonii* × *P. nigra*. The PPO gene may be significantly related to poplar defense against *L. dispar* attack.**

**Key words**: Lipoxygenase, polyphenol oxidase, *Populus simonii* × *Populus nigra*, *Lymantria dispar* feeding, mechanical wounding.

## INTRODUCTION

Physical, chemical and biological stresses induce defensive mechanisms in plants (Somssich and Hahlbrock, 1998; Chaman et al., 2003). Some secondary metabolites such as phenylpropanoids, *o*-diphenols and *o*-diquinones are involved in plant defenses against pathogens and pests (Dixon and Paiva, 1995; Karban and Baldwin, 1997), the levels of which are often mediated by defense-related enzymes, such as phenyalanine ammonia-lyase (PAL), lipoxygenase (LOX) and polyphenol oxidase (PPO) (Constabel et al., 1996; Li and Steffens, 2002; Chaman et al., 2003; Sha et al., 2005).

LOXs (EC1.13.11.12) belong to a family of non-heme iron-containing fatty acid dioxygenases and widely exist throughout plants and animals. LOXs catalyze the hydroperoxidation of *cis, cis*-1,4-pentadiene structures present in unsaturated fatty acids in plants such as linoleic acid, α-linoleic or arachidonic acid (Cheng et al., 2006). Depending on the region-specificity of the oxygen added to linoleic acid, plant LOXs are classified as 9-LOX or 13-LOX; furthermore, based on the N-terminal chloroplast transit peptide sequence, plant LOXs can be further classified as types 1 or 2 lipoxygenases (Feussner and Wasternack, 2002). Studies of insect-plant interaction suggest that phenylpropanoid compounds are involved in plant defenses against pests (Dixon and Paiva, 1995; Chaman et al., 2003; Han et al., 2009, Gosset et al., 2009; Copolovici et al., 2010) and LOX has a role in pest resistance, wound response and pathogen infection (Bostock and Stermer, 1989; Stout and Duffey, 1996; Karban and Baldwin, 1997). Another defensive enzyme PPO (EC1.14.18.1 or EC1.10.3.2) is an enzyme broadly distributed among plants and oxidizes phenolic compounds to quinones, reactive molecules that can interact with many biological molecules (Mayer and Harel, 1979). PPO is also a major protein acting against diverse diseases and insect pests and is induced in plants by wounding system in and the octadecanoid pathway

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(Constabel et al., 1995, 1996; Pinto et al., 2008). Insect feeding and herbivore attack is frequently associated with plant wounding and also allows plants to use defenses that are ineffective against pathogens (Chaman et al., 2003).

The Asian gypsy moth (*Lymantria dispar*) is a key lepidopterious pest species of forests around the world. It is estimated that worldwide at least 500 species of plants are hosts to *L. dispar* (Lazarević et al., 1998). Among many plants, poplars are a preferred host to *L. dispar*. To gain some insight into key defense enzymes in poplar (*Populus simonii* × *Populus nigra*) involved in resistance to pest insects, we investigated the effects of *L. dispar* feeding and mechanical wounding on LOX and PPO activity and their mRNA expression levels in poplar.

## MATERIALS AND METHODS

### Plants and *L. dispar*

The plants used were poplar (*P. simonii* × *P. nigra*), which is a primary host plant of *L. dispar* in forestry. Plants were grown in a mixture of turfy peat and sand (2:1 v/v) in a greenhouse with 75% relative humidity and an average temperature of 24℃. Fully expanded leaves were treated by feeding and wounding at the two-month-old stage. No chemical sprays were applied to the plants before and during the experiments. *L. dispar* (L.) eggs were collected in a mixed forest, predominantly *Betula platyphylla* from the Forestry Center in Northeast Forestry University, China, in February. The egg masses were kept in the refrigerator at 4℃ before they were set for hatching over February to April. The individuals were reared at 25℃ and a 14/10 h light/dark photoperiod. Transparent plastic bottles (9.0 cm in diameter, 14 cm in depth) with a vented lid were used to rear larvae. Humidity in the plastic cups was maintained by botanical sponges soaked in water, which also kept the poplar leaves fresh. Healthy third-instar *L. dispar* larvae were used in this experiment.

### Treatment

Poplars (two-months-old, each with 6 to 10 tender branches) with the same growth potential were used for the experiment. Intact healthy poplars were kept as controls (without wounds and pest insect damage), artificially damaged plants were prepared by punching 10 holes (1 cm in diameter) per poplar leaf and *L. dispar* feeding was achieved by placing four third-instar larvae per poplar leaf. Every treatment was repeated three times. To prevent escape of *L. dispar* larvae, the poplars were caged with a fine nylon net. The experimental setup was maintained for 1 to 3 days. Activity and mRNA expression of LOX and PPO were measured 6, 12, 24, 48 and 72 h after treatment. Leaves were harvested and immediately stored at -70℃ until activity assays and RNA isolation.

### Enzyme assays

LOX activity was assayed by measuring absorption at 234 nm arising from the formation of conjugated double bonds during the reaction. Poplar leaves (approximately 1.0 g) were cut in small pieces and ground in 5 ml 0.1 M phosphate buffer (pH 7.0) containing 1% (w/v) polyvinylpyrrulidone on ice. The slurry was centrifuged at 15,000 g for 30 min at 4℃. The supernatant was used as an extract solution to measure enzyme activity. The linoleic acid substrate for activity measurement was made according to the method of Surrey (1964). The reaction mixture contained extraction solution (50 µl) and linoleic acid (0.5 ml) and was incubated at 25℃ for 5 min. 1 ml of 60% ethanol solution was added into the reaction products and absorption measured at 234 nm, an increase of 1.00 $min^{-1}$ was defined as one enzymatic activity unit.

The extraction and assays of the PPO activity were according to the method of Mazzafera and Robinson (2000). The leaves were cut in small pieces and ground in extraction buffer (100 mM Na-Pi buffer, pH 7.0, containing 2% ascorbic acid, 5 mM dithioerythritol and 20% polyvinyl polypyrrolidone, using 5 $mL \cdot g^{-1}$ of leaves). The homogenized tissue was centrifuged at 25,000 g for 20 min at 4℃. PPO activity was assayed with an aliquot from supernatant (300 µl) in 0.3 M catechol. The reaction product was measured at 405 nm in an UltroSPec 4300 (GE, USA) over 3 min, one enzymatic activity unit is defined as $A_{405}$ increasing 1.00 $min^{-1}$. Every enzyme activity measure was repeated three times and the samples were conducted in triplicate.

### RNA extraction, cDNA synthesis, LOX and PPO cloning

Total RNA was extracted by the CTAB method and total RNA was digested with DNase to remove DNA contamination. The RNA was quantified by measuring the absorbance at 260 nm and the quality was checked by electrophoresis through agarose gels stained with ethidium bromide. Total RNA (4 µg) was reverse transcribed with Oligo (T) primer by using reverse transcription system (Promega, Madison, WI) following the manufacturer's protocols.

Two gene-specific primers originated from poplars (GenBank/EMBL Accession nos. DQ131178, DQ131179 and AF263611) were designed for amplifying the open reading frame of LOX and PPO genes. LOXS: 5'-TCAAGGTCATCGCTGGTA-3'; LOXA: 5'-GTGT AACAGTCTGTAAATTG-3'; PPOS: 5'-TCACCGTAATGTCCACCT-3'; PPOA: 5'-GTCTCAATCGTCCCAGCT-3'.

PCR was carried out in a Biometra Tgradient 96 Thermocycler (Biometra, Germany). The reactions were performed in a 20 µl mixture, which contained 2 µl of 10 × PCR buffer, 0.4 µl of 10 mM dNTP, 1 µl of specific primers (10 µM), 0.2 µl cDNA (equivalent to 0.08 µg of total RNA) and 2 U of *Taq* polymerase (Sangon). The cycling parameters were 94℃ for 3 min, followed by 40 cycles of 94℃ for 30 s; 56℃ for 30 s; 72℃ for 2 min; and a final extension for 7 min at 72℃. PCR products were separated, purified and ligated into the pGEM T-easy vector (Promega) and then, transformed into JM109 *Escherichia coli* cells. The positive clones from LB plates were subjected to colony PCRs (Sambrook et al., 1998). Sequence analysis was performed by dideoxynucleotide chain termination method. Clones were sequenced with SP6 and T7 sequencing primers in an automated fluorescence sequencing system, ABI377 DNA sequencer (Applied Biosystems, Foster City, CA).

### Real-time PCR

To investigate the expression differences of LOX and PPO genes induced by *L. dispar* feeding and mechanical wounding, the expressions of the two genes were analyzed using real-time PCR. *P. simonii* × *P. nigra* were treated with third-instar *L. dispar* larvae feeding or punching for 6, 12, 24, 48 and 72 h, respectively. Total RNA from harvested leaves was isolated and digested with DNase I (Promega) to remove DNA contamination. Approximately, 0.5 µg of total RNA was reverse transcribed to cDNA using a 10 µl of an oligodeoxythymidine primer following the PrimeScript™ RT reagent kit (TaKaRa) protocol. The synthesized cDNA was diluted with 90 µl of water and used as the template in real-time RT-PCR. PCR primers were designed to amplify target cDNA fragments (Table 1).

**Table 1.** Nucleotide sequences of primers used in RT-PCR to analyze gene expression induced by *L. dispar* feeding and mechanical wounding.

| Gene | Forward primer (5'- 3') | Reverse primer (5'- 3') | Product size (bp) |
|---|---|---|---|
| Lipoxygenase (FJ864335) | TTGAACTGACTCGCCCACC | AAGCTGCCGATTCGTTGC | 206 |
| Polyphenoloxidase (FJ864337) | TGCCTATTGCGATGATGC | CTGGTCGTAGAGTGGAGATT | 235 |
| TUA (CA822230,CA825391) | AGGTTCTGGTTTGGGGTCTT | TTGTCCAAAAGCACAGCAAC | 185 |
| EF1β (BI125345) | AAGAGGACAAGAAGGCAGCA | CTAACCGCCTTCTCCAACAC | 145 |
| UBQ(BU879229) | GTTGATTTTTGCTGGGAAGC | GATCTTGGCCTTCACGTTGT | 193 |

TUA, UBQ and EF1β were used as internal references to normalize the amount of total RNA present in each reaction. Every treatment was repeated three times and the samples were conducted in triplicate on a MJ Research Opticon$^{TM2}$ machine (MJ Research). PCR was performed in a total volume of 20 µl containing 10 µl of QuantiTect SYBR-green PCR master mix (Qiagen), 0.5 µM of forward and reverse primers and 2 µl cDNA template (equivalent to 0.05 µg of total RNA). The amplification was completed with the following cycling parameters: 94°C for 30 s, followed by 45 cycles of 94°C for 12 s; 60°C for 30 s; 72°C for 45 s; and l s at 78.5°C for plate reading. After the cycling protocol, the final step was applied to all reactions by continuously monitoring the fluorescence through the dissociation temperature of the PCR product at a temperature transition rate of 0.1°C $s^{-1}$ to generate a melting curve. All samples were also electrophoresed in agarose gels to verify implication of the target fragments. The expression levels of the clones were calculated from the threshold cycle according to $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

#### Statistical analysis

Activity differences between the feeding and wounding and control treatments were evaluated with Tukey's multiple comparison tests. The mRNA relative expression level differences between feeding and wounding were analyzed by Student's t-test. All data were analyzed using a statistical software package (Graphpad instat).

## RESULTS

### Effects of larval *L. dispar* feeding and mechanical wounding on the LOX activity and gene expression

The partial sequences of the LOX gene were cloned from *P. simonii × P. nigra* (FJ864335). LOX activity and mRNA expression level induced by *L. dispar* feeding and mechanical wounding during 72 h are shown in Figure 1. LOX activity was not obviously induced by the *L. dispar* feeding and mechanical wounding treatments (Figure 1A). After 12 h, LOX activity induced by feeding was at a maximum, 1.17-fold of controls. Moreover, the LOX activity was partially inhibited by wounding, at 0.73- and 0.84-fold of controls at 48 and 72 h, respectively.

The mRNA relative expression level of LOX in poplar leaves induced by *L. dispar* feeding was not significantly different from controls (0.68 to 1.09-fold, Figure 1B). For wounding, the maximum induction of mRNA expression was only 1.53-fold of controls at 24 h.

### Induction of PPO activity and expression of PPO gene in leaves submitted to *L. dispar* feeding and mechanical wounding

The partial sequences of PPO gene (FJ864337) were obtained from *P. simonii × P. nigra*; the PPO activity and expression induced by *L. dispar* feeding and mechanical wounding at different times are shown in Figure 2. After *L. dispar* feeding, the PPO activity consistently increased with time while there was no significant variation in the controls. PPO activity in *L. dispar*-attacked poplar leaves was 26.32-fold that of controls after 72 h. Meanwhile, wounding clearly increased PPO activity at 12 and 48 h. To further investigate gene transcript levels, we examined poplar leaves treated with *L. dispar* feeding and mechanical wounding (Figure 2B). During the 72 h treatment, the mRNA relative expression level induced by feeding gradually increased and reached 45.10-fold of controls at 72 h and was significantly higher than the mechanical wounding treatment during 12 to 72 h. Compared with controls, the effect of wounding on the mRNA expression level was not significant except at 12 and 48 h.

## DISCUSSION

Attacks that change physiological or structural characteristics of plants included biotic and abiotic effects such as insect feeding, pathogen infection and mechanical wounding. The biochemical mechanism of plant defense induced by these stimuli has been extensively studied in plant-attacker interactions in some crops (Argandoña, 1994; Cabrera et al., 1995; Chaman et al., 2003; Kempema et al., 2007; Han et al., 2009), whereas herbivore-induced biochemical changes in forest plants are a relatively new research area (Wang and Constabel, 2004; Melo et al., 2006).

The LOX activity in some plants was induced by attacks such as insect feeding, disease infection and mechanical damage (Hui et al., 2003; Schmidt et al., 2005; Ralph et al., 2006; Kempema et al., 2007). However, we found that *L. dispar* feeding and wounding did not increase LOX activity and expression but instead, partially decreased them in *P. simonii × P. nigra*. This

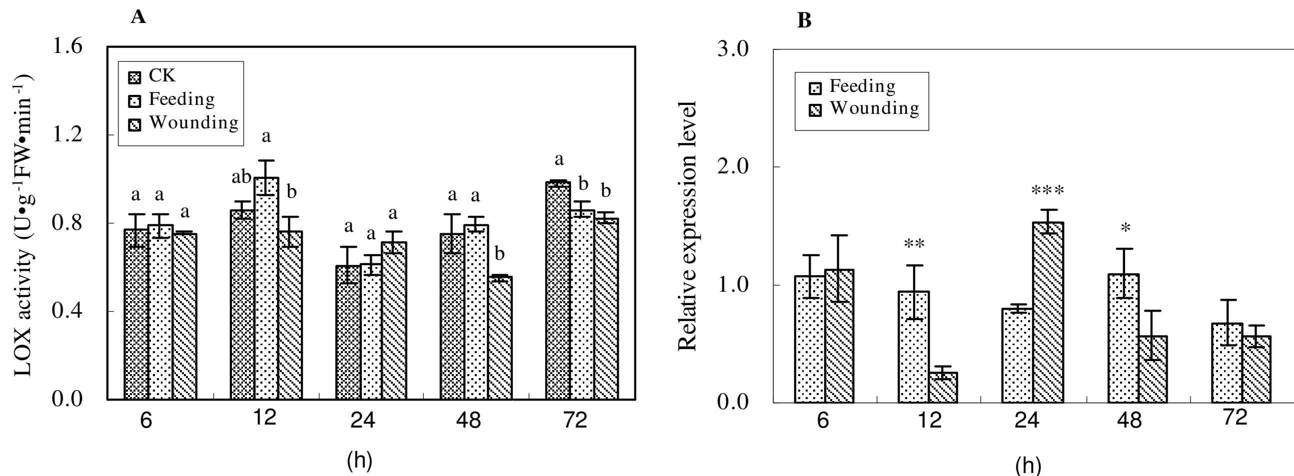


**Figure 1.** LOX activity (A) and mRNA relative expression level (B) in poplar submitted to *L. dispar* feeding and mechanical wounding. Different and same small letters above histogram indicate significant (P < 0.05) and no significant differences (P > 0.05), respectively, between the feeding and wounding and control treatments with Tukey's multiple comparison test. ***, ** and * indicate extremely significant (P < 0.001), very significant (P < 0.01) and significant (P < 0.05) differences, respectively, between feeding and wounding by Student's t-test.

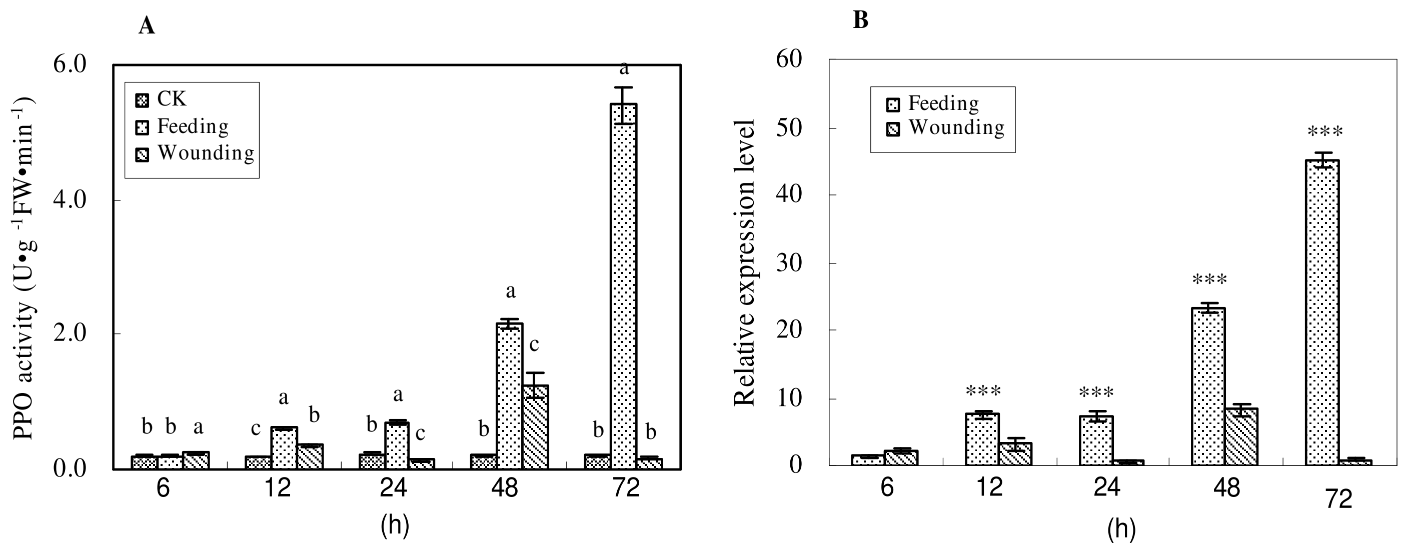


**Figure 2.** PPO activity (A) and mRNA relative expression level (B) in poplar submitted to *L. dispar* feeding and mechanical wounding. Different and same small letters above histogram indicate significant (P < 0.05) and no significant differences (P > 0.05), respectively, between the feeding and wounding and control treatments with Tukey's multiple comparison test. ***, ** and * indicate extremely significant (P < 0.001), very significant (P < 0.01) and significant (P < 0.05) differences, respectively, between feeding and wounding by Student's t-test.

phenomenon was also reported in the caterpillar *Helicoverpa zea* feeding on tomato, which induced a weak transient accumulation of LOX mRNA that was detectable within 24 h of challenge (Fidantsef et al., 1999). LOX expression induced in some plants may be not follow a strict pattern because of the type of biological stress, such as insect or others.

In plants, PPO has been related to defense mechanisms against pathogens and insects (Melo et al., 2006). PPO have been confirmed an anti-nutritive role in defense against leaf-eating insects (Duffey and Felton, defense against leaf-eating insects (Duffey and Felton, 1991; Constabel et al., 1996). In this study, PPO was most strongly induced by *L. dispar* feeding in hybrid poplar *P. simonii* × *P. nigra*. For 72 h, the PPO activity and expression continually increased and reached 26.32- and 45.10-fold, respectively, of controls at 72 h. Over-expressed PPO could oxidize more phenolic compounds

to quinones responding to *L. dispar* feeding.

Some key enzymes including PAL, LOX and PPO were confirmed to play roles in the plant defense pathway. However, these are intricate mechanisms since it was unclear what signals were generated to engage different local and systemic stress responses following pathogen and insect attack in plants (Fidantsef et al., 1999; Qin and Gao, 2005). To investigate roles of some key defense enzymes in plant resistance, some research has begun using transgenic plants overexpressing key enzymes gene (Wang and Constabel, 2004). Transgenic hybrid aspen (*Populus tremula* × *Populus deltoides*) plants overexpressing a hybrid poplar (*Populus trichocarpa* × *P. deltoides*) *PtdPPO1* gene showed high PPO enzyme activity, *PtdPPO1* mRNA levels and PPO protein accumulation and significant resistance to forest tent caterpillar (*Malacosoma disstria*) (Wang and Constabel, 2004). Whereas, Barbehenn et al. (2007) reported transgenic PPO-overexpressing poplar (*P. tremula* × *Populus alba*) and treating poplar foliage with purified mushroom PPO produced limited effects on tree-feeding caterpillars (*L. dispar and Orgyia leucostigma*) consumption and growth rates. Therefore, the defensive mechanisms need to be explored in the further.

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