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ORIGINAL ARTICLE

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Polyphenol oxidase overexpression in transgenic *Populus* enhances resistance to herbivory by forest tent caterpillar (*Malacosoma disstria*)

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Abstract In order to functionally analyze the predicted defensive role of leaf polyphenol oxidase (PPO; EC 1.10.3.1) in *Populus*, transgenic hybrid aspen (*Populus tremula* × *P. alba*) plants overexpressing a hybrid poplar (*Populus trichocarpa* × *P. deltoides*) *PtdPPO1* gene were constructed. Regenerated transgenic plants showed high PPO enzyme activity, *PtdPPO1* mRNA levels and PPO protein accumulation. In leaf disk bioassays, forest tent caterpillar (*Malacosoma disstria*) larvae feeding on PPO-overexpressing transgenics experienced significantly higher mortality and reduced average weight gain compared to larvae feeding on control leaves. However, this effect was observed only when older egg masses were used and the resulting larvae showed reduced growth and vigor. In choice tests, no effect of PPO overexpression was detected. Although PPO in poplar leaves is latent and requires activation with detergents or trypsin for full enzymatic activity, in caterpillar frass the enzyme was extracted in the fully activated form. This activation correlated with partial proteolytic cleavage, suggesting that PPO latency and activation during digestion could be an adaptive and defense-related feature of poplar PPO.

Keywords Forest tent caterpillar · Herbivore defense · Plant–insect interaction · *Populus* · Polyphenol oxidase · Transgenic aspen

Abbreviations CaMV: Cauliflower mosaic virus · DOPA: Dihydroxyphenylalanine · FTC: Forest tent caterpillar · GUS: β-Glucuronidase · LPI: Leaf plastochron index · PPO: Polyphenol oxidase · SDS: Sodium dodecyl sulfate

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Introduction

Polyphenol oxidase (PPO) catalyzes the oxidation of *o*-diphenols to *o*-diquinones (diphenolase activity; EC 1.10.3.1), and in some plants may also perform the *o*-hydroxylation of monophenols (monophenolase activity; EC 1.14.18.1) (Vaughn and Duke 1984). The *o*-quinones are very reactive, and rapidly polymerize and alkylate cellular constituents. This often leads to cross-linking of phenols, proteins, and other cellular constituents, accompanied by conspicuous browning of tissues and extracts (Duffey and Felton 1991). The formation of black or brown quinone adducts is considered very detrimental in the food processing industry and is a primary reason for many studies on the properties of PPO (Mathew and Parpia 1971; Steffens et al. 1994). Transgenic technology is one approach for inhibiting the effects of PPO on browning reactions; transgenic apple and potato with antisense suppression of PPO show greatly reduced browning due to their reduced PPO activity (Murata et al. 2000; Coetzer et al. 2001).

Despite the attention PPO has received from food technologists, physiological functions of PPO have only recently been addressed. Many potential physiological roles for PPO are found in the literature, but to date the most convincing case has been made for a function of PPO in defense against pests and pathogens (Steffens et al. 1994; Constabel et al. 1996). Most significantly, tomato plants overexpressing PPO exhibit greater resistance to *Pseudomonas syringae*, as measured by bacterial growth, compared with control plants (Li and Steffens 2002). Although the mechanism underlying this effect is unclear, most biological effects of PPO are based on the reactive nature of the *o*-quinones produced. PPO has also been shown to be effective in defense against

small insects such as aphids. *Solanum berthaultii* leaves are covered with PPO-containing glandular trichomes; these are easily broken by aphids, mixing PPO with its substrates and resulting in a sticky exudate, which entraps the insects and prevents them from feeding (Kowalski et al. 1992).

A mechanistically different, anti-nutritive role for PPO has been proposed in defense against leaf-eating insects (Duffey and Felton 1991; Constabel et al. 1996). Leaf PPO is widely distributed in the plant kingdom, so that it could have more widespread importance in defense. In tomato, tobacco and poplar plants, wounding and herbivory stimulate a plant-wide induction of defense genes and proteins, including a suite of protease inhibitors as well as PPO, suggesting a role of this protein in defense against leaf herbivory (Felton et al. 1989; Bergey et al. 1996; Constabel and Ryan 1998). The effect of high PPO levels against leaf-eating herbivores is proposed to reside in the propensity of PPO-generated *o*-quinones to covalently modify and cross-link dietary proteins during feeding, resulting in decreased amino acid assimilation (Felton et al. 1989, 1992). Since the amino acids most susceptible to attack by *o*-quinones (lysine, histidine, cysteine, and methionine) are generally limiting in herbivore diets, the effect of these modifications can be significant (Felton et al. 1992). However, despite indirect evidence linking high PPO in the diet to reduced insect nutrition and performance, these effects of PPO have never been demonstrated directly using transgenic plants.

We previously cloned a wound-inducible leaf PPO cDNA from hybrid poplar (*Populus trichocarpa* × *P. deltoides*) and trembling aspen (*P. tremuloides*) (Constabel et al. 2000; Haruta et al. 2001a). The high levels of expression and systemic inducibility of leaf PPO by herbivores and wounding suggest a role of this gene in insect defense. Hybrid poplar and trembling aspen are economically important world-wide for pulp production, and trembling aspen is a key tree species of the boreal forest in western North America and is subject to dramatic defoliator attacks. To obtain direct proof of the anti-herbivore role of PPO in *Populus* and to determine its potential for biotechnological application in forestry and agriculture, we overexpressed hybrid poplar PPO in transgenic *P. tremula* × *P. alba* plants. These transgenics demonstrate the negative effect of PPO on forest tent caterpillar (FTC) health, and also provided insight into the activation of latent PPO in the caterpillar gut.

Materials and methods

Plant transformation and maintenance

The hybrid poplar (*Populus trichocarpa* × *P. deltoides*) *PtdPPO1* cDNA (Constabel et al. 2000) was PCR-amplified and linker sites were engineered for subcloning into pBI-525, between a double cauliflower mosaic virus (CaMV) 35S promoter with the AMV RNA4 trans/enhancer sequence and a nopaline synthase (*nos*) terminator region (Datla et al. 1993). Both sense and antisense plasmids were constructed. The overexpression cassettes (35S promoter/enhancer with the *PtdPPO1* coding sequence and the *nos* terminator) were then subcloned into the binary plasmid pRD400 (Datla et al. 1992), with the neomycin phosphotransferase II (*nptII*) gene as a selectable marker. The entire PPO coding sequence and vector junctions were verified by sequencing. The resulting binary vectors, pRD400-SPPO and pRD400-ASPPO, which contained the *PtdPPO1* cDNA in sense and antisense directions, respectively, were transferred into the disarmed *Agrobacterium tumefaciens* strain C58 pMP90 (Koncz and Schell 1986) by electroporation (McCormac et al. 1998). A 35S promoter-GUS binary plasmid (pRD410) (Datla et al. 1992), was also obtained for use as a control construct. Transformation of hybrid aspen (*Populus tremula* × *P. alba* clone INRA 717I-B4) was done according to Leplé et al. (1992) using stems and petioles from in vitro-grown plantlets. After regenerating shoots reached a height of 2–3 cm, they were transferred to root-inducing medium. After plantlets had rooted and reached a height of approximately 10 cm, they were planted in Sunshine Mix #4 (Sungro, Seba Beach, AB, Canada) plus slow-release nutrients (0.458 g l^{-1} superphosphate 0–20–0 (Green Valley Surrey, Canada), –2g l^{-1} Micromax Micronutrients (Scotts-Sierra, Marysville, OH, USA) , 8–6–12 NPK + micronutrients 4.75 g l^{-1} Dolomite lime (IMASCO, Surrey, Canada), and 8.9 g l^{-1} controlled release (Acer, Delta, BC, Canada). All plants were maintained in the greenhouse at the Centre for Forest Biology at the University of Victoria.

Enzyme assays, western blotting, and phenolic analysis

For PPO activity assays, leaves or FTC frass were ground in 100 mM sodium phosphate buffer (pH 7.0) with 0.1% Triton X-100. Extracts were centrifuged at 13,000 rpm and the supernatant was tested for PPO activity using the DOPA (dihydroxyphenylalanine) assay as described previously (Constabel et al. 2000). One enzyme unit was defined as the activity producing a change of 1.0 absorbance unit/min at 490 nm. The protein content was determined by the method of Bradford using bovine serum albumin (BSA) as a standard. For western blots and protein analyses, soluble proteins were separated on SDS–PAGE (12% acrylamide) gels and visualized by Coomassie Blue staining, or transferred to PVDF membranes (BioRad, Mississauga, Canada) for detection using polyclonal antibodies raised against recombinant *PtdPPO1* protein. Immunocomplexes were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (BioRad).

To measure PPO-mediated browning, leaf tissue was ground in 100 mM citrate phosphate buffer (pH 6.0). The supernatant was incubated overnight at room

temperature and the absorbance at 690 nm recorded. This wavelength was chosen as it represented a broad peak in the absorbance spectrum. To quantify total phenolics in control and transgenic foliage, we used the Folin–Ciocalteau method (Singleton and Rossi 1965). Fresh leaf samples (25 mg) were extracted in 10 ml aqueous methanol (80%), centrifuged, and the clarified extract (100 µl) was added to 500 µl Folin–Ciocalteau reagent (Sigma, Oakville, Canada) and mixed. After the addition of 2.5 ml Na_2CO_3 solution (1.8 M), the total volume was brought up to 5 ml with water and the mixture incubated for 20 min. Following centrifugation at 4,000 rpm for 5 min, the absorbance at 735nm was recorded. Tannic acid (Sigma) was used as a standard. All experiments and analyses of transgenic plants were repeated at least three times.

Northern and Southern analyses

For northern blots, total RNA was isolated from young leaves using the procedure described previously (Haruta et al. 2001b). Ten µg total RNA extracted from transgenic and wild-type lines was subjected to electrophoresis on 1.2% w/v agarose denaturing formaldehyde gels, and subsequently transferred onto Hybond N^+ membranes (Amersham, Baie d'Urfé, Canada) using standard procedures (Sambrook and Russell 2001). Membranes were hybridized with the ^{32}P-labelled full-length *PtdPPO1* cDNA using standard procedures (Church and Gilbert 1984) and detected with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, USA).

For Southern analysis, genomic DNA was extracted from untransformed and transgenic aspen lines as previously described (Constabel et al. 2000). Aliquots containing 10 µg genomic DNA were *Eco*RI-digested (Amersham), subjected to electrophoresis on 0.8% w/v agarose gels and transferred onto Hybond N^+ membranes. Hybridization was performed using a 620-bp gel-purified 35S CaMV promoter fragment or the full-length *PtdPPO1* cDNA.

Forest tent caterpillar feeding bioassays

FTC egg bands were obtained from Bob McCron, Canadian Forest Service, Great Lakes Forestry Centre, Sault Ste. Marie, Canada, and stored at −2°C until used. Insect bioassays were conducted in 9-cm-diameter plastic petri dishes lined with moistened filter paper (Whatman#1, Ann Arbor, USA). All hybrid aspen trees used in the experiments had at least 25 leaves. Leaves were designated by the leaf plastochron index (LPI; Larson and Isebrands, 1971); the first developing leaf of lamina length ≥20 mm was designated as the index leaf (LPI 0), and all other leaves were numbered basipetally thereafter. Preliminary tests indicated that leaf toughness and FTC feeding preference were very much dependent on leaf age. Therefore, all leaves used for bioassays were chosen for comparable size, LPI, and toughness.

Choice bioassays were carried out using leaf disks (1.5 cm diameter) punched from a leaf at LPI 12 of control and PPO-overexpressing transgenic lines, and anchored in alternating order with minuten pins. Four third-instar FTC larvae were placed in the center of the dish, and allowed to feed for 48 h. Leaf consumption was recorded with an area meter (LI-3100 Area meter; Li-Cor) or as the weight of the leaf disks before and after feeding. A feeding preference index (FPI), varying from −1.0 to 1.0 (zero indicating no preference), was calculated as follows: FPI = (amount of low-PPO leaf consumed − amount of high-PPO leaf consumed)/(amount of preferred leaf consumed).

No-choice bioassays were also carried out using disks (1.5 cm diameter) punched from either control or PPO-overexpressing hybrid aspen leaves. Depending on the experiment, 2–8 independent PPO-overexpressing and an equal number of control lines were used, with 4–5 replicate dishes for each line. Just-hatched FTC larvae (5–8, depending on size) were placed on 4–6 disks from leaves of LPI 11, which were renewed every 2 days with leaf disks from the next LPI. For a given experiment, all bioassay dishes contained the same number of leaf disks and FTCs. Larvae were weighed every 2 days, and for some experiments the leaf disks were also weighed or the disk area scanned to record leaf consumption. Each experiment lasted 2 weeks, and survivorship and molting in each petri dish were monitored daily, although only final weights and survival are reported here. The average larval survivorship and weight gain within each petri dish was used for statistical analysis, and a one-way ANOVA was performed on the average 2-week weight gain and mortality data using StatsDirect Software (Cheshire, UK).

Results

Transformation and characterization of transgenic *Populus*

In order to test the functional roles of PPO in *Populus*, we overexpressed the hybrid poplar (*P. trichocarpa* × *P. deltoides*) *PtdPPO1* cDNA in transgenic hybrid aspen (*P. tremula* × *P. alba*). Preliminary analysis of leaves of 25 independent transformants indicated up to 50-fold greater PPO activity relative to untransformed controls. Individuals derived from the same transformed line all exhibited very similar levels of PPO activity, and this activity remained stable through multiple rounds of in vitro micropropagation. By contrast, plants transformed with antisense PPO constructs showed no alteration of PPO levels compared to controls. PCR and Southern analyses clearly showed that these plants were indeed transformed and therefore these plants were used for controls in bioassay experiments.

Nine PPO-overexpressing transgenics, as well as one β-glucuronidase (GUS) and one *PtdPPO1*-antisense transgenic line, were chosen for more detailed molecular analyses. PCR analysis using *nptII* gene-specific oligonucleotide primers revealed that the putative transformants all contained this marker gene (data not shown). Foliage from leaves of the highest PPO-overexpressing lines showed PPO activity in the range of 20–40 Units/mg protein, approximately two times greater than that seen in wounded hybrid poplar leaves (Constabel et al. 2000). GUS-transformed, PPO-antisense, and untransformed control plants all had similar and very low PPO activity (Fig. 1a). Southern blots using a CaMV 35S promoter probe confirmed that the GUS, antisense PPO, and PPO-overexpressing transgenics contained one to several copies of this transgene, while in the untransformed line no hybridization signal was detected (Fig. 1c). Re-hybridizing the Southern blot using the *PtdPPO1* cDNA as a probe resulted in the same banding pattern, with the addition of the band corresponding to the endogenous PPO gene (data not shown).

Northern analysis confirmed that the elevated PPO activity in the transgenics was due to the PPO transgene, as levels of PPO mRNA were generally proportional to PPO activity (Fig. 1b). Likewise, western blots using a PPO-specific antibody also showed strongest signals in protein extracts of plants with higher PPO activity. In the lines showing highest overexpression, a series of smaller bands was also detected by the antibody; these likely represent partially degraded PPO, resulting from the very high levels of transgene expression (Fig. 1d). In addition, these westerns showed a faint PPO band in the control and antisense plants, migrating slightly higher than the ectopically expressed PPO. The same band appeared to be present in PPO-overexpressors as well, but was masked by the overexpressed PPO. Based on its constitutive expression and slower migration, this band likely corresponds to the endogenous PPO-2 protein, distinct from the overexpressed *PtdPPO1* gene product, PPO-1 (Wang and Constabel 2003). The putative PPO-2 band had the same intensity in all antisense and control plants tested, and appeared not to be affected by the antisense *PtdPPO1* construct (see Discussion).

To further characterize the transgenics, we compared PPO expression in leaves of different ages along the axis of saplings, in both PPO-overexpressing and untransformed controls. As expected, higher levels of PPO activity were observed in leaves of all developmental stages in the transgenics relative to controls (Fig. 2a). Other independent transgenic lines showed the same pattern (data not shown). Interestingly, PPO activity increased with leaf age (greater LPI), in both, transgenics and controls (Fig. 2a). Western and northern analyses again confirmed that the increased PPO activity in both control and transgenic lines correlated with PPO protein and mRNA levels (Fig. 2b,c). The northern blots also showed that PPO transcript levels are higher in older leaves, suggesting that the higher PPO activity in older leaves is due to higher rates of transcription or greater transcript stability in these leaves.

The role of PPO in tissue browning predicts that PPO-overexpressing leaf extracts should brown or

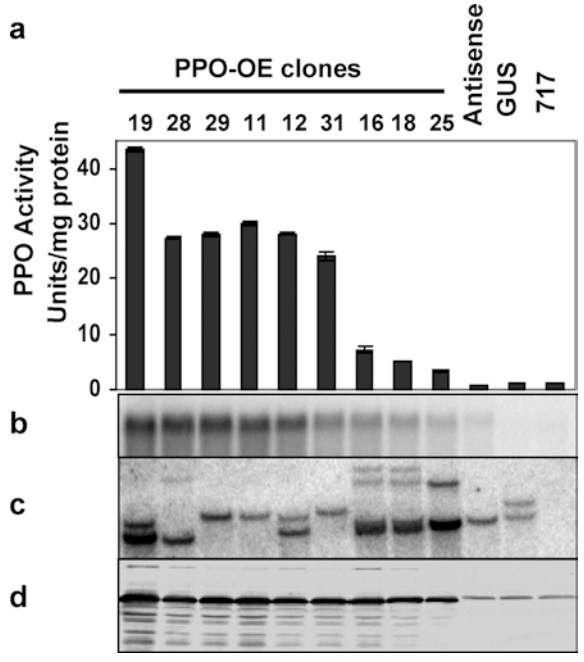

Fig. 1a–d Characterization of PPO expression levels in control and PPO overexpressing transgenics of hybrid aspen (*Populus tremula* × *P. alba*). Leaves at LPI 12 from nine independent PPO-overexpressing lines (PPO-OE), one PPO antisense line, one GUS line and one control hybrid aspen line (717) were analyzed. **a** PPO activity was measured using the DOPA assay. Each measurement was repeated three times and means ± SD are shown. **b** The same tissues as in **a** were analyzed by Northern blotting to show PPO transcript levels. **c** Genomic Southern blot analysis of plants in **a** using a CaMV 35S promoter fragment as a probe. **d** Western blot of the same plants using a polyclonal PPO antibody to show PPO protein levels

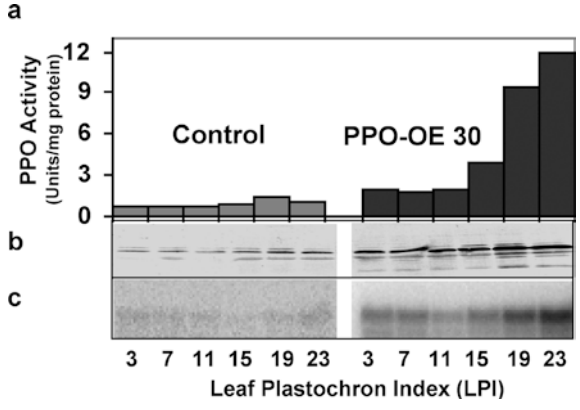

Fig. 2a–c PPO expression in hybrid aspen leaves of different ages sampled from the axis of an untransformed control and a representative PPO-overexpressing (PPO-OE) transgenic plant. Leaves from positions LPI 3 to LPI 23 were analyzed for PPO activity using the DOPA assay (**a**), for PPO protein by western blotting (**b**), and for PPO transcript levels by northern blotting using the *PtdPPO1* cDNA as a probe (**c**)

darken more rapidly than control extracts. To test for such autonomous browning, we prepared fresh leaf extracts of PPO-overexpressing and control plants and incubated these at room temperature (Fig. 3a). Overnight incubation of PPO-overexpressing extracts resulted in strong browning which could be inhibited by the PPO-specific inhibitor tropolone (Kahn and Andrawis 1985), whereas no browning was observed in the control extracts. Absorbance measurements (690 nm) of fresh extracts showed no difference between transgenics and controls, but after the overnight incubation a dramatic increase in A_{690} was seen in the transgenic sample. By contrast, only a small increase in absorbance was visible in the control (Fig. 3b). Tropolone was again effective in inhibiting this difference, thus demonstrating that PPO was responsible for the pronounced darkening. The total phenolic content did not differ significantly between control and transgenic leaves (Fig. 3c). The rapid autonomous browning in transgenic extracts is an important observation, as it suggests that PPO substrates are present in sufficient quantity to sustain high rates of phenol oxidation and quinone formation. Therefore, such plants should be useful for testing the effect of PPO-overexpression on the performance of leaf-eating insects.

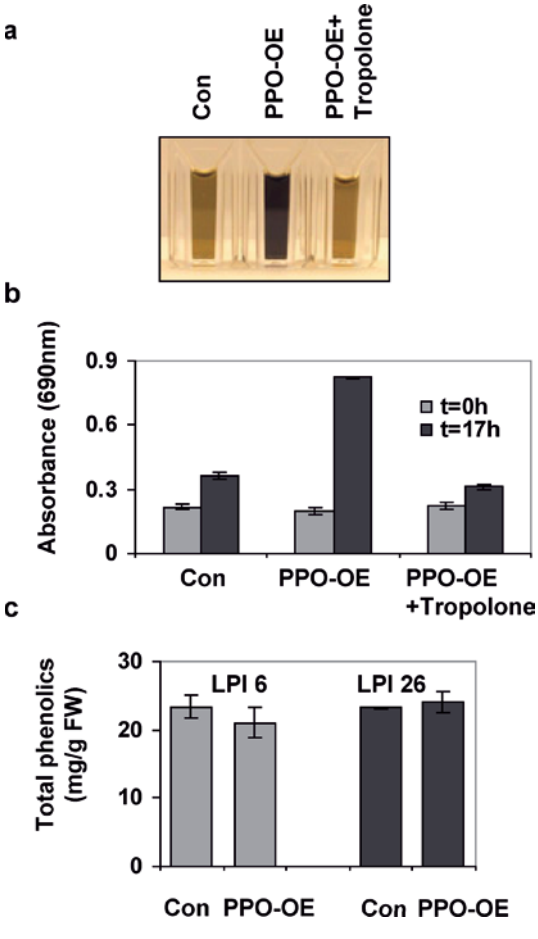

Fig. 3a–c Browning and total leaf phenolics in leaf extracts prepared from untransformed control (Con) and PPO-overexpressing (PPO-OE) hybrid aspen. **a** Photograph illustrating darkening after 17 h incubation of extracts at room temperature. **b** A_{690} of extracts seen in **a** before and after the incubation. **c** Total phenolic content of control and transgenic leaves as measured by the Folin–Ciocalteau method. Triplicate measurements were taken and means ± SD are shown

Forest tent caterpillar (FTC) bioassays

To determine if elevated PPO was detrimental to larval performance or feeding, we conducted a series of bioassay experiments using FTC larvae and leaf disks cut from transgenic and control foliage. We first conducted choice tests using alternating leaf disks. No consistent preference for or against any of the high-PPO lines was detected in nine independent experiments; rather, there was a random feeding bias which likely depended on which type of leaf disk the larvae first encountered (Fig. 4). We concluded that PPO does not have a strong influence on FTC feeding choice.

Next, we conducted a series of no-choice tests comparing FTC performance on control and PPO-overexpressing transgenics. In the first two no-choice experiments, conducted in August 2002, larval weight gain after 2 weeks was not significantly different between controls and PPO overexpressors (Table 1). Likewise, we detected no differences in leaf area consumed from either group of plants; the leaf area consumed from control vs. high-PPO foliage was 28.6 ± 6.3 cm^2 vs. 30.8 ± 7.4 cm^2, and 15.8 ± 4.3 cm^2 vs. 16.5 ± 2.4 cm^2 for experiments 1 and 2, respectively. This indicated that larvae feeding on PPO-overexpressing transgenics did not consume more leaf tissue in order to gain the same weight compared to FTCs feeding on control plants. Since leaf consumption paralleled larval growth, we monitored only the latter in subsequent experiments. Survival was almost 100% in both larval populations in both experiments (Table 1). In experiment 3, FTCs feeding on PPO-overexpressing leaf disks performed more poorly than those feeding on control disks, both in terms of survival and weight gain, although ANOVA

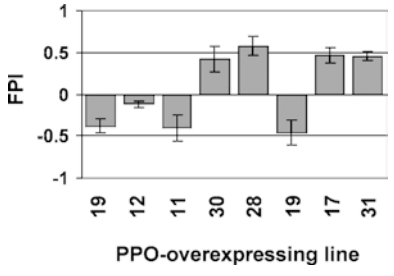

Fig. 4 Choice test comparing FTC feeding preference on leaf disks of PPO-overexpressing and control hybrid aspen. Third-instar FTC larvae were allowed to feed for 48 h on both types of leaf disk, and the amount of foliage consumed determined as described in Materials and methods. FPI, feeding preference index. An index of "0" indicates no preference, while a negative index indicates preference for the PPO-overexpressors

Table 1 Forest tent caterpillar (FTC) performance in no-choice tests on leaf disks from PPO-overexpressing and control hybrid aspen (*Populus tremula* × *P. alba*). First-instar FTC larvae were fed PPO-overexpressing (PPO-OE) and control leaf disks for 14 days as described in Materials and methods. Leaf disks were renewed every 2 days. Egg bands (collected during the winter months) were stored at −2°C until being transferred to ambient temperature on the experimental dates indicated

Hatch date	FTC performance	Treatment		ANOVA		
		Control	PPO-OE	*F*	*P*	*df*
Expt. 1: 01/08/02	Weight (mg)	17.4 ± 4.0	19.0 ± 6.3	0.59	0.45	1, 22
	Survival (%)	95.8 ± 6.2	99.0 ± 3.6	2.30	0.14	1, 22
Expt. 2: 21/08/02	Weight	15.5 ± 5.1	15.8 ± 2.9	0.035	0.85	1, 22
	Survival	98.3 ± 5.8	96.7 ± 7.8	0.35	0.56	1, 22
Expt. 3: 05/09/02	Weight	5.4 ± 1.3	4.6 ± 1.0	1.67	0.22	1, 14
	Survival	92.5 ± 14.9	87.5 ± 14.9	0.45	0.51	1, 14
Expt. 4: 16/09/02	Weight	3.7 ± 0.5	2.9 ± 0.6	23.8	< 0.0001	1, 38
	Survival	75.0 ± 8.9	70.0 ± 12.1	2.2	0.15	1, 38
Expt. 5:17/10/02	Weight	2.5 ± 0.5	1.4 ± 0.6	32.7	< 0.0001	1, 28
	Survival	55.8 ± 14.8	34.1 ± 18.6	12.5	0.0015	1, 28
Expt. 6: 10/07/03	Weight	10.9 ± 2.3	11.9 ± 1.6	2.62	0.11	1, 46
	Survival	81.9 ± 9.7	83.3 ± 12.0	0.196	0.66	1, 46
Expt. 7: 28/09/03	Weight	5.4 ± 1.0	3.26 ± 0.6	118.4	< 0.0001	1, 62
	Survival	68.4 ± 12.3	41.4 ± 8.7	102.9	< 0.0001	1, 62

indicated that this difference was not significant. However, in the subsequent experiments 4 and 5 (late September and October 2002), PPO-overexpressing transgenic leaf disks had a stronger negative effect on growth and survival of FTCs relative to controls (Table 1). This was particularly evident in experiment 5, where survival was reduced to 34% for transgenics compared to 56% for controls (Table 1). In this experiment, at the end of 14 days the larvae reared on high-PPO foliage gained only one-half as much weight as FTCs reared on control leaves (Table 1). In both experiments 4 and 5, there was clearly lower survivorship and slower growth of larvae on both types of foliage compared to earlier experiments, indicating reduced vigor of the FTC larvae. We note that at the beginning of experiment 5, the FTC egg masses had been stored for over 6 months, and it is likely that this extended time in storage reduced the health and vigor of the hatched FTC. We confirmed these results by repeating experiments in 2003 with a new batch of FTC egg bands. As before, we detected no differences in FTC performance on PPO-overexpressing and control leaf disks in experiments carried out during the summer (experiment 6, July 2003), but did find pronounced differences in both survival and weight gain when the experiment was repeated in September/October 2003 (Table 1). Therefore, these experiments demonstrate that PPO can have a direct effect on FTC, and that this effect becomes apparent when FTCs are hatched from aged egg bands with reduced vigor (see Discussion).

Detection of PPO activity in FTC frass

To be an effective anti-nutritive defense, PPO must be at least partially resistant to digestion and inactivation by proteolytic enzymes of the insect gut. To test the stability of PPO in the insect digestive system, we collected frass from PPO overexpressor-fed FTC larvae and from control-fed larvae for PPO analysis. We had previously determined that, like many plant PPOs, poplar PPO is latent and requires either detergents such as SDS, or partial proteolysis using trypsin or other proteases, for activation (Constabel et al. 2000; Wang and Constabel 2003). Therefore we measured PPO activity in the presence and absence of SDS, which would test if PPO in frass had already been activated by passage through the insect gut. No PPO activity was detected in control leaf frass, but significant PPO activity was present in frass from larvae fed on both high and moderately overexpressing lines (Fig. 5a). Interestingly, PPO protein in FTC frass was fully active, and SDS did not increase PPO activity. This suggested that PPO had already been activated by factors within the gut, most likely by proteolytic enzymes. For comparison, extracts of the same leaves that had been used to generate the frass were also assayed for PPO activity; similar levels of PPO were

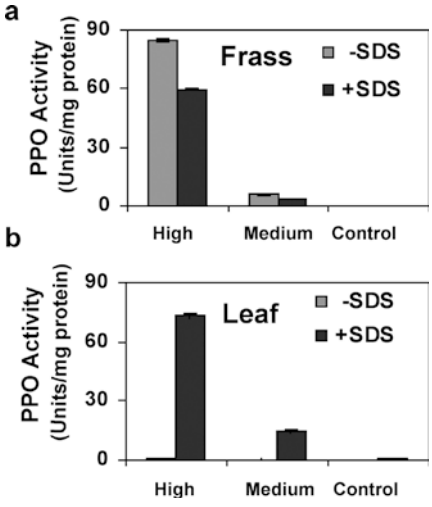

Fig. 5a,b Effect of SDS on PPO activity in FTC frass, and transgenic and control leaves of hybrid aspen. Extracts of FTC frass (**a**) and leaf tissues (**b**) derived from one high-overexpressing PPO (High) transgenic, one medium PPO-overexpressing transgenic (Medium), and one untransformed control line were analyzed for PPO activity (Control). DOPA assays were carried out with or without 0.15% SDS in the assay buffer

detected in these extracts compared to frass extracts, but only if SDS was present in the assay buffer (Fig. 5b). This confirmed the latency of the enzyme.

To determine if the activation of PPO in the caterpillar gut might be due to partial proteolysis of PPO, frass protein was analyzed by SDS–PAGE and western blotting. Both endogenous PPO and overexpressed PPO were clearly detected by western blot in control and transgenic extracts, respectively, but with different expression levels as expected (Fig. 6a, left lanes). In frass extracts, bands corresponding to PPO were again detected; however, here the major PPO band migrated faster, at approximately 40 kDa (Fig. 6a, right lanes). The shift in mass is likely due to partial proteolysis by gut enzymes, and this may be the mechanism of activation of PPO observed in Fig. 5. Activation of PPO by protease digestion has been demonstrated previously (Robinson and Dry 1992), and in our earlier work with poplar PPO protein we were able to activate latent PPO with trypsin (Wang and Constabel 2003). The intensity of the PPO bands in frass indicated that a significant proportion of leaf PPO remained relatively intact after digestion. Coomassie-stained SDS gels corroborated this observation; overexpressed PPO was visible as a minor band among the other proteins in transgenic leaf extracts (Fig. 6b, left lanes), and a faint band of similar intensity which corresponds to the processed PPO, was seen in the trangenic frass extract (Fig. 6b, right lanes). In frass, however, most of the other proteins seen in leaves had disappeared, presumably due to the digestive process, whereas PPO remained visible. Therefore, it appears that PPO is more resistant to digestive enzymes than most other leaf proteins.

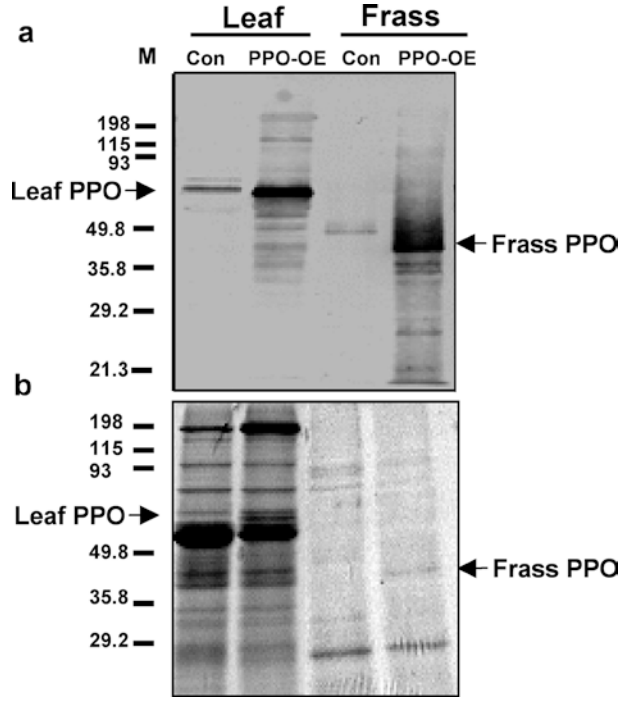

Fig. 6a,b Proteolytic processing of hybrid aspen leaf PPO protein after passage through FTC digestive tract. **a** Western blot analysis showing PPO protein in transgenic PPO-overexpressing (PPO-OE) and untransformed (Con) leaves and FTC frass. **b** Replicate SDS gel as in **a**, stained with Coomassie Blue to reveal total protein

Discussion

PPO-overexpressing hybrid aspen plants

In this study, we used transgenic plants to demonstrate the effects of the proposed defense and anti-herbivore enzyme, PPO. The wound- and herbivore-inducible *PtdPPO1* gene from hybrid poplar was placed under the control of the double CaMV 35S promoter and a partial alfalfa mosaic virus translational enhancer (Datla et al. 1993). Our results confirmed the efficacy of this enhanced promoter in conferring high levels of constitutive transgene expression in leaf tissues of hybrid poplar (Figs. 1, 2). PPO expression levels were dramatically higher (up to 50-fold) compared to untransformed controls or GUS transgenics. However, no phenotypic abnormalities were observed for any PPO-overexpressing plants.

The successful overexpression of PPO was contrasted by the antisense PPO transgenics, which did not show any phenotype or reduced PPO levels, despite both PCR and Southern analyses confirming they had been transformed successfully. All antisense plants analyzed had similar PPO activity compared to untransformed or GUS-transformed controls (Fig. 1 and data not shown). Likewise, no difference was observed in wounding experiments. However, we have noted that in the 717I-B4 hybrid aspen genotype, wounding does not induce strong PPO expression; therefore, it is likely that the endogenous PPO levels of this genotype were too low for antisense suppression to have had a significant and detectable impact. On western blots, the expression of the constitutive PPO-2 protein, faintly visible as a slightly slower migrating PPO band, was not affected by the antisense *PtdPPO1* transgene (Fig. 1d). We recently isolated a cDNA corresponding to PPO-2 from hybrid poplar (*PtdPPO2*), where it is expressed primarily in conducting organs such as petioles and stems (Wang and Constabel 2004). This PPO shares only 66% nucleotide identity with the *PtdPPO1* cDNA used in our transgenics, and this level of sequence similarity would not have been sufficient for antisense suppression of *PtdPPO2*.

In autonomous browning experiments, we found that extracts of PPO-overexpressing poplar leaves turned brown more rapidly than those from control leaves (Fig. 3). This demonstrated that the endogenous phenolic substrate pool was sufficiently large to sustain rapid phenolic oxidation for a significant time, even though total phenolic levels were no greater than in untransformed controls. A similar phenotype was described for PPO-overexpressing tomato plants, where more rapid browning was also observed in transgenics,

but total phenolics were not altered (Li and Steffens 2002). This implies that it is PPO, rather than its substrate, that normally limits phenolic oxidation following tissue damage. From a biotechnological perspective, it suggests that overexpression of PPO alone could have a significant impact on improving oxidative defenses. In poplar, the endogenous PPO substrates have not yet been unambiguously identified. We previously suggested that in trembling aspen, breakdown products derived from tremulacin, salicortin, or other phenolic glycosides are potential PPO substrates (Haruta et al. 2001a), and future experiments will address the issue of PPO substrates in more detail.

Folin-Ciocalteau assays indicated that the levels of total phenolics in the PPO-overexpressing transformants and controls are similar (Fig. 3c), confirming previous observations that PPO does not appear to influence overall phenolic synthesis in transgenics (Li and Steffens 2002). Although PPO in some plants has recently been shown to have a role as a specific hydroxylase in the biosynthesis of aurones and lignans (Nakayama et al. 2000; Cho et al. 2003), our *PtdPPO1* data suggest a biosynthetic role is unlikely for *PtdPPO*. Earlier suggestions that PPOs might be required in the general phenylpropanoid pathway for the 3′-hydroxylation of *p*-coumaric acid have been ruled out by the identification in *Arabidopsis* of a P450-type hydroxylase that is responsible for this step (Humphreys and Chapple 2002).

Effects of PPO overexpression on herbivores

Although we detected no effects of high PPO on FTC in choice tests, our no-choice bioassay data clearly indicated that high PPO activity in PPO-overexpressing *Populus* can reduce FTC performance (Table 1). However, significant effects on survival and development were detected only when FTC larvae were hatched later in the season (September/October), when egg masses had been stored for at least 6 months. Even when fed with control leaf disks, FTC larvae grew more slowly and had higher mortality as the season progressed (Table 1). The hatch and development rate of FTC is known to decrease with time in storage (Bob McCron, personal communication). This suggests that decreased vigor might have made the FTC more vulnerable to the detrimental effects of PPO in our experiments. We observed this egg band age-dependent PPO sensitivity in two consecutive years with two different batches of egg bands. Therefore, we conclude that in poplar, PPO can act as a defense against FTC under certain conditions. To our knowledge, this is the first use of transgenic plants to demonstrate an antiherbivore role for a plant PPO.

The observation that PPO can be effective against already weakened FTCs is important because in nature, insect herbivores may be confronted with a number of stresses simultaneously. For example, in the boreal forest of western Canada, FTCs are commonly exposed to nuclear polyhedrosis virus, which weakens larvae and may lead to increased susceptibility to plant defenses (Ebling and Kaupp 1997). Furthermore, PPO is induced together with other defense proteins; there are at least four different wound-induced trypsin inhibitors, as well as a number of chitinases and other defense proteins in hybrid poplar (Parsons et al. 1989; Bradshaw et al. 1990; Christopher et al. 2004). In trembling aspen, wounding also leads to induction of condensed tannins (Peters and Constabel 2002). Therefore, in herbivore-induced foliage, PPO is only one of a number of constitutive and induced defenses that may work synergistically against FTC and other herbivores. The combined effects of antiinsect factors (Bt toxin and phytochemical defenses) on gypsy moth larvae were recently demonstrated in transgenic *Populus* (Kleiner et al. 2003).

The conclusion that PPO acts like a defense protein in poplar is substantiated by our finding that this enzyme is resistant to proteolytic enzymes of the caterpillar gut (Fig. 6). The PPO protein band remained visible on Coomassie-stained gels after passage through the FTC digestive system, while the majority of other proteins disappeared or were reduced in abundance. Even predominant protein bands such as ribulose bisphosphate carboxylase had disappeared entirely from the frass (Fig. 6b). Enzyme assays indicated that PPO was still active, further demonstrating the resistance of PPO to digestive enzymes. Interestingly, PPO activity extracted from leaves is latent and usually requires activation by detergents or other factors such as proteolytic cleavage (Constabel et al. 2000; Wang and Constabel 2003). By contrast, PPO extracted from frass was fully active and could not be activated further with SDS; evidently, passage through the FTC digestive tract overcame this latency and activated poplar PPO. The mechanism by which this occurs could be partial proteolysis, as the PPO visible in the frass appears to have been proteolytically cleaved and migrated faster than PPO extracted from leaves (Fig. 6). Proteolytic processing as a means of activating PPO is well known, and in grape the in vivo active form lacks the C-terminal portion of the protein (Robinson and Dry 1992). Therefore, the latency of PPO, which has so long perplexed plant biochemists, can here be seen as an adaptative strategy in defense, keeping the enzyme inactive until it is in the insect gut. This would ensure that the PPO-mediated oxidative reactions and quinones are produced within the insect, where the potential impact is maximized.

Our data suggest that PPO can be added to the list of potential transgenes for use in genetically engineering pest resistance into *Populus*. Other successfully overexpressed genes for *Populus* plant protection include those encoding the *Bacillus thuringiensis* (Bt) d-endotoxin (Robison et al. 1994), a soybean Kunitz trypsin inhibitor (Confalonieri et al. 1998), an *Arabidopsis* cysteine proteinase inhibitor (Delledonne et al. 2001), and tryptophan decarboxylase (Gill et al. 2003). Before PPO-overexpressing plants can be deployed for biotechnology, their effectiveness should be demonstrated

under field conditions. Nevertheless, the results of this study suggest an important potential role of PPO in anti-herbivore defense.

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