Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates

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The biochemical anti-herbivore defense of trembling aspen (*Populus tremuloides* Michx.) was investigated in a molecular analysis of polyphenol oxidase (PPO; EC 1.10.3.2). A PPO cDNA was isolated from a trembling aspen wounded leaf cDNA library and its nucleotide sequence determined. Southern analysis indicated the presence of two PPO genes in the trembling aspen genome. Expression of PPO was found to be induced after herbivory by forest tent caterpillar, by wounding, and by methyl jasmonate treatment. Wound induction was systemic, and occurred in unwounded leaves on wounded plants. This pattern of expression is consistent with a role of this enzyme in insect defense. A search for potential PPO substrates in ethanolic aspen leaf extracts using electron spin resonance (ESR) found no pre-existing diphenolic compounds. However, following a brief delay and several additions of oxygen, an ESR signal specific for catechol was detected. The source of this catechol was most likely the aspen phenolic glycosides tremulacin or salicortin which decomposed during ESR experiments. This was subsequently confirmed in experiments using pure salicortin.

Introduction

Trembling aspen (*Populus tremuloides* Michx.) is an ecologically and economically important forest tree found throughout North America. It is a host for a number of defoliating insects, which at times may cause substantial damage (Peterson and Peterson 1992). In the boreal forest of Canada, for example, the forest tent caterpillar (*Malacosoma disstria*; FTC) can completely defoliate large areas of forest during periodic outbreaks. The loss of forest productivity because of defoliating insects is a major motivation for investigating how trembling aspen–insect interactions are mediated and for elucidating the endogenous defense systems of trembling aspen and other forest trees. A better understanding of defense in trembling aspen may also contribute to our understanding of the forces driving FTC population cycles (Haukioja 1980).

In trembling aspen, both protein- and secondary metabolite-based defenses have been described. Herbivore-inducible trypsin inhibitor genes were recently cloned in this laboratory, and the trypsin inhibitor protein was shown to accumulate in leaves within 2 days of damage (Haruta et al. 2001). This induction is systemic, or plant-wide, suggesting that this is an active defense, which may reduce subsequent damage. In addition, trembling aspen leaves constitutively accumulate high levels of condensed tannins and phenolic glycosides, which appear to play a role in aspen defense against insects (Lindroth and Hwang 1996). In particular, two complex glucosides of salicyl alcohol, salicortin and tremulacin, have been shown to be detrimental to several lepidopteran defoliators (Clausen et al. 1989, Lindroth and Bloomer 1991, Lindroth and Kinney 1998). Although the mechanism of toxicity has not been clearly established, these compounds are unstable and may be broken down during insect feeding by esterases and β-glucosidases (Clausen et al. 1989, Julkunen-Tiitto and Meier 1992). Their biological activity has therefore been proposed to be a result of smaller toxic products such as phenol and catechol (Clausen et al. 1989). However, salicortin can also act as an inactivator of β-glucosidase from *Agrobacterium faecalis* (Zhu et al. 1998), a finding that emphasizes the complex nature of these phenolics.

Catechol and other diphenolic compounds are readily oxidized by the enzyme polyphenol oxidase (PPO), a process

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Abbreviations – ESR, electron spin resonance; FTC, forest tent caterpillar; MeJa, methyl jasmonate; PPO, polyphenol oxidase.

which enhances the anti-insect activity of phenolics by producing highly reactive *o*-quinones (Felton et al. 1989, Duffey and Felton 1991). Preliminary tests had indicated that trembling aspen leaves contain significant levels of PPO (J. Patton and C. P. Constabel, unpublished data). The possible involvement of PPO in phenolic glycoside toxicity and aspen defense prompted us to investigate PPO in trembling aspen. PPO is a copper-containing enzyme that catalyzes the oxidation of *o*-diphenols to *o*-quinones using molecular oxygen (diphenolase activity; EC 1.10.3.2; reviewed in Steffens et al. 1994). The polymerization and cross-linking of the *o*-quinone products lead to the commonly observed browning of injured plant tissues and extracts (Joslyn and Pontig 1951). The *o*-quinones also readily attack and alkylate a variety of biologically important molecules including amino acids and proteins; this is the basis of the anti-nutritive function of PPO against chewing insects (Felton et al. 1989, Duffey and Felton 1991). Cysteine, lysine, histidine, and methionine residues are preferentially alkylated, which prevents their assimilation (Felton et al. 1992, Hurrell and Finot 1984). As these are essential amino acids, a diet rich in PPO can lead to nutritional deficiencies and a suppression of larval growth (Felton et al. 1989, Duffey and Stout 1996). In tomato, the importance of PPO in defense was confirmed by the finding that it is induced by the tomato herbivore defense signal systemin (Constabel et al. 1995). Other species in which PPO is systemically wound-induced include potato, tobacco, and hybrid poplar (Thipyapong et al. 1995, Constabel and Ryan 1998, Constabel et al. 2000).

PPO cDNAs have been isolated from a number of plants, including hybrid poplar (Constabel et al. 2000), and biochemical studies have established the relatively broad substrate specificity of this enzyme (reviewed in Mayer and Harel 1979). The availability of the hybrid poplar PPO cDNA provided an opportunity to investigate PPO in trembling aspen, a species with well-characterized phenolic secondary metabolites. First, we cloned trembling aspen PPO and established that its expression is herbivore- and wound-induced, indicating it is likely to play a defensive role in this forest tree. We then made use of ESR spectroscopy to identify possible diphenolic PPO substrates. Our results indicate that the major detectable diphenolic compound available as a PPO substrate is catechol, which appears to be derived from phenolic glycosides. Based on these results, we propose that PPO contributes to trembling aspen defense by enhancing the toxicity of phenolic glycoside-derived catechol.

Materials and methods

Plant material and treatments

Native trembling aspen (*Populus tremuloides* Michx.) were obtained locally and grown in peat (Terra-lite Redi-Earth, WR Grace, Ajax, Ontario, Canada). All plant material was maintained in the University of Alberta Biotron's growth chambers under 16 h of light (250 μmol m^{-2} s^{-1}) at 18°C and 75% relative humidity. Plants were watered daily with a solution containing 1 g l^{-1} 20-20-20 fertilizer (Plant-Prod, Plant Products Co., Brampton, Ontario, Canada). Experiments were generally carried out with 10–12-week-old plants having 20–25 leaves, although for some experiments, larger plants were substituted. Leaves were numbered sequentially beginning with the youngest unfolded leaf, and leaves 9–15 were used for experiments.

Three types of induction treatments were performed. Wounding treatment was carried out by crushing leaf margins with a hemostat 3 times at 2-h intervals. Plants were exposed to methyl jasmonate (MeJa) by placing them in sealed glass boxes with 20 μl of a 10% solution of MeJa in ethanol on a cotton wick. Forest tent caterpillar (*Malacosoma disstria*; FTC) treatment was performed by placing plants in 75-cm cages with 10 fourth and fifth instar larvae. Leaves were harvested 24 h after wounding and MeJa treatments and 36 h after FTC treatment, then frozen in liquid N$_2$ and stored at -80°C until analyzed. For time course experiments, 6 leaves per plant were wounded, and both wounded and upper unwounded leaves were harvested at various times. All experiments were performed at least 3 times.

cDNA library screening, sequencing, and hybridization analyses

A Lambda ZAP II cDNA library (Stratagene, La Jolla, CA, USA) was screened using a ^{32}P-labeled poplar PPO cDNA (Constabel et al. 2000) according to standard methods. Positive plaques were purified, excised in vivo, and sequenced in both directions with the Thermosequenase dye terminator cycle sequencing system (Amersham, Baie d'Urfé, QC, Canada).

For Southern blots, 10 μg of genomic DNA isolated using the method of Doyle and Doyle (1990) was digested with 50 U of *Hin*dIII, *Xba*I, *Bam*HI, *Eco*RI, or *Eco*RV (Gibco/BRL, Rockville, MD, USA) for 30 min at 37°C. An additional 25 U of the restriction enzymes was added and digestion allowed to proceed for 24 h. Restriction fragments were desalted by ethanol precipitation and Southern blots carried out with a full-length ^{32}P-labeled cDNA using standard procedures (Sambrook et al. 1989). Hybridization signals were detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) or recorded by autoradiography. RNA was extracted from 500 mg crushed leaves using the extraction protocol of Chang et al. (1993), with the addition of one phenol and one phenol/chloroform extraction step following the LiCl precipitation. For northern analyses, 20 μg of total RNA was electrophoresed through 1.2% agarose-formaldehyde gels, blotted, and hybridized as described above.

Analysis of trembling aspen phenolics

Leaves were ground in 4 volumes 95% ethanol using a mortar and pestle and extracted overnight. The extracts were then filtered (Whatman No. 1) and used directly in ESR experiments. Twenty microliters of the extract was mixed with 5 μl 0.1–0.2 *M* NaOH, whereafter 10 μl of the mixture in a capillary was introduced into the magnetic resonance cavity and electron spin resonance (ESR) spectra

recorded. Spectra were run on a Bruker ER300 spectrometer with 100 kHz modulation frequency. All ESR spectra were obtained at room temperature. Pure salicortin was a gift from Dr T. Clausen, University of Alaska.

Results and discussion

Cloning and sequence analysis of a trembling aspen PPO cDNA

We screened a trembling aspen cDNA library constructed from wounded aspen leaves, using a hybrid poplar PPO cDNA as a probe. From the large number of positive plaques obtained, 3 were purified, the plasmids excised, and subjected to DNA sequence analysis. The largest clone was selected, and its entire sequence determined on both strands. Database comparison confirmed its identity as trembling aspen PPO, and it was named PtPPO. The full-length cDNA was 1922 bp in length and included an open reading frame of 563 amino acids with a predicted molecular mass of 64.5 kDa. Removal of the predicted chloroplast transit peptide (Fig. 1) would give rise to a mature protein of 59 kDa. The deduced amino acid sequence shows significant identity with other plant PPOs, in particular those from the woody plants hybrid poplar (91.9%), apple (56.6%), apricot (54.2%), and grape (54.0%). The copper-binding domains within the sequence (Fig. 1) are more conserved; for example, the CuA and CuB regions of PtPPO show 86.5% and 76.3% identity with those of apple, respectively.

Analysis of the PtPPO gene family

To determine if PtPPO belongs to a multigene family, Southern blot analysis was carried out. As trembling aspen from natural populations (as we are using in this study) have high levels of heterozygosity, we tested two aspen genotypes; both gave the identical banding pattern (Fig. 2). Southern blots, washed at high stringency, showed two bands when DNA was digested with HindIII and XbaI, and 4 bands after EcoRV digestion (Fig. 2). The PtPPO sequence contains a single internal EcoRV site but no HindIII or XbaI sites; furthermore, it is unlikely to contain introns based on genomic sequence data of tomato PPO (Hunt et al. 1993). This indicates that two copies of PPO gene are present in the aspen genome. We conclude that each band most probably represents a different PPO gene, rather than different alleles at the same locus, as in wild aspen most alleles would be variable and produce restriction fragment polymorphisms. In hybrid poplar, PPO is also present as a small gene family with at least two members (Constabel et al. 2000). In apple, there are 4 PPO genes (Boss et al. 1995). In contrast, other species such as tomato have been found to contain as many as 7 PPO genes, although only one is wound-induced (Hunt et al. 1993). In hybrid poplar, preliminary analyses suggest there are at least one wound-inducible and one constitutively expressed PPO isoform (J. Wang and C. P. Constabel, unpublished data). It will be interesting to compare expression of individual PPO genes in both trembling aspen and hybrid poplar.

Induction of PtPPO expression by wounding, herbivory, and methyl jasmonate

Preliminary experiments indicated that undamaged leaves contain little detectable PPO mRNA when analyzed by northern blot, but that it accumulates following tissue damage. We therefore tested different types of induction treatments for an effect on PPO mRNA levels. Feeding by FTC for 36 h resulted in a dramatic increase in PPO mRNA, migrating at approximately 2.1 kb, as did mechanical wounding with a hemostat to simulate chewing insect attacks (Fig. 3). In contrast, no PPO mRNA was detected in control leaves. These experiments demonstrated the inducibility of PtPPO by simulated and real herbivory, a first indication of a defensive role of PPO in trembling aspen. Methyl jasmonate (MeJa), an inducer for wound and stress responses in many plant species including hybrid poplar (Weiler 1997, Constabel et al. 2000), also strongly induced PtPPO mRNA (Fig. 3).

Fig. 1. Nucleotide and predicted amino acid sequence of PtPPO. Double-underlined sequence corresponds to the predicted chloroplast transit peptide; single-underlined sequences are copper-binding domains. The PtPPO nucleotide sequence has been deposited in GenBank, accession no. AF 368291.
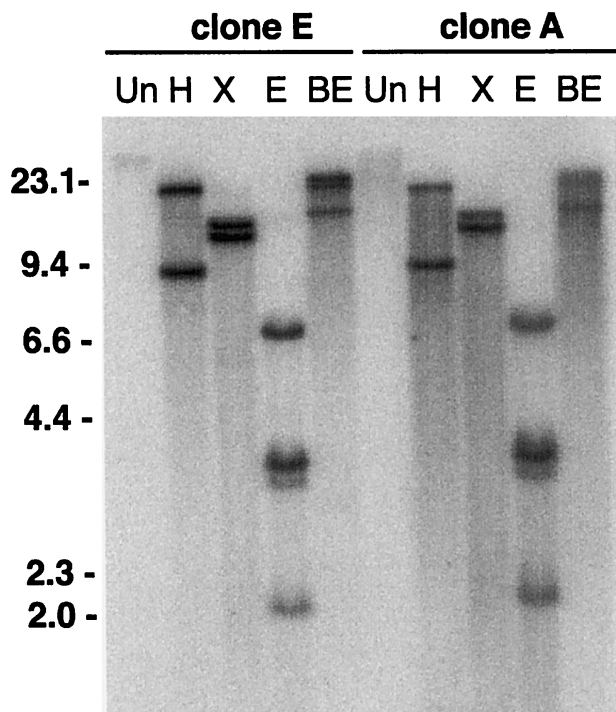
Fig. 2. Southern analysis of the PtPPO gene family in trembling aspen. Genomic DNA from two different genotypes was digested with restriction enzymes, electrophoresed and blotted according to standard protocols. Clones E and A represent different genotypes of aspen. Un, undigested DNA; H, *Hin*dIII; X, *Xba*I; E, *Eco*RV; BE, *Bam*HI and *Eco*RI.

The induction of PPO mRNA was characterized further. To study the kinetics of PtPPO mRNA induction, we carried out time course experiments. As previous work on defense gene induction indicated that trembling aspen can respond to tissue damage with a systemic defense response (Haruta et al. 2001), we monitored PPO expression in both wounded and unwounded leaves on wounded plants. The accumulation of PtPPO mRNA in the wounded leaves was first detected 6 h after wounding and reached a maximum
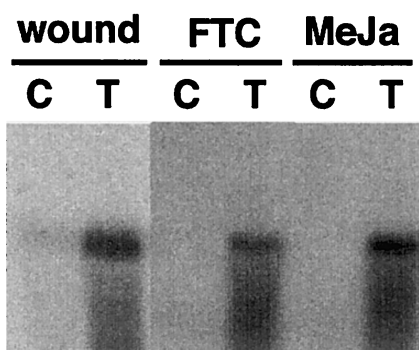

Fig. 3. Induction of PtPPO mRNA by mechanical wounding, FTC treatment, and MeJa treatments. Plants were treated as described in Materials and methods, and leaves harvested 24 h (wounding and MeJa treatment) or 36 h (FTC herbivory) later for RNA extraction and northern analysis. Twenty micrograms of total RNA was separated on agarose gels, blotted, and hybridized with ^{32}P-labeled PtPPO cDNA. C, control; T, treated.
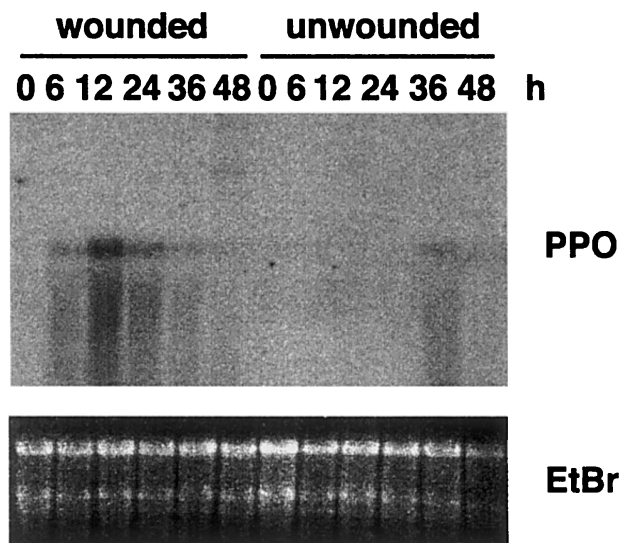
Fig. 4. Time course of wound-induced PtPPO gene expression. Leaves were wounded as described in Materials and methods, and harvested at the indicated times and analyzed by northern blot. Unwounded leaves are from wounded plants. Upper panel, image of blot hybridized with ^{32}P-labeled PtPPO cDNA. Bottom panel, the ethidium bromide stained gel as a loading control.

between 12 and 24 h (Fig. 4). In unwounded (systemic) leaves, PtPPO induction was observed after 36 h. The delay in systemic PPO expression may be a result of the additional time required for the wound signal to move to the distal leaves. Induction of PPO in the systemic leaves was weaker than in the wounded leaves (Fig. 4) but was clearly demonstrated in several independent experiments. When wound induction of PtPPO was monitored in leaves of differing ages, a strong dependence on leaf age was observed. Young leaves showed a stronger response to wounding than older leaves, with very old leaves not responding to wounding with PPO induction at all (data not shown). This pattern of expression is likely to be important in defense, as young leaves are more vulnerable to many herbivores than are older, tougher leaves (Coley 1980). Induction of PPO activity in aspen leaves following wound treatment was also observed, with an average induction of approximately two-fold (not shown). While relatively modest, this increase was consistently observed in several independent experiments, indicating that the observed induction of PPO mRNA does result in increased PPO protein accumulation as well. The PPO assays were complicated by significant inter-plant variability and a high level of background PPO activity from constitutive PPO expression. In some control leaves, PPO activity was as high as 800 μmol substrate oxidized min mg^{-1} soluble protein (using 3,4-dihydroxyphenylalanine as the substrate), which made it difficult to measure a strong increase in PPO activity above the basal level. PPO assays using methyl catechol gave the same results (not shown). Therefore, monitoring PPO mRNA levels was considered to be a more sensitive indicator for PPO induction.

The inducible PPO gene expression we have observed is consistent with a role for PPO in trembling aspen defense against herbivores, and parallels the pattern of expression

for Kunitz trypsin inhibitors (Haruta et al. 2001). Like other plant species, trembling aspen appears to induce a number of defense genes in response to herbivore attack, which are likely regulated by a signaling system that includes jasmonates and octadecanoid signaling molecules (Weiler 1997). Overall, the pattern of expression of PPO in trembling aspen is similar to that of hybrid poplar PPO (Constabel et al. 2000). Significant PPO expression may be common within the Salicaceae, as in a *Salix* species very high PPO levels were also measured although no wound induction was observed (Constabel and Ryan 1998). Systemic induction of PPO mRNA was also previously reported in potato and tomato (Thipyapong et al. 1995, Constabel et al. 1996).

Analysis of trembling aspen phenolics as PPO substrates

We next investigated potential PPO substrates in trembling aspen leaves. To identify potential diphenolic compounds in crude extracts, we made use of ESR spectroscopy. This technique is a powerful tool in the detection and identification of simple diphenols in crude extracts, as demonstrated in an examination of phenolics from leaves of 365 species in the Lamiaceae (Pedersen 2000). Aromatic compounds with free *o*- or *p*-dihydroxy groupings (*o*- or *p*-hydroquinone) are oxidized in base to semiquinone radicals, which are then detected and identified, even in crude extracts, by way of their unique spectra (Pedersen 1978). Usually, the oxidation of existing quinols to semiquinone radicals occurs immediately because of the presence of molecular oxygen from the atmosphere, the oxygen being simultaneously reduced to superoxide.

When we first applied this ESR method to ethanolic aspen leaf extracts, no diphenolics were detected. Only after adding extra oxygen to the solution by letting air bubbles pass through the capillary sample did a weak spectrum consisting of 9 lines become apparent, which became stronger with additional additions of oxygen (Fig. 5). This spectrum had an intensity sequence 1:2:1:2:4:2:1:2:1 and hyperfine splitting constants $a(3) = a(6) = 0.66$ G and $a(4) = a(5) = 3.66$ G, all of which are diagnostic for catechol (1,2-dihydroxybenzene). The identical spectrum was obtained with the pure compound (data not shown), so that we were able to unambiguously identify the spectrum from the

Fig. 5. ESR semiquinone spectrum showing the unique 9-line profile obtained from a crude aspen leaf extract. An indistinguishable spectrum is obtained from a sample of pure catechol, and from pure salicortin, as described in Results. Bar = 2 G.
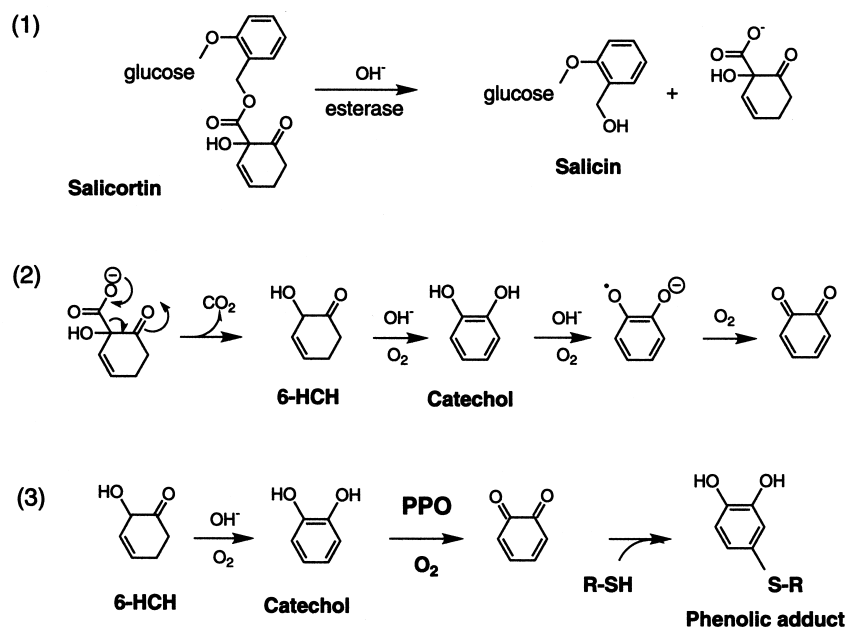
aspen extract as being derived from catechol. In the ESR run with pure catechol, however, the signal was generated instantaneously and without a delay or additional oxygen. These results suggested that exposure of the leaf extracts to the basic ESR reaction mixture in the presence of O_2 released catechol from some progenitor compounds, possibly the aspen phenolic glycosides salicortin and tremulacin. Catechol is an excellent substrate for trembling aspen PPO (J. J. Patton and C. P. Constabel, unpublished data). Furthermore, it had previously been demonstrated that under basic conditions, catechol is formed as a decomposition product of salicortin and tremulacin (Pearl and Darling 1971).

In order to determine if the catechol detected could be generated from the aspen phenolic glycosides, pure salicortin was analyzed by ESR as above. As with the leaf extracts, no catechol was detected in the first ESR run. However, after 4–6 additions of air through the capillary, the identical 9-line spectrum seen earlier appeared, growing in intensity with extra additions of oxygen. Thus, in both crude and pure samples tested by way of the ESR technique we get the same course of 'events' (Fig. 6, reactions 1 and 2). The catechol spectrum appears most clearly in an alkaline environment (pH > 10) with sufficient oxygen, and it increases in intensity with additional oxygen. These experiments suggested that the catechol generated in aspen extracts by our experimental conditions is indeed derived from the salicortin and, most likely, tremulacin as well. Although we have not tested tremulacin using ESR, it is very similar to salicortin and is subject to the same decomposition pattern (Julkunen-Tiitto and Meier 1992; see below).

Whether these reactions all occur spontaneously in the basic environment of lepidopteran insect midguts (around pH 10 for FTC; Barbehenn and Martin 1994) is not yet clear; however, there is evidence that the breakdown of salicortin and tremulacin also occurs enzymatically. Clausen et al. (1989) showed that when trembling aspen leaves are crushed, 6-hydroxy-2-cyclohexenone (6-HCH) is produced from salicortin and tremulacin by an unidentified enzyme. Furthermore, Julkunen-Tiitto and Meier (1992) found that 6-HCH is released from salicortin after incubation with plant β-glucosidase, and from both salicortin and tremulacin after treatment with mammalian esterases. 6-HCH is unstable and easily converted to catechol under the mildly alkaline conditions found in lepidopteran midguts (Fig. 6; Clausen et al. 1989). Therefore, catechol may be continuously generated from salicortin or tremulacin during herbivory; we propose that this catechol is the substrate for aspen PPO. Oxidation of catechol by PPO then results in *o*-quinone formation, subsequent alkylation of dietary protein, and ultimately, in reduced insect nutrition and health (Fig. 6, reaction 3; Duffey and Stout 1996).

It is significant that we found no evidence for the presence of any other diphenolic compounds in aspen leaves using the ESR method. Therefore, we suggest that the major PPO substrate in aspen leaves is the catechol formed as a decomposition product of the phenolic glycosides. Our proposal, that PPO is involved in aspen herbivore defense based on the oxidation of salicortin- and tremulacin-derived catechol,

Fig. 6. The production of catechol from salicortin and its oxidation during ESR experiments and defense reactions. (1) Salicortin is hydrolyzed by a strong base, or by an esterase during tissue maceration and herbivory. (2) Catechol and its semiquinone are formed from unstable intermediates by strong base (pH > 10) during ESR experiments. The semiquinone is detected based on characteristic ESR spectra. (3) Reactions proposed to occur during insect feeding in lepidopteran guts (pH ~ 10). 6-HCH is converted to catechol, which is subsequently oxidized by PPO. The resulting o-quinone alkylates a free sulfhydryl group, for example, a cysteine residue within polypeptides, resulting in a protein with one or more phenolic adducts. Free amino groups on amino acids and proteins may also be alkylated (not shown).

Summary and conclusions

In conclusion, we have cloned and sequenced a trembling aspen PPO cDNA. Using northern analyses, we have demonstrated that PPO gene expression is induced systemically by wounding, by forest tent caterpillar feeding, and by MeJa. We propose that the major in vivo PPO substrate is catechol released from the phenolic glycoside salicortin, and that release of catechol and subsequent PPO oxidation is a possible mechanism of toxicity of trembling aspen phenolic glycosides. Plant defense in trembling aspen therefore involves both protein- and phytochemical-based components. Such complexity as a result of an interaction of phytochemicals and plant proteins in a defense context is becoming increasingly apparent in many plant-herbivore interactions (Duffey and Stout 1996). The presence of well-characterized phytochemicals and inducible protein-based defenses makes trembling aspen an interesting experimental system for studies of plant–herbivore interactions.

adds an additional layer of complexity to trembling aspen chemical ecology. Although salicortin and tremulacin of aspen have been extensively studied as defensive agents, their effects on herbivores are poorly understood. This report suggests a new mechanism, extending the hypothesis originally put forward by Clausen et al. (1989). These authors had suggested that it is the decomposition products (especially 6-HCH and catechol), rather than the glycosides themselves, that are responsible for phenolic glycoside toxicity. Although catechol has anti-insect activity on its own (Clausen et al. 1989, Duffey and Stout 1996), its effects would be enhanced by oxidation to the o-quinone with its strong alkylating activity (Felton et al. 1992). PPO, both constitutive or induced, would therefore be important for the defensive potential of phenolic glycosides, compounds that are very abundant in trembling aspen foliage (up to 7% dry weight; Lindroth and Hwang 1996). Definitive proof of our hypothesis will clearly require additional experiments. However, our data do provide a model that can be tested by other methods, such as direct quantitation of both the phenolic glycosides and their proposed decomposition products.

One can speculate that such enzyme-mediated enhancement of chemical toxicity is a strategy by which plants can modulate the strength of their phytochemical defense. PPO induction by herbivory may thus increase the defensive potential of the leaf without requiring the up-regulation of an entire secondary metabolic pathway. Phenolic glycosides such as salicortin and tremulacin are widespread in the Salicaceae (Palo 1984). Many species within these genera also contain significant levels of PPO activity, although enzyme levels and wound inducibility is variable. Given the potential connection between phenolic glycosides and PPO demonstrated here, it will be interesting to see if high PPO activity and high phenolic glycoside levels commonly co-occur in this plant family.

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