University of Alberta

Analysis of the Expression and Function of Polyphenol Oxidase in Hybrid Poplar

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of Doctor of Philosophy

in

Molecular Biology and Genetics

Department of Biological Sciences

Edmonton, Alberta
Spring, 2004

Your file Votre référence
ISBN: 0-612-96330-6
Our file Notre référence
ISBN: 0-612-96330-6

Abstract

In order to probe the physiological functions and expression of polyphenol oxidase (PPO) in hybrid poplar (*Populus trichocarpa x P. deltoides*), full-length cDNAs of three tissue-specific *PtdPPOs* were obtained and characterized. Based on the results of northern blots using gene-specific probes, *PtdPPO1* is a wound-inducible leaf PPO; *PtdPPO2* is a constitutive and wounding- inducible PPO mainly found in conducting organs; and *PtdPPO3* is root-specific and does not respond to stresses tested. Distinct distribution among hybrid poplar organs in addition to their differential responses to wounding and defense elicitors indicate that these three PPOs may perform different physiological roles in hybrid poplar. In order to directly test the defensive role of PPO in herbivore defense, *PtdPPO1*-overexpressing hybrid aspen (*Populus tremula x P. alba*) was constructed and forest tent caterpillar (FTC) was tested for their survivorship and weight gain when reared on those transgenics. We found that PPO1 negatively affected the growth and survivorship of FTC when the FTC larvae were hatched from old egg mass, which had low vigor. This confirmed the anti-herbivore role of PPO1 in hybrid poplar. In order to investigate the biochemical properties of hybrid poplar PPOs, PPO1 and PPO2 proteins were partially purified through hydrophobic chromatography. Biochemical analysis showed that these two PPO proteins exhibited somewhat different enzymatic properties such as storage stability, heat sensibility, SDS activation, pH optima and substrate preferences. In summary, this work thoroughly investigated the expression and induction patterns of different PPO genes in hybrid poplar, directly confirmed the defensive role of PPO1 in *Populus* herbivore defense and revealed that the three different PPO proteins in hybrid poplar possess distinct enzymatic properties.

Acknowledgements

I would like to extend great appreciation to my supervisor Dr. C. Peter Constabel, for his guidance and support of this research work. To the Constabel lab, Ian Major, Robin Mellway, Dr. Miyoshi Haruta, Dr. Manoela Miranda, Dr. Mary Christopher, Joe Patton, Nicole Dafoe, thanks for the all the help and advice you always give to me. Thanks to my family, my husband, Mom, Dad, and my brother, for the love, encouragement over the past five years that I have always received and relied on.

This research was funded by the Natural Sciences and Engineering Research Council of Canada (NSERC). I gratefully acknowledge funding from a FS Chia Fellowship and teaching assistantship from the University of Alberta.

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List of Abbreviations

4-CH	4-Cinnamic Acid Hydroxylase
ABA	Abscisic Acid
AMV	Alfalfa Mosaic Virus
BCIP	5-Bromo-4-Chloro-3-Indoyl Phosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine Serum Albumin
BZIP	Basic Domain Leucine Zipper
CaMV	Cauliflower Mosaic Virus
CTAB	Cetyltrimethylammonium Bromide
DEAE	Diethylaminoethyl
DOPA	Dihydroxyphenylalanine
ERF	Ethylene-Responsive-Element-Binding Factors
FC	Furanocoumarins
FTC	Forest Tent Caterpillar
GUS	β-Glucuronidase
HPLC	High Performance Liquid Chromatography
ISR	Induced Systemic Resistance
JA	Jasmonate
JGI	Joint Genome Institute
LOX	Lipoxygenase
LPI	Leaf Plastochron Index
MeJA	Methyl Jasmonate
MeSA	Methyl Salicylic Acid
NBT	Nitroblue Tetrazolium Chloride
NO	Nitric Oxide
NPT	Neomycin Phosphotransferase
PAL	Phenylalanine Ammonia Lyase
PCR	Polymerase Chain Reaction
PI	Protease Inhibitor
PPO	Polyphenol Oxidase

PPO-OE	PPO Overexpressing
PR	Pathogenesis-related
PVPP	Polyvinylpolypyrrolidone
RACE	Rapid Amplification of cDNA Ends
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcriptase PCR
SA	Salicylic Acid
SAR	Systemic Acquired Resistance
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS Polyacrylamide Gel Electrophoresis
TNB	2-Nitro-5-Thiobenzoic Acid
VOCs	Volatile Organic Compounds

Chapter 1 General Introduction

Plants have developed sophisticated defensive adaptations to different stresses including herbivore and pathogen attack. Such adaptations include stress-induced proteins and secondary metabolites. One such enzyme is polyphenol oxidase (PPO), which is found in many plant species and whose activity often increases following injury or stress. It was previously shown that following herbivore damage, the nutritional quality of tomato foliage decreases, in part due to elevated PPO activity (Duffey and Felton, 1991). Recently, it was also shown that overexpression of polyphenol oxidase in transgenic tomato plants results in enhanced bacterial disease resistance (Li and Steffens, 2002). However, the defensive function of PPO still needs to be confirmed in other plants, in particular because levels of PPO expression can vary widely (Constabel and Ryan, 1998). The objective of this work is to use several approaches to further characterize PPO in hybrid poplar (*Populus trichocarpa x P. deltoides*), with the ultimate aim of better understanding the physiological functions of PPO in this economically important model system for tree biology. In order to achieve this aim, I (1) cloned multiple PPO cDNAs from hybrid poplar and characterized their individual expression and induction patterns under normal or stress conditions (Chapter Two), (2) experimentally overexpressed the level of PPO protein in transgenic *Populus* and assessed its subsequent protective effect against insect herbivores (Chapter Three), and (3) examined the biochemical properties of two partially purified PPO isoforms from poplar shoots (Chapter Four). In this chapter, I review the literature relevant to this project, covering three areas: (1) plant polyphenol oxidases, (2) plant defense against herbivores and pathogens, and (3) hybrid poplar as a model system for tree biology. Further background information is included in the introductory section of each chapter. In Chapter Five, I draw some general conclusions and discuss the project's overall significance.

1.1 Plant Polyphenol Oxidase (PPO)

Polyphenol oxidase (PPO) and its phenolic substrates are widespread among higher plants and fungi. Although the oxidative browning caused by the PPO-generated quinones are sometimes desirable for food products such as raisins, the browning of

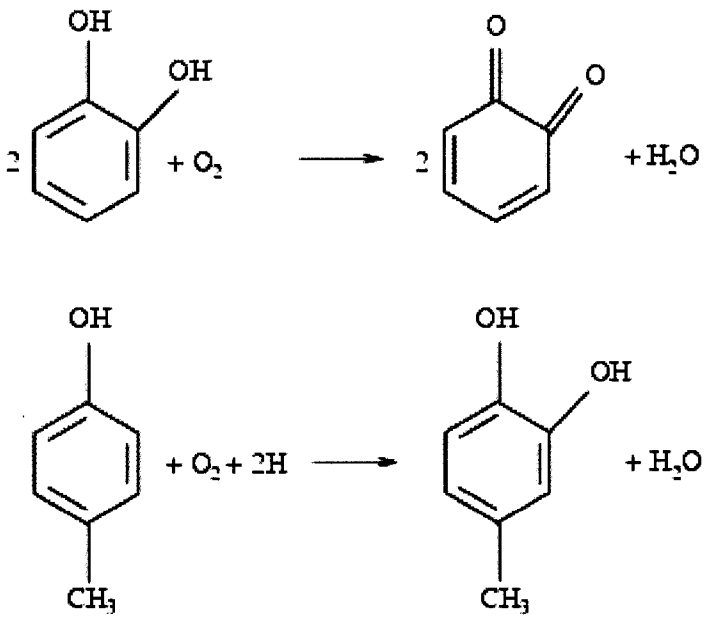
damaged plant tissue has been a big concern in the food industry for many years (Vamos-Vigyazo, 1981). The purpose of this section is to provide basic background on the enzymatic properties of PPO and review current progress towards understanding its expression patterns and functions in different plant species.

1.1.1 Enzymatic Properties of PPO

Polyphenol oxidase (PPO) catalyzes the oxidation of *o*-diphenols to *o*-diquinones [EC 1.10.3.2] as well as the *o*-hydroxylation of monophenols [EC 1.14.18.1] (Vaughn and Duke, 1984) (Fig. 1). PPOs may exhibit either or both of these activities, but the monophenol oxidase activity is often labile or requires priming with reducing agents or trace amounts of *o*-diphenols (Gowda and Paul, 2002; Steffens et al., 1994). Regardless of the substrates utilized, *o*-quinones are the final reaction products. PPO-generated quinones are highly reactive, electrophilic molecules, which covalently modify and crosslink a variety of cellular constituents, including nucleophiles of proteins such as sulfhydryl, amine, amide, indole, and imidazole substituents (Duffey and Felton, 1991). The formation of quinone adducts (usually brown or black colored) represents the primary detrimental effect of PPO in food processing and the post-harvest physiology of plant products (Mathew and Parpia, 1971). Because of the considerable economic and nutritional loss caused by PPO enzymatic browning in the food industry, numerous studies have been devoted to the study of the biochemical and catalytic properties of PPOs. Recently, transgenic apple and potato containing antisense PPO genes have been shown to have reduced PPO activity and, consequently, exhibit a lower browning potential than control plants (Coetzer et al., 2001; Murata et al., 2000). This technology is applicable to a wide range of other crops where enzymatic browning has a deleterious effect on product quality, storage or processing. PPOs from plants and fungi are bicopper metalloenzymes and the most highly conserved features observed in PPO sequences are the two Cu^{++} binding sites, called CuA and CuB. The active site of PPO consists of a pair of copper ions, which are each bound by three conserved histidine residues (His) (Gaykema et al., 1984). This copper pair is the site of interaction of PPO with both molecular oxygen and its phenolic substrates. For example, there is 92%

Figure 1: Reactions catalyzed by PPO.

(A) Diphenolase (catechol oxidase) activity. (B) Monophenolase (tyrosinase, cresolase) activity.



amino-acid sequence similarity in the CuA binding regions between broad bean and tomato PPO, but only 42% sequence similarity overall (Van Gelder et al., 1997). In plant PPO, a third histidine-rich domain, CuC, was identified near the carboxy terminus, which is absent in fungal PPO (Steffens et al., 1994).

Although PPOs extracted from some plant sources, such as bean, corn, and tomato are fully active, a frequent feature of plant PPO is its latency (Constabel et al., 1996). Latent PPO enzyme can be activated by aging; for example, during senescence of spinach leaf, an increase in PPO activity was shown to be due to enzyme activation (Meyer and Biehl, 1981). During PPO extraction procedures, a number of processes such as acid shock or release of endogenous proteases can also activate the latent form of PPO. A broad spectrum of exogenous substances can activate PPO in crude tissue preparations, such as ammonium sulfate (Swain et al., 1966), detergents (Espín and Wichers, 1999; Moore and Flurkey, 1990), and protease (Robinson and Dry, 1992). This activation by several seemingly unrelated substances is generally attributed to conformational changes or solubilization of the enzyme, or the removal of an inhibitor (Van Gelder et al., 1997). In the case of *Vicia faba* (broad bean) PPO, it was shown that the active site is blocked in the absence of SDS, but not in its presence (Moore and Flurkey, 1990). Although *in vivo* regulatory mechanisms of PPO are as yet unknown, it is thought that endogenous protease(s) might be involved. In grapevine, it has been shown that the active 40 kDa form of PPO is synthesized as a inactive precursor protein of 60 kDa, and normal processing is interrupted in a mutant resulting in the accumulation of the 60 kDa inactive form of PPO (Rathjen and Robinson, 1992).

1.1.2 Plastidic Localization and Processing of PPO

There is a general agreement in the literature that plant *PPO* is a nuclear-encoded gene and its protein product is targeted to the internal lumen of thylakoid membranes in plastids (Keegstra et al., 1989; Vaughn et al., 1988). PPO is present in a variety of tissue-specific plastids including amyloplasts, leucoplasts, etioplasts and chromoplasts (Vaughn and Duke, 1984), as well as in the plastids of epidermal cells and glandular trichomes (Yu et al., 1992). As with all nuclear-encoded plastid enzymes, plant PPO precursor polypeptides possess N-terminal plastid transit peptides. The peptide bond cleaved by the

processing proteases was identified as alanine-serine or alanine-alanine in all plant PPO preproteins studied to date (Van Gelder et al., 1997). Based on the model by Douwe de Boer and Weisbeek (1991), it is thought that PPO transit peptide is characterized by the presence of three domains (Douwe de Boer and Weisbeek, 1991; Joy et al., 1995). The high content of hydroxylated amino acids at the N terminus is a common and specific feature of chloroplast-targeted proteins. The middle section is more loosely conserved and called the 'n'-region. The final 25 amino acids at the C-terminal form a hydrophobic domain, which is the thylakoid transfer domain (lumen targeting). PPO transit peptides contain regions for two-step processing to a mature protein. For instance, the 67 kDa tomato PPO preprotein is first imported into the stroma, then processed to 62 kDa by a stromal peptidase. Subsequently, translocation into the lumen, which is light dependent, involves processing to yield a 59 kDa soluble product (Sommer et al., 1994).

Although almost all plant PPOs characterized to date contain transit peptides, the deduced N-terminal sequence of aureusidin synthase, a polyphenol oxidase homolog in snapdragon (*Antirrhinum majus)* does not contain any plastid and thylakoid targeting features (Nakayama et al., 2000). Therefore, this aureusidin synthase, which catalyzes the hydroxylation and/or oxidative cyclization of chalcones during aureusidin synthesis, is unlikely to be localized in plastids (Nakayama et al., 2000). Instead, this enzyme is more likely to occur in vacuoles, where aureusidin synthase substrates are found (Nakayama et al., 2000). No transit peptides have been detected in fungal PPOs and they are thus thought to be cytoplasmic (Van Gelder et al., 1997).

Due to its localization in plastids, under normal circumstances plant PPO is separated from its phenolic substrates. Only in damaged cells, in which the physical barriers between the substrates in the vacuole and enzyme in the plastids are broken down, can PPO oxidize phenolic substrates (Vaughn et al., 1988). The identity of PPO phenolic substrates varies from one plant species to another and substrate concentration could also change at different developmental stages. For example, catechins and dactylifric acids are the principal polyphenolic enzymatic browning substrates present in dates, particularly in green dates (Hasegawa and Maier, 1980), and their concentration decreases steadily during dates fruit ripening and storage as browning proceeds (Hasegawa and Maier, 1980). Chlorogenic acid is one of the major *o*-diphenolic

substrates of PPO in tomato leaves (Li and Steffens, 2002), and chlorogenic acid and catechin are major polyphenols in apple fruit. In aspen leaves, no evidence for the presence of any diphenolic compounds was found using ESR (electron spin resonance); however, it was suggested that the major PPO substrate in aspen leaves is catechol formed as a decomposition product of the phenolic glycosides tremulacin or salicortin (Haruta et al., 2001b).

1.1.3 Spatial and Temporal Regulation of PPO

The expression level of PPO varies in different organs, tissues, and cell types. In tomato, the most abundant PPO transcripts were found in the mesophyll of young leaves, phloem cells in the stem, type VI trichomes, floral meristem, pollen mother cells, pollen tubes, style, and ovules in the flower (Shahar et al., 1992; Thipyapong et al., 1997). PPO activity in potato (*Solanum tuberosum*) was high in stolons, tubers, roots, and flowers but low in leaves and stems (Hunt et al., 1993). In addition, PPO activity was greatest at the potato tuber exterior, including the skin and cortex tissue 1 to 2 mm beneath the skin (Hunt et al., 1993). As in tomato, potato flowers had high PPO activity throughout development, particularly in the anthers and ovary (Hunt et al., 1993). In grape, high levels of PPO gene expression were found in young developing berries, leaves and roots (Dry and Robinson, 1994). In strawberry fruit, PPO was localized almost exclusively in the cortex and to a lesser extent in the pith (Lopez-Serrano and Barcelo, 2001). In apple fruit, however, PPO activity was localized mainly near the core (Murata et al., 1993).

PPO expression is not only controlled spatially, but typically depends on the developmental age of the tissues. At different developmental stages, PPO activity can be regulated at the transcriptional levels or by the stability and turnover rate of PPO protein. For many reported plant PPOs, the mRNA level has been shown to be highest in young tissues and in meristematic regions of flowers and fruits, with PPO gene expression generally decreasing during development and maturation of plant tissues (Chevalier et al., 1999; Dry and Robinson, 1994; Hunt et al., 1993; Shahar et al., 1992). The abundance of PPO protein in tubers and fruits at early stages of development along with high levels phenolic substrates has led to suggestions of a possible role in making the unripe fruit and storage organs unpalatable to predators (Mayer and Harel, 1981). In addition to the

transcriptional control of PPO expression, protein stability and turnover rate also affect PPO level at specific developmental stages. In tomato, PPO mRNA is detectable only at the first leaf node, but not in older parts of the plant. Likewise, the amount of detectable PPO protein, and PPO activity, diminishes rapidly from the apex in tomato as it ages (Shahar et al., 1992; Thipyapong et al., 1997). In potato, although the leaf PPO mRNA is also developmentally regulated and only detectable in young foliage (apical leaf nodes 1-3), the protein profile of PPO remains constant from the apical node through the eleventh leaf node, which indicates slow turnover of potato PPO protein (Hunt et al., 1993). Broad bean, however, exhibits a different kind of expression pattern in that its PPO transcripts are present throughout leaf development, and abundant PPO protein can be found in mature leaves (Cary et al., 1992). Spatial and temporal regulation of PPO expression also exists in monocots. In sugarcane, a C4 grass, PPO mRNA level and enzyme activity is highest in the growing point and declines down the stalk (Bucheli et al., 1996). In wheat kernels, PPO activity is high at the early stages of kernel development and decreases with kernel maturation (Kruger, 1976).

Differential expression of PPO among various plant tissues and developmental stages suggests a complex pattern of PPO mRNA accumulation and protein turnover. More intriguingly, distribution and regulation of PPO have been found in some plant species to be based on the complex and overlapping expressions of different genes (see 1.1.4). However, to date only a few plants have been studied at this level.

1.1.4 Differential Expression of PPO Gene Family Members

In some plant species, PPO genes were shown to be present as a multigene family (Cary et al., 1992; Newman et al., 1993; Thygesen et al., 1995), whereas in others only a single PPO gene has been identified (Dry and Robinson, 1994). Tomato PPOs are encoded by a seven-member multigene family (*PPO A, A', B, C, D, E* and *F*) clustered within a 165-kb locus on chromosome 8 (Newman et al., 1993). These seven PPO sequences are highly conserved within their coding regions, but their 5' flanking regions are divergent (Newman et al., 1993). Further studies demonstrated that tomato PPO gene family members are differentially expressed in both vegetative and reproductive tissues (Thipyapong et al., 1997). For example, *PPO B* is the most abundant in young leaves,

while *PPO D* is only expressed in type VI trichomes (Thipyapong et al., 1997). As in tomato, there appear to be a number of different PPO genes in potato plants. Five PPO cDNAs have been cloned from potato tuber, each with a specific spatial and temporal pattern of expression (Thygesen et al., 1995). The observation that plants control PPO gene expression with different genes exhibiting distinct patterns of spatial and temporal expression has led to the speculation that individual PPO genes possess physiologically distinct roles (see 1.1.6). Newman et al. (1993) classified the seven PPO genes of tomato into three classes on the basis of restriction fragment length polymorphisms. Later, because of the extensive homology between individual potato and tomato PPO genes, Thygesen et al. (1995) assigned the five potato tuber PPOs to three tomato PPO classes and attempted to attribute some functional significance to this conservation based on their tissue specificity. Thipyapong and Steffens (1997) isolated and analyzed the promoters responsible for regulating the tissue-specific expression of PPO genes and found that only one of the seven tomato PPO genes responds to pathogen and wounding, while the majority is regulated by developmental signals.

Unlike tomato and potato, in most plant species multiple PPO genes still remain to be characterized, and expression studies are based on individual genes. For example, using an apricot fruit PPO cDNA fragment as a probe, PPO transcripts were not detectable in leaves, wounded leaves, and stems, even though they contain high PPO activity (Chevalier et al., 1999). This discrepancy may be explained by the presence of other highly divergent PPOs in these tissues, not detectable with the fruit PPO probe. The finding of the existence of multiple differentially expressed PPO genes within one plant species is both exciting and challenging. On one hand, it provides an excellent opportunity to discover the physiological roles of PPOs in plants based on their tissue-specificity and distinct regulation. On the other hand, it implies that PPO is a versatile enzyme and may display unexpected functions.

1.1.5 Inducibility of PPO by Stress and its Implication for Defense

Inducibility of PPO by abiotic stresses has been demonstrated in some plant species. For example, treatment of tomato roots with heat or chloroform results in elevated PPO levels (Gentile et al., 1988). Resistance to cadmium accumulation in

members of the Nymphaeaceae family has also been connected to the plants' ability to induce PPO activity in epidermal structures and therefore trap the cadmium crystals by polymerized phenols (Lavid et al., 2001).

Mechanical wounding has been shown to induce PPO activity in many plants. In some cases, the latency of PPO makes it difficult to determine whether wounding induces PPO activity *via* enzyme activation or gene expression and *de novo* protein synthesis. For example, in midribs of lettuce, wounding caused an exponential increase in PPO activity due to the activation of latent PPO (Cantos et al., 2001). With the recent availability of cloned PPO genes from many species, it has been shown that induction of PPO by wounding is often regulated at the transcriptional level. For example, expression of PPO mRNA is strongly up-regulated in response to chilling and wounding in pineapple roots, leaves, inflorescence tissues, and developing fruits (Stewart et al., 2001). Apple PPO was induced at the transcriptional level in wounded fruit tissues, as well as in peel tissue showing the symptoms of superficial scald, a post-harvest disorder (Boss et al., 1995). Wounding lower leaves of young tomato plants dramatically induced PPO activity in both wounded and unwounded leaves (i.e. systemic induction) (Constabel et al., 1995), which was later demonstrated to be due to the transcriptional induction of the tomato *PPO F* gene (Thipyapong and Steffens, 1997). Systemic induction of PPO activity based on increased levels of PPO mRNA was also observed in potato (*Solanum tuberosum*) leaves in response to wounding (Thipyapong et al., 1995).

PPOs can be induced by herbivore feeding and pathogen infestation in some species, and this provides some evidence for PPO's role in plant defense response. For example, tomato PPO activity is inducible by *Pseudomonas syringae* infestation (Bashan et al., 1987). In the leaves of hybrid poplar (*Populus trichocarpa x P. deltoides*) and trembling aspen (*Populus tremuloides*), both mechanical wounding and herbivore feeding could induce PPO expression at the transcriptional level (Constabel et al., 2000; Haruta et al., 2001b). In some plant species, it has been observed that the induction of PPO mRNA by wounding or biotic stresses was much more dramatic in young tissues than in old tissues (Constabel et al., 2000; Haruta et al., 2001b; Thipyapong et al., 1995) and it was suggested that only those tissues developmentally competent to express PPO mRNA are

capable of responding to a systemic wounding signal by the increased accumulation of PPO mRNA (Thipyapong et al., 1995).

In addition to mechanical wounding, herbivore feeding and pathogen infestation, signaling molecules involved in plant defense have also been tested for their ability to induce PPO expression. In tobacco and tomato, methyl jasmonate (MeJA) can induce an increase of PPO activity up to 24-fold and 46-fold, respectively (Constabel and Ryan, 1998). Exposure of coffee leaves to MeJA increased PPO activity 1.5–4-fold (Mazzafera and Robinson, 2000). PPO synthesis is also induced systemically in tomato by supplying excised plants with systemin, a plant peptide hormone and a critical mobile defense signaling molecule (Constabel et al., 1995). In prosystemin-overexpressing tomato plants, PPO activity was elevated up to 70-fold compared to control leaves (Constabel et al., 1995; McGurl et al., 1994). In mutations that suppress *35S::prosystemin*-mediated phenotypes, systemin-induced PPO gene expression is blocked (Howe et al., 1996). These results indicated that induction of PPO in tomato relies on the peptide systemic wounding signal systemin.

The induction of PPO in some plant species by stress and signaling molecules supports previous suggestions that PPO activity functions as an enzymatic defense. However, PPO induction does not appear to be universal. In plants with little or no induction of PPO by wounding or treatment with MeJA (Constabel and Ryan, 1998), the relationship between PPO and plant defense is still an open question.

1.1.6 Proposed Physiological Functions for Plant PPO

Due to its inducibility by many environmental factors including wounding, herbivore feeding, pathogen attack, and plant defense signaling molecules, PPO has been implicated as an important defensive protein. However, its induction by such stresses is only supporting, but not conclusive, evidence for its defensive function. Although examples of correlations between PPO activity and resistance could be found in coffee varieties resistant to coffee rust (Maxemiuc-Nacache and Dietrich, 1985) and mung bean varieties resistant to *Rhizoctonia solani* (Arora and Bajaj, 1985), there are other studies in which no correlation or negative correlations have been obtained between PPO activity and disease resistance. Thus, no generalizations can be made regarding PPO and disease

resistance based on different PPO levels and resistance or susceptibility. PPO function remained somewhat ambiguous until recently, when the defensive role against pathogen of PPO was directly demonstrated in transgenic tomato plants that overexpressed a potato PPO cDNA under control of the cauliflower mosaic virus (CaMV) 35S promoter. The transgenic plants showed up to 30-fold increases in PPO transcript level and 5- to 10-fold increases in PPO activity and immunodetectable PPO. As expected, these PPO-overexpressing transgenic plants oxidized the endogenous phenolic substrate pool at a higher rate than control plants. More importantly, these PPO-overexpressing tomato plants exhibited greatly increased resistance to *P. syringae*, which demonstrated the importance of PPO-mediated phenolic oxidation in restricting plant disease development.

Besides its anti-pathogen role, an antiherbivore function of PPO protein has been widely accepted in at least two cases. The first one is the role of PPO in polymerization of trichome exudates (Kowalski et al., 1992). In this case, contact of *Solanum* type-A trichomes by small-bodied insect pests such as aphids or leafhoppers causes the membrane-enclosed head of the trichome to rupture (Kowalski et al., 1992). Trichome contents coat the legs and mouthparts of the insect and immediately begin to polymerize, brown and harden (Kowalski et al., 1992). As the insect breaks additional trichomes, the dark polymerized accretions on the tarsi and mouthparts increase dramatically, disrupting insect feeding by restricting movement, occluding the mouthparts, or entrapping the insect on the leaf surface (Kowalski et al., 1992). Disease incidence of *Phytophthora infestans* among wild potato hybrid lines has also been shown to be correlated negatively with type-A trichome density and with PPO activity of type A trichome glands (Lai et al., 2000).

A second possible role played by PPO in herbivore defense is its antinutritive effect against leaf-feeding insects. Upon insect wounding and feeding, PPO-generated quinones can react with the nucleophilic amino acids of dietary proteins, reducing the digestibility, palatability, and nutritive value of the plant tissue to herbivores (Duffey and Felton, 1991; Felton et al., 1989; Mayer and Harel, 1981). The action of PPO in this role has been most thoroughly explored in a model suggested by Felton et al. (1989). The primary targets of quinones formed by PPO are nucleophilic amino acids, including His, Cys, Met, Trp, and Lys, whose low abundance limits insect growth on plant diets (Duffey

and Felton, 1991). Covalent modification of these essential amino acids further decreases their nutritional availability to herbivores and may result in poorer herbivore performance (Felton et al., 1989). In support of this hypothesis, Duffey and Felton (1991) showed that relative growth rates of S*podoptera exigua* (beet armyworm) and *Heliothis zea* (tomato fruitworm) on artificial diet were significantly decreased when the protein source was previously alkylated by incubation in media containing chlorogenic acid, a common PPO substrate, and PPO protein. Similarly, when the protein component of an artificial diet was composed of tomato leaf protein from homogenates in which PPO activity was not inhibited, relative growth rates of both *S. exigua* and *H. zea* were significantly decreased relative to insects reared on diets in which tomato leaf protein was isolated in the presence of PPO inhibition (Duffey and Felton, 1991).

Besides its roles involved in plant defense, other functions have also been proposed for PPO. Due to its ability to carry out the hydroxylation of many phenolic compounds *in vitro*, it has been assumed that PPO was involved in synthesis of these compounds *in vivo*. Nevertheless, complete elimination of PPO activity from the tissue of developing mung bean seedlings by treatment with tentoxin, a fungal toxin inhibiting the transfer of PPO into thylakoid membrane and its processing into a active form, had no effect on the synthesis of endogenous phenolic compounds including caffeic acid or the orthohydroxylated flavonoids, rutin and delphinidin (Duke and Vaughn, 1982). Recently, a *p*-coumarate 3-hydroxylase (C3H) was identified and shown to actively convert the 5-*o*-shikimate and 5-*o*-D-quinate esters of *p*-coumaric acid into their corresponding caffeic acid conjugates (Schoch et al., 2001). The characterization of C3H enzyme in the synthesis of normal lignin and secondary metabolites in *Arabidopsis* supported the previous conclusion that PPO is not required for the hydroxylation of *p*-coumaric acid.

Due to its localization in mesophyll thylakoid membranes and its high K_m for O_2, PPO was proposed to function in regulating plastidic oxygen levels (Vaughn and Duke, 1984). Co-fractionation of *Vicia faba* PPO with PSII suggested that PPO may also be involved in some aspects of chloroplast metabolism (Lax and Vaughn, 1991). In addition, PPO may interact with PSII through competition with the 23 kDa protein of the oxygen-evolving complex for transport into the lumen (Hind et al., 1995). However, no suitable PPO substrate in chloroplasts has been found and a proposed role for PPO in

photosynthesis also fails to account for the abundance of the enzyme in non-photosynthetic organs such as roots, tubers, and flowers (Constabel et al., 1996). In a genetically modified PPO-null plant, total abolishment of PPO activity did not have any effects on the normal functions of chloroplast, which argues against a possible role of PPO in photosynthesis (Steffens et al., 1994).

PPO is the central topic of this thesis, and my doctoral research has focused on studying PPO, in particular as a potential defensive protein in hybrid poplar. Plant defense is a complicated system and PPO is only one of many defense mechanisms. To better understand the context of PPO protein in plant defense, in the next section, some general background on plant defense will be presented.

1.2 Overview of Plant Defense

In their natural environments, plants rely on both preformed and inducible defense systems to defend themselves against biotic attackers including viruses, bacteria, fungi, nematodes and insects. Structural attributes are always used by plants to protect themselves from attackers by impeding pathogen or anthropod access to tissues. Pre-existing defense structures such as surface waxes, thick epidermal cell walls, position of stomata and lenticels, and leaf hairs are involved in pathogen defense, while thorns, silica and trichomes are typically involved in herbivore defense. Preformed structural barriers are the first line of plant defense and they are undoubtedly important in preventing disease and herbivory. However, constitutive defensive structures can be overcome and therefore plants also rely on biochemical defense mechanisms to protect themselves. Biochemical defense will be presented below, based on either preformed and inducible plant secondary metabolites or defensive proteins.

1.2.1 Preformed and Inducible Secondary Metabolites Involved in Defense

Under normal growing conditions, plants synthesize a broad range of secondary metabolites, such as alkaloids and terpenoids that are toxic to herbivores and pathogens, and, thus, believed to act as defense compounds. These compounds often accumulate and are stored in plants, so that when attacked, the plant already has the means to deter, or kill the pathogen or herbivore. In general, plant secondary metabolites that have negative

effects on the growth, development or survival of another organism can be regarded as toxins (Wittstock and Gershenzon, 2002). While the modes of action of many other toxic secondary metabolites still await discovery, the mechanisms of action of some plant secondary metabolites are well known. For example, saponins are glycosylated triterpenoid, steroid, or steroidal alkaloid molecules that occur constitutively in many plant species (Osbourn, 1996). Because of their capability to disrupt cellular membranes, saponins have been implicated as antimicrobial phytoprotectants (Osbourn, 1996).

A wide variety of secondary compounds are constitutively produced by a subset of plant tissues or cells to serve as defensive agents against herbivores. Their chemical complexity is one of the greatest challenges facing herbivorous insects (Li et al., 2002b). These secondary metabolites are usually toxic compounds that have antixenotic (i.e. deterring herbivore colonization of plants) or antibiotic (i.e. deterring herbivore growth, reproduction, development, or survival) effects (Walling, 2000). Phenylpropanoids (including simple phenolics, flavonoids, and isoflavonoids), polyacetate, terpenoid, and alkaloid classes of metabolites are the major secondary metabolite groups involved in antiherbivore defense. A good example is nicotine, the infamous alkaloid component of tobacco. Nicotine is synthesized in roots and transported to shoots, and then accumulates in foliage (Waller and Dermer, 1981), where it is insecticidal and inhibits feeding of herbivores.

Defensive chemicals are thought to be costly for plants because of the resources consumed in their biosynthesis, their toxicity to the plant itself or the ecological consequences of their accumulation (Purrington, 2000). One way for a plant to reduce these costs is to synthesize defense compounds only after initial damage by herbivores or pathogens. It has been suggested that plant parts that are of high fitness value or that are under a high risk of attack may be best protected by constitutive defenses, whereas others may be better defended by induced responses (Wittstock and Gershenzon, 2002). For example, the reproductive organs of wild parsnip are attacked very frequently by herbivores and these organs accumulate high constitute levels of the toxic furanocoumarin, xanthotoxin, which do not increase following artificial damage. In contrast, the roots of wild parsnip are rarely attacked and have only low constitutive

levels of xanthotoxin, but these increase readily upon wounding (Wittstock and Gershenzon, 2002; Zangerl and Rutledge, 1996).

Phenolic and phenylpropanoid metabolites and terpenoids are the two most common types of phytochemicals induced by herbivore attack and pathogen infestation. Phenolic compounds are derived from the shikimic acid and phenylpropanoid pathways. One group of the best-known phenolic defense metabolites is furanocoumarins (FCs), which are tricyclic aromatic derivatives of the phenylpropanoid pathway. FCs can act as herbivore repellents and feeding deterrents (Klocke et al., 1989) and phototoxins by UV radiation-promoted cross-linking of DNA pyrimidines (Sastry et al., 1997). In addition, some linear FCs disrupt the detoxification capability of insects by acting as suicide substrates for cytochrome P-450 monooxygenases, an enzyme system involved in detoxification processes in insects (Neal and Wu, 1994).

Tannins are large polyphenolics that have been associated with induced herbivore defense, and are often considered general feeding deterrents in plant-herbivore interactions. Hydroxyl groups of tannins interact with proteins, denaturing and precipitating them in solution (Hagerman and Butler, 1991); thus, ingestion of tannin-rich diets has generally been considered to depress insect growth rate and food utilization efficiencies (Hagerman and Butler, 1991). Another complex phenolic polymer, lignin, is found predominantly in the secondary cell wall of xylem and other vascular cells. Wound-induced accumulation of lignin is particularly prevalent in organs such as tubers (Borg-Olivier and Monties, 1993), fruits (Hyodo et al., 1993) and barks (Thomas et al., 1995), tissues with complex wound-repair mechanisms.

Plants are not only able to synthesize individual defense metabolites with diverse chemical structures but also produce complex mixtures of defense compounds. For example, the terpenoids form a major group of phytochemicals induced by herbivores in conifers. Many conifer species respond to bark beetle attack by secreting oleoresin at the wound site and the induced oleoresin biosynthesis in grand fir has been demonstrated as a defense against bark beetles (Steele et al., 1995). Oleoresin is composed of mainly monoterpenes, sesquiterpenes and diterpenoid resin acids. These chemicals are toxic to both beetles and their pathogenic fungal associates (Raffa, 1991). After the monoterpenes are volatilized, the diterpene acids harden to seal off the injury and entrap the beetles

(Raffa, 1991). In addition, the monoterpene compounds emitted from some species can serve as attractants for beetle predators and parasitoids (De Moraes et al., 1998) and therefore decrease herbivore populations on plants. In the next section, this volatile-induced tritrophic interaction between plants, herbivores and natural enemies of insect herbivores will be discussed in more detail.

1.2.2 Insects can Induce Systemically Released Volatile Compounds

Healthy plant leaves normally release small quantities of volatile organic compounds (VOCs), but when damaged by herbivores, plants can release larger quantities or different profiles of volatiles, which have recently been implicated in herbivore defense. In some cases, these compounds are released when feeding ruptures pre-existing internal or external secretory structures in which the volatiles are synthesized and stored. In other cases, volatiles are formed at the moment of damage. The chemical identity of the VOC varies with plant species and with the type of damage or herbivorous insect species. The indirect defenses involving herbivore-induced plant volatiles have been implicated in three areas as illustrated by the following examples. First, the green-leaf volatile (Z)-3-hexenyl acetate emitted by tobacco after damage was found to deter female *Heliothis virescens* moths from laying eggs on injured plants (De Moraes et al., 2001). Second, terpenes derived from isoprenoid pathways play a major role in attracting predators and parasitoids to the infested plant to reduce the herbivore load in plants (Frey et al., 2000; Pare and Tumlinson, 1999). Third, volatiles have the intriguing potential to function as herbivore signals to nearby plants, which in turn can activate their own defense. A recent field investigation showed that *N. attenuata* plants growing adjacent to wounded *Artemisia tridentata* (sagebrush) suffered reduced levels of herbivore damage and exhibited increased levels of PPO compared to *N. attenuata* plants growing adjacent to undamaged sagebrush (Karban et al., 2000), which indicated that plant volatiles released after wounding or herbivory could be used as defense priming signals in plant neighborhoods. Plant volatiles as indirect and inducible defense strategies have received much attention in recent years and the tritrophic strategy could provide the basis for new approach in sustainable biological control of agricultural pests (Kessler and Baldwin, 2002).

1.2.3 Defense Proteins in Plants

In addition to the induction of phytochemicals, plants under stress also increase the expression of a vast array of enzymes and other proteins including transcriptional factors. For biotechnology, defense proteins have an advantage over phytochemicals in that only one gene encoding a protein needs to be isolated and used to genetically engineer plants for enhanced pathogen/pest resistance. Several groups of defense proteins have been well characterized in plant-herbivore and plant-pathogen interactions, and in this section some of them will be introduced.

In herbivore defense, one of the most studied antiherbivore proteins are proteinase inhibitors (PIs), which have been found in a wide range of species (Ryan, 1990). PIs are widely present in plants, in particular in storage organs, and are known to be inducible by injuries, such as insect damage. PI proteins can tightly bind proteolytic enzymes and thereby inhibit their activity. High concentrations of PI are thought to cause a lack of available amino acids for insect herbivores, which may lead to oversecretion of trypsin and further loss of sulfur amino acids (Ryan, 1990). Based on the type of protease to which they are targeted, PIs are classified as serine PIs, cysteine PIs, aspartic PIs and metallo PIs. Biochemical tests demonstrated that 5-10% trypsin inhibitor in artificial diet can efficiently inhibit the growth of insect herbivores (Birk, 1985; Gatehouse and Boulter, 1983; Shunkle and Murdock, 1983). Transgenic tobacco plants expressing a cowpea trypsin inhibitor have been shown to be more resistant to insect herbivores than wild type plants (Hilder et al., 1987). Tobacco hornworm larvae feeding on transgenic tobacco plants expressing a potato trypsin inhibitor were severely inhibited in growth compared to larvae feeding on control plants (Johnson et al., 1989). In recent years, growing research on plant proteinase inhibitors has confirmed their important role in plant defense and the research on systemically induced PI accumulation led to the identification of the systemic wound signal systemin in tomato plants (Pearce et al., 1993) (see 1.2.6).

A major set of herbivore- and wound-inducible defense proteins are oxidative enzymes including lipoxygenases, polyphenol oxidases, and peroxidases. Lipoxygenases (LOX) are primarily induced by pathogen attack, and to a lesser extent by wounding and herbivore damage (Hildebrand et al., 1988). The function of LOX in the defense against

pathogens seems to be related to the synthesis of a number of different compounds with signaling functions (Creelman and Mullet, 1997; Parchmann et al., 1997), antimicrobial activity (Croft et al., 1993; Weber et al., 1999), or to the development of the hypersensitive responses (HR) (Rusterucci et al., 1999). In antiherbivore defense, LOX can have a direct antinutritive effect resulting from the destruction of polysaturated fatty acids (Duffey and Stout, 1996) or from the reactions of the LOX-generated fatty acid hydroperoxides with essential amino acids in dietary proteins (Duffey and Felton, 1991). LOX is also required for the synthesis of JA, so play a central role in herbivore defense signaling (Bell et al., 1995). Besides LOX, other oxidases including polyphenol oxidase, peroxidase and ascorbate oxidase also play an important role in antiherbivore defense. All oxidative enzymes potentially have an antiherbivore effect based on their ability to destroy essential nutrients in insect diets. As mentioned previously, the products of the PPO-catalyzed reactions are highly reactive quinones, which can spontaneously polymerize and crosslink with other biomolecules (Felton et al., 1989). Another oxidative enzyme, peroxidase, is implicated in the generation of phenoxy radicals, which can polymerize nonenzymatically to form lignin polymer (Douglas, 1996) and crosslink cell wall carbohydrates and proteins (Cassab and Varner, 1988). Besides of their role in fortifying the plant cell wall, peroxidase is capable of oxidizing a variety of phenolics, and much like PPO, their presence in insect diets can lead to alkylation of dietary proteins (Duffey and Felton, 1991). Ascorbate is a potent antioxidant and free radical scavenger, which can counteract the effect of the quinones. The induction of ascorbate oxidase by herbivory and wounding has been observed and its presence in insect diets might therefore enhance the effectiveness of other oxidative enzymes (Duffey and Felton, 1991; Duffey and Stout, 1996).

Stress gene induction occurs primarily at the level of transcription, and the transcriptional control of stress-responsive gene expression is a crucial part of plant responses to a range of biotic stresses. There has been a lot of progress in recent years on characterizing transcription factors involved in the expression of stress-related genes in plants. The *Arabidopsis* genome codes about 1500 transcription factors, which often belong to large gene families. Four main families of transcription factors in *Arabidopsis* are ethylene-responsive-element-binding factors (ERF), basic-domain leucine-zipper

(bZIP), WRKY proteins and the MYB proteins (Singh et al., 2002). Specific transcription factors show enhanced expression and/or DNA-binding activity following induction by a range of pathogens, defense signals and wounding. For example, one tomato ERF transcription factor, PTI4 protein can be specifically phosphorylated by PTO kinase and this phosphorylation enhances the binding of PTI4 to the GCC box. When the *Pto* and *Pti4* genes were overexpressed in tomato leaves, a concomitant increase in the expression of GCC-box regulated PR genes was observed (Gu et al., 2000).

As mentioned previously (section 1.2.1), plant secondary metabolites exhibit apparent roles in stress protection. Therefore, enzymes involved in the production of secondary metabolites can be considered as one group of stress-inducible proteins. Phenolics and phenylpropanoids form one of the largest classes of plant phytochemicals. Phenylalanine ammonia lyase (PAL) is the entry point enzyme into phenylpropanoid metabolism. Induction of PAL and the followed increase of phenolic compounds concentration is a common response to pathogen attack, herbivory and wounding (Hahlbrock and Scheel, 1989). Other core enzymes involved in the phenylpropanoid pathway, such as 4-cinnamic acid hydroxylase (4-CH) (Frank et al., 1996), 4-coumarate CoA ligase (Smith et al., 1994) are also known to be induced at the transcriptional level by pathogen, herbivores and wounding. Besides producing a variety of toxic secondary phenolic compounds (see 1.2.1), the same enzymes are also responsible for the production of lignin or lignin-like polymers, which are important for cell wall reinforcement.

Plants respond to pathogen disease and herbivorous insects in different ways, which is demonstrated by the induction of different sets of defensive proteins. Pathogenesis-related (PR) proteins represent the major quantitative changes in soluble proteins during pathogen defense responses. PRs are typically resistant to acidic pH and proteolytic cleavage and, thus, survive in the harsh environments in which they occur, including vacuolar compartments, or cell wall, or intercellular spaces (Bowles, 1990). Since the discovery of the first PRs in tobacco, many other similar proteins have been isolated from tobacco and other plant species, including dicots and monocots. For example, chitinases and glucanases in plants are two well-known PR proteins with the capability to degrade fungal cell wall polysaccharides, which arrest or severely impair

fungal growth (Collinge et al., 1993; Melchers et al., 1994). For example, in tobacco, PR-1 proteins include PRs of 15-17 kDa molecular mass, whose biological activity has been shown to have antifungal activity. PR-5 proteins can be acidic-neutral or very basic, with extracellular and vacuolar localization, respectively. Several members of the PR-5 group from tobacco and other plant species were shown to display significant *in vitro* activity of inhibiting hyphal growth or spore germination of various fungi.

1.2.4 Jasmonate and Salicylate as Global Signals for Defense Gene Expression

Plant defense responses to wounding and herbivore attack are regulated by signal transduction pathways that operate both at the site of wounding and in undamaged distal leaves. Intensive research in recent years on plant defense signaling pathways has focused on low molecular mass regulators including salicylic acid (SA), jasmonic acid (JA), reactive oxygen species (ROS), nitrite oxide (NO) and ethylene. Here, I will mainly discuss the SA and JA-signaling pathways because of their essential roles in pathogen and herbivore defense, respectively, as these signal molecules are known to regulate PPO expression in tomato (Constabel et al., 1996; Thipyapong and Steffens, 1997). However, it should be kept in mind that other signaling molecules are also involved in plant defense and that these pathways do not function independently, but rather influence each other through a complex network of regulatory interactions.

1.2.4.1 Jasmonates in Plant Defense

A wealth of information exists on the role of jasmonates in plant responses to wounding and insect attack. Jasmonic acid (JA), and its methyl (MeJA) and other conjugates are collectively referred as jasmonates. Jasmonates are important regulators involved in multiple aspects of plant development such as fruit ripening, production of viable pollen, root growth, tendril coiling (Creelman and Mullet, 1997). Of particular relevance here, jasmonates halp activate plant defense mechanisms in response to herbivore wounding and various pathogens. Jasmonic acid is a 12-carbon fatty acid-derivative, which is synthesized *via* the octadecanoid pathway from the 18-carbon linoleic acid, and MeJA is formed by esterification by JA carboxyl methyltransferase (JMT). The enzymes involved in jasmonate biosynthesis are generally up-regulated by

wounding, or treatment with JA itself (Leon and Sanchez-Serrano, 1999; Mueller, 1997) resulting in a positive feedback signaling system, amplifying the initial signal.

Several lines of evidence support the important roles of jasmonates in plant defense. First, jasmonates accumulate in wounded plants (Creelman et al., 1992) and in plants or cell cultures treated with elicitors of pathogen defense (Gundlach et al., 1992). Second, jasmonates activate genes encoding proteinase inhibitors that help protect plants from insect damage (Johnson et al., 1989). Third, jasmonates also activate expression of genes encoding antifungal proteins, such as thionin (Becker and Apel, 1992) and osmotin (Xu et al., 1994). Furthermore, jasmonates induce genes involved in phytoalexin biosynthesis (*CHS, PAL, HMGR*) (Choi et al., 1994; Creelman et al., 1992) and phenolics involved in plant defense (Doares et al., 1995b).

The central roles in plant defense of JA and MeJA have been most conclusively demonstrated in plant mutants impaired in jasmonate biosynthesis and signaling perception. For example, the *def1* tomato mutant, which does not up-regulate levels of jasmonic acid after wounding, also produces lower levels of PIs, and is more susceptible to attack by lepidopteran insects (Howe et al., 1996). *A. thaliana* mutants impaired in JA production or perception exhibit enhanced susceptibility to a variety of fungal and bacterial pathogens (Staswick et al., 1998; Stintzi et al., 2001; Vijayan et al., 1998). Likewise, several mutants that exhibit enhanced or constitutive JA responses have been recently shown to exhibit enhanced resistance to necrotrophic pathogens (Ellis and Turner, 2001; Hilpert et al., 2001; Jensen et al., 2002; Xu et al., 2001).

Jasmonates are responsible for the induction of many defense-related genes including those involved in jasmonate biosynthesis, secondary metabolism, cell-wall formation, and those encoding stress-protective and defense proteins (Cheong and Choi, 2003). Interestingly, genes involved in photosynthesis, such as ribulose bisphosphate carboxylase/oxygenase, chlorophyll a/b-binding protein, and light-harvesting complex II are found to be down-regulated by jasmonate application (Cheong and Choi, 2003). Jasmonates have also been connected to systemic defense responses. The best-studied examples are the accumulation of PI in distal parts of wounded plants (Leon et al., 2001), and induced systemic resistance (ISR) to pathogens observed after exposure to specific biotic stimuli (Pieterse et al., 1998). Recently, JA or a derivative was suggested to act as a

long-distance transmissible signal for wound signaling (Li et al., 2002a). In addition, MeJA has also been suggested to function as an airborne signal between different plants in one neighborhood or between different parts of the same plant (Seo et al., 2001). The possible role of jasmonates as a long-distance signal will be discussed more in section 1.2.6.

1.2.4.2 Salicylic Acid in Plant Defense

Salicylic acid (SA) is common throughout the plant kingdom and is known as a regulator for physiological processes such as thermogenesis or plant defense. While JA is thought to be the central signaling molecule in plant-herbivore and some plant-pathogen defenses, SA is regarded as a global and central regulator of many defense genes in plant-pathogen defense. Increases in the endogenous levels of SA in pathogen-inoculated plants coincide with the elevated expression of genes encoding PR proteins and the activation of disease resistance (Durner, 1997; Shulaev et al., 1995). Preventing SA accumulation, by degrading it to catechol in transgenic plants that express the bacterial salicylate hydroxylase gene (*NahG*) (Delaney et al., 1994; Gaffney et al., 1993) or by blocking SA synthesis (Nawrath and Metraux, 1999; Wildermuth et al., 2001), effectively blocks the activation of SA-dependent defense responses. By contrast, the exogenous application of SA or its synthetic functional analog benzo (1,2,3) thiadiazole-7-carbothioic acid *S*-methyl ester (BTH) results in the activation of *PR* gene expression and enhanced resistance to pathogens (Shah, 2003).

SA is synthesized *via* the phenylpropanoid pathway in which phenylalanine (Phe) is converted to cinnamic acid (CA) and CA is transformed to either *ortho*-coumaric acid or benzoic acid, both compounds are precursors of SA (Lee et al., 1995). However, this pathway cannot account for all of the SA in plant cells, suggesting the presence of an alternative biosynthesis pathway for SA (Shah, 2003). Similar to JA, the volatile derivative of SA, methyl salicylate (MeSA), is also involved in plant defense. MeSA was shown to function as an airborne signal, which activates the expression of defense-related genes in tobacco (Shulaev et al., 1997).

1.2.4.3 Interaction of JA, SA and other Signals

To date, SA and JA have been known to play key roles in various aspects of plant defense. However, SA, JA and other important endogenous plant signaling molecules, do not function independently, but rather control and potentiate each other's activities. Plant stress responses involve the interaction of several signals to fine-tune its defense by upregulating different sets of overlapping genes against different aggressors (Reymond and Farmer, 1998). Interactions among JA, SA, ethylene and other signaling molecules may explain the specificity of the responses (Kessler and Baldwin, 2002). For example, during pathogenesis, a plant might need to channel a large amount of energy into the synthesis of pathogen-inducible gene products (PR proteins, etc.), simultaneously suppressing the expression of wound-inducible genes directed against other pests. Consistent with this, the inhibitory effect of SA on JA-regulated gene expression has been previously reported for the induction of proteinase inhibitors in wounded tomatoes (Doares et al., 1995a). Similarly, genes activated by JA are hyper-inducible in transgenic plants expressing *NahG*, therefore fail to accumulate wild-type levels of SA during pathogenesis (Reymond and Farmer, 1998).

Furthermore, the actions of JA and SA are modulated by other plant growth and defense regulators. For example, ABA has an early role in activation of the octadecanoid pathway to produce JA (Carrera and Prat, 1998; Chao et al., 1999). Ethylene can potentiate the level of expression of many defense genes in different plants. The sensitivity of *Arabidopsis* plants to SA-inducible PR1 accumulation is enhanced by ethylene (Lawton et al., 1994). In tobacco, the genes encoding PR1b and PR5 are synergistically induced by MeJA and ethylene, or by the combination of MeJA and SA (Xu et al., 1994). In tomato, ethylene co-operatively stimulates the expression of wound- and JA-inducible proteinase inhibitor genes (O'Donnell et al., 1996). In the case of PPO, different injuries, SA, ethylene, and jasmonates elicited distinct, cell-specific and developmental stage-specific patterns of tomato *PPO F* expression, which reflect the ability of plants to fine-tune their defense gene expression by potentiating or inhibiting different signaling pathways.

Much of what we currently know about JA, SA and other signaling molecules comes from studies on *Arabidopsis* and tomato. However, there are several discrepancies

between the proposed signaling pathways of these species, and it may reflect gaps in knowledge or reveal fundamental differences in mechanism. Studies of the defense signaling pathway in other plant species therefore are in high demand.

1.2.5 SAR and ISR in Pathogen Defense

Mechanical wounding, pathogen or insect attack, and application of signaling molecules such as SA and JA not only activate plant defense responses locally, but can also induce plant defense in tissues far from the invasion site and even in neighbouring plants. This plant-wide defense induction is referred as systemic acquired resistance (SAR) and is always accompanied by the induction of some defense-related marker genes, such as those encoding PR proteins, PIs and PPOs. SAR ensures that the plant is primed and can therefore respond more effectively to subsequent attacks. The type of induced systemic responses is determined by the type of attacking organism. Induced systemic responses to pathogens and herbivores are distinct and their respective signaling cascades result in the local and systemic accumulation of different sets of defense-response RNAs and proteins (see 1.2.3). Overall, the phenomenon of SAR can be compared to immunization in animals and humans, although the underlying mechanisms are different. SAR resistance is expressed against a broad spectrum of organisms, which may differ from the SAR-inducing organism. In cucumber, for example, a primary inoculation with the fungus *Colletotrichum lagenarium,* the causal agent of anthracnose, induces SAR against a dozen diseases caused by fungal, bacterial, and viral pathogens (Kessmann et al., 1994). The time needed for the establishment of SAR depends both on the plant and the type of inducing organism, and usually takes 5-7 days. However, a very rapid induction was reported for cucumber, where SAR sets in as early as 7 h after a primary inoculation with *Pseudomonas syringae* (Smith and Metraux, 1991).

Another interesting case of acquired resistance was described more recently using plant growth promoting rhizobacteria (PGPR) colonizing the rhizosphere as biocontrol agents. PGPR applied to the soil remain localized at the root surface and induced resistance in the leaves or stem (Liu, 1995). To differentiate this form of acquired resistance from SAR, it has been termed induced systemic resistance (ISR) (Pieterse et al., 1996b). The induction of resistance in parts remote from the site of primary

inoculation is postulated to result from the translocation of an unknown systemic signal produced at the site of primary infection. Progress has been made in unraveling the signals involved in SAR, and it appears that SA is required, but is not the systemically mobile compound of this process. In plants transformed with the *NahG* gene, the SA levels are low and SAR is blocked, which indicates that SA is required for SAR induction (Delaney et al., 1994; Gaffney et al., 1993). ISR occurs in *nahG* plants, indicating that it is not a SA-dependent phenomenon (Pieterse et al., 1996a). Rather, ISR appears to be JA- and ethylene-dependent and the observation that ethylene induce ISR in *jar1* mutants led to the hypothesis that ISR requires a JA signal followed by an ethylene signal (Pieterse et al., 1998).

1.2.6 Systemin, Jasmonates, and the Systemic Herbivore Defense Response

When tomato leaves are damaged by herbivores or mechanical wounding, defense genes encoding PPO and PI proteins are activated in a plant-wide (systemical) manner within hours. Early events in the tomato signaling pathway involves systemin, an 18-amino-acid peptide hormone, which is derived from prosystemin, a 200-amino-acid precursor by proteolytic processing (Ryan and Pearce, 1998; Ryan, 2002). Prosystemin is present at low constitutive levels in leaf tissue; it lacks a signal peptide sequence or other targetting information, and is probably present in the cytoplasm of cells (Ryan and Pearce, 1998). Systemin is produced at wound sites, and after translocation via the phloem, systemin is perceived by the systemin receptor SR160, a Leu-rich repeat receptor kinase on the plasma membrane (Scheer et al., 2003). After systemin binding, a complex wound cascade is initiated by activating a phospholipase A_2, which releases linolenic acid from the plasma membrane, supplying the substrate for the octadecanoid pathway (Ryan, 2000) and the subsequent biosynthesis of jasmonic acid (Lee and Howe, 2003). Systemin systemically regulates the activation of over 20 defensive genes including PIs and PPO in tomato plants in response to herbivore and pathogen attacks.

A critical role for prosystemin and systemin in signaling defense genes has been established using transgenic tomato plants. Plants expressing a fused gene comprised of a prosystemin cDNA in its antisense orientation, driven by the CaMV 35S are severely impaired in their systemic induction of both proteinase inhibitor I and II proteins in

response to wounding (McGurl et al., 1994). The plants were also severely compromised in their ability to defend themselves against attacking *Manduca sexta* larvae (Orozco-Cardenas et al., 2001). Supplying antisense plants with systemin resulted in the expression of the defensive genes, confirming that the plants were capable of responding to the wound signal, although they could not produce it. The characteristics of systemin, including its potency, its mobility in the plant, the wound-inducibility of the prosystemin gene, and the effects of the antisense prosystemin gene have led to the conclusion that systemin is a systemic wound hormone that plays a central role in regulating the expression of defense genes in response to pest attacks (Ryan, 2000). However, this view has now been modified (see below).

Systemin has been identified so far only in members of the subtribe *Solaneae* of the *Solanaceae* family, including tomato, potato, black nightshade, and pepper (Constabel et al., 1998). In tobacco, a solanaceous species that does not express a systemin precursor gene nor responds to systemin, when transformed with the tomato systemin receptor gene, expresses the gene product and the gain in function when induced by systemin (Scheer et al., 2003). This indicates that early steps of the systemin signaling pathway found in tomato are present in tobacco cells (Scheer et al., 2003).

Recently, Li et al., used two tomato mutants defective in the wound-signaling pathway in a reciprocal grafting experiment to provide evidence that JA or a derivative may also act as a long-distance signal for wound signaling (Li et al., 2002a). Based on this result, a more attractive amplification hypothesis was suggested by Ryan and Moura (2002). In this hypothesis, both systemin and JA can be released at wound sites and move away from the site in a systemic manner, but with the smaller soluble JA likely being more mobile. Localized production of systemin at the site of wounding may induce the synthesis of JA/MeJA, which in turn could promote gene expression in neighboring cells (Ryan and Moura, 2002).

1.2.7 Genetically Engineered Plant Defense

With the accumulation of knowledge on plant defense responses and signaling pathways, genetic transformations is opening the possibility to directly transfer the resistance trait into elite lines of plant species within one generation. For example,

overexpression of the *NPR1* gene, a single component in the induced resistance signaling pathway, yielded resistance to multiple pathogens (Cao, 1998). Successful disease control has also been achieved by the overexpression of a few specific defense genes. For example, the tomato *Pto* gene specifies race-specific resistance to the bacterial pathogen *Pseudomonas syringae* pv tomato. Overexpression of *Pto* in tomato caused elevated expression of *PR* genes and displayed significant resistance to bacterial pathogens compared to nontransgenic lines (Tang et al., 1999). The constitutive expression of chitinase and glucanase has also provided enhanced control of several fungal diseases (Lan et al., 2000). Prior to the work described here, PPO-overexpressing tomato was the first and so far, the only PPO-transformed plant species exhibiting greater resistance to a bacterial pathogen. As a confirmed anti-pathogen protein in tomato, PPO has the potential to be genetically engineered into plants to provide the host with better resistance against pathogens and perhaps insects.

1.3 Hybrid Poplar as a Model System in Tree Biology

PPO enzymes have been purified and studied in a variety of fruits, crops, and vegetables, but few studies have been carried out in trees. Trees are subject to attacks from a large array of insect pests and pathogens and should thus be very useful for studies of defense. I decided to study PPO thoroughly and systemically in the model system, hybrid poplar, as this plant contains high levels of wound-inducible PPO and phenolics (Constabel and Ryan, 1998; Constabel et al., 2000).

The genus *Populus* comprises about 30 woody perennial species of great economic and environmental value and is represented by familiar trees such as as poplars, cottonwoods, and aspens. In contrast to other model plants, the genus *Populus* has genuine commercial value as a tree for wood, pulp and paper. *Populus* has also been used as a model system for addressing biological questions related to long generation intervals and outcrossing mating systems as is typical of trees. The biological attributes of *Populus* as a model system in forest tree biology include rapid growth, rapid seed formation, small genome size (2C = 1.2 pg) (Bradshaw and Stettler, 1993), and identical chromosome number of all species ($2n = 38$). Tremendous natural variation among species or

populations exist in *Populus,* and new hybrid can be created by interspecific hybridization that is straightforward between many species (Stettler et al., 1980).

While many aspects of tree biology are common to all plants, and hence can be studied in very tractable model species such as *Arabidopsis*, some unique facets of tree anatomy and physiology must be investigated in trees themselves. A large number of these unique characters are related to their perennial growth habit. Extensive formation of secondary xylem (wood) is perhaps the most obvious, but other characters include leaf and flower phenology, seasonal reallocation of nutrients, cold hardiness, iterative development of a complex crown form, and juvenile-mature phase change. In contrast to agronomic crops, relatively few physiologically based growth models have been developed for trees, largely due to a lack of fundamental physiological information and the difficulty of working with complex perennial. Just as in forest genetics, poplars have been adopted by tree physiologists as a model system.

Because of their small genome size, short rotation cycle, fast growth rate and capacity for micropropagation, *Populus* species are suitable models for genetic engineering of deciduous trees. All of the routine methods of plant transformation (*Agrobacterium* mediated, direct DNA transfer, electroporation) have been tried and have proven successful with *Populus* (Campbell et al., 2003). The current emphasis, however, is on *Agrobacterium*-mediated transformation and on improving regeneration efficiency in commercially relevant genotypes as not all are equally susceptible to transformation. Currently, there are at least four areas of interest in *Populus* transgenic research including control of flowering (Weigel and Nilsson, 1995), herbivore and pathogen resistance (Wang et al., 1996), herbicide resistance (Strauss et al., 1997) and the manipulation of lignin quality and quantity during wood formation (Franke et al., 2000). The entire 550Mbp *Populus* genome is being sequenced by the Joint Genome Institute (http://genome.jgi-psf.org/poplar0/poplar0.home.html) and will be the first woody plant whose genome will be known in such detail. The DNA sequence will be used to define genes that give forest trees some of their specialized attributes, such as wood formation, vegetative dormancy, crown architecture, juvenile-mature transition and more relevant to my work, pathogen and herbivore resistance.

1.4 Herbivore and Pathogen Defense in Populus

Populus are susceptible to a wide range of diseases and insect infestations. The fungi that affect *Populus* species are extremely diverse; for example, more than 250 species of fungi are known to be associated with decay of *P. tremuloides* alone in North America (Lindsey and Gilbertson, 1978). At least 300 species of insects have been reported on living on *P. tremuloides* (Davidson and Prentice, 1968). Variation in resistance to pathogens and herbivores among *Populus* clones has been observed frequently. The production of defense compounds is thought to be an important component of resistance against insect herbivores. For example, high concentrations of phenolic glycosides have strong and typically negative effects on herbivore development (Hemming and Lindroth, 1995; Hwang and Lindroth, 1997). In insect feeding experiments, it was shown that artificial diets containing phenolic glycosides have negative effects on larval performance of forest tent caterpillar and gypsy moth (Lindroth and Bloomer, 1991; Lindroth and Hemming, 1990). These phenolic glycosides are expected to be metabolized in herbivore guts and their enzymatically decomposed products analyzed in the feeding tests also showed negative effects on larval growth.

Condensed tannins, flavonoid polymers, and other products of phenylpropanoid metabolism are also important for phytochemical defense in trembling aspen. The phenolic groups of tannins bind to proteins and this is the basis of their antiherbivore functions, which include reduced digestibility of proteins or interactions with proteins in the herbivore digestive tract. Recently, it has been shown that genes involved in tannin biosynthesis are induced by wounding and herbivore feeding in trembling aspen (Peters and Constabel, 2002), which indicates that tannin-based antiherbivore mechanism also exists in *Populus*.

Besides the induction of secondary metabolites, defensive proteins are also involved in anti-pathogen and antiherbivore defense in *Populus*. Proteinase inhibitor proteins (Hollick and Gordon, 1993), chitinase (Clarke et al., 1994), polyphenol oxidase (Constabel et al., 2000) are all induced in wounded *P. trichocarpa x P. deltoides* (TD) leaves. Trypsin inhibitor and polyphenol oxidase protein have been shown to accumulate in *Populus* leaves within two days of herbivore damage and these inductions are systemic and can be mimicked by application of MeJA, which suggested that the octadecanoid

pathway is involved in the wound-induction of defenses in *Populus* (Constabel et al., 2000; Haruta et al., 2001a).

Due to its economic value as a short rotation biomass tree, its representative role in current tree research and more importantly, high PPO constitutive levels and inducibility by stresses in some hybrid poplar species, I decided to use this plant species to study the physiological functions of PPO, a potential defensive protein involved in pathogen and herbivore defense of plant.

1.5 Objective of this Research

The objective of my project was to investigate the expression and functions of polyphenol oxidases in hybrid poplar. To achieve this objective, three major approaches were employed. (1) New *PtdPPO* genes encoding more PPO isoforms were cloned from hybrid poplar (*Populus trichocarpa x P. deltoides*) and their gene-specific expression patterns were investigated at tissue and organ levels, as well as their inducibility under normal or stressed growing conditions. (2) Transgenic hybrid aspen, which overexpresses *PtdPPO1*, was developed and tested for the effect on herbivore performance. Transgenic *Populus* varying only in the level of PPO expression provides an experimental platform that allows direct testing of PPO function on herbivores. (3) Two PPO protein isoforms were partially purified and their enzymatic properties including substrate preference and activation requirement were analyzed.

In the following three chapters, I will describe these three main components of my project separately. Briefly, the key findings include (1) the differential expression and induction patterns of three *PtdPPO* genes; (2) the negative correlation between *PtdPPO1* expression level, and herbivore growth and survival rate; and (3) the distinct biochemical properties of two partially purified PPO proteins. Altogether, my research supports the idea that PPO in hybrid poplar is positively involved in antiherbivore defense. However, due to their differential expression and induction patterns of different *PtdPPO* genes, as well as their distinct biochemical properties, it is likely that PPO proteins in hybrid poplar also perform other physiological functions.

Chapter 2: Three Polyphenol Oxidase Genes from Hybrid Poplar Are Differentially Expressed During Development and After Wounding

2.1 Introduction

Polyphenol oxidase (PPO) is a copper-containing enzyme which uses molecular oxygen to oxidize *o*-diphenols to *o*-quinones (diphenolase activity; EC 1.10.3.1), and which in some plants can also hydroxylate monophenols to *o*-diphenols (monophenolase activity; EC 1.14.18.1). Quinones are reactive compounds responsible for the damage-induced browning of many fruits and vegetables, and PPO activity is, thus, often associated with damaged and diseased plants (Mathew and Parpia, 1971; Steffens et al., 1994). Due to its location in the thylakoid, PPO had once also been postulated to play a role related to photosynthesis (Vaughn et al., 1988), but it is now becoming accepted that PPO is involved in plant defense against insects and pathogens (Constabel et al., 1996; Li and Steffens, 2002; Steffens et al., 1994). The antiherbivore role of PPO was first demonstrated in tomato by Felton et al. (1989), who showed that PPO-generated quinones could alkylate dietary proteins and reduce their nutritive value for insect pests. Further evidence for a function of PPO in the defense against insects came from the discovery of PPO induction by systemin through the octadecanoid pathway (Constabel et al., 1995; Constabel et al., 1996). It should be noted, however that some species such as willow and soybean have very high constitutive PPO activities, and show no significant PPO induction by wounding or methyl jasmonate treatment (Constabel and Ryan, 1998). A different defensive role of PPO which acts against small-bodied pests, such as aphids, was shown for *Solanum berthaultii* trichomes, where as much as 50-70% of the total protein can be PPO (Kowalski et al., 1992). When small insects break the trichomes, PPO is mixed with its substrate, and PPO-generated quinones polymerize, entrapping the insects (Kowalski et al., 1992). PPO has also been shown to be important in pathogen defense, as transgenic tomato plants which overexpress PPO show enhanced resistance to the bacterial pathogen *Pseudomonas syringae* (Li and Steffens, 2002). In addition, in some species PPOs may also have biosynthetic functions based on their hydroxylase activity; this was recently demonstrated for PPO-like hydroxylases, which are involved in the biosynthesis of aurones and lignans (Cho et al., 2003; Nakayama et al., 2000).

In plants, PPOs are often encoded by mid-sized gene families. In potato, five PPO genes were characterized and these genes have complex tissue-specific patterns of

expression with high levels of transcript commonly found in flowers, young leaves, trichomes, roots, and tubers (Thygesen et al., 1995). In tomato, there are at least seven PPO genes expressed in various tissues including leaves, stems, flowers, roots and trichomes (Newman et al., 1993; Thipyapong et al., 1997). Interestingly, only one tomato PPO gene is wound- and stress-inducible, and while the other six PPO family members are regulated developmentally (Thipyapong and Steffens, 1997). In tobacco, a stigma/style-specific PPO gene (*tobP1*) is expressed exclusively in flowers, and is not inducible in mature leaves by wounding (Goldman et al., 1998). Recently, four different PPO cDNAs were isolated from banana, which are differentially expressed in various vegetative tissues as well as fruits and flowers (Gooding et al., 2001).

A wound- and herbivore-inducible leaf PPO (*PtdPPO1*) has been isolated from hybrid poplar, *Populus trichocarpa x P. deltoides* (Constabel et al., 2000). *PtdPPO1* was proposed to be a component of the inducible defense against leaf-eating herbivores, as it is up-regulated by MeJA and co-ordinately expressed with other defense genes such as trypsin inhibitor (Constabel et al., 2000). Western blots using a polyclonal antibody raised against recombinant PPO1 suggested that stems and petioles express a different PPO (Wang and Constabel, 2003). In this chapter, I describe the isolation of the gene encoding this stem PPO, as well as an additional *PtdPPO* gene encoding a root PPO. The results demonstrate very distinct and organ-specific expression of all three hybrid poplar *PtdPPO* genes.

2.2 Material and Methods

2.2.1 Plant materials and treatments

Hybrid poplar (*Populus trichocarpa x P. deltoides*) clone H11-11 was propagated and grown in the University of Victoria's Forest Biology greenhouse facility under long-day conditions and fertilized daily as described previously (Constabel et al., 2000). Plants were about two months old and typically had at least 25 leaves when used. Leaves were numbered from the apical meristem downward according to the leaf plastochron index (LPI) (Maksymowych and Erickson, 1960), with the first developing leaf with a lamina of length ≥20 mm designated as the index leaf (LPI 0). Trees were wounded by crushing

leaves at the margins with pliers, or by puncturing stems with a sterile needle. For MeJA treatment, whole trees were sprayed twice at 2 h intervals with 10μM MeJA dissolved in 0.1% Triton X-100. Induced tissues were harvested after 24 hours unless stated otherwise. Young leaves and stems in this study refer to the tissue at LPI 2-4, and old leaves and stems refer to tissues at LPI 16. Young root samples were root tips (5 cm length, approximately 1mm diameter), and old root samples consisted of major roots (at least 5 mm diameter) collected near the crown. All tissue samples were frozen in liquid N_2 and stored at -80°C until extraction.

2.2.2 Isolation of stem- and root-specific PPO cDNAs

Partial cDNA sequences corresponding to *PtdPPO2* and *PtdPPO3* were first amplified using PPO-specific degenerate primers (Constabel et al., 2000) with stem RNA as the RT-PCR template. The resulting fragments (~270bp in length were purified from the gel (Qiaquick Gel Extraction Kit, Qiagen, Mississauga, Canada) and cloned into the pGemT Easy Vector (Promega, Nepean, Canada). Sequence analysis allowed these fragments to be clustered into three groups representing three *PtdPPO* genes. Gene-specific primers were then constructed based on these fragments in both forward and reverse directions. 3' RACE was carried out using a Lock-Dock system (Borson et al., 1992) and downstream gene specific primers (5'-AAGGAGATGCGAATCCAG-3' for *PtdPPO2* and 5'-ATATTCCACACGGTCCAG-3' for *PtdPPO3*). To obtain the 5' cDNA ends, unannotated sequence data of the *Populus* genome project was searched at the Joint Genome Initiative, US-DOE *Populus* database (http://genome.jgi-psf.org/poplar0/poplar0.home.html), and two sequences in the *P. trichocarpa* genome corresponding to *PtdPPO2* and *PtdPPO3* were identified. A series of partially overlapping sequence runs corresponding to the two new *PtdPPOs* were constructed, and used to design primers 5' to the coding sequences. These primers (5'-GTATCCAAGAATCCTTCACCC-3' for *PtdPPO2* and 5'-CCATGGCAAAAGGTGTTCAG-3' for *PtdPPO3*) were then used to amplify the remainder of the PPO cDNAs from stem and root total RNA. Final PPO sequences were sequenced from both directions using M13-F and M13-R primers and assembled into full-length cDNA contigs using DNAStar software (DNASTAR Inc., Madison, WI).

Sequence alignments and distances were calculated for three full-length PPOs at the nucleic acid and amino acid levels using MEGALIGN (DNASTAR). Similarity = 100X Consensus Length/(consensus length + mismatches +gaps). Phylogenetic analysis was carried out using Clustal X on a Linux computer system.

2.2.3 Northern and Southern blot hybridization using gene-specific PPO probes

Total RNA was isolated from plant tissues using the procedure described previously (Haruta et al., 2001). Aliquots containing 10μg total RNA was electrophoresed on 1.2% agarose denaturing formaldehyde gels, and subsequently transferred onto Hybond$^+$ membranes (Amersham, Baie d'Urfé, Canada). Three gene-specific probes were made based on the 3'-end sequences, which included the 3'-UTR of the PPOs plus a small fragment of coding sequences. The PCR primers used to amplify the gene-specific probes were 5'-ATCTGGAAGCTGAAGGAG-3' for *PtdPPO1*, 5'-GTTGAGGGTGATGACAGT-3' for *PtdPPO2* and 5'-CCCAGGTCTAATAGTGTT-3' for *PtdPPO3*, paired with the SP6 primer on the T-vector in each case. The sizes of three gene-specific probes are 290bp (*PtdPPO1*), 310bp (*PtdPPO2*) and 350bp (*PtdPPO3*), respectively. Probes were made by Amersham Rediprime II kit using the random-priming method, which involves the co-denaturation of a template DNA with random sequence hexmeric oligonucleotides. The random sequence oligo hybridize to random sites along the template strand and the sites of hybridization serve as priming site for exo-DNA polymerase fragments. While the DNA pol fragment extends the oligo, the Redivue®-Deoxycytidine 5' [α-^{32}P]-triphosphate in the polymerase buffer is corporated into the probe product. Hybridizations were performed at 65°C in 0.5 M phosphate buffer, pH 7.0, with 2 mM EDTA, 1% BSA (w/v), 7% (w/v) SDS for 24 hours and were washed at high stringency at 65°C according to Church and Gilbert (1984) in 0.04 M phosphate buffer, pH 7.0, with 1% (w/v) SDS, 2 mM EDTA for two times (half an hour each time). Signals were detected and analysed on a PhosphorImager (Molecular Dynamics, Sunnyvale). For Southern blots, genomic DNA extracted from *P. trichocarpa* or hybrid poplar H11-11 was performed as previously described (Constabel et al., 2000). Aliquots containing 10 μg genomic DNA were digested with 60 Units *Eco*RI or *Hin*dIII (Amersham), analyzed on 0.8% agarose gels, and transferred onto Hybond$^+$ membranes. Hybridizations were

performed with full-length *PtdPPO1* probe, *PtdPPO2* gene-specific probe and *PtdPPO3* gene-specific probe, respectively.

2.2.4 PPO activity assays, protein determination and immunoblotting

Poplar tissue was ground in 100 mM $NaPO_4$ buffer, pH 7.0, with 0.1% Triton X-100 and PVPP (polyvinylpolypyrrolidone). After centrifugation, the supernatant was used for protein determination using bovine serum albumin as a standard (Bradford, 1976). PPO activity was determined by the DOPA (dihydroxyphenylalanine) assay as described previously (Constabel et al., 2000). For western blots, 10 μg of total protein was loaded onto SDS-PAGE gels and then transferred to PVDF membranes (BioRad, Mississauga, Canada). Detection was carried out using polyclonal antibody raised against the recombinant PPO1 protein (Christopher and Constabel, unpublished data). Immunocomplexes were detected using goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (BioRad) together with reagents 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT).

2.2.5 Cell culture and elicitor treatment

Hybrid poplar suspension cells were obtained from Dr. Carl Douglas (University of British Columbia, Vancouver, Canada) and maintained according to Haruta and Constabel (2003). Partially acid-hydrolyzed chitosan was obtained from Dr. Armand Seguin (Canadian Forest Service, QC). For northern analysis, 3-d-old cultures were treated with the elicitors for a 3 h period. Treatments consisted of final concentrations of 1mM salicylic acid, 50 μM MeJA (Bedoukian Research, Danbury, CT), 0.5 μg/mL chitosan, 0.1% (v/v) *Phytophthora megasperma* (*pmg*) elicitor (Lisker, 1977; Kuc, 1995), or 1% *P. radiosa* extract. Treated cells were harvested by centrifugation at 1,600*g* for 15 min, frozen in liquid nitrogen, and stored at -80°C until analyzed.

2.2.6 Tissue printing and localization of PPO in stem section

Fresh stem and petiole sections were briefly pressed onto PVDF membranes and were incubated in 0.1 M sodium phosphate buffer, pH 6.5, containing 0.1 M catechol (Avdiushko et al., 1993). The incubating buffer also included 470 units/ml laccase and 1

mM CTAB to inhibit the peroxidase and laccase activity possibly present in plant tissue, respectively. After the development of intense yellow staining, blots were washed with distilled water and dried. 20 mM 3,4-dihydroxyphenylalanine (DOPA) also was used as an alternate substrate. PPO-specific inhibitor, tropolone, was added to the incubating buffer at the concentration of 100 μM to comfirm the specificy of PPO staining. The histochemical localization of PPO was performed by incubating tissue printings with the PPO antibody and developed as described for western blots. Trypsin inhibitor antibody was used to replace PPO antibody in this part as the negative control.

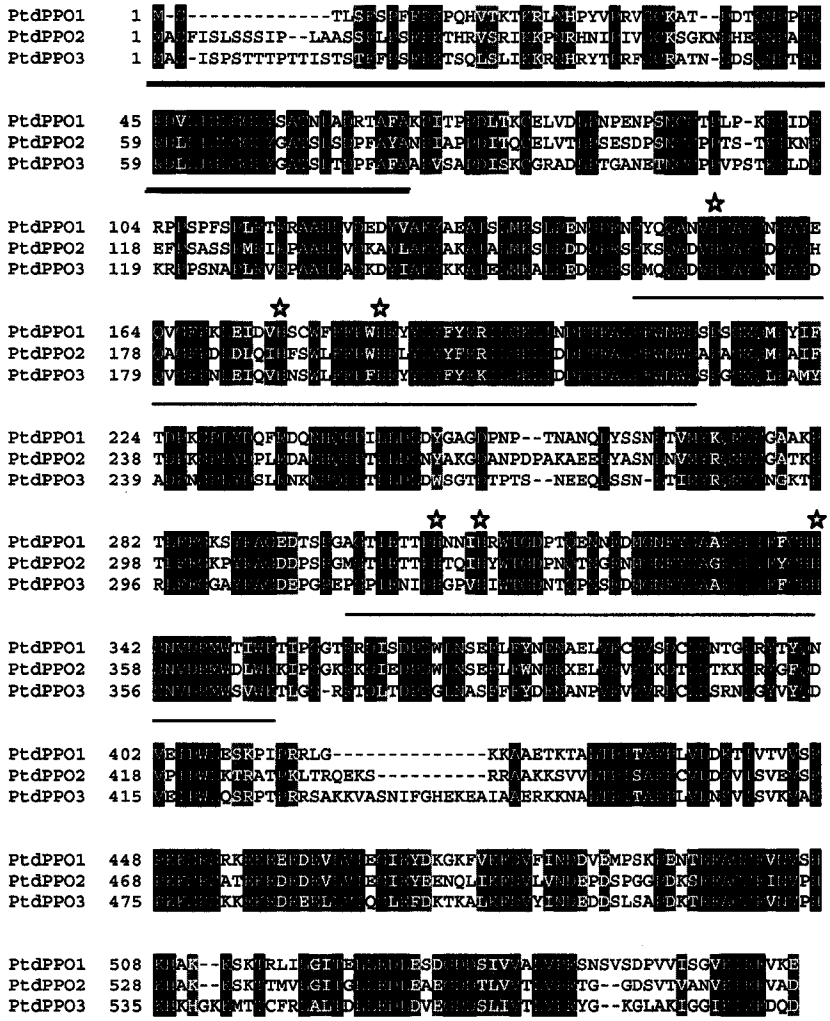
2.3 Results

2.3.1 Hybrid poplar contains at least three distinct PPOs

In order to isolate cDNAs encoding PPO2 protein from hybrid poplar, we used an RT-PCR strategy with RNA isolated from stems as a template. Degenerate primers corresponding to the conserved copper-binding regions of PPO were used to amplify short fragments. After sequence verification, these fragments were clustered into three groups, representing the previously isolated *PtdPPO* (here named *PtdPPO1*) as well as two new *PtdPPO* genes. To obtain their entire coding sequences, we used 3' RACE and the available sequence data at the Poplar Genome Project (http://genome.jgi-psf.org/poplar0/poplar0.home.html) to design additional 5' primers, which enabled us to clone and sequence the entire coding sequences of two new poplar PPO cDNAs, which we named *PtdPPO2* and *PtdPPO3* (see Materials and Methods). The *PtdPPO2* and *PtdPPO3* cDNA sequences were 1950 bp and 2038 bp long, respectively (Fig. 1). Conceptual translation of the *PtdPPO2* and *PtdPPO3* coding sequences predicted proteins of 581 and 590 amino acids with molecular weights of 65 kDa and 66.2 kDa, respectively. Two conserved copper-binding domains, each with three highly conserved His residues that complex the catalytically active Cu^{2+}, are also present (Fig. 1). The predicted PPO2 and PPO3 proteins both included an N-terminal transit peptide of 81 and 82 amino acid residues, which target the protein into the plastids (Fig. 1). These also contain thylakoid targeting sequences, consisting of an N-terminal hydroxy amino acid-rich region required for import into the stroma, followed by a hydrophobic core domain

Figure 1: Comparison of the deduced amino acid sequences of three hybrid poplar *PtdPPO* cDNAs.

The stars indicate conserved His residues. The thylakoid transit peptides are double underlined, and the copper-binding domains are single underlined.



ending in the Ala-X-Ala consensus (Robinson and Mant, 1997; Steffens et al., 1994). The three PPOs share less than 66% identity among themselves at both the nucleic acid and amino acid levels (Table 1), clearly indicating that these cDNAs represent distinct genes rather than allelic variants.

2.3.2 The three poplar PPOs are part of small gene families

Previous experiments had suggested that PPO activity was found in a variety of poplar tissues, and that different PPO isoforms accumulate in different tissues (Wang and Constabel, 2003). To test for differential expression of the three *PtdPPO* genes in hybrid poplar, gene-specific probes for three *PtdPPOs* were generated from the sequences. These were composed of their 3'-UTRs plus a short (65-150 bp) coding sequence of the cDNAs. In this region, the three PPOs shared the least similarity and cross hybridization was negligible (Fig. 2A). I carried out Southern blots on genomic DNA restricted with *Eco*RI and *Hin*dIII. These enzymes do not cut within *PtdPPO1* cDNA sequence, but cut once within *PtdPPO2* (*Eco*RI) and *PtdPPO3* (*Hin*dIII). However, the sites are not found in the sequences corresponding to the gene-specific probes, so that each gene copy should only be recognized once in Southern blots. In addition to the poplar TD hybrid, I analyzed genomic DNA of its *P. trichocarpa* female parent in parallel. As expected, the banding pattern for the parental genotype was simpler than the hybrid, reflecting the more divergent alleles of the TD hybrid. Hybridization with *PtdPPO1* cDNA confirmed that three to four *PtdPPO1* genes exist in the hybrid poplar genome (Fig. 2B (a) (Constabel et al., 2000). When the same Southern blot was hybridized with the *PtdPPO2* and *PtdPPO3* gene-specific probes, very different hybridizing band patterns were observed (Fig. 2B, panels b & c), which confirmed that the probes recognized different *PtdPPO* genes. Hybridization of restricted genomic DNA with the *PtdPPO2* gene-specific probe resulted in five to seven bands in the *P. trichocarpa* parent, while hybridization with the *PtdPPO3* gene-specific probe resulted in only one or two bands (Fig 2). I conclude that both *PtdPPO1* and *PtdPPO2* are present as a small gene family in the poplar genome, while *PtdPPO3* may be represented by only one or two genes.

Table 1

Similarity among three hybrid poplar PPO sequences at both the nucleotide and amino acid levels.

	PtdPPO1	PtdPPO2	PtdPPO3
PtdPPO1	100	63.4	59.3
PtdPPO2	66.0	100	56.8
PtdPPO3	65.8	61.5	100

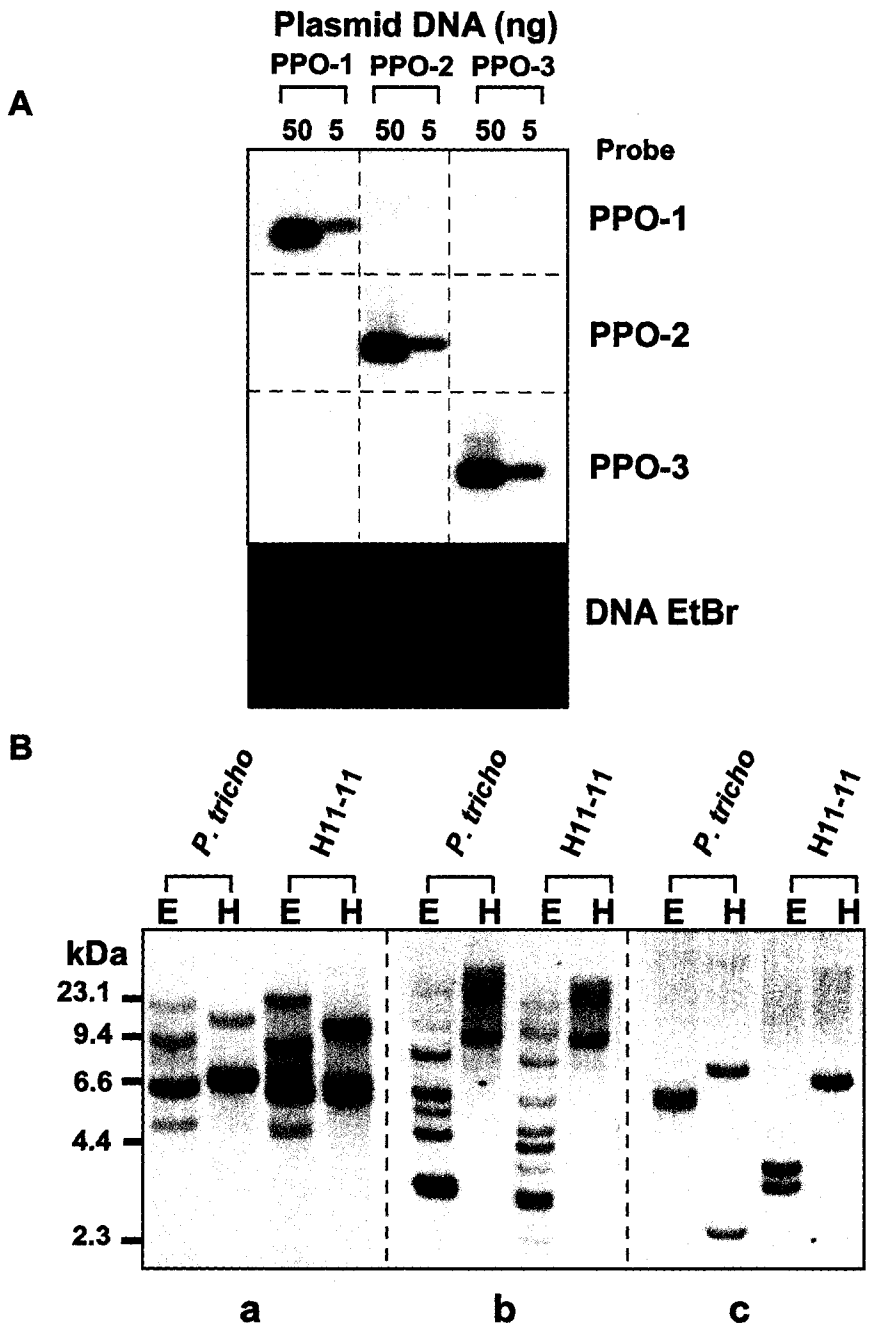
Amino Acid

Nucleotide

Calculated by MEGALIGN, DNAStar software. Similarity compares sequences directly, without accounting for phylogenetic relationships.

Figure 2: Gene-specific PPO probes and Southern analysis of three *PtdPPO* genes in poplar.

(A) Plasmid DNA (50ng and 5ng) corresponding to the three *PtdPPOs* were hybridized with three PPO gene-specific probes. (B) Genomic Southern analysis of *P. trichocarpa* and TD hybrid HY11-11. Ten micrograms of restricted genomic DNA was hybridized with (a) full-length *PtdPPO1* probe; (b) *PtdPPO2* gene-specific probe; and (c) *PtdPPO3* gene-specific probe. E, *Eco*RI; H: *Hin*dIII.



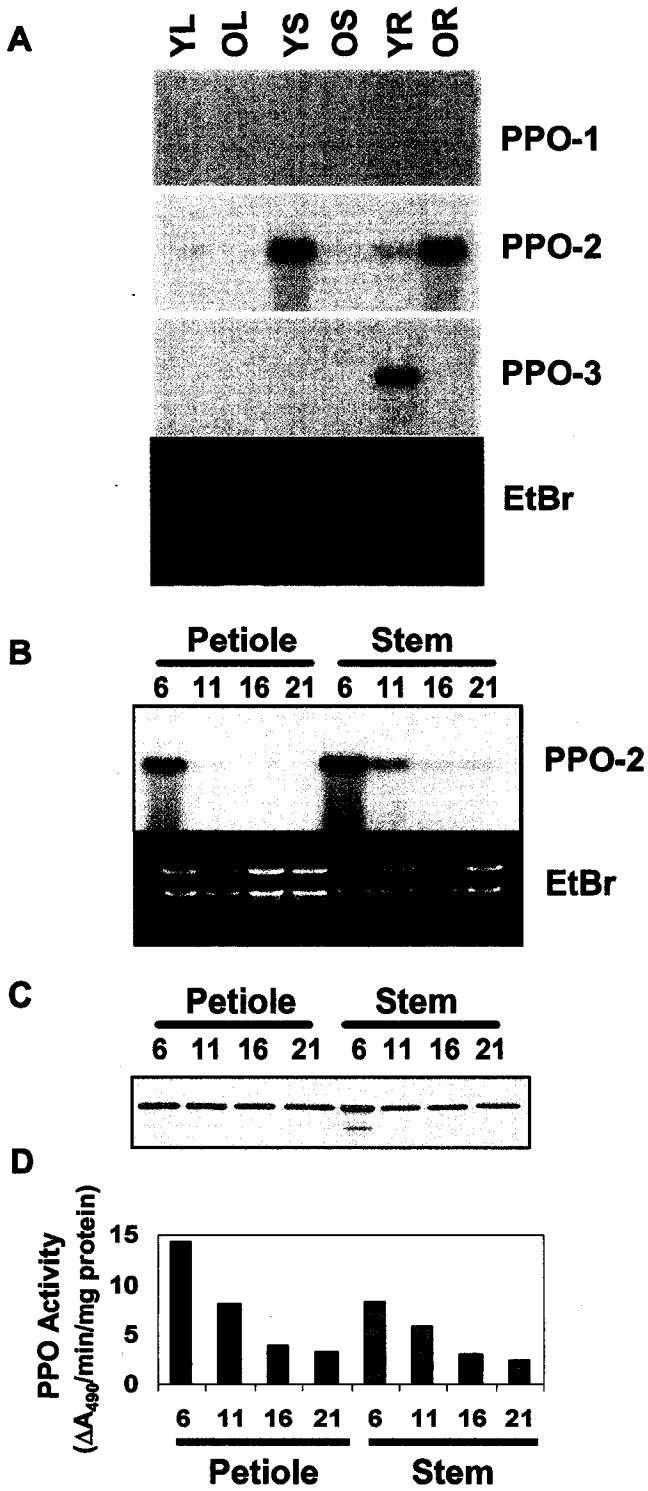
2.3.3 The three PtdPPO genes show differential expression in development and following stress

In normally growing hybrid poplar, *PtdPPO1* transcripts could not be detected in any vegetative organs tested, i.e. in leaves, stems and roots (Fig. 3A). By contrast, *PtdPPO2* transcript was detected and most abundant in young stems and old roots, but less so in old stems and young roots (Fig. 3A). Expression of *PtdPPO2* in young leaf tissue was barely detectable, and may be due to the presence of veins in the sample. *PtdPPO3* was expressed only in young roots, and was not detectable in any other plant organs tested (Fig. 3A). Thus, the three *PtdPPO* genes have distinct constitutive expression patterns in different vegetative tissues of hybrid poplar: *PtdPPO1* is not constitutively expressed in vegetative tissues, *PtdPPO2* is exclusively expressed in conducting organs and *PtdPPO3* is root-specific.

To determine if PPO is up-regulated in younger tissues as has been described for potato (Hunt et al., 1993), I investigated the developmental regulation of *PtdPPO2* expression in petioles and stems along the axis of the plant, which represents a tissue-age gradient. Northern blots showed that the transcript level of *PtdPPO2* was significantly expressed in younger petioles and stems (LPI 6) (Fig. 3B). However, when PPO protein levels were compared in the same tissues on western blots, PPO levels were much more consistent among various tissue ages and even old tissues (LPI 21) had high levels of PPO (Fig. 3C). This distribution of PPO was confirmed using PPO activity assays, although younger tissues did show higher PPO activity than the older ones (Fig. 3D). Overall, my results would suggest that PPO2 protein is primarily synthesized in young petioles and stems, but it is very stable and thus still present in older tissues.

Since it has been established that *PtdPPO1* is inducible by wounding and MeJA in leaves (Constabel et al., 2000), I used northern analysis to test for a response of *PtdPPO2* and *PtdPPO3* to wounding and MeJA. Plants were wounded on leaves and stems as described in Material and Methods, and wounded leaf and stem tissues were harvested after 24 h. I also collected root samples from the wounded plants, but since only above-ground parts of the saplings were wounded these are considered systemically wound-induced. As I had found previously, *PtdPPO1* transcripts accumulated in wounded leaves after mechanical wounding, and the induction was stronger in young

Figure 3: Expression of three *PtdPPO* genes in various hybrid poplar organs.
(A) Total RNA was isolated from young leaf (YL), old leaf (OL), young stem (YS), old stem (OS), young root (YR), and old root (OR). Northern blots were hybridized with three *PtdPPO* gene-specific probes, respectively and rRNA served as loading control. (B) Expression of *PtdPPO2* in petiole and stem at different developmental stages. Petiole and stem were collected from different internode positions. (C) Same tissue samples as in (B) was analyzed in western blot hybridized with polyclonal PPO1 antibody. 10μg total protein was loaded to each lane. (D) PPO activity measured by DOPA assay on petioles and stems at different developing ages.



leaves than in old leaves (Fig. 4). *PtdPPO2* also responded to mechanical wounding by increasing transcript levels, and this inducibility was observed in both stems and roots, but not in leaves. In stems, the induction was more dramatic in young tissues, whereas in roots, the induction was stronger in older tissues (Fig. 4). No induction of *PtdPPO3* was detected in root tissues, but after wounding, a very low induction of *PtdPPO3* could be seen in stems (Fig. 4).

Jasmonates are key signals involved in plant defense, and we therefore tested the inducibility by MeJA of the three *PtdPPO* genes. As previously reported, *PtdPPO1* was highly inducible by MeJA in leaf tissues, although I also noted very faint expression in young stems and old roots (Fig 5). By contrast, *PtdPPO2* was dramatically induced by MeJA in stem and old root tissues. *PtdPPO3* expression in roots, however, did not respond to MeJA application (Fig. 5). The induction pattern following MeJA treatment was almost identical to that observed after wounding (compare Fig. 4 and 5). I conclude that, *PtdPPO1* is inducible and leaf specific, *PtdPPO2* is expressed in stems and roots, and is inducible in these tissues, whereas *PtdPPO3* is root-specific and not induced by either MeJA or wounding.

2.3.4 PtdPPO genes are differentially regulated by various elicitors in poplar cell culture

Because of the inducible nature of PPO, I also wanted to test the responses of three *PtdPPO* genes to pathogen stress. For practical reasons, I chose to use a suspension culture, but one derived from the same TD poplar hybrid (de Sá et al., 1992). Cell cultures have been widely used to mimic defense gene induction (Andi et al., 2001; Haruta and Constabel, 2003; Sasabe et al., 2000) and have the advantage of consisting of relatively homogenous cells and being very sensitive to pathogen elicitors compared to whole plants. *PtdPPO2* was highly induced by MeJA in cell culture, as it was in the whole-tree experiments (Fig. 6A). *PtdPPO2* was also induced by a crude extract of the aspen pathogen *P. radiosa*, but to a lesser extent than with MeJA. *PtdPPO2* was only slightly induced by the other elicitors, *Phytophthora megasperma*-derived crude elicitor (*pmg*), which is a nonhost pathogen elicitor, and chitosan (Fig. 6A). By contrast, salicylic acid (SA) repressed the expression of *PtdPPO2* after 3 hours treatment (Fig.

Figure 4: Northern blot analysis of three *PtdPPO* gene expression in response to mechanical wounding.

Leaf and stem tissues were directly wounded and collected 24 hours after wounding. Root tissue was collected from leaf-damaged plants and considered as systemically wounded. Young and old tissue refer to leaves at LPI 2-4 and LPI 16, respectively. C: control; W: wounded; S: systemic.

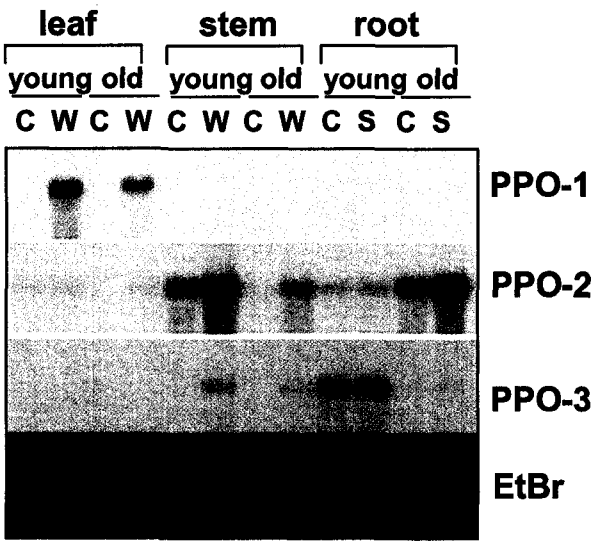


Figure 5: Northern blot analysis of *PtdPPO* gene expression in response to MeJA. A whole sapling was sprayed with MeJA and tissue collected after 24 hours. C: control; MJ: MeJA treated. Tissue types are as described in Fig 4.

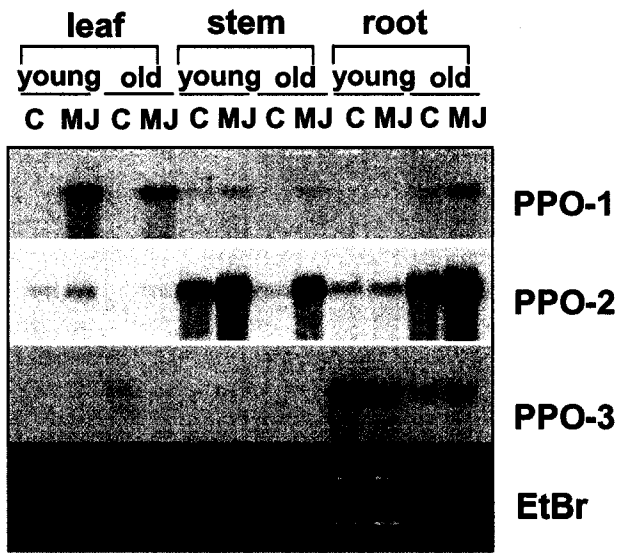
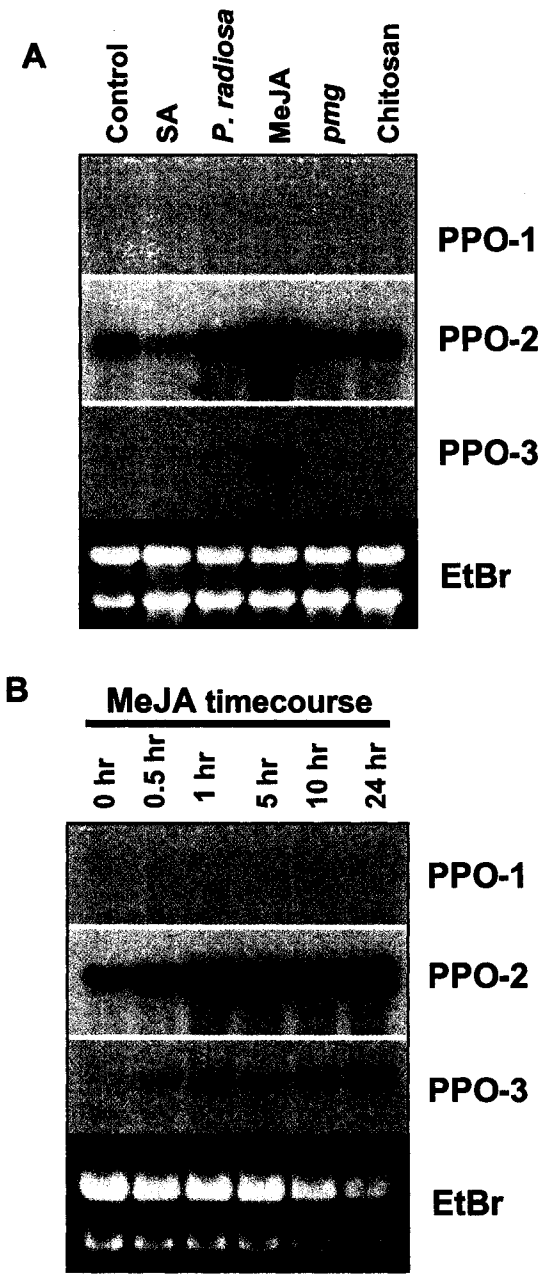


Figure 6: Responses of three *PtdPPO* genes to various elicitors in poplar suspension cell culture.

(A) Northern blot analysis of three *PtdPPO* genes in cell culture in response to different elicitors. Cells were elicited with 1mM SA, 50 μM MeJa, 0.5 μg/mL chitosan, 0.1% (v/v) *Phytophthora megasperma (pmg)* elicitor and 1% *P. radiosa* extract and harvested after 3 hours by centrifugation. (B) Time course of the induction of three PPOs in cell culture by 50 μM MeJA.

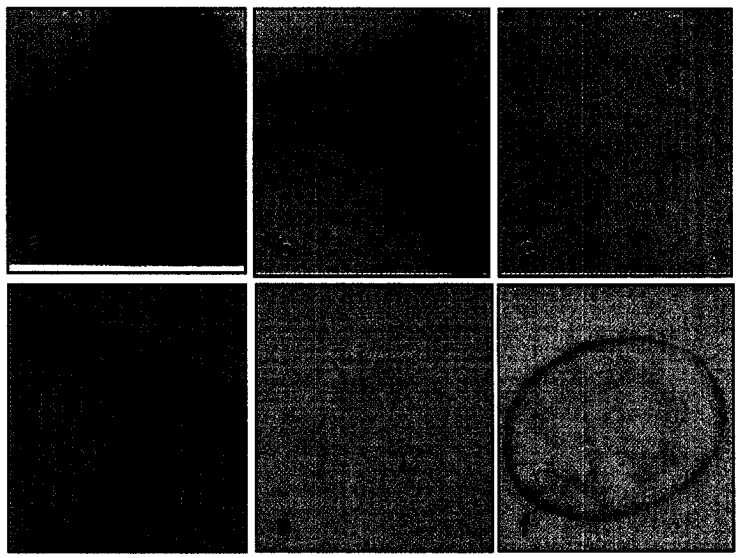


6A). A similar though less pronounced SA-mediated repression of *PtdPPO2* transcripts was also observed in whole-plant experiments, in which 1mM SA was applied to hybrid poplar trees (data not shown). Compared to *PtdPPO2*, *PtdPPO3* was induced to a lesser degree by MeJA in cell cultures (Fig. 6A). I did not detect expression of *PtdPPO1* in cell cultures either before or after any of the elicitor treatments, despite the strong inducibility of this gene in leaves.

2.3.5 PtdPPO2 is localized to subepidermal cells in stem tissue

The cell-type localization of PPO2 protein in hybrid poplar stem and petiole sections was visualized by enzyme activity staining or antibody recognition after tissue printing. Fresh cross sections of stems and petioles were blotted onto PVDF and then incubated with PPO substrates or PPO antibody. For young stem tissue at LPI 2-3, PPO activity was exclusively localized in the subepidermal cells, which was demonstrated by the dark pigments accumulation in these cells (Fig 7A). For older stem tissue below LPI 5, besides the subepidermal cells, PPO activity can also be found in phloem fiber cells and xylem cells (Fig 7B). The oxidation of PPO substrates in subepidermal cells could be totally abolished by the addition of PPO inhibitor, tropolone, which confirmed the specificity of this enzyme staining method (Fig 7C). Tissue prints were also immunolocalized with PPO antibody and same result was obtained. PPO protein was detected in the subepidermal layers and phloem fiber cells (Fig 7D). When trypsin inhibitor antibody was used as a control, no epidermis-specific PPO could be detected (Fig 7E). A section of petiole was also tested with the PPO antibody after tissue printing, and as in the stem, the petiole PPO protein is closely connected with the subepidermis (Fig 7F). When the tissue printing was stained for total protein, subepidermal cell layer did not show higher protein levels compared to other cell types (data not shown), which demonstrated that localization of PPO in this cell type was not due to a higher concentration of protein. Both phloem fibers and xylem cells were highly stained for lignin using phloroglucinol method (data not shown) (Speer, 1987). Because lignin in poplar tissue is closely connected with the activity of laccase, which is able to catalyze the oxidation of PPO substrates, staining in the phloem fibers and xylem cells may be due to activity of laccase, instead of PPO. Therefore, I conclude that PPO protein is highly

Figure 7: Cell-type localization of PPO protein in tissue prints of stem and petiole tissue. Cross-sections of stem and petiole were printed on PVDF membrane and then incubated with PPO substrates or with PPO antibody. (A) Tissue printing of young stem (LPI 3) cross-section was incubated in activity staining buffer with 10mM catechol. (B) Tissue printing of older stem (LPI 10) cross-section was incubated in activity staining buffer with 20mM DOPA. (C) Tissue printing of older stem (LPI 10) cross-section was incubated in activity staining buffer with 20mM DOPA and 1 mM tropolone. (D) Tissue printing of stem cross-section was incubated with PPO antibody then developed for coloration as described for western blot. (E) Same as (D), except stem cross-section was incubated with trypsin inhibitor antibody. (F) Same as (D), except tissue printing of petiole cross-section was used.



expressed in the subepidermal cells in stem and petiole and possibly in the phloem fiber and xylem cells.

2.4 Discussion

2.4.1 The hybrid poplar PPO gene family

PPOs have been extensively studied in plants from a variety of perspectives, and evidence for role of PPO in pest and pathogen defense has been accumulating (Constabel et al., 1995; Duffey and Felton, 1991; Li and Steffens, 2002). We previously isolated the herbivore-inducible hybrid poplar leaf *PtdPPO*, encoding PPO1 and renamed here as *PtdPPO1*, and based on its expression we proposed that it acts in defense against insect herbivores (Constabel et al., 2000). Subsequent experiments revealed the presence of a second protein isoform, PPO2, which was expressed in stems, petioles, and midveins but not leaves. I partially purified both the leaf and the stem PPO, and found they had distinct substrate specificities and other biochemical properties (Wang and Constabel, 2003). Here I report the cloning of the corresponding cDNA (*PtdPPO2*), as well as a third *PtdPPO* cDNA, the root-specific *PtdPPO3*. I found that the three *PtdPPO* genes show very distinct and organ-specific patterns of expression, and also show very different responses to inducers of the defense response.

The specific expression of these *PtdPPOs* facilitated the isolation of partial *PPO* cDNAs from stem RNA by RT-PCR. After establishing that these sequences were distinct from *PtdPPO1*, they were extended to the entire coding region using both 3'RACE and using gene-specific primers based on genomic sequence data from the *P. trichocarpa* genome sequencing project (http://genome.jgi-psf.org/poplar0/poplar0.home.html). As a result, two new hybrid poplar *PtdPPO* genes, *PtdPPO2* and *PtdPPO3*, were characterized, with predicted MWs of 56.3 kDA and 57.4 kDa, respectively. On western blots, the PPO2 protein expressed in stems migrated at approximately 66 kDa, which is somewhat higher than PPO1 (Wang and Constabel, 2003). Nevertheless, I believe that PPO2 isolated from stems is encoded by *PtdPPO2*, given that this is the only *PtdPPO* gene we found to be expressed in petioles and stems (Figures 3, 4 & 5). The discrepancy in MW may be due to migration artifacts or

additional processing of polypeptide as seen in grape (Rathjen and Robinson, 1992). In *Vicia faba*, the deduced molecular mass of mature PPO (58 kDa) is less than the fully denatured PPO isolated from *V. faba* choloroplasts (ca. 65 kDa).

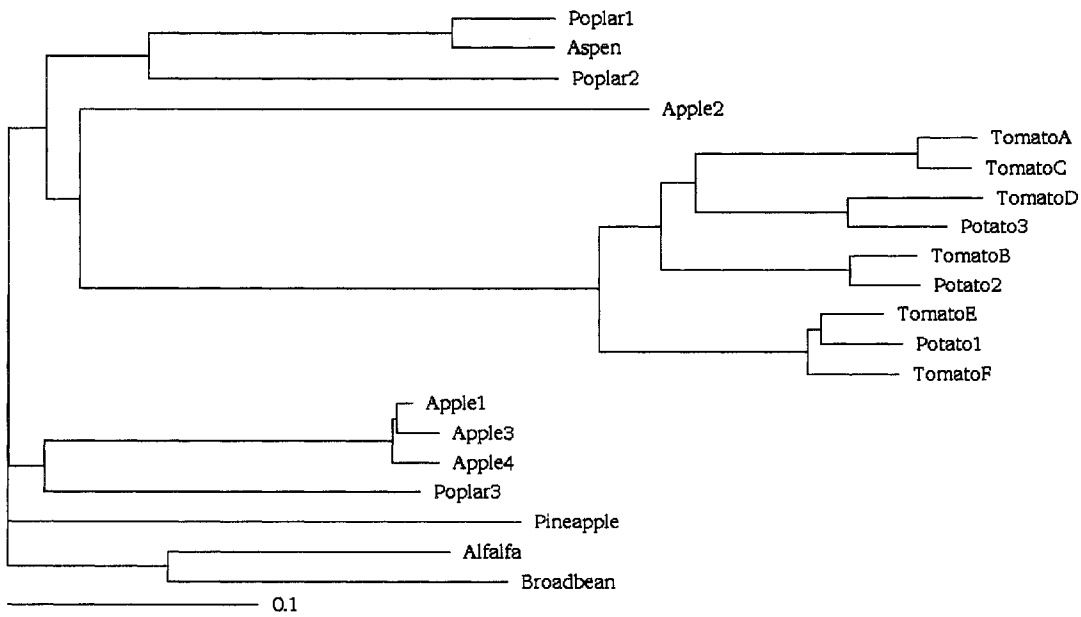
Since I am working with an interspecific hybrid, the cDNAs we cloned could originate from either a *P. trichocarpa* or *P. deltoides* parent. When I compared the first 200 bp at the 5'-end of our cDNA sequences to corresponding genomic *P. trichocarpa* sequence from the *Populus* sequencing project, I found sequences which were 100%, 99%, and 98% identical with *PtdPPO1*, *PtdPPO2* and *PtdPPO3*, respectively. These high similarities suggest that the three hybrid poplar cDNAs genes originate from the *P. trichocarpa* genome. Like most dicot PPOs, it appears that poplar PPOs contain no introns, as none were detected during our analysis of the *P. trichocarpa* genomic data (data not shown). Our Southern analysis can therefore be interpreted more easily, and suggest that both *PtdPPO1* and *PtdPPO2* belong to small gene families with three or four, and four to six members, respectively. By contrast, there are only one or two *PtdPPO3*-type genes in the genome. These ranges of gene family size are roughly consistent with 2-D gel electrophoresis and PPO western blot analysis, where I could detect up to five PPO1, six PPO2, and two PPO3 isofroms (data not shown). Tomato contains at least seven PPO genes, and five PPO cDNAs have been isolated from potato (Hunt et al., 1993; Thygesen et al., 1995). Interestingly, in phylogenetic analysis (Fig 8), the three *PtdPPO* genes do not cluster as a group, unlike the seven characterized tomato PPO genes which are all closely related and physically linked on chromosome 8 (Newman et al., 1993). In my phylogenetic analysis, *PtdPPO1* and *PtdPPO2* both group together with the *P. tremuloides PtPPO1* ortholog. However, *PtdPPO3* is more closely related to several apple *PPO* genes, one of which was found to be wound-inducible in apple fruit (Boss et al., 1995). Therefore, among the different species, there appears to be no correlation of primary sequence type and wound-induced expression.

2.4.2 Expression and possible functions of hybrid poplar PPOs

The expression patterns of three *PtdPPO* genes were surprisingly specific, with *PtdPPO1* expressed only in induced leaves, and *PtdPPO3* only in roots. *PtdPPO2*

Figure 8: Phylogenetic tree of three hybrid poplar PPOs and other plant species-related PPO proteins.

For construction of the tree, we used the mature PPO amino acid sequences of each PPO protein without gaps. The matrix of sequence similarities was calculated with CLUSTAL program from the CLUSTAL X package (Thompson et al., 1997) and submitted to a neighbor-joining analysis to generate a branching pattern. The consensus tree was drawn using the TreeView program (version 0.3, Roderic D.M. Page, University of Glasgow, UK). Scale bar of "0.1" means 0.1 nucleotide substitutions per site. The actual value will depend on the branch lengths in the tree.GenBank Accession Numbers of sequences used are Tomato A (*Lycopersicon esculentum*, Q08303), Tomato B (*Lycopersicon esculentum* Q06355), Tomato C (*Lycopersicon esculentum* Q08305), Tomato D (*Lycopersicon esculentum* Q08306), Tomato E (*Lycopersicon esculentum* Q08307), Tomato F (*Lycopersicon esculentum* Q08296), Potato1 (*Solanum tuberosum* Q06355), Potato2 (*Solanum tuberosum* T07097), Apple1 (*Malus domestica* P43309), Apple2 (*Malus domestica* AAK56323), Apple3 (*Malus domestica* BAA21677), Apple4 (*Malus domestica* BAA21676), Aspen (*Populus tremuloides* AAK53414), Alfalfa (*Medicago sativa subsp. sativa* AAP33165), BroadBean (*Vicia faba* S24758), Pineapple (*Ananas comosus* AAK29782), Poplar1 (*Populus balsamifera subsp. trichocarpa x Populus deltoides* AF263611).



expression was more general, being expressed in organs with conducting functions (root, petiole, midvein, and stem), as well as in cell culture. This organ-specificity is in contrast to similar studies on other species such as tomato, where most of the PPO genes are expressed in a variety of tissues including leaf, root, stem, and reproductive tissues. In potato, PPO expression was also more gene-specific, but unlike our results, most genes were expressed in several different tissues. One exception appears to be the highly root-specific expression of one of the potato PPOs (Thygesen et al., 1995), much like *PtdPPO3*. Tight tissue-specific regulation could be indicative of some differentiation of function of the PPO isoforms.

In previous experiments using western blots, I did not detect wound-induction of PPO2 (Wang and Constabel, 2003); this discrepancy is likely due to the significant level of PPO constitutively present in stem tissues, which can obscure a small increase, and by the greater sensitivity of northern blot analysis. *PtdPPO3* was not wound- or MeJA-induced in roots, and only very slightly MeJA-induced in cell cultures. Wound-induction is a common, though by no means a universal property of plant PPOs, and wound-induced PPO were described in tobacco, tomato, and apple (Boss et al., 1995; Constabel and Ryan, 1998). Interestingly, Thipyapong et al. (1997) found that among the seven tomato PPO genes, only *PPO F* was wound- and MeJA-induced. This PPO also responded to a number of pathogen-related signals including salicylic acid and ethylene. In addition to MeJA, *PtdPPO2* was induced only slightly by elicitors derived from pathogens of poplar (Fig 7), as well as flg22, a bacterial pathogen-derived peptide elicitor (data not shown). By contrast, salicylic acid reduced *PtdPPO2* expression in cells (Fig 7) and whole plants (data not shown), and may demonstrate the interaction, or cross-talk, of signal pathways involved in wounding and pathogen defense. The inhibitory effect of SA on JA-regulated gene expression has been previously reported for the induction of proteinase inhibitors in wounded tomatoes as well as in the *Arabidopsis* defense response (Doares et al., 1995a; Kunkel and Brooks, 2002).

The induction by wounding is consistent with a role for PPOs in defense. We argued previously for a role of PPO1 in defense against folivorous insects based on its pattern of expression (Constabel et al., 2000); by analogy, PPO2 might be effective against stem boring insects. Defense of the stem is likely of paramount importance for the

plant, to protect the vital conducting tissues, and this could explain why plants maintain high constitutive levels of PPO activity in petioles, stems and roots. The induction of *PtdPPO2* by pathogen elicitors also argues for a potential role of this PPO in pathogen defense. In tomato, the importance of PPO-mediated phenolic oxidation in restricting plant disease development has been clearly demonstrated in PPO-overexpressing transgenics (Li and Steffens, 2002).

Determination of cell-type-specific expression patterns may provide additional insight into the roles of specific PPOs. In tomato, PPOs accumulate abundantly in both the internal and the external primary phloem as well as the secondary phloem (Thipyapong et al., 1997). Here I found that in young stems, PPO2 protein was mainly expressed in the subepidermal cells in stems and petioles (Fig 7). In older stems, PPO activity was also be detected in phloem and xylem cells, but this was less conclusive due to the possibility of laccase in those cells (Fig 7).

PtdPPO3 was found to be root-specific, and the role of PPO in roots has not been extensively investigated. Other distinguishing characteristics of this PPO include greater sequence divergence from the other poplar *PtdPPOs*, and a lack of strong stress-inducibility. To determine if PPO3 is also distinct in its biochemical properties, I performed PPO assays with crude young root extracts, where the predominant isoform is expected to be PPO3. Preliminary data on substrate preferences indicated that PPO3 had a higher preference towards chlorogenic acid, and a lower preference towards caffeic acid, compared to PPO1 and PPO2 (data not shown). In addition, PPO3 in crude extracts showed significant activity in the absence of SDS, unlike the other isoforms (data not shown). Therefore, PPO3 appears to be distinct from the other PPOs at several levels; we speculate that this may reflect other, perhaps non-defensive function. For example, a PPO homolog involved for aurone biosyntheis in flower coloration was recently characterized in *Antirrhinum majus* (snapdragon) (Nakayama et al., 2000). Similarly, the expression of Virginian pokeweed PPO specially localized in ripened fruits was suggested to be highly related to the accumulation of betalains (Joy et al., 1995). Future work will focus on cell-type localization of the hybrid poplar PPOs and their functional analysis, from both defense and developmental perspective.

Chapter 3 Polyphenol oxidase overexpression in transgenic *Populus* leaves enhances resistance to forest tent caterpillar (*Malacosoma disstria*) herbivory

3.1 Introduction

Polyphenol oxidase (PPO) catalyzes the oxidation of *o*-diphenols to *o*-diquinones, and in some plants may also perform the *o*-hydroxylation of monophenols, which are immediately oxidized to the *o*-diquinones (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 to investigate the effects of PPO; transgenic apple and potato with antisense suppression of PPO show greatly reduced browning due to their reduced PPO activity (Coetzer et al., 2001; Murata et al., 2000).

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 can be made is a function of PPO in defense against pests and pathogens (Constabel et al., 1996; Steffens et al., 1994). Most significantly, tomato plants overexpressing PPO have been generated, and these exhibited greater in resistance to *P. syringae,* as measured by bacterial growth, compared with control plants (Li and Steffens, 2002). Although the mechanism underlying this effect has not been investigated, most are based on the reactive nature of the *o*-quinones produced. In pest defense, PPO has been convincingly shown to be effective in defense against small insects such as aphids. *Solanum berthaultii* leaves are covered with PPO-containing glandular trichomes; when damaged by insects such as aphids, the PPO is mixed with phenolic substrates and immediately begins to polymerize. The resulting sticky exudate entraps the insects and prevents them from feeding *via* occlusion of their mouthparts (Kowalski et al., 1992).

An adaptive role for PPO has also been proposed as an antinutritive defense against leaf-eating insects (Constabel et al., 1996; Duffey and Felton, 1991). Leaf PPO is widespread in the plant kingdom, and unlike PPO-containing trichomes, such a defensive

role could have widespread importance. In tomato plants, wounding and herbivory stimulates a plant-wide induction of defense genes and proteins including a suite of proteinase inhibitors as well as PPO, suggesting role of this protein in defense against leaf herbivory (Bergey et al., 1996; Felton et al., 1989). This response is regulated by the 18-amino-acid peptide wound hormone called systemin (McGurl et al., 1992), and transgenic plants that overexpress prosystemin, the systemin precursor, exhibit extremely high constitutive expression of polyphenol oxidase and show a strong browning phenotype in damaged leaves and extracts (Constabel et al., 1995; Constabel et al., 1996). Expression of an antisense prosystemin gene in tomato plants reduces resistance toward *Manduca sexta* larvae, and this is likely due to the absence of the combination of induced defense proteins (Orozco-Cardenas et al., 1993). 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 crosslink dietary proteins during feeding, resulting in decreased amino acid assimilation (Felton et al., 1989; Felton et al., 1992). Since the amino acids most susceptible to attack by *o*-quinones (lysine, histidine, cysteine, and methionine) are generally limiting in herbivore diet, the effect of these modifications can be significant (Felton et al., 1992). Despite good indirect evidence linking high PPO in the diet to reduced insect nutrition and performance, the effects of PPO have never been demonstrated directly, for example using transgenic plants.

We previously cloned a wound-inducible *PPO* from hybrid poplar (*Populus trichocarpa x P. deltoides*) and trembling aspen (*P. tremuloides*) (Constabel et al., 2000; Haruta et al., 2001b). High levels of expression and systemic inducibility by herbivores and wounding are most consistent with the role of these genes 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 subject to dramatic defoliator attacks (Osier and Lindroth, 2001). To obtain direct proof of the antiherbivore role of PPO in *Populus*, and to determine its potential for biotechnological applications in forestry and agriculture, I overexpressed hybrid poplar *PtdPPO1* in transgenic *P. tremula x alba* plants. High-PPO foliage from these transgenics showed enhanced resistance to forest tent caterpillar (FTC), *Malacosoma disstria*. These plants also provided insight into the activation of latent PPO in the caterpillar gut.

3.2 Material and Methods

3.2.1 Plant transformation and maintenance

The hybrid poplar *PtdPPO1* cDNA (Constabel et al., 2000) was PCR-amplified and linker sites engineered for subcloning into pBI-525, between a duplicated-enhancer cauliflower mosaic virus 35S promoter with the AMV RNA4 trans/enhancer sequence and a nopaline synthetase terminator region (*NOS*) (Datla et al., 1993b). Both sense and antisense constructs were constructed. The overexpressing cassettes (35S promoter/enhancer + PPO coding sequence + *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, in which *PtdPPO1* cDNA was 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 obtained for use as a control construct. Transformation of hybrid aspen (*Populus tremula x P. alba* clone INRA 717I-B4) was done according to Leplé et al. (1992) using stems and petioles from *in vitro* grown plantlets. Transformed calli were separated from petiole and stem segments and transferred to medium containing 100 mg/L kanamycin in the light for regeneration of shoots. After 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 planting mix (Sunshine Mix #4; Sungro Inc., ON, Canada) plus slow-release nutrients (0.458 g/L Superphosphate 0-20-0, 1.2 g/L 18-6-12+ micronutrients, 4.75 g/L Dolomite lime, and 8.9 g/L Osmocote 18-7-12; Acer, Westgro Sales Inc., BC, Canada). All plants were maintained in the Bev Glover Greenhouse of the Centre for Forest Biology at the University of Victoria.

3.2.2. Enzyme assays, western blotting, and phenolic analysis

For PPO activity assays, leaves or FTC frass were ground in 100 mM sodium phosphate buffer with 0.1% Triton X-100, pH 7. Extracts were centrifuged at 13,000 rpm and the supernatant tested for PPO activity using the DOPA (dihydroxyphenylalanine) assay as described previously (Constabel et al., 2000). One enzyme unit was defined as 0.1 ΔA490/min. The protein content was estimated by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard. For western and protein analyses, soluble proteins were separated on SDS-PAGE (12% w/v acrylamide) gels and visualized by Coomassie blue staining, or transferred to PVDF membranes (BioRad, Mississauga, Canada) for detection using polyclonal antibody raised against recombinant PPO1 protein. Immunocomplexes were detected with alkaline phosphatase conjugate (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 690nm was recorded. To quantify total phenolics in control and transgenic foliage, we used the Folin Ciocalteau method as described previously (Singleton and Rossi, 1965). A 25mg fresh leaf samples were extracted in 10ml MeOH (80% v/v). The clarified extract (100µl) was added to 500µl Folin-Ciocalteu reagent (Sigma, Oakville, Canada) and mixed. After the addition of 2.5ml Na_2CO_3 (20% w/v), the total volume was brought up to 5 ml with water and sit for 20 minutes. Following centrifugation at 3220*g* for 5 minutes, the absorbance at 735 nm was recorded. Tannic acid (Sigma) was used to make a standard curve. All analyses were replicated at least three times.

3.2.3 Northern and Southern analysis

For northern blots, total RNA was isolated from young leaves using the procedure described previously (Haruta et al., 2001b). Ten micrograms of total RNA extracted from transgenic and wild-type lines were electrophoresed on 1.2% (w/v) agarose denaturing formaldehyde gels, and subsequently transferred onto Hybond$^+$ membranes (Amersham, Baie d'Urfé, Canada) using standard procedures (Sambrook and Russell, 2001). Membranes were hybridized with a ^{32}P-labelled, full-length *PtdPPO* fragment using standard procedures (Church and Gilbert, 1984) and detected with a PhosphorImager (Molecular Dynamics, Sunnyvale, USA) as previously described (2.2.3).

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), electrophoresed on 0.8% (w/v) agarose gel and transferred onto Hybond$^+$ membranes. Hybridization was performed using a 620-bp, gel-purified duplicate-enhancer CaMV 35S promoter fragment or the *PtdPPO* probe.

3.2.4 Forest tent caterpillar feeding bioassays

FTC egg bands were obtained from Bob McCron, Insect Production, Canadian Forest Centre, Sault Ste. Marie, ON, Canada and stored at -2°C until used. Feeding preference tests 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 experiments were about two-month old and had at least 25 leaves. Leaves were designated by the leaf plastochron index (LPI) (Maksymowych and Erickson, 1960); the first developing leaf of lamina length ≥20-mm, was designated as the index leaf (LPI 0), and all other leaves were numbered accordingly.

For no-choice experiments, each petri dish held 4 leaf disks of 2.1 cm^2 punched from either low-PPO or high-PPO hybrid aspen saplings, with 4-5 replicate dishes for each tree depending on the experiments. All the PPO-overexpressing transgenics used in feeding bioassays are high-PPO expressors and have PPO levels close to each other. Just-hatched FTC larvae were fed with starting disks from leaves of LPI 11, which were renewed every two days with leaves at the next LPI. Larvae were weighed, and the leaf discs were weighed to record leaf consumption every two days. Each no-choice consumption lasted two weeks and survivorship and molting in each petri dish were monitored daily. To test the difference in weight gain and mortality of FTC reared on low-PPO and high-PPO aspens, one-way-ANOVA was performed on the FTC average weight gain and mortality data. All analysis was performed with EXCEL (Microsoft, Seattle, USA).

Choice experiments were carried out using interveinal leaf disks of 1.5 cm^2 punched from leaf at LPI 12 of wild-type and PPO-overexpressing transgenic lines, and were anchored in alternating order with minuten pins through the filter paper. Four third-

instar FTC larvae were placed in the center allowed to feed and removed after 48 hours. Leaf consumption was recorded with an area meter (LI-3100 Area meter, LI-COR Inc. USA) or as the weight loss of the leaf discs 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).

3.3 Results

3.3.1 Transformation and characterization of transgenic aspens

In order to test the functional roles of PPO, we overexpressed the *PtdPPO* cDNA at high levels in transgenic hybrid aspen by using an overexpression vector containing the double CaMV 35S promoter sequence as well as the AMV translational enhancer sequence (Datla et al., 1993b). I transformed the *P. tremula x P. alba* clone INRA 717I-B4 due to its susceptibility to *Agrobacterium* and low basal levels of PPO activity and inducibility. Over 30 independent kanamycin-resistant putative transformants were produced. Preliminary analysis of leaves of 25 transformants indicated up to 50-fold greater PPO activity relative to untransformed controls. Individuals derived from the same transformed line exhibited very similar levels of PPO, and PPO activity remained stable through multiple rounds of *in vitro* micropropagation. Antisense PPO-transformed plant showed no significantly altered PPO levels, despite substantial antisense mRNA levels on northern blots (Fig 1). Likewise, no significant differences in PPO activity between antisense and control plants could be detected following wounding. As a result, I included the antisense plants as low-PPO trees along with the untransformed controls. Nine PPO overexpressing transgenics, as well as one GUS transgenic and one PPO-antisense transgenic were chosen for further analysis. PCR analysis using *NPTII* gene-specific oligonucleotide primers revealed that the putative transformants all contained this marker gene (data not shown). Foliage from leaves at LPI 12 from the PPO-overexpressing lines showed PPO activity in the range of 35-435 units/mg protein, while GUS transgenic, PPO-antisense and untransformed controls all had similar and very low PPO activity (~10 units/mg protein) (Fig 1A). *PtdPPO* was originally cloned from

hybrid poplar (*P. trichocarpa x P. deltoides*) and in this *Populus* species, PPO activity after wounding is significantly induced in leaves, particularly in young tissues (from ~10 units to 200 units/mg protein at LPI 5). Therefore, constitutive PPO activity in transgenic aspens constructed in this study is even higher than that in wounded TD hybrid poplar leaves. Southern blots using a 35S promoter probe confirmed that these GUS, antisense PPO, and PPO-overexpressing plants contained one to several copies of this promoter sequence, while in the untransformed line no hybridization signal was detected (Fig 1B). Rehybridizing this blot using the *PtdPPO* gene 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 also confirmed that the elevated PPO activity in the trangenics was due to the PPO transgene, as levels of PPO mRNA were generally proportional to PPO activity (Fig 1C). Likewise, western blots using a PPO-specific antibody also showed strongest signals in protein extracts of plants with higher PPO activity. These blots also showed a faint band in the control and antisense plants, migrating slightly higher than the ectopically expressed PPO. Based on its constitutive expression and slower migration, this band likely corresponds to the endogenous PPO2 protein (Wang and Constabel, 2003). It appeared not to be affected by the antisense PPO, as a number of different lines showed the same band intensity (Fig 1).

To further characterize the transgenics, I compared PPO expression in leaves of different ages along the axis of saplings, in both PPO-overexpressors 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 exactly the same pattern (data not shown). Interestingly, PPO activity increased with leaf age, in both transgenics and controls (Figure 2A). Western and northern analysis again confirmed that the increased PPO activity in both control and transgenic lines correlated with PPO protein and mRNA levels (Figure 2B and 2C). The northern blots also suggested that PPO transcript levels are higher in older leaves,

Figure 1: Characterization of PPO expression level in control and PPO-overexpressing transgenics.

Leaves at LPI 12 from nine independent PPO overexpressing transgenics, one PPO antisense transgenic, one GUS transgenic and one control hybrid aspen were analyzed for PPO activity using DOPA assay (each unit equals to 0.1 ΔA_{490}/min). (A) Each measurement was repeated three times and standard deviation (SD) was shown as error bars. (B) The same leaf tissues as in (A) were further analyzed in Southern blots using the duplicate CaMV 35S promoter fragment as probe; (C) Northern blot for PPO transcriptional level using full-length PPO cDNA fragment as probe; and (D) Western blot for PPO protein level using polyclonal PPO antibody.

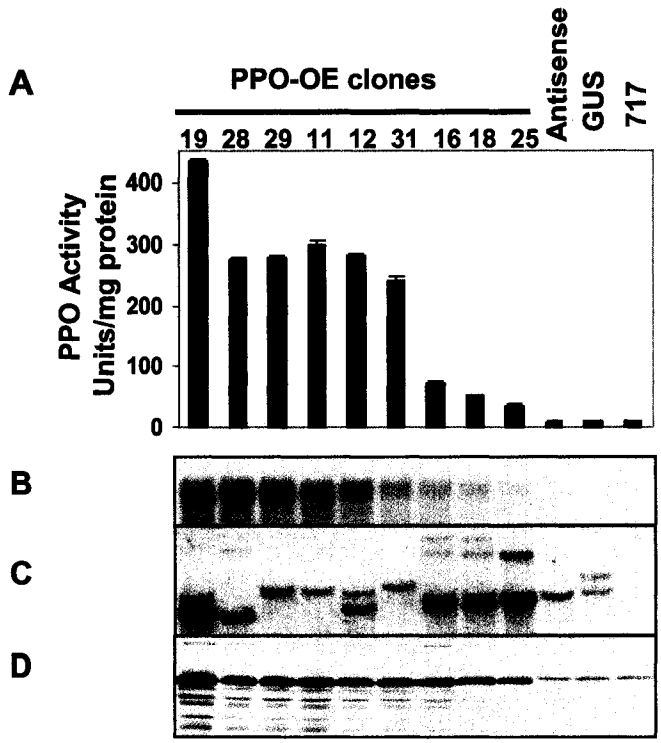
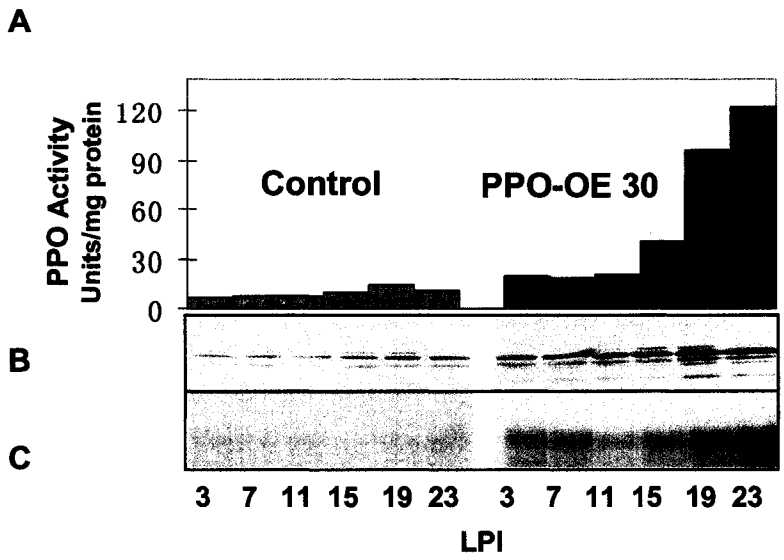


Figure 2: Analysis of PPO expression in leaves of different ages along axis of trees in both a control and PPO overexpressing trangenic (#30). (A) Leaves from LPI 3 to LPI 23 were analyzed for PPO activity in DOPA assay. Same tissue as in (A) was analyzed in (B) PPO protein level in western blots using polyclonal PPO antibody; (C) PPO transcriptional level in northern blots using full-length PPO cDNA as probe.



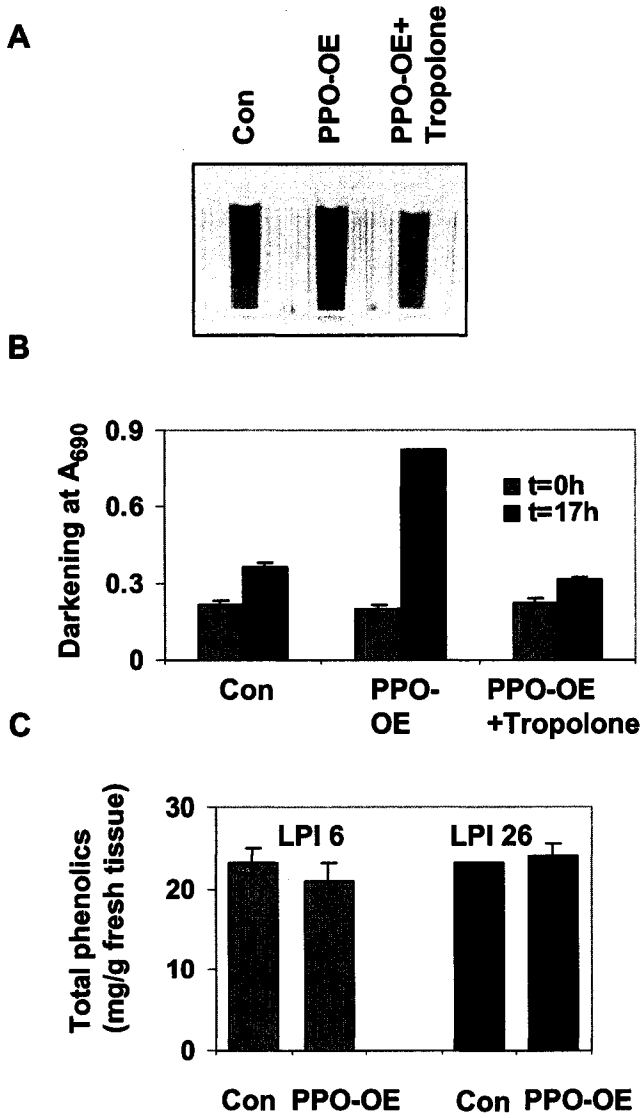
indicating that the higher PPO activity in older leaves may be due to higher rates of transcription in these leaves. The role of PPO in tissue browning predicts that PPO-overexpressing leaves should brown or darken more rapidly than controls. To test for such autonomous browning, I incubated fresh leaf extracts of PPO-overexpressor and control plants at room temperature (Fig 3A). Overnight incubation of extract from plants overexpressing PPO, but not extract from controls, resulted in strong browning, which could be inhibited by tropolone, a specific PPO inhibitor (Kahn and Andrawis, 1985). Absorbance measurements (690 nm) in fresh extracts showed no difference between transgenics and controls, but after the 17-h, room temperature incubation, a dramatic increase in A_{690} was seen in the transgenic sample whereas only a small increase was visible in the control (Fig 3B). Tropolone was again effective at inhibiting this browning, thus demonstrating that the high PPO levels were responsible for the pronounced browning. The content of total phenolics in leaves, as measured by the Folin assay, appeared not to differ significantly between controls and transgenics (Fig 3C). The rapid autonomous browning in transgenic extracts is an important observation, as it suggests that PPO substrates are not limiting in these plant extracts. This indicates that the high PPO transgenics contain sufficient phenolic substrates to initiate artificially high rates of autonomous phenol oxidation and quinone formation. Therefore they should be ideal for testing the effect of this phenomenon on folivorous insects.

3.3.2 FTC feeding experiments

In order to determine if elevated PPO was detrimental to larval performance or feeding, I conducted a series of bioassay experiments using FTC larvae (just hatched) and leaf disks. Preliminary tests indicated that leaf toughness and FTC feeding preference, was very much dependent on leaf age. Therefore, identical leaves of the same size and LPI were always chosen for comparative experiments. I first conducted choice tests using alternating leaf disks (see Materials and Methods), with several larvae feeding in each petri dish for 48 h. No preference could be detected in nine repeat experiments using several different paired plant types. Thus, it is concluded that PPO does not have a strong influence of the FTC feeding choice (data not shown).

Figure 3: Tissue browning and total phenolics content in leaf extracts from both control and PPO-overexpressing transgenics.

(A) Photo of darkening of leaf extract after 17 hours incubation at room temperature. (B) Same extract as in (A) was measured for their A_{690} before and after darkening process. (C) Content of total phenolics in leaves at LPI 6 and LPI 26 was analyzed using Folin-Ciocalteu method for control and PPO transgenics. These experiments were repeated three times with identical results. The means (±SD) are shown.



Next, I conducted a series of no-choice tests on low-PPO and high-PPO trees, performed between July and October, 2002. Each experiment consisted of several low-PPO and high-PPO transgenic lines (3-8 of each), for each tree, at least four replicate dishes containing 4-6 leaf discs, together with 5-8 first instar FTC larvae were used. In the first two no-choice experiments carried out during August 2002, no significant differences were detected in terms of larval average weight after two weeks between low-PPO and high-PPO groups (Table 1). Likewise, in these experiments I detected no differences in leaf area or leaf biomass consumed from either group of plants, indicating that FTC feeding on PPO-overexpressing transgenics did not consume more leaf tissue in order to gain the same weight compared to FTC feeding on low-PPO plants. Survival was almost 100% in both larval populations in both experiments (Table 1). In experiment 3, FTC on PPO-overexpressing plants performed more poorly than those on low-PPO plants, both in terms of survival and final average weight, although ANOVA indicated that this was not significant. During subsequent experiments 4 and 5 (late September and October 2002), the PPO-overexpressing transgenics had a strong negative effect on growth and survival of FTC relative to controls (Table 1). This was particularly evident in experiment 5, where survival was reduced to 21 % for high-PPO group compared to 67 % for low-PPO group (Table 1). In this experiment, at the end of 14 days, the average weight of larvae reared on high-PPO foliage was only one third as much the weight of FTC reared on low-PPO leaves, and experienced three times higher mortality (Table 1). In both experiments 4 and 5, there was clearly lower survival and much slower growth of larvae regardless of the foliage they fed. At the beginning of experiment 5, the FTC egg masses had been stored for over six months, and we hypothesized that this extended time in storage reduced the health and vigor of the hatched FTC larvae. To confirm our results, we repeated our bioassays the following year with a new batch of FTC egg bands. As before, I detected no differences in FTC performance on low-PPO and high-PPO leaf disks during experiments carried out in the summer (experiment 6, July 2003), but did find pronounced differences in both survival and average weight when the experiment was carried out later in September through October 2003 (Table 1). These experiments suggest that PPO can have a direct effect on FTC, in particular when FTC are stressed or have reduced growth due to other factors (see Discussion).

Table 1

Forest tent caterpillar (FTC) performance in no-choice tests on hybrid aspen clone.

	FTC performance	Treatment		ANOVA		
Hatch date*	**in 14 days**	**Controls****	**Transgenics*****	**F**	**P**	**df**
2002-08-01	Weight	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
2002-08-21	Weight	15.5±5.1	15.8±2.9	0.04	0.85	1, 22
	Survival	98.3±5.8	96.7±7.8	0.35	0.56	1, 22
2002-09-05	Weight	5.4±1.3	4.6±1.0	1.68	0.22	1, 14
	Survival	92.5±14.9	85.0±14.9	0.45	0.51	1, 14
2002-09-16	Weight	3.7±0.5	2.9±0.6	23.9	<0.0001	1, 38
	Survival	75.0±8.9	70.0±12.1	2.21	0.15	1, 38
2002-10-17	Weight	2.5±0.5	1.4±0.6	32.7	<0.0001	1, 28
	Survival	55.8±14.8	34.2±18.6	12.5	<0.001	1, 28
2003-07-10	Weight	10.8±2.8	11.8±1.7	2.62	0.11	1, 46
	Survival	81.1±10.7	83.3±12.6	0.19	0.66	1, 46
2003-09-28	Weight	5.4±1.0	3.3±0.6	91.8	<0.0001	1, 62
	Survival	68.4±12.3	41.4±8.7	102.9	<0.0001	1, 62

First instar FTC larvae were fed on high-PPO and low-PPO leaf discs as described in Experimental procedures. * FTC egg bands were obtained in May 2002 and 2003 and then stored at -2°C until being used on the hatch date. ** Low-PPO plants included untransformed controls and PPO antisense transgenics, which also showed unaltered (control) PPO expression level. *** Transgenics were PPO-overexpressing clones, which showed high PPO expression levels.

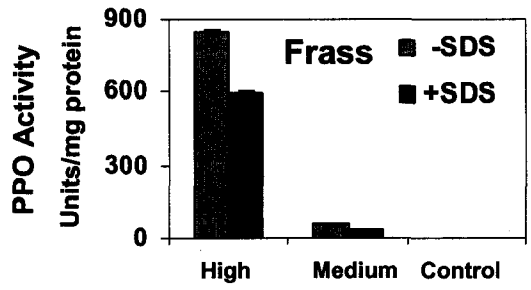
3.3.3 Detection of PPO activity in FTC frass

To be an effective anti-nutritive defense, PPO is expected to be at least partially resistant to digestion and inactivation by enzymes of the insect gut. Therefore, frass was collected from FTC larvae that had fed on PPO-overexpressing leaves (both high and medium expressors), as well as control leaves. I measured PPO activity in the presence and absence of SDS, in order to test if the PPO in the frass had been activated from its latent state by passage through the insect gut. It had been previously determined that like many plant PPOs, poplar and aspen PPO is latent and requires either detergents such as SDS or proteolytic activation (Constabel et al., 2000; Wang and Constabel, 2003). No PPO activity was detected in control leaf frass, but significant PPO activity was present in frass from both high and moderately overexpressing lines (Figure 4A). Interestingly, PPO protein in FTC frass was fully active, and adding SDS to the assay did not increase measurable PPO activity, suggesting that the proteolytic gut enzymes may have activated the enzyme. For comparison, extracts of the same leaves that had been used to generate the frass were assayed for PPO activity; very similar levels of activity were detected in these extracts if SDS was present in the assay buffer. Almost no detectable PPO activity could be measured without SDS, illustrating the latency of the enzyme (Fig 4B).

To test if the activation of PPO in the caterpillar guts was due to partial proteolysis of PPO, frass protein was analyzed by SDS/PAGE and PPO western blotting. In control and transgenic leaf extracts, both endogenous and overexpressed PPO were clearly detected at the expected mass of 59 and 66 kDa, respectively, although at very different expression levels (Fig 5A, left lanes). In frass extracts, bands corresponding to PPO were again detected and at comparable relative intensities; however, after passage through the caterpillar gut, the main PPO band migrated at approximately 40 kDa (Fig 5A, 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 in the insect. Activation of PPO by protease digestion has been observed previously (Robinson and Dry, 1992), and in my earlier work with semi-purified PPO, I demonstrated that trypsin was able to activate the latent enzyme (Wang and Constabel, 2003). The intensity of the bands confirms my assay results indicating that a significant proportion of leaf PPO remains intact during digestion. Coomassie-stained SDS gels corroborated this observation; overexpressed PPO

Figure 4: Effect of SDS on PPO activity in FTC frass and transgenic *Populus* leaves. Leaf tissues from one high-PPO expressor, one medium-PPO expressor, and one control tree, as well as the frass collected from the FTC feeding on these trees, were analyzed for PPO activity with or without 0.15% SDS. (A) PPO activities in the FTC frass extracts were measured by the DOPA assay with and without SDS; (B) PPO activities in extracts of the same leaves were measured by the DOPA assay with and without SDS.

A



B

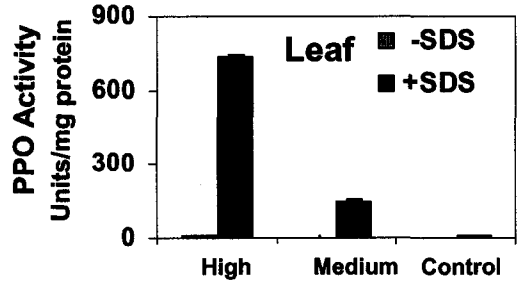
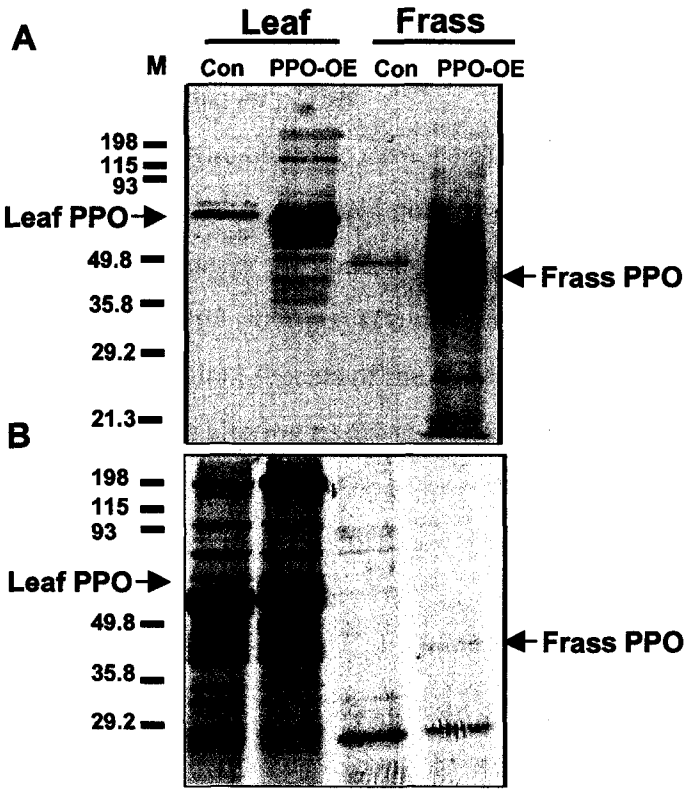


Figure 5: Proteolytic processing of *Populus* leaf PPO protein after passage through FTC digestive tract.

Total proteins were extracted from poplar leaves (PPO transgenic and control poplar) and FTC frass (FTC fed on PPO transgenic or control poplar). Protein concentration was checked using Bradford assay and same amount of total protein was loaded on comparative lanes. (A) PPO protein was detected by PPO antibody on western blot. The left two lanes show leaf PPO protein in control and PPO overexpressors and the right two lanes show their respective processed leaf PPO protein in FTC frass. (B) The same samples as in (A) were stained for total protein using Coomassie Blue.



was visible as a discrete yet minor band among the other leaf proteins in overexpressor extracts. A similarly faint band corresponding to the processed PPO was seen in frass extract; however, most other leaf proteins had dissappeared from the extract, presumably due to digestive enzymes. Therefore, it appears that PPO is more resistant to digestive enzymes compared to most other leaf proteins.

3.4 Discussion

3.4.1 PPO-overexpressing hybrid aspen plants

In this study, I used transgenic plants to demonstrate the effects of the proposed defense and antiherbivore protein, PPO. A hybrid poplar wound- and herbivore-inducible PPO gene (*PtdPPO1*) was placed under the control of the double CaMV 35S promoter and successfully transformed into hybrid aspen (*Populus tremula x P. alba)* using the method of Leplé et al. (1992). Tandem repeats of the CaMV 35S promoter are known to enhance gene transcription without altering tissue-specific expression patterns (Kay et al., 1987). In addition, the construct included a 44-bp segment of the alfalfa mosaic virus translational enhancer region, which increases expression levels in transient expression systems, possibly by enhancing mRNA stability (Bekkaoui et al., 1990; Datla et al., 1993a). My results confirmed the efficacy of this enhanced promoter to confer high levels of constitutive transgene expression in leaf tissues of hybrid poplar (Figures 1 and 2). PPO expression levels were dramatically higher (up to 50-fold) compared to untransformed controls or GUS transgenics; however, no growth abnormalities were observed for any PPO-overexpressing plants.

By contrast, my PPO antisense transgenics did not show any clear phenotype, despite confirmed antisense mRNA expression, and all antisense plants analyzed had similar PPO activity compared to the untransformed or GUS-transformed controls. Even after wounding of leaves, the antisense plants had similar PPO levels to controls. However, I previously observed that wounding does not induce strong PPO expression in the B717 hybrid aspen genotype; therefore it is possible that antisense suppression was simply too low to detect. The faint constitutive PPO2 band visible in western blots also appeared to be unaffected in the antisense plants (Figure 1, panel D). I recently isolated a

cDNA likely corresponding to this band, and the coding sequence shares only 66% amino acid identity with the *PtdPPO1* transgene, which is too divergent for the antisense strategy to have been effective on *PtdPPO2*.

Although high constitutive levels of PPO activity were consistently observed in all leaves of transgenics, the highest PPO activity was always found in the old leaves, regardless of which transgenic line was analyzed (Fig 2 & data not shown). Surprisingly, this correlation of higher PPO activity with tissue age was also observed in untransformed control plants, although to a much lesser degree due to low levels of constitutive PPO. This pattern is also roughly reflected on northern blots, in which three independent transgenic lines also showed highest PPO mRNA in the old leaves (Fig 2 & data not shown). By contrast, in PPO-overexpressing transgenic tomato plants, the expression of a potato PPO gene driven by the CaMV 35S promoter is down-regulated with increasing leaf age (Li and Steffens, 2002). One possible explanation for this difference is distinct activities of the 35S promoter in different species. A GUS gene expressed in *Nicotiana tabaccum* L. plants under the control of the 35S promoter was affected by ontogenic development of the leaves and GUS activity in ontogenetically younger leaves was almost ten times higher than in ontogenetically older leaves (Pret'ová, 2001). In addition to differences in promoter activity, high PPO levels in old leaf tissues are also be the result of the high stability and slow turnover rate of PPO protein observed in other work.

In the autonomous browning experiments, extracts of PPO-overexpressing poplar leaves turned brown more rapidly than the control leaves (Fig 3). This demonstrates that the endogenous phenolic substrate pool was sufficiently large to sustain more rapid phenolic oxidation for a significant time, even though total phenolic levels were very similar to those 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). The implication of this experiment is that it is PPO, rather than substrate, that normally limits phenolic oxidation during tissue damage; from a biotechnological perspective, this suggests that overexpression of PPO alone will have a significant impact on improving oxidative defenses. Furthermore, regulatory genes may be used to upregulate entire branches of the

phenylpropanoid biosynthetic pathway (Bovy et al., 2002). In the future, such genes could potentially be used in combination with PPO to further enhance pest resistance. In poplar, the endogenous PPO substrate has not been identified, although it has been previously suggested that in trembling aspen, breakdown products derived from tremulacin, salicortin, or other phenolic glycosides are potential PPO substrates (Haruta et al., 2001b). Future experiments would address the issue of substrates in more detail.

Folin assays indicated that the level 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 has recently been shown to have a role as a hydroxylase in the biosynthesis of specific phenolics such as aurones and lignans (Davin and Lewis, 2000; Nakayama et al., 2000), this does not appear to be the case for poplar PPO. The earlier suggestion that PPOs might be required in the general phenylpropanoid pathway in catalyzing the 3'-hydroxylation of *p*-coumaric acid has been disproven by the identification of the enzyme(s) carrying out this step (Humphreys and Chapple, 2002). Therefore, my data support the idea that the primary role of poplar PPO is in defense reactions following tissue disruption.

3.4.2 Effects of PPO overexpression on herbivores

My no-choice bioassay data suggest that high PPO activity in PPO-overexpressing plants reduces FTC performance (Table 1). However, significant effects on survival and development were detected only when FTC larvae were hatched in the late season (September and October), and egg masses had been stored at -2°C for over six months. Even when fed low-PPO leaf disks, FTC larvae grew more slowly and had higher mortality as the season progressed, suggesting that egg mass age is an important factor (Table 1). The hatch rate and development of FTC is known to decrease with time in storage (B. McCron, personal communication), suggesting that decreased vigor may have made the FTC more vulnerable to the detrimental effects of PPO in my experiments. I observed an identical pattern over two consecutive years with two different batches of egg bands, and, thus, I conclude that poplar PPO can have negative effects on FTC and that *Populus* PPO acts a defense protein. The idea that PPO is particularly effective

against already weakened FTC is consistent with the observation that plant defense against herbivory is a multi-component process. In hybrid poplar, PPO induction is coordinate with the induction of at least four different trypsin inhibitors, as well as a number of chitinases and other proteins (Bradshaw et al., 1990; Parsons et al., 1989). In trembling aspen, wounding also leads to induction of condensed tannins, and there are a number of other uncharacterized induced proteins and defenses (M Christopher, M Miranda, IT Major, CP Constabel, personal communication). Therefore, in herbivory-induced foliage, PPO is only one of a number of defenses, which likely all work together synergistically. Additional stresses of FTC in the boreal forest are chronic infections of virus, which typically weaken larvae and can lead to increased susceptibility to plant defenses (Donaghue and Hayashi, 1972; Huang et al., 1999). One reason for this complexity is that different defense mechanisms are likely to work differently against different herbivores. Ultimately, it will be important to test our transgenic plants against a diversity of pest insects.

The idea that PPO is a defense protein in poplar is further substantiated by my finding that this enzyme is very resistant to proteolytic enzymes of the caterpillar gut (Fig 5). The PPO protein band remained visible on Coomassie-stained gels after passage through the FTC digestive system, while the majority of proteins were proportionally much more reduced in intensity, and some predominant protein bands such as ribulose bisphosphate carboxylase had dissappeared entirely. PPO assays indicated that the PPO was still active, confirming the resistance of this enzyme to digestive enzymes. I note that poplar leaf PPO activity remains latent during enzyme extraction, and usually requires activation by detergents or other factors such as proteolytic cleavage (Constabel et al., 2000; Wang and Constabel, 2003). Since PPO extracted from frass was fully active and could not be activated further with SDS, it is clear that passage through the digestive tract of FTC has activated latent poplar PPO. The mechanisms by which this occurs is as yet unclear, but I note that the predominant form of PPO visible in the frass appears to have been proteolytically cleaved and migrates much faster than PPO extracted from leaves (Fig 5). Proteolytic processing as a means of activating PPO is well known, and in grape, the *in vivo* active form has a C-terminal portion of the protein removed (Robinson and Dry, 1992). In this context, the latency of PPO which has so long perplexed plant

biochemists, can be seen as an adaptative strategy in defense, by ensuring that the oxidative reactions and reactive quinones are produced within the insect.

The data suggest that PPO can be added to the list of potential transgenes for use in genetically engineering pest resistance into *Populus* species. Other successfully employed genes for *Populus* plant protection include those encoding the *Bacillus thuringiensis* (Bt) d-endotoxin (Robison et al., 1994), an *A. thaliana* cysteine proteinase inhibitor (Delledonne et al., 2001), and tryptophan decarboxylase (Gill et al., 2003). In conclusion, the results in this study suggest an important role of PPO-catalyzed phenolic oxidation in limiting the access of herbivore insect to their diet food, and provide evidence for the involvement of PPO in antiherbivore defense.

Chapter 4: Biochemical characterization of two differentially expressed polyphenol oxidases from hybrid poplar

4.1 Introduction

Polyphenol oxidase (PPO, EC 1.10.3.1) is a common enzyme which uses molecular oxygen to oxidize *o*-diphenols to *o*-quinones, and in some plants also hydroxylate monophenols to *o*-diphenols (monophenolase activity; EC 1.14.18.1). The quinones are reactive compounds which are responsible for the damage-induced browning of many fruit and vegetables (Steffens et al., 1994). Typically, plant PPOs have broad substrate specificities and are able to oxidize a variety of *o*-diphenols. While the physiological function of PPO in many plants is unresolved, in some species there is strong evidence for a role of PPO in defense against insects as well as pathogens (reviewed in Constabel et al., 1996; Steffens et al., 1994). In tomato, PPO induction by the wound signal systemin and the octadecanoid pathway provided strong evidence for a defensive function of PPO against insects (Constabel et al., 1995). A clear role of PPO in pathogen defense was demonstrated by Li and Steffens using PPO-overexpressing transgenic tomato plants, which show enhanced resistance to *Pseudomonas syringae*. An intriguing feature of PPO is that while this enzyme has been found in many plant species, its expression patterns and levels may differ widely between species (Constabel and Ryan, 1998). This suggests that other physiological roles for PPO still remain to be discovered; for example, a hydroxylation reaction during aurone biosynthesis was recently found to be carried out by a PPO-like enzyme (Nakayama et al., 2000).

Plant PPOs are usually encoded by mid-sized gene families. For example, in tomato there are at least seven PPO genes (Newman et al., 1993), and in potato, five PPO genes were characterized (Thygesen et al., 1995). The genes can have complex temporal and tissue-specific patterns of expression, with high levels of transcript commonly found in flowers, young leaves, trichomes, roots, and tubers (Thipyapong et al., 1997; Thygesen et al., 1995). Only one member of the tomato PPO gene family is wound- and stress-induced (Thipyapong and Steffens, 1997). As in higher plants, multiple forms of polyphenol oxidases have also been described in fungi (Zhang et al., 1999).

Previously, a wound- and herbivore-inducible leaf specific PPO from hybrid poplar (*Populus trichocarpa x P. deltoides)* was cloned and proposed to be a component of the inducible defense against leaf-eating herbivores (Constabel et al., 2000).

Additional experiments with PPO-specific antibodies showed the presence of two differentially expressed PPO proteins in hybrid poplar. In order to learn more about these PPO isoforms and their possible functions, both hybrid poplar PPOs were partially purified and characterized.

4.2 Materials and Methods

4.2.1 Plant material

Saplings of poplar hybrid H11-11 (*Populus trichocarpa x P. deltoides*) were propagated and grown in environmental chambers with 16 h days at 21°C as described previously (Constabel et al., 2000). *P. trichocarpa* (clone 93-968) was obtained from Dr. Carl Douglas, University of British Columbia, Vancouver, Canada, and *P. tremula x P. nigra* (clone 305-40) was obtained from Dr. Michael Carlson, British Columbia Ministry of Forests, Vernon, Canada. Plants were about two months old when used. To obtain wounded leaf samples, leaves were mechanically wounded by crushing leaf blades at the margins with pliers. Each leaf was wounded three times at 2 h intervals. Vascular tissue for purification included the stem and petioles between internode 1 and internode 10. All tissue samples were frozen in liquid N_2 and stored at -80°C until use.

4.2.2 PPO assay and protein determination

Unless noted otherwise, all chemicals were purchased from Sigma (Oakville, Ontario). For routine measurements, PPO activity was measured spectrophotometrically by following the conversion of DL-DOPA (dihydroxyphenylalanine) to dopaquinone at 490 nm using a Shimadzu Model UV-1601PC spectrophotometer. DOPA (25 mM) was dissolved in 100 mM citrate phosphate buffer (pH 5.0) with 0.15% SDS and 470 units/ml catalase. Catechol was used as a substrate at a concentration of 10 mM, and the change in absorbance monitored at 410 nm. The assay buffer was aerated for 5 min prior to the assay. For some assays, a coupled TNB (2-nitro-5-thiobenzoic acid) assay was used (Esterbauer et al., 1977). The TNB standard reaction mixture contained 0.1 M citrate–phosphate buffer (pH 6.0), 0.05 mM TNB, and 0.15% SDS. The reaction was initiated by the addition of PPO enzyme, and the decrease in absorbance due to oxidation of TNB

was followed at 412 nm. Under the conditions used, reaction rates were linear for at least 2 min. Approximately equal amounts of PPO1 and PPO2 activity were used for all comparative experiments. Depending on the experiment, between 20 and 60 units of PPO were used per assay, where one unit is defined as the amount of enzyme converting 1 μmol DOPA to dopaquinone per min, calculated using a molar extinction coefficient of 3600 M^{-1} cm^{-1} (Burton and Kirchmann, 1997). Because the TNB assay is more sensitive, lower amounts of enzyme activity were required for this assay. Protein concentration was determined with the Bradford reagent using bovine serum albumin as a standard (Bradford, 1976). All PPO assays were performed in triplicate and means reported. Variation was less than 5% between assays. All experiments were repeated at least three times.

4.2.3. Extraction and partial purification of PPO isoforms

Ground frozen plant material (3 g) was suspended in 30 ml of 100 mM sodium phosphate buffer (pH 7.0) containing 1% Triton X-100, 80 mM ascorbic acid, 2% PVP-40, 2% Amberlite XAD-4, 5% PVPP, and proteinase inhibitor cocktails (Roche Molecular Biochemicals, Montreal), and was stirred on ice for 20 min. The homogenate was centrifuged at 16,000 *g* at 4 °C for 20 min and the supernatant used as the crude extract. Proteins were precipitated sequentially from the crude extract using 40 and 90% $(NH_4)_2SO_4$. Following centrifugation, the protein pellet was suspended in 10 ml of 0.5 M $(NH_4)_2SO_4$ in 10 mM sodium phosphate buffer, pH 7.0. The redissolved protein was loaded on a Phenyl Sepharose CL-4B column (diameter 1.5 cm, length 13 cm) equilibrated with the same buffer and eluted at a flow rate of 45 ml h^{-1}. After washing away unbound proteins with the equilibration buffer, the PPO activity was eluted using a linear gradient (0.5–0 M) of $(NH_4)_2SO_4$ in 10 mM sodium phosphate buffer (pH 7.0), followed by distilled water. The absorbance at 280 nm was monitored with a single path UV monitor and the PPO activity was determined on each 1.5 ml fraction using the DOPA assay. Fractions containing PPO activity were pooled, and the pooled fractions were dialyzed at 4°C overnight against 100 mM sodium phosphate buffers pH 7.0. The dialyzed samples were aliquoted and kept at -20°C for use in further experiments.

4.2.4. Immunoblotting

SDS-PAGE and immunoblotting was carried out using standard procedures (Harlow, 1988). Polyclonal anti-PPO antiserum was raised against recombinant PPO1 protein (Christopher and Constabel, unpublished data). The antiserum was diluted 1:15,000 for immunostaining of the blots. Antibody binding was visualized using goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate in a dilution of 1:3000 (BioRad, USA) and alkaline phosphatase color development reagents 5-bromo-4-chloro-3-indoyl phosphate (BCIP) and nitroblue tetrazolium chloride (NBT).

4.2.5. In-gel staining of PPO activity

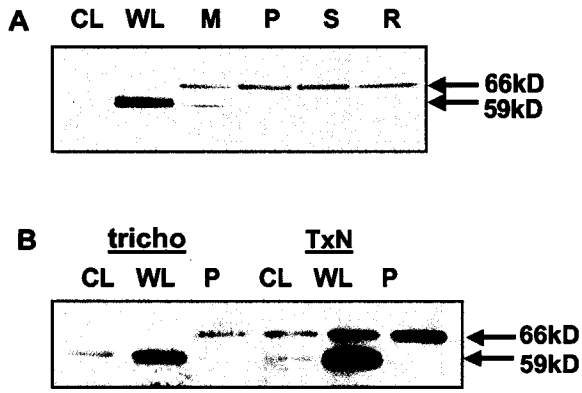
Partially purified PPO1 and PPO2 (10 µl each), unboiled and without reducing agent, were applied to SDS-polyacrylamide gels and electrophoresed in the presence of SDS. Following electrophoresis, PPO activity was visualized by staining the gels with 25 mM DL-DOPA in 10 mM sodium phosphate buffer (pH 6.0) containing 0.15% (w/v) SDS and 470 units/ml catalase. Gels were stained until no further increase in color was apparent, usually within 30 min. The gels were dried between cellophane sheets and photographed.

4.3 Results

4.3.1. Hybrid poplar contains two major PPO isoforms with distinct patterns of expression

In the course of characterizing a wound-induced PPO cDNA, *PtdPPO* from *Populus trichocarpa x P. deltoides* (Constabel et al., 2000), I observed that in addition to the inducible leaf PPO activity, hybrid poplar contained high levels of PPO activity in petiole and stem tissue. To further characterize this PPO activity, an antibody was raised against recombinant PPO1 (M. Christopher and C.P. Constabel, unpublished data) and then used to perform western blots with different poplar sapling tissues (Fig 1A). While there was no PPO band in control leaves, the antibody recognized a protein migrating at 59 kDa in wounded leaves. Based on its induced expression, size, and recognition by the

Figure 1: Western blot analysis of PPO in different tissues of hybrid poplar.
Tissues were extracted and blotted as described in Materials and Methods, and analyzed using an antibody raised against recombinant PPO1. (A) Analysis of *P. trichocarpa x P. deltoides* tissues. (B) Analysis of the *P. trichocarpa* parent and a *P. tremula x P. nigra* hybrid. CL, Control leaf; WL, Wounded leaf; M, Midvein; P, Petiole; S, Stem; R, Root.



PPO antibody, this band likely represents the product of the wound-induced *PtdPPO* gene. Furthermore, in PtdPPO-overexpressing transgenic canola plants, the same 59 kDa band was present while being absent in controls, providing direct proof that *PtdPPO* encodes the 59 kDa PPO (C.P. Constabel, unpublished data). The 59 kDa band was not present in petioles, stems, and roots, and was only faintly visible in midvein samples of *P. trichocarpa x P. deltoides* (Fig 1A). In these tissues, however, the antibody recognized a different protein band, migrating at approximately 66 kDa. The intensity of this band correlated well with PPO activity in crude extracts made from these tissues (data not shown), suggesting that this protein represents a second PPO isoform. Additional western blots with the *P. trichocarpa* parental clone, as well as an unrelated *P. tremula x P. nigra* hybrid, also showed the induced 59 kDa band in wounded leaves and the 66 kDa PPO band in petioles (Fig 1B). In addition, the 66 kDa band was present in control and wounded leaves of the *P. tremula x P. nigra* hybrid, and small amounts of the 59 kDa band were present in control leaves of both this and the *P. trichocarpa* genotype. We concluded that the 66 kDa PPO band does not represent a second parental allele found only in hybrids, but rather represents a second PPO isoform encoded by a second gene. The wound-induced leaf isoform was named PPO1, and the constitutive isoform PPO2. Wounding of the PPO2-expressing tissues did not increase expression levels discernably on western blots (data not shown).

4.3.2. Partial purification of PPO isoforms

The distinct patterns of expression of the two PPO isoforms prompted us to undertake partial purification of both proteins, in order to compare their enzymatic properties. Wounded leaf tissue, and stems and petioles were used as starting material for extraction of PPO1 and PPO2, respectively. Since *Populus* is known to contain high levels of phenolic compounds, PVP-40, Amberlite XAD-4, and PVPP were included in the extraction buffer as phenolic adsorbants. Proteinase inhibitor cocktails were also added, as these were found to preserve the integrity of the PPO protein as visualized on western blots. Preliminary experiments suggested that while the starting material for PPO extraction was highly enriched for either of the PPOs, it contained small amounts of the other isoform. Therefore, the protein extracts were fractionated using ammonium sulfate

precipitation, followed by column chromatography for further purification. Anion exchange chromatography on DEAE cellulose was not successful in separating the isoforms (data not shown). However, a hydrophobic interaction column (Phenyl Sepharose CL-4B), was very useful for separating the two PPO isoforms, and allowed me to eliminate the small amount of PPO2 from the leaf PPO1 preparation (Fig 2 A, B). Western blot analysis of the extracts during this procedure confirmed that the final preparation contained only a single PPO protein band (Fig 2C).

The semi-purified PPO preparations were analyzed using *in situ* PPO-staining in non-denaturing SDS-polyacrylamide gels. This technique is based on the observation that plant PPOs retain full activity in the presence of SDS; poplar PPO is extracted in a latent form but is activated by SDS (Constabel et al., 2000). When analyzed on non-denaturing SDS-polyacrylamide gels, only single PPO activity bands were detected for both PPO1 and PPO2 preparations, which confirmed successful chromatographic separation of the PPO isoforms (Fig 3). Interestingly, the difference in migration between the two PPOs as seen on western blots was also retained, with PPO1 migrating faster than PPO2. I concluded that each of our semi-purified preparations contained only one of the two PPO isoforms, and that these were suitable for further comparisons.

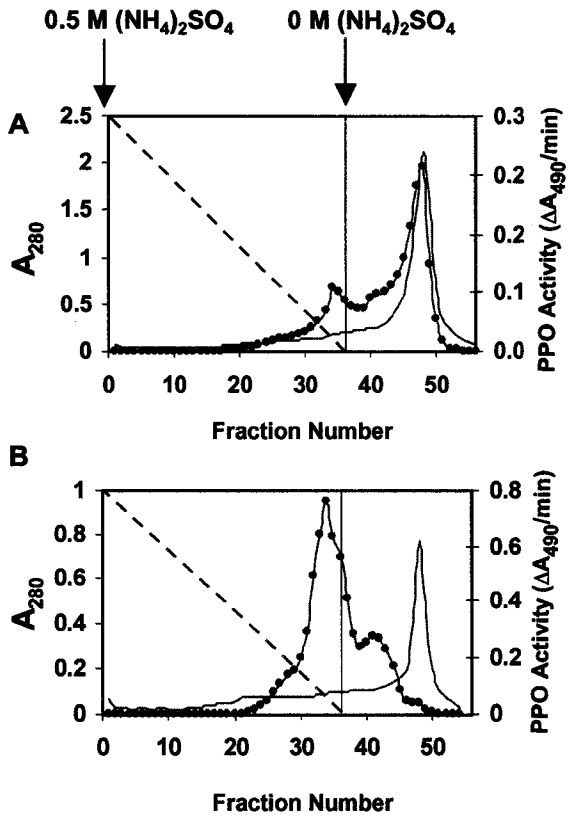
4.3.3. PPO stability, heat tolerance and pH optima

To investigate the stability of both PPO isoforms during storage, aliquots of both enzyme preparations were kept at -20°C, and PPO activity was measured using 25 mM DOPA as substrate. PPO1 activity decreased to 43% after one week, and continued to decrease until after 4 weeks at -20°C, only 8% of the original activity remained. By contrast, for PPO2 no loss of activity occurred in the same 4-week experimental period. Thus, PPO2 was more stable in storage than PPO1. Because of this differential stability, fresh PPO preparations were used for all subsequent experiments.

In order to study the tolerance of the PPO isoforms to heat treatment, both PPO preparations were incubated at temperatures between 40–80°C. Aliquots were removed periodically, cooled on ice, and then assayed for PPO activity. Incubation at temperatures between 40–70°C stimulated PPO1 activity. This transient temperature-induced stimulation of PPO was not observed for PPO2, and PPO2 activity decreased

Figure 2: Elution profiles of two major hybrid poplar PPO proteins on Phenyl Sepharose CL-4B column and western blot analysis showing the major steps of the purification process of both PPO isoforms.

(A) 40-90% $(NH_4)_2SO_4$ precipitated crude extract of wounded poplar leaf was suspended in 0.5 M $(NH_4)_2SO_4$, 10 mM $NaPO_4$ buffer, pH 7.0, and then loaded on the Phenyl Sepharose CL-4B column and eluted with a linear gradient (0.5-0 M) $(NH_4)_2SO_4$ followed by distilled water. A_{280} was monitored and PPO activity was determined on each 1.5 ml fraction using DOPA assay. (B) Same procedure was repeated as in (A) except the total protein was extracted from poplar stem tissue. (C) Aliquots of active fractions were applied to SDS-PAGE gels, blotted, and analysed using the PPO antibody. 1, crude extract; 2, 40–90% $(NH_4)_2SO_4$ precipitate; 3, semi-purified PPO from Phenyl Sepharose CL-4B column.



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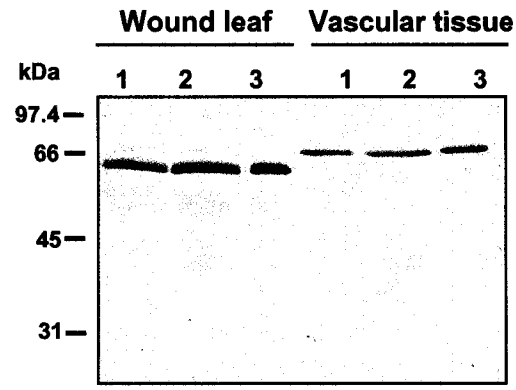
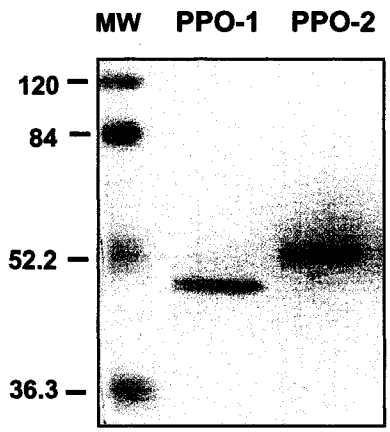


Figure 3: PPO activity-stained gel of the two partially purified PPO isoforms. Proteins were electrophoresed on 12% SDS-PAGE gels without prior boiling and without ß-mercaptoethanol. Following electrophoresis, PPO activity was visualized using 25 mM DOPA in 100 mM sodium phosphate buffer. MW indicates prestained molecular weight marker.



progressively during incubation at 70°C. Between 40–60°C PPO2 was not inactivated, but maintained 100% of its activity for the full 1 h period. Both isoforms were inactivated rapidly at 80°C, again with PPO2 being more sensitive. Therefore, PPO1 appeared to be more tolerant of high temperature incubations, and was even activated at high temperatures compared to PPO2. Plant PPOs generally appear to be robust enzymes; recently, a latex PPO from *Hevea brasilensis* was also shown to be very resistant to heat treatment, and at 60 °C this PPO retained 80% of the activity (Wititsuwannakul et al., 2002).

When the pH activity profiles of the poplar PPO isoforms were compared, they both showed broad pH preferences (Fig 4). Using catechol as the substrate, PPO1 had the highest activity at pH 6, whereas the optimum for PPO2 was pH 7. PPO1 had a somewhat narrower pH range than PPO2, which retained significant activity even at pH 4. The only previous report of poplar PPO, obtained from senescent *P. nigra* leaves, found the highest activity at pH 7.5, but also reported a similarly broad pH preference (Trémolières and Bieth, 1984).

4.3.4. Substrate specificities and K_m determinations

A major objective of this work was to identify potential differences in substrate preference between PPO1 and PPO2, since these may provide clues as to physiological function. A number of substrates were tested using the 2-nitro-5-thiobenzoic acid (TNB) assay, in which the PPO-generated quinones react with TNB and yield colorless Michael-type adducts (Esterbauer et al., 1977). This spectrophotometric assay monitors the consumption of TNB, and therefore allows for the direct comparison of enzyme rates with different substrates. PPO1 showed the highest rates of oxidation with methyl catechol, followed by catechin>chlorogenic acid>DOPA>catechol>caffeic acid, in decreasing order (Fig 5). This substrate profile is generally consistent with our previous analysis of PPO wound-induced leaves (Constabel et al., 2000). Minor differences were observed, likely because earlier data were obtained using crude leaf extracts. For PPO2, the substrate preferences were catechol=methyl catechol>>chlorogenic acid>caffeic acid>>catechin>DOPA (Fig 5). Methyl catechol was clearly a good substrate for both enzymes, but surprisingly, catechol was a much poorer substrate for PPO1 than for PPO

Figure 4: Effect of assay buffer pH on activity of PPO1 and PPO2.
100 mM citrate phosphate buffer was used from pH 4.0 to pH 6.5 and 100 mM sodium phosphate buffer was used from pH 6.5 to pH 8.0. The pH optimum of PPO1 and PPO2 activity was measured using 10 mM catechol and the TNB assay.

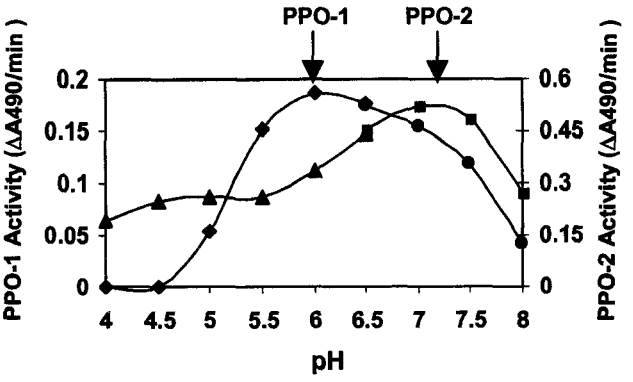
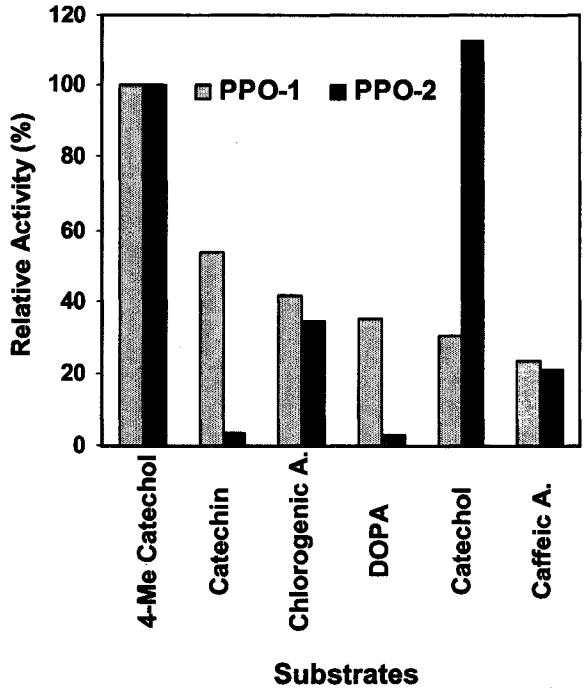
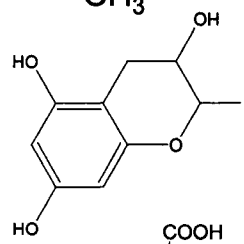
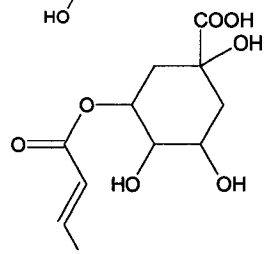
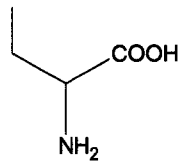
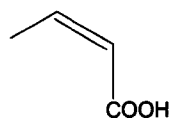
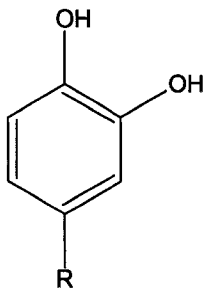


Figure 5: Substrate preferences of PPO1 and PPO2 for some common diphenols. PPO activity was measured in TNB assays with 5 mM substrate, 0.15% SDS at pH 6. Activities for each substrate are shown as percentages of the activity for 4-methyl catechol.



Substrates	R
Catechol	H
4-Methyl catechol	CH_3
Catechin	
Chlorogenic acid	
DOPA	
Caffeic acid	



2. Conversely, catechin and DOPA were good substrates for PPO1 but not for PPO2. Overall, I concluded that PPO2 has a greater substrate specificity than PPO1. As a control, for all experiments and substrates we confirmed that the measured activity was inhibited by the PPO inhibitor tropolone (Kahn and Andrawis, 1985).

The pattern of substrate preference was confirmed by K_m values determined for the best PPO substrates (Table 1). Both PPOs had the lowest K_m with methyl catechol, 0.6 mM and 0.5 mM, respectively. However, PPO2 had a much larger range of K_m values compared to PPO1, consistent with the results of substrate preference experiment. Again, the most dramatic differences were found for catechin, DOPA, and catechol, confirming previous findings. Trémolières reported K_m values for *P. nigra* leaf PPO of 5.0 mM for catechol, chlorogenic acid and methyl catechol. I obtained similar values for PPO1 (leaf PPO) as these authors, with the exception of our calculated K_m for methyl catechol of 0.6 mM (Table 1). This difference could be due to their use of senescent poplar leaves as a source of PPO. In general, the K_m values obtained are in the range commonly observed in plant PPOs with these substrates (Billaud et al., 1996; Fujita et al., 1991; Robert et al., 1996). No appreciable enzyme activity was detected with either PPO isoform using the monophenol 4-hydroxyanisol (Espín, 1998), suggesting that there is no monophenolase activity associated with either PPO.

Tropolone is a very potent PPO inhibitor (Kahn and Andrawis, 1985) and was the most effective inhibitor for both PPOs, with less than 0.5% of either PPO activity remaining at 200 μM (Table 2). Kojic acid (Chen et al., 1991) and ferulic acid (Walker and Mccallion, 1980) were also effective inhibitors of both PPOs. Therefore, both PPOs showed comparable sensitivity to common PPO inhibitors. Cetyltrimethylammonium bromide (CTAB), an inhibitor of laccase (*p*-diphenol oxidase), did not have an inhibitory effect on either poplar PPO isoform (data not shown).

To determine if PPO1 and PPO2 differ in their ability to be activated by SDS, both enzymes were assayed at different SDS concentrations. PPO1 is fully activated with approximately 4 mM SDS, whereas PPO2 had already achieved maximal activity at 1 mM (Fig 6). Therefore, PPO2 appears to be more sensitive to SDS. The identical results were obtained when the experiment was repeated using crude enzyme extracts, indicating

Table 1

Kinetic studies[a] on the K_m of PPO1 and PPO2

	K_m (mM)	
Substrates	**PPO1**	**PPO2**
4-Methyl catechol	0.6	0.5
chlorogenic acid	3.5	6.5
catechin	3.7	24.3
catechol	6.6	0.89
DOPA	21.1	79.5

[a]Michaelis-menten constants (K_m) were determined using five substrates at their different concentrations. K_m value of PPO for each substrate was calculated from plot of 1/V vs. 1/[S] by the method of Lineweaver and Burk.

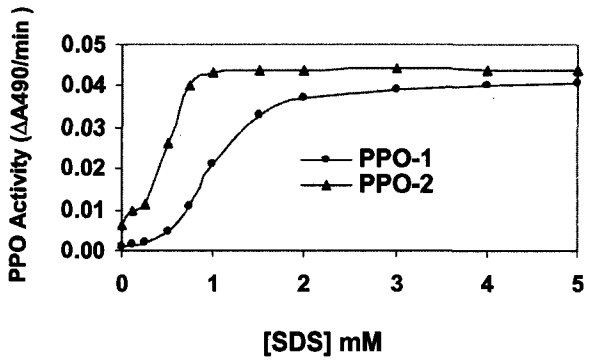
Table 2

Comparative study on effects of various inhibitors on PPO activity[a]

	Remaining PPO Activity (%)	
Inhibitors	**PPO1**	**PPO2**
Tropolone (200 μM)	0.43	0.25
Kojic acid (1 mM)	6.11	13.5
Ferulic acid (1 mM)	38.4	68.8

[a]The inhibition of PPO activity was tested on 20 μl of each partial purified PPO isoform in standard TNB assay using 25 mM DOPA as substrate. The 100% activity of each isoform preparation is 0.211 for PPO1 and 0.072 for PPO2 in ΔA490/min.

Figure 6: Effect of SDS on PPO1 and PPO2 activation.
SDS was added to the assay buffer at different concentrations and PPO activity was determined immediately using a direct assay with 25 mM DOPA as substrate.



that this effect is not dependent on different total protein concentrations (data not shown). There was no inhibitory effect of SDS concentrations greater than 10 mM (data not shown), although such a decrease in activity has been reported for fungal tyrosinases (Perez-Gilabert et al., 2001). Proteases such as trypsin are also known to activate latent PPOs (Steffens et al., 1994), and both poplar PPO isoforms were activated by trypsin to 70% of maximal activity (data not shown). Analysis by SDS-PAGE and western blotting indicated that trypsin-treatment of PPO resulted in migration on SDS-PAGE at approximately 45 kDa. It thus appears that poplar PPO, like the enzyme from grape, has a protease sensitive site, and that removal of a C-terminal extension exposes the active site or activates it via a conformational shift (Dry and Robinson, 1994).

4.4. Discussion

Higher plant PPOs are often encoded by multigene families with complex regulation, and different tissues can express very distinct sets of PPO isoforms (Thipyapong and Steffens, 1997; Thygesen et al., 1995). The significance of this complexity is not clear, in particular as the catalytic differences between these PPO isoforms have rarely been investigated. In hybrid poplar there are at least two PPO genes, and western blot analysis confirmed the presence of two major PPO protein isoforms (Fig 1). PPO1 was detected primarily in wound-induced leaves, whereas PPO2 is expressed constitutively in midveins, petioles, stems and roots. I partially purified both PPO isoforms from poplar tissues in order to characterize these enzymes biochemically and determine differences, which could provide clues as to differences in function.

Both PPO isoforms differed in stability, heat sensitivity, pH optimum, and activation by SDS; overall, the results are generally typical of plant PPOs, which are stable enzymes and have broad pH optima. Although both poplar PPOs are extracted in a latent form and require activation, PPOs from other plant sources are extracted in fully active form, for example from apricot (Chevalier et al., 1999). The most striking differences between PPO1 and PPO2 were observed in substrate preference. My results indicate that PPO1 accepts a broader range of substrates compared to PPO2, as both catechin and DOPA are good PPO1 sustrates (Table 1). Interestingly, PPOs from a number of plants are not able to oxidize catechins (Ho, 1999; Wititsuwannakul et al.,

2002). I speculate that this substrate difference between poplar PPO1 and PPO2 is indicative of differences in function. PPO1 is wound-induced and active during tissue disruption due to insect feeding, where cross-linking and amino acid alkylation by the PPO-generated *o*-quinones can lead to a loss of amino acid assimilation by the insect gut. Similarily, PPO1 may also be involved in pathogen defense. In these defense situations, one might expect selection for an enzyme with a broad range of acceptable substrates, such as PPO1. Greater specificity, as observed with PPO2, might indicate a separate physiological function, one that requires oxidation of particular phenolic substrates. The *in vivo* substrates of hybrid poplar PPO are as yet unknown. However, in *P. tremuloides*, there is indirect evidence that the abundant phenolic glycosides tremuloidin and salicortin are broken down in insect guts, releasing catechol as a possible PPO substrate (Clausen et al., 1989; Haruta et al., 2001b). By contrast, in tomato leaves the most likely *in vivo* PPO substrate is chlorogenic acid (Li and Steffens, 2002).

Although PPO has been purified from many sources, in only a few studies have different PPO isoforms from the same species been compared directly. Ho (1999) characterized four PPO isoforms from orchid root, and Wititsuwannakul (2002) compared two PPOs from *Hevea brasiliensis* latex. In both of these studies, the PPO isoforms compared were very similar. By contrast, our data indicate that poplar PPO1 and PPO2 are quite distinct and show clear differences in substrate preference; together with their distinct patterns of expression, this may be indicative of differences in function. While PPO1 is hypothesized to be an inducible anti-nutritive defense against folivore insects, the role of PPO2 is unknown. It is possible that PPO2 has a role in sealing of wounds, in pathogen defense, or other processes, and experiments to study PPO expression in poplar following stress treatments are under way. In addition, other novel roles for PPO have recently been discovered, for example in hydroxylation reactions during the biosynthesis of aurones in snapdragon flower pigments (Nakayama et al., 2000). Future work should focus on the cell-type localization of both PPO isoforms and on the identification of the PPO2 encoding gene.

Chapter 5 General Discussion

The goal of my project was to characterize hybrid poplar PPO at several levels in order to address the question of the physiological functions of PPOs in *Populus*. This problem was approached using three different strategies designed to answer the following questions (1) How many *PtdPPO* genes exist in hybrid poplar? How are they expressed in various tissues and organs? How do they respond to wounding and defense signaling molecules? Do they respond to these environmental cues in the same way or differently? (2) What is the effect of overexpressing the wound-inducible PPO1 protein in hybrid poplar by genetic engineering? Will it provide a protective effect against herbivores for the transgenic trees? (3) What are the biochemical properties of the major PPO protein isoforms expressed in hybrid poplar? Is it possible to separate them and therefore compare their respective biochemical properties? The answers to these questions are the first steps in elucidating PPO functions in hybrid poplar, and add valuable information to the current knowledge of plant PPO. In addition, this research will help to understand the defense mechanisms that forest trees have evolved to protect themselves from biotic enemies in their environments. In this section of my thesis, I will present an overall summary on the results of the previous three chapters, and discuss the significance of this work as well as the future research that can be carried out.

5.1 PPOs in Hybrid Poplar: Expression, Induction and Function

Three PPO genes of hybrid poplar (*Populus trichocarpa x P. deltoides*) were investigated, and their respective expression and induction patterns were studied using gene-specific probes. Two of these genes were newly identified and cloned as part of this work. Most plant species have been shown to possess multiple PPO genes although only one PPO message was found in grapevine (Dry and Robinson, 1994). For example, at least three PPOs were characterized in *Vicia faba* (Cary et al., 1992), four PPOs in apple (Boss et al., 1995), seven PPOs in tomato (Newman et al., 1993), two PPOs in potato leaves (Hunt et al., 1993) and five PPOs in potato tubers (Thygesen et al., 1995). With multiple PPO genes identified within one plant species, it is important to use gene-specific probes when the expression of an individual PPO genes is studied. This work

demonstrated the existence of multiple PPO genes in hybrid poplar, namely *PtdPPO1*, *PtdPPO2* and *PtdPPO3*, and their distinct organ-specific expression patterns and differential responses to mechanical wounding and MeJA application. In this section, the expression, induction, protein biochemical properties of the three PPO proteins and their possible physiological roles in hybrid poplar be discussed individually.

5.1.1 Stress Inducibility and Antiherbivore Role of PtdPPO1 in Poplar Leaves

Previous work in the Constabel lab showed that *PtdPPO1* mRNA and enzyme activity is dramatically induced in hybrid poplar *(P. trichocarpa x P. deltoides)* leaves by wounding, MeJA and herbivory (Constabel et al., 2000). At that time, the physiological functions of PPOs in plant growth and its involvement in herbivore defense were implicated in some plant species, but only demonstrated clearly in two cases. One is the entrapping ability of PPO-generated quinones in wild potato trichomes (Kowalski et al., 1992) and the other is the anti-nutritive effect of leaf-derived quinones in herbivore diets (Duffey and Felton, 1991) (see section 1.1.6). In order to directly test the proposed antiherbivore effect of PPO in hybrid poplar, PPO-overexpressing transgenic hybrid aspen (*Populus tremula x P. alba*) were constructed and tested in forest tent caterpillar (FTC) bioassays. PPO-overexpressing *Populus* seriously affected the survivorship and growth rate of FTC when late-season hatched larvae were used. The negative effect on herbivore performance was not observed for early-season hatched FTC larvae. This result indicated that FTC may have the ability to adapt to PPO activity under normal growing conditions and, therefore, is not affected by high levels of this enzyme in the transgenic *Populus*. Aspen, the native host for FTC, contains high levels of PPO, an observation which may support this idea (Haruta, 2001). Adaptation of insect herbivores to defensive proteins has been reported in transgenic tobacco plants. In this case, the insects compensated for the overexpression of trypsin inhibitor in transgenic tobacco with a significant induction of leucine aminopeptidase-like and carboxypeptidase A-like activities (Lara et al., 2000). Therefore, FTC may adopt similar constitutive or induced detoxification mechanisms to deal with high levels of plant PPOs. Recently, it was shown that lepidopteran larvae can overcome the effects of dietary oxidized phenolics by maintaining reducing conditions in the gut (Barbehnen et al., 2001).

Results obtained using transgenic plants also indicated that the action of defensive proteins is likely to be synergistic, and PPO activity probably is only one of many potential defense mechanisms. It has been shown that in wounded and herbivore attacked trembling aspen leaves, the induction of PPO is often accompanied with the upregulation of other defensive proteins such as trypsin inhibitor. Overexpression of a single defensive protein may not be enough to inhibit the attack of herbivores. In order to overcome the diversity of herbivore adaptation mechanisms, it will be important to express various antiherbivore protein encoding genes in the same transgenic plant rather than to use individual components.

In addition to constructing PPO-overexpressing transgenic *Populus* and, therefore, confirming its antiherbivore function directly, this work also investigated other characteristics of PPO1 as a defensive protein. First, there is no constitutive transcriptional expression of *PtdPPO1* in various organs tested and its induction by real herbivory, simulatory herbivory and herbivore-defense signaling molecules only occurs in leaf tissues. Such a strict correlation of its expression with herbivory and specific tissue type also supports its defensive role in protecting *Populus* against leaf-eating herbivores. Second, when the biochemical properties of PPO1 protein were compared with the constitutively expressed PPO2 and PPO3 proteins, it was found that PPO1 have a wider range of substrate preference. This is consistent with the expectation for PPO1 in defense situations to oxidize a broad range of acceptable substrates to generate *o*-quinones and therefore cross-link and alkylate the amino acids in insect gut. The ability of PPO1 to oxidize a wide range of diphenolic substrates means that this PPO enzyme can utilize phenolic substrates from different sources and therefore maximally its antiherbivore effects, which can be regarded as one of its evolutionary advantages.

5.1.2 PtdPPO2 Expression and its Potential Functions

Unlike PPO1, *PtdPPO2* is a constitutively expressed PPO in hybrid poplar midveins, petioles, stems and roots. In normally growing hybrid poplars, this PPO protein is responsible for the constitutive PPO activity and natural tissue browning in stems and petioles. Although expression of *PtdPPO2* is under temporal regulation and young stem and primary roots exhibits the highest mRNA level, this enzyme seems to be quite stable

and maintains fairly high levels throughout ages. Besides its high constitutive level in various tissue types, *PtdPPO2* in also inducible by wounding and MeJA at the transcriptional level and this induction only happens in conducting organs (roots, stems and petioles), where it is also normally present. Most studies on wound-inducible PPO have involved leaves and there are few instances of wound induction of PPO in stems and roots.

High constitutive levels of *PtdPPO2* and its induction by stress in conducting organs imply the importance of this PPO enzyme in sealing wounds and protecting the conducting organs against sap-sucking herbivores. Preliminary *in situ* staining experiments on stem tissue printing, using PPO-specific substrates or a PPO polyclonal antibody, suggested that PPO2 protein is associated with epidermal cells of stems and petioles. Most aphids, mealy bugs, leafhoppers, psyllids, and whiteflies stylets must traverse the cuticle, epidermis, and mesophyll to establish feeding sites in vein of the phloem. During this process, epidermal cell structures may be destroyed and high levels PPO in these cells could react with its phenolic substrates and therefore produce highly reactive quinones to help sealing the wounds, crosslink cellular macromolecules and prevent insect's further attack. To date, based on the limited number of studies currently available, it has been suggested that some piercing/sucking insects induce the defense-signaling pathways most commonly activated by bacterial, fungal, and viral pathogens (Walling, 2000). Interestingly, when hybrid poplar cell culture was treated with various pathogen elicitors, only *PtdPPO2* responded by dramatic mRNA induction rapidly. This suggests that PPO2 may also be a pathogen defense-related protein, in addition to its action against conducting organ-attacking insects in petioles and stems.

As a constitutive and highly expressed PPO protein in hybrid poplar, biochemical analysis showed that PPO2 has a narrower range of substrate than PPO1 (PPO2 have a much lower affinity towards DOPA and catechin compared to PPO1). Therefore, it is possible that the endogenous substrates found in stems and petioles differ from that found in leaves. The precise substrates must still be determined.

5.1.3 Root-specific PtdPPO3 and its Non-Responsiveness to Stress

PtdPPO3 is a root-specific PPO and coexists with *PtdPPO2* in roots. *PtdPPO2* is mainly expressed in older primary roots, whereas *PtdPPO3* is mainly expressed in the young root tips. Unlike *PtdPPO1* and *PtdPPO2*, *PtdPPO3* is not responsive to wounding and MeJA signalings, and thus it may perform physiological role other than defense. Such functions of PPO have been revealed in other plant species in recent years. For example, a PPO homolog is responsible for flower coloration in *Antirrhinum majus* (snapdragon) (Nakayama et al., 2000). Similarly, the expression of Virginian pokeweed PPO was localized to ripened fruits and was suggested to be highly related to the accumulation of betalains (Joy et al., 1995). Very recently, an enantio-specific PPO was purified from the creosote bush (*Larrea tridentata*). This PPO was shown to have a central role in the biosynthesis of the 8-8' linked lignans and this result provides an example of additional roles for PPO. Although the significance of a root-specific PPO in hybrid poplar remains to be determined, based on that fact that it is not inducible by systemic wounding and MeJA application, *PtdPPO3* gene may have functions involved in normal root development other than defense.

PPO3, when assayed in crude extracts, appears to exhibit some distinct biochemical properties when compared to PPO1 and PPO2. For example, PPO3 exhibits higher affinity towards chlorogenic acid and more interestingly, unlike PPO1 and PPO2, whose activities totally rely on SDS, PPO3 retain 10-20% total activity even without SDS depending on the substrates. These different enzymatic characteristics of PPO3 from other two PPOs support the idea that it performs physiological functions other than defense.

5.2 Significance of this Work

The aim of this work was to study PPO in hybrid poplar in order to obtain some insight into its physiological function. Several questions including gene expression and induction under normal or stressed conditions, the effect of PPO-overexpressing plants on insect herbivores, and protein biochemical characteristics were addressed to give a general picture of PPO expression and function. The significance of this work can be concluded as several points. (1) In the past, much attention has been given to the

defensive role of PPO in plants, however, there has been no enough consideration of the effect other than defense of PPO. Identification and characterization of different PPO gene family members could be very important in documenting their respective functional contributions to one specific plant species. This work revealed the unique expression patterns of multiple PPOs in one plant species, and included the isolation of two new poplar PPO cDNAs. Such clear-cut, non-overlapping organ-specific distribution of three PPO genes as seen here has not been observed in other plant species. This makes hybrid poplar an ideal experimental system for studying the distinct functions of different PPOs. Parallel comparison of the gene-specific constitutive and inducible expressions of three different PPO genes provided convincing evidence that different PPO genes are associated with distinct physiological functions. (2) This work is the first one to directly prove the antiherbivore role of PPO using transgenic plants. In addition, this work contributed to the knowledge of PPO antiherbivore function by revealing the conditionality of this defensive protein. Adaptibility and susceptibility of herbivore to high levels of PPO under normal and stressed growing conditions, respectively, was revealed for the first time in this work, and thus, added new information to the PPO literature. Although the negative effects on growth and survival of forest tent caterpillar were subtle, the finding that larvae with reduced vigor are susceptible to PPO-mediated defense is consistent with the idea that there are multiple defenses that work in concert and illustrates the complex co-evolutionary relationship between herbivores and plants. (3) In literature, there are many reports on PPO gene cloning or biochemical characterization of purified PPO proteins, however, very few work carried out both. In this work, two major PPO isoforms were partially purified and their respective biochemical properties were compared. In addition, their encoding cDNAs were characterized, permitting a multilevel comparison of hybrid poplar PPOs. Such paraelle analyses of PPO on both molecular level and biochemical level allowed us to obtain a more comprehensive picture about PPO and represents the first step to obtain a better understanding of their functions.

5.3 Suggestions for Future Study

Work in this thesis included an investigation project of *PtdPPO* gene expression and protein properties. As any other scientific research, this work also provides opportunities for further research on hybrid poplar PPO. Due to its ease for transformation, hybrid aspen (*Populus tremula x P. alba*, clone INRA 717I-B4) was chosen to construct transgenic plants. However, this poplar hybrid is not the natural preferred host for forest tent caterpillar. It may be better to use other *Populus* species and hybrids to construct PPO transgenic trees and if do so, a good candidate could be the trembling aspen (*P. tremuloides*), which is the preferred native host for FTC. Currently, PPO-overexpressing transgenic hybrid aspen (*Populus tremula x P. tremuloides*) T89 has been obtained *via* collaborations with other laboratories. These would also be used in next FTC bioassays. Since this hybrid appears to be more palatable to these herbivores, they may produce a stronger antiherbivore effect of PPO. In addition, other defoliators and other types of herbivore insects, including sap-sucking insects, should be tested in bioassays. Pathogens, in particular phytopathogenic bacteria should also be tested for the disease-resistance of the high-PPO expressors, as the literature suggests. Although the negative impact of high levels of PPO on the performance of FTC in our studies was dependant on egg mass age, it does not exclude the possibility that PPO can have a stronger effect towards other insect herbivores.

Although this work has isolated three distinct PPO genes, Southern blots (page 43, Chapter 2, figure 2) and 2-D gel (data not shown) indicated that there are more uncharacterized PPO genes in hybrid poplar. More rounds of PCR on hybrid poplar cDNA need to be carried out and the cDNA could be made from RNA extracted from different tissues at different developmental stages or in response to external stimuli, so that these genes do not elude us. It is worth pointing out that so far, no PPO gene expressed in hybrid poplar flowers has been characterized and it would be very interesting to do so. The DOE Joint Genome Institute provides a 6X draft sequence of the *P. trichocarpa* clone Nisqually-1 and annotation is currently underway. In my work, BLAST searches with hybrid poplar *PtdPPO1*, *PtdPPO2*, and *PtdPPO3* sequences yielded highly homologous counterparts in JGI genomic clones, indicating that they are alleles of the *P. trichocarpa* female parent. Further BLAST search of the complete

Populus genome is an obvious strategy to find all other poplar PPO genes. After obtaining all the PPO genes in hybrid poplar, their respective gene-specific probes could be made and gene-specific expression and induction of these newly identified PPO genes could be figured out easily. These results will add to the current data presented in this work and provide an integrate picture of PPO gene expression in hybrid poplar.

In this study, PPO1 and PPO2 proteins were partially purified from wounded leaf and stem, respectively by the use of chromatography. With the discovery of the root-specific PPO3 protein, it will also be informative to purify this PPO isoform and study its biochemical properties, especially its substrate preference. In addition to partially purify the different PPO protein isoforms, the *in vivo* PPO substrates also need to be identified. In aspen, it appears that the major detectable diphenolic compound available as a PPO substrate is catechol, which may be derived from phenolic glycosides such as salicortin and tremulacin (Haruta et al., 2001b). However, more detailed chemical analysis is required to confirm these and other potential PPO substrates. Due to the very different tissue distribution and induction patterns of three PPOs, it is intriguing to investigate their respective *in vivo* substrates using HPLC techniques in different organs. At the same time, HPLC can also be used on PPO-overexpressing transgenic poplars to better analyze the effect of high levels of PPO on the composition of endogenous polyphenolic pools.

In this thesis, cell-type localization of PPO2 in stem and petiole was investigated using enzyme staining and antibody localization after tissue printing, which provided interesting but preliminary results. For future work, it would be important to carry out *in situ* hybridization using the gene-specific probes. This should provide more accurate and detailed information about the cell-type localization of PPO2.

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Appendix 1: Recipe for Hybrid Populus Tissue Culture Mediums

ACM3 medium (mg L^{-1})

Macronutrients	
NH_4NO_3	400
$Ca(NO_3)_2.4H_2O$	556
K_2SO_4	990
$CaCl_2.2H_2O$	96
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Iron	
Sodium-Ferric EDTA	30
Micronutrients	
$MnSO_4$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	0.83
$Na_2MO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Vitamins	
Thiamine.HCl	0.1
Nicotinic acid	0.5
Pyridoxine.HCl	0.5

Hormones	
Indolebutyric acid (IBA)	0.5
Acetic acid (NAA)	0.02
Sugars	
Sucrose	20000
PH	5.5-5.7
Phytoagar	7000

M medium (mg L^{-1})

Macronutrients	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2$	332.2
$MgSO_4$	180.7
KH_2PO_4	170
Iron	
$FeSO_4.7H_2O$	27.8
Micronutrients	
H_3BO_3	6.2
Na-EDTA	37.26
$MnSO_4.H_2O$	16.9
$ZnSO_4 .7H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025

Vitamins	
Nicotinic acid	1
Pyridoxine·HCl	1
Thiamine·HCl	1
Calcium pantothenate	1
Amino acids	
L-Cysteine	1
Biotin	0.01
L-glutamine	200
Sugars	
Sucrose	30000
Agar	7000

M1/2 medium (mg L^{-1})

As M but with half strength macro-nutrients

Sucrose	20000

M1 medium (mg L^{-1})

As M but with NAA 10 μm and 2iP 5μM

M2 medium (mg L^{-1})

As M1 but with Carbenicillin 500mg/L, Cefotaxime 250 mg/L

M3 medium (mg L^{-1})

As M but with Carbenicillin 500 mg/L, Cefotaxime 250 mg/L, Thidiazuron 0.1 μM

Appendix 2: Protocol for Transgenic Hybrid Aspen Construction

Plant culture

INRA clone 717 1-B4 (*Populus tremula x P. alba*) is normally cultured *in vitro* on ACM3 medium through nodal propagation. Poplar plantlets were maintained on this medium and grown at 25°C in 16 h light/8 h dark photoperiod.

Agrobacterial strains and plastids

A. tumefaciens strain C58/pMP90 is a disarmed derivative of the nopaline C58 strain. The binary vectors pRD400PPOS and pRD400PPOA were two plasmids with PPO cDNA inserted into the pRD400 in the sense and antisense directions, respectively. The vectors were transferred to the *Agrobacterium* by electroporation method.

Plant transformation and regeneration

1. Transformation of poplar clone 717 1-B4 was accomplished by co-cultivating stem or petiole explants with *A. tumefaciens* strains. Inter-nodal stem segments were cut half-length longitudinally.
2. Pre-conditioning: Stem and petiole explants were preincubated on solidified M1 medium for 48 hours at 24°C in darkness.
3. Co-cultivation: For co-cultivation, a two-day culture of agrobacteria was used to prepare 25 ml of M liquid suspension to appropriate concentration about 5x 10^8 cfu/ml corresponding to an OD_{660nm} of 0.3. About 50 explants were dipped into 25 ml of the bacterial suspension in petri dish and slowly stirred on shaker. After 16 hours, the explants were blotted on sterile paper to remove excess bacteria and placed onto solidified M1 medium for 48 hours of additional co-cultivation.
4. Decontamination: The explants were washed 3 times 5 minutes in a tetracycline solution (25mg/l) and 3 times 5minutes in sterile water, with vigorous stirring.

The explants were then transferred onto M2 medium for 2 weeks in darkness at 24°C.

5. Regeneration: Calli initially isolated were cut into pieces of about 2 mm in diameter and transferred to standard light conditions on solid M3 medium containing 100 mg/l kanamycin for induction and selection of transformed shoots.
6. Rooting: The shoots regenerated from calli were excised when they were approximately 1 cm long and transferred to M1/2 medium supplemented with 100 mg/l kanamycin for rooting. After about 20 days the rooted and elongated shoots were micropropagated on the same medium.
7. The rooted plantlets were removed from the agar medium, put into soil supplemented with slow-releasing nutrients and kept in mist chamber. After two weeks, the plants were transferred into big pots and put in greenhouse with continuous water irrigation.

Appendix 3: Sequence Data for Hybrid Poplar PPO genes

PtdPPO1 cDNA sequence (Constabel, 2000)

ATGTCCACCTTATCTTTCAGTCCTTTCTTTCCAAAACCACAGCATGTTAC
CAAAACCAAAAGGCTGAATCACCCATACGTCCCTAGAGTTTCATGCAAAG
CCACAGATGATACACAAAACCCTCCCACGAGAAGAGACGTCCTCATCGGT
CTAGGAGGCCTATATAGCGCTACCAATCTTGCTGACCGAACTGCCTTTGC
TAAGCCAATCACCCCTCCAGACTTGACCAAATGTGAGTTGGTGGACTTAC
CAAACCCCGAAAATCCATCAAACTGTTGCACTCCATTACCCAAAAAGATC
ATAGACTTCAGACCTCCTTCTCCATTCTCCCCGCTACGCACTAGACGTGC
GGCCCATTTAGTTGACGAAGACTACGTCGCCAAATATGCTGAAGCCATTT
CCTTAATGAAAAGTCTCCCCGAAAATGATCCACGTAACTTCTACCAACAA
GCCAACGTACATTGTGCCTATTGCAATGGTGCCTACGAACAAGTGGGGTT
TCCAAAATTAGAAATTGATGTTCATTCCTGTTGGTTCTTCTTTCCTTGGC
ACAGATACTATCTTTACTTCTATGAAAGAATCTTGGGCAAACTTATTAAT
GACCCAACTTTTGCTCTGCCTTTCTGGAACTGGGATTCCCCCAGTGGTAT
GCAAATGCCTTACATCTTTACCGACCCTAAATCTCCACTCTATGACCAGT
TCCGCGACCAGAATCACCAACCTCCTATATTGCTAGATCTTGATTATGGA
GCGGGAGACCCCAACCCCACAAACGCAAATCAATTATACTCTAGTAATCT
TACAGTAATGTACAAGCAAATGGTGTCTGGTGCAGCAAAACCAACACTCT
TTTTTGGAAAATCATACCGTGCTGGTGAAGATACTAGTCCTGGAGCTGGG
ACGATTGAGACCACCCCTCACAATAATATTCACAGATGGACCGGTGATCC
AACTCAAGAAAATAACGAAGACATGGGCAATTTTTACTCAGCTGCGAGAG
ATCCAATATTTTTTTGTCATCACTCAAATGTTGACCGAATGTGGACGATA
TGGAAGACTATACCTGGAGGAACTAGGAGGGATATCAGTGATCCTGATTG
GCTTAATTCAGAGTTTCTTTTCTATAATGAGAATGCAGAGCTTGTTCGTT
GTAAGGTTAGCGATTGTCTTGACAATACAGGGCTAAGGTATACTTATCAA
AATGTTGAAATTCCTTGGCTAGAATCAAAACCAATTCCGAGAAGGTTGGG
AAAGAAAGCAGCTGAAACAAAAACTGCATTAACACCGATCACTGCATTCC
CTTTAGTCTTAGACAAAACCATAGTCACTGTAGTTTCAAGACCAAAGAAA
TCAAGAAGCAGGAAAGAGAAAGAAGAGGAAGATGAAGTTTTAGTGATAGA
AGGGATAGAATACGACAAAGGCAAATTCGTGAAGTTTGACGTGTTCATAA
ATGATGACGTTGAGATGCCTTCTAAACCAGAAAATACAGAGTTTGCTGGG
AGCTTTGTTAATGTTTCTCATAAGCATGCGAAGAAGTCCAAGACAAGATT
GATATTGGGGATTACAGAATTGTTGGAAGACTTAGAAAGTGATGGTGATG
ATAGCATTGTGGTGGCTTTGGTGCCCAGGTCTAATAGTGTTAGTGATCCT
GTTGTCATCTCTGGCGTTAAGATTGAGTTTGTTAAAGAGTGATGATTATC
GAGGACATCTCGTTTTTGCTTTTCCTTCAGGAGATCGATGTAGGTCTTTT
AGTGTTTGTCTGGGTGCCTTGCTGCTTAATATCATGCACGTGTAATTCAA
CTCTGTACTTTTTTATTTAACAATCTACAATTAAAGTAATGGCCGCTGAT
TATTTTTTTTAA

PPO1 amino acid sequence

MSTLSFSPFFPKPQHVTKTKRLNHPYVPRVSCKATDDTQNPPTRRDVLIG
LGGLYSATNLADRTAFAKPITPPDLTKCELVDLPNPENPSNCCTPLPKKI
IDFRPPSPFSPLRTRRAAHLVDEDYVAKYAEAISLMKSLPENDPRNFYQQ
ANVHCAYCNGAYEQVGFPKLEIDVHSCWFFFPWHRYYLYFYERILGKLIN
DPTFALPFWNWDSPSGMQMPYIFTDPKSPLYDQFRDQNHQPPILLDLDYG
AGDPNPTNANQLYSSNLTVMYKQMVSGAAKPTLFFGKSYRAGEDTSPGAG
TIETTPHNNIHRWTGDPTQENNEDMGNFYSAARDPIFFCHHSNVDRMWTI
WKTIPGGTRRDISDPDWLNSEFLFYNENAELVRCKVSDCLDNTGLRYTYQ
NVEIPWLESKPIPRRLGKKAAETKTALTPITAFPLVLDKTIVTVVSRPKK

SRSRKEKEEEDEVLVIEGIEYDKGKFVKFDVFINDDVEMPSKPENTEFAG
SFVNVSHKHAKKSKTRLILGITELLEDLESDGDDSIVVALVPRSNSVSDP
VVISGVKIEFVKE

PtdPPO2 cDNA sequence

ATGGCTTCCTTTATCTCTCTTTCCAGTAGCATCCCTTTAGCTGCCTCCTC
TTTCTTGCCTTCCTTCCCGAAAACACACCGAGTTTCCAGAATTAAAAAGC
CAAACCGTCACAATATCCCGATTGTTTCTTGCAAATCAGGCAAGAATGAT
CACGAACAAAACCCTGCCACCAGAAGAGATCTGCTCATTGGTCTCGGTGG
ACTCTATGGAGCAACTAGTCTTAGTGATCCATTTGCCTATGCTAACCCCA
TTGCACCCCCAGACATAACCCAATGTGAGCTAGTTACCCTGCCATCCGAA
AGTGACCCCTCGAACTGTTGCCCTCCAACATCCACAAAGATCAAAAACTT
CGAATTCCCTTCTGCGTCCTCCCCAATGCGCATAAGGCCTGCTGCTCATT
TAGTTGATAAAGCCTACTTAGCTAAATACGCCAAAGCCATTGCACTGATG
AAAAGTCTTCCTGACGATGATCCACGTAGCTTCAAGAGCCAAGCCGACGT
TCATTGTGCTTATTGTGATGGTGCTTATCACCAAGCAGGCTTTCCTGATT
TAGACCTTCAAATTCACTTCTCGTGGCTCTTCTTTCCCTGGCATAGACTC
TATTTATACTACTTTGAAAGAATCTTGGGTAAACTGATTGATGATCCAAC
TTTCGCTTTGCCTTTCTGGAACTGGGATGCCCCTGCCGGCATGCAAATGC
CAGCCATTTTTACTGACCCCAAATCACCACTTTATGACCCCCTTCGCGAT
GCGAATCACCAACCTCCGACATTGCTTGATCTTAATTACGCAAAAGGAGA
TGCGAATCCAGACCCTGCAAAAGCAGAGGAATTGTATGCAAGCAATCTTA
ACGTAATGTACAGGCAAATGGTGTCCGGTGCCACGAAGCCTACTCTCTTT
TTTGGAAAACCATATCGTGCTGGTGATGATCCAAGTCCTGGAATGGGTAC
AATTGAGACCACCCCACACACTCAGATTCACTACTGGACCGGCGACCCCA
ATCAAACTAACGGGGAAAATATGGGCAATTTTTACTCAGCAGGGAGAGAT
CCCATATTTTATTGTCATCACTCGAACGTCGACCGAATGTGGGACTTGTG
GAAGAAAATACCAGGAGGCAAGCGAAAGGATATCGAGGATCCTGATTGGC
TTAATTCAGAGTTTCTTTTCTGGAATGAGAATNAAGAGCTGGTTCGAGTA
AAGGTTAAAGATACTCTTGACACCAAGAAGCTAAGATATGGCTTTCAAGA
TGTTCCTATTCCTTGGCTGAAAACTAGAGCAACACCAAAATTAACAAGGC
AGGAAAAATCACGTCGTGCAGCTAAAAAAAGCGTTGTATTAACACCAATT
AGTGCATTCCCNTGTGTCTTGGATAAAGTCATAAGTGTGGAGGTTTCCAG
GCCAAAGAAATCAAGAAGCGCGACGGAGAAAGAAGATGAAGATGAAGTTT
TGGTTATTGAAGGGATTGAGTATGAAGAAAATCAATTAATTAAGTTCGAT
GTCCTCGTCAACGATGAACCTGATTCACCTGGTGGACCAGACAAGTCCGA
GTTTGCAGGAAGTTTCATCAATGTGCCTCACAAGCATGCAAAAAAATCAA
AGACAACTATGGTATTGGGGATTACAGGATTGTTGGAAGATCTGGAAGCT
GAAGGAGATGATACCCTTGTGGTGACTTTAGTGCCTCGGACTGGTGGTGA
TTCTGTTACTGTTGCTAATGTCAAGATTGAGTTTGTCGCTGACTGATCAT
GGTGATGAGAGGACATATGCTACCATTTTCTTTTCTTTTCTTTTTTTCCT
TTCGCCTTTTCTTGTTTTCTCTCTTTTCTATGCTAGTATTCAGTGAGTTC
TCTTTTTTCTTTCAAGATGCTCAACTAATGGACCGTATTTCAATTTAAAA
AAAAAAAAAAAA

PPO2 amino acid sequence

MASFISLSSSIPLAASSFLPSFPKTHRVSRIKKPNRHNIPIVSCKSGKND
HEQNPATRRDLLIGLGGLYGATSLSDPFAYANPIAPPDITQCELVTLPSE
SDPSNCCPPTSTKIKNFEFPSASSPMRIRPAAHLVDKAYLAKYAKAIALM
KSLPDDDPRSFKSQADVHCAYCDGAYHQAGFPDLDLQIHFSWLFFPWHRL
YLYYFERILGKLIDDPTFALPFWNWDAPAGMQMPAIFTDPKSPLYDPLRD
ANHQPPTLLDLNYAKGDANPDPAKAEELYASNLNVMYRQMVSGATKPTLF

FGKPYRAGDDPSPGMGTIETTPHTQIHYWTGDPNQTNGENMGNFYSAGRD
PIFYCHHSNVDRMWDLWKKIPGGKRKDIEDPDWLNSEFLFWNENXELVRV
KVKDTLDTKKLRYGFQDVPIPWLKTRATPKLTRQEKSRRAAKKSVVLTPI
SAFPCVLDKVISVEVSRPKKSRSATEKEDEDEVLVIEGIEYEENQLIKFD
VLVNDEPDSPGGPDKSEFAGSFINVPHKHAKKSKTTMVLGITGLLEDLEA
EGDDTLVVTLVPRTGGDSVTVANVKIEFVAD

PtdPPO3 cDNA sequence

ATGGCTTCTATCTCTCCTTCCACCACCACGCCAACCACCATCTCTACCTC
CACTTTCTTCCCTTCATTCCCTAAAACATCCCAACTTTCCTTGATCAAGA
AGAGAAACCATCGTTATACCCCTAGATTTTCATGCAGAGCCACAAATGAT
GATTCGCAAAACCCTACCACTAGAAGAGATTTACTTATTGGTCTAGGAGG
CCTATATGGGGCAACAAGTCTTACTGATCCATTTGCCTTCGCTGCGCCAG
TGTCTGCTCCTGATATCTCCAAATGTGGCAGAGCAGACTTGCCCACCGGT
GCGAATGAAACAAACTGTTGCCCTCCAGTACCATCTACCAAGATCCTAGA
CTTCAAGCGCCCTCCTTCAAATGCCCCATTACGTGTTAGACCAGCAGCTC
ATTTAGCTGATAAGGACTACATAGCTAAATACAAGAAAGCCATTGAGCTC
ATGAAAGCTCTCCCTGAAGATGATCCACGTAGCTTCATGCAACAAGCCGA
TGTTCATTGTGCTTATTGTAATGGTGCTTATGACCAAGTGGGGTTTCCTA
ATTTAGAAATTCAAGTTCACAACTCTTGGCTCTTCTTTCCTTTCCATAGA
TACTATCTTTACTTCTATGAGAAAATCTTAGGCAAATTGATTGATGATCC
AACTTTTGCTTTGCCTTTCTGGAACTGGGATTCTCCTGGTGGCATGCAAT
TGCCAGCCATGTATGCCGATCCTAATTCACCACTGTATGATAGTCTCCGC
AACAAGAATCACCAACCACCAACATTGCTTGATCTTGATTGGAGTGGCAC
AGACACCCCCACTTCAAACGAAGAGCAGTTATCTAGCAACCTTACCATTA
TGTACAGGCAAATGGTGTCCAATGGAAAGACTCCTAGGCTCTTTTTCGGT
GGTGCATACCGTGCTGGTGATGAACCAGGCCCTGAACCAGGTCCAATTGA
GAATATTCCACACGGTCCAGTTCATATCTGGACAGGTGACAACACTCAAC
CAAACTCTGAAGACATGGGCAATTTCTACTCAGCTGCTAGAGATCCAATC
TTTTTCTGTCATCACTCGAACGTTGACCGAATGTGGTCAGTATGGAAGAC
ATTAGGAGGCAGGCGAACTGACCTGACTGACCCTGATGGGCTTAATGCCT
CATTCTTTTTCTACGATGAAAATGCCAATCCTGTTCGTGTCAAGGTTAGA
GATTGCCTGGATTCAAGAAACCTAGGATATGTTTATCAAGATGTTGAAAT
TCCTTGGCTACAATCCAGGCCAACACCAAGAAGGTCAGCCAAGAAAGTGG
CTAGCAATATCTTTGGCCATGAAAAGGAAGCAATTGCAGCTGAAAGGAAA
AAGAACGCATTGACACCAATCACTGCTTTCCCTCTAGTGTTGAATAAAGT
GATCAGTGTTAAGGTTGCAAGGCCAAAGAAATCGAGAAGCAAAAAGGAGA
AAGAAGACGAGGAGGAGCTTCTGGTAATTCAAGGGCTTGAGTTTGACAAA
ACTAAAGCTTTGAAATTTGATGTGTACATTAATGATGAGGATGATTCCCT
TAGTGCACCAGACAAGACAGAGTTTGCTGGAAGTTTTGTGAATGTGCCCC
ATAAGCACAAACATGGGAAGAAAATGACTACATGTTTCAGGTTGGCTCTT
ACAGATCTCTTGGAGGATTTGGATGTTGAGGGTGATGACAGTCTGATTGT
GACTTTGGTGCCAAGGTATGGCAAGGGTCTAGCAAAAATTGGCGGCATCA
AGATTGAGTTTGATCAAGATTAATCGATGATCATCAAGAACATGTTCGGA
GATTTGTGCGTGGAGCAATTGGATTAGCATGATGTGATTTTCCTTTTTCT
TCTCTTAATTTGTTTTTTTAATCATTTTGCTATCATATGTGCCCATATGG
CAAGTGGCAATAAGGACATATGTATGTTCGTGTCTTATTTGCAGCAAATA
AAGCAAGATCGATTTCACTGAAAAAAAAAAAAAAAA

PPO3 amino acid sequence

MASISPSTTTPTTISTSTFFPSFPKTSQLSLIKKRNHRYTPRFSCRATND
DSQNPTTRRDLLIGLGGLYGATSLTDPFAFAAPVSAPDISKCGRADLPTG
ANETNCCPPVPSTKILDFKRPPSNAPLRVRPAAHLADKDYIAKYKKAIEL
MKALPEDDPRSFMQQADVHCAYCNGAYDQVGFPNLEIQVHNSWLFFPFHR

YYLYFYEKILGKLIDDPTFALPFWNWDSPGGMQLPAMYADPNSPLYDSLR
NKNHQPPTLLDLDWSGTDTPTSNEEQLSSNLTIMYRQMVSNGKTPRLFFG
GAYRAGDEPGPEPGPIENIPHGPVHIWTGDNTQPNSEDMGNFYSAARDPI
FFCHHSNVDRMWSVWKTLGGRRTDLTDPDGLNASFFFYDENANPVRVKVR
DCLDSRNLGYVYQDVEIPWLQSRPTPRRSAKKVASNIFGHEKEAIAAERK
KNALTPITAFPLVLNKVISVKVARPKKSRSKKEKEDEEELLVIQGLEFDK
TKALKFDVYINDEDDSLSAPDKTEFAGSFVNVPHKHKHGKKMTTCFRLAL
TDLLEDLDVEGDDSLIVTLVPRYGKGLAKIGGIKIEFDQD

Appendix 4: Average Weight and Survival of FTC after 14-day in No-choice Feeding Bioassay Using Low-PPO and High-PPO Hybrid Aspens.

2002-08-01		Low-PPO controls			High-PPO transgenics		
	Replicates	717 (1)	717 (2)	717 (3)	S19	S12	S28
Weight (mg)	1	21.64	11.75	21.99	13.84	23.53	18.39
	2	21.10	16.41	13.26	15.63	15.58	15.90
	3	15.08	22.73	20.16	28.83	12.90	15.60
	4	13.35	17.26	13.60	29.81	12.15	26.05
Survival (%)	1	100	100	87.5	100	100	100
	2	87.5	100	87.5	100	100	100
	3	100	87.5	100	100	87.5	100
	4	100	100	100	100	100	100

2002-08-21		Low-PPO controls			High-PPO transgenics		
	Replicates	717 (1)	717 (2)	717 (3)	S12	S31	S18
Weight (mg)	1	17.00	18.00	13.70	19.48	14.56	14.16
	2	28.48	14.86	18.72	12.68	19.52	15.68
	3	10.96	13.32	11.18	19.85	15.22	12.72
	4	16.44	15.14	8.52	14.80	12.12	19.32
Survival (%)	1	100	100	80	100	100	100
	2	100	100	100	100	100	80
	3	100	100	100	80	100	100
	4	100	100	100	100	100	100

2002-09-05		Low-PPO controls		High-PPO transgenics	
	Replicates	717 (1)	717 (2)	S12	S28
Weight (mg)	1	4.54	4.84	3.00	4.03
	2	7.23	3.94	5.42	5.20
	3	6.38	7.15	5.23	3.90
	4	4.92	4.08	6.12	4.00
Survival (%)	1	100	100	100	80
	2	60	100	100	60
	3	100	80	80	100
	4	100	100	100	80

2002-09-16		Low-PPO controls				High-PPO transgenics			
	Replicates	717(1)	717(2)	A5	A1	S19	S12	S18	S29
Weight (mg)	1	4.17	4.13	3.70	4.43	2.65	2.53	3.98	2.00
	2	2.83	3.10	3.73	3.20	2.28	3.27	3.17	2.45
	3	3.38	3.05	3.33	4.03	2.47	2.13	3.23	3.17
	4	3.55	4.25	4.87	4.05	3.10	2.25	2.35	3.40
	5	3.50	3.70	3.30	4.40	2.63	3.30	3.03	4.00
Survival (%)	1	60	60	80	60	80	80	80	60
	2	80	80	80	80	80	60	60	80
	3	80	80	80	60	80	80	60	60
	4	80	80	60	80	40	80	80	60
	5	80	80	80	80	80	60	60	80

2002-10-17		Low-PPO controls			High-PPO transgenics		
	Replicates	717	A1	A2	S11	S19	S28
Weight (mg)	1	2.98	2.02	2.00	0.70	1.08	1.03
	2	2.84	1.63	2.57	1.01	1.50	1.40
	3	3.38	2.68	2.68	1.95	1.53	1.93
	4	2.48	2.32	1.87	1.58	1.43	2.07
	5	2.25	2.70	2.40	1.10	0.00	2.20
Survival (%)	1	75	62.5	25	50	50	37.5
	2	62.5	37.5	62.5	12.5	25	12.5
	3	50	50	75	25	50	50
	4	62.5	75	50	37.5	12.5	62.5
	5	50	62.5	37.5	37.5	0	50

2003-07-10		Low-PPO controls					
	Replicates	717 (1)	717 (2)	717 (3)	717 (4)	A5	A2
Weight (mg)	1	12.37	10.08	11.76	9.86	15.45	12.22
	2	10.16	11.52	11.38	7.65	8.22	7.22
	3	11.04	9.72	13.03	7.10	15.86	11.64
	4	11.52	11.91	12.22	12.02	10.72	8.10
Survival (%)	1	100.0	83.3	83.3	83.3	66.7	83.3
	2	83.3	83.3	83.3	66.7	83.3	100.0
	3	83.3	83.3	66.7	83.3	83.3	83.3
	4	83.3	66.7	100.0	83.3	83.3	66.7
		High-PPO transgenics					
	Replicates	S11	S30	S28	S19	S17	S31
Weight (mg)	1	13.28	9.66	10.80	11.32	14.12	11.35
	2	12.02	12.44	12.97	9.32	13.87	9.15
	3	11.42	11.35	11.45	12.26	12.18	12.63
	4	12.18	15.03	11.08	8.88	13.82	12.10
Survival (%)	1	83.3	83.3	83.3	83.3	83.3	100.0
	2	100.0	83.3	100.0	83.3	100.0	100.0
	3	100.0	66.7	66.7	83.3	66.7	66.7
	4	66.7	83.3	66.7	83.3	83.3	83.3

2003-09-28		Untransformed controls							
	Replicates	717(1)	717(2)	717(3)	717(4)	717(5)	717(6)	A5	A2
Weight (mg)	1	3.90	6.00	4.68	7.23	5.68	6.40	5.22	4.14
	2	8.25	5.60	5.78	5.30	4.93	5.87	5.03	3.50
	3	6.38	4.92	4.48	5.13	6.28	6.18	3.95	5.53
	4	4.60	4.60	6.17	5.58	5.73	5.63	5.05	6.09
Survival (%)	1	87.5	50	75	75	75	75	62.5	62.5
	2	62.5	62.5	50	75	75	75	75	87.5
	3	50	62.5	50	37.5	75	62.5	50	75
	4	75	75	75	75	75	75	62.5	87.5
		High-PPO transgenics							
	Replicates	S28	S30	S17	S29	S12	S19	S11	S31
Weight (mg)	1	3.18	3.70	2.28	3.60	3.05	3.75	2.87	3.38
	2	3.43	3.43	3.85	2.03	2.37	2.60	3.27	4.17
	3	3.77	3.05	2.53	3.45	3.48	4.10	3.47	2.45
	4	3.50	3.63	3.27	3.65	2.93	4.05	2.70	3.45
Survival (%)	1	50	50	37.5	50	50	50	37.5	50
	2	50	37.5	37.5	37.5	37.5	50	50	37.5
	3	37.5	25	50	25	50	25	37.5	50
	4	37.5	37.5	37.5	25	37.5	50	37.5	50