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THE MOLECULAR BASIS FOR OZONE SENSITIVITY IN HYBRID POPLAR

DISertation

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

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ABSTRACT

Ozone is a major gaseous pollutant known to contribute to forest decline. Although the physiological and morphological responses of forest trees to ozone have been well characterized, little is known about the molecular basis for these responses. To determine the underlying molecular mechanisms involved in ozone tolerance in tree species, comparisons were made between ozone-tolerant (NE-245) and ozone-sensitive (NE-388) hybrid poplar clones. Gene expression analysis using marker genes for known plant defense response pathways resulted in the finding that ozone-, pathogen-, and wound-induced expression of both salicylic acid- (SA) and jasmonic acid- (JA) regulated defense genes is attenuated in NE-388. Experiments using exogenous SA and methyl jasmonate (MeJA; a biologically active methyl derivative of JA) demonstrated that the attenuated expression of defense response genes in this clone was due to an insensitivity to both SA and JA. This finding was confirmed by quantitative analysis of endogenous levels of SA and JA that determined that both clones made similar amounts of these molecules in response to ozone. TUNEL assays were performed to detect DNA fragmentation associated with programmed cell death (PCD) that is characteristic of SA-dependent activation of a hypersensitive
response (HR). DNA fragmentation was detected in both ozone-treated and pathogen-inoculated tissue from the tolerant clone but not in the sensitive clone. In addition to providing supporting evidence for the insensitivity of NE-388 to SA, these results also indicate that the mechanism of lesion formation differs between the two clones. Cell death in the tolerant clone is caused by the induction of an SA-dependent PCD pathway, while cell death in the sensitive clone is likely to be necrosis caused by the lack of induction of antioxidant defenses to a level that is sufficient to prevent the formation of toxic ozone-induced active oxygen species. Treatment of these clones with MeJA prior to ozone fumigation resulted in a decrease in visible symptom development only in the tolerant clone, indicating that the SA-mediated activation of PCD may be inhibited by JA. Finally, experiments were performed that ruled out a deficiency in ethylene biosynthesis as having a role in the SA/JA-insensitivity of the ozone-sensitive clone.
Dedicated to my parents,

my husband

and

my children.
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CHAPTER I.

INTRODUCTION

The Impact of Ozone as a Pollutant

During the Industrial Revolution large-scale industrial complexes were developed that were dependent on burning fossil fuels for the production and transport of goods. The burning of these fossil fuels resulted in the production of photochemical smog, a mix of noxious gases and particles, several of which including ozone are now known to be phytotoxic. As early as the mid-1950's reports of stippling of grape leaves were recorded, a classic symptom of what is currently known to be due to ozone damage (Richards et al., 1958). Of the various pollutants studied to date, ozone is credited with having caused more significant damage to both natural and cultivated crop plants in industrialized nations than any single pollutant (Krupa and Kickert, 1989; Heagle 1989). It has been estimated that a 40% decrease in ambient ozone would have the net benefit of increasing crop yield by 3 billion dollars per year (Adams et al., 1990). Ozone is also believed to be a major contributing factor to forest decline (Preston and Tingey, 1988). The role of ozone in forest decline is particularly important due to not only the impact on biodiversity through the potential loss of habitat, but
also because of the role forests play in sequestering carbon and reducing CO₂ levels in the atmosphere. A decrease in the ability of a healthy, vigorously growing forest to serve as a CO₂ sink could potentially contribute to an increase in global warming.

Much of the impact of ozone on forest trees stems from the resulting alterations of basic metabolic processes including reducing photosynthetic rate (Reich and Amundson, 1985; Coleman et al., 1995b), decreasing ribulose bisphosphate carboxylase/oxygenase (Rubisco) quantity and activity, reducing foliar conductance (Pell et al., 1992; Farage and Long, 1995; Paakkonen et al., 1996), and accelerating leaf senescence (Coleman et al., 1995b). Altered patterns of carbon allocation have also been reported, resulting in a reduction in winter storage pools (Coleman et al., 1995a). These alterations of critical metabolic processes can lead to an increased susceptibility to biotic and abiotic stressors and contribute to forest decline (McLaughlin, 1985; Schmeiden and Wild, 1995).

Rapid increases in industrialization and other human activities have contributed significant amounts of gaseous pollutants to the troposphere during the twentieth century. Ambient concentrations of ozone have increased 1-2 % per year during the past 20 years in both Europe and the United States (Stockwell et al., 1997) and show no indication of leveling, particularly in developing industrialized areas (Chameides et al., 1994). Ozone is produced in both the troposphere and the stratosphere. Stratospheric ozone is generated
through photolysis of molecular oxygen by ultraviolet solar radiation and actually serves as a shield preventing harmful UV irradiation from reaching the earth's surface- the so-called 'ozone layer'. Ironically, tropospheric ozone is toxic to biological organisms. Ozone in the troposphere is produced by oxygen/ozone equilibrium reactions involving NO₂/NO (Fig. 1.1.A) and also results from reactions involving the photo-oxidation of various pollutants including carbon monoxide (Fig. 1.1.B), unburned hydrocarbons such as methane (Fig. 1.1.C), formaldehyde and other non-methane organic compounds (Fig. 1.1.D; Rao et al., 1999).

**Acute vs. Chronic Responses**

Since the initial observations of the phytotoxicity of ozone, there has been widespread investigation of the plant physiological processes effected by ozone under both laboratory and field conditions. There are two different types of plant responses to ozone, typically described as being acute and chronic. Acute stress from high concentrations of ozone (150-300 ppb), even for short periods of time (4-6 h), generally results in the rapid occurrence of visible injury to the leaf surface. Chronic ozone stress results from exposure to low concentrations (≤100 ppb) over a long period of time (days to months) and depending on the plant species, may result in symptoms such as chlorosis and premature senescence (Pell et al., 1997). Although little to no visible injury may occur, chronic ozone exposure results in biochemical and physiological changes that lead to reduced
(A) \( \text{NO}_2 + \text{O}_2 + \text{hv} \leftrightarrow \text{NO} + \text{O}_3 \)

(B) \( \text{CO} + 2\text{O}_2 + \text{hv} \leftrightarrow \text{CO}_2 + \text{O}_3 \)

(C) \( \text{CH}_4 + 4\text{O}_2 + 2\text{hv} \rightarrow \text{HCHO} + \text{H}_2\text{O} + 2\text{O}_3 \)

(D) \( \text{RH} + 4\text{O}_2 + 2\text{hv} \rightarrow \text{R’CHO} + \text{H}_2\text{O} + 2\text{O}_3 \)

Figure 1.1. Chemical reactions of ozone formation in the troposphere.
vigor and growth (Heath and Taylor, 1997). Acute ozone exposures have also been found to contribute to reduced crop yield (Heagle, 1989).

**Initial Site Reactions**

In spite of the discovery of ozone as a phytotoxic agent and the numerous studies describing the physiological impact of ozone exposure on plants, the sequence of events from exposure to the development of symptoms is not well understood. It is believed that ozone enters the mesophyll through the stomata, and studies by Laisk et al., (1989) suggest that it does not penetrate deep into the intercellular spaces but likely decomposes at the cell wall and plasma membrane. Based on studies by Weiss (1935), it was proposed that ozone dissociates in aqueous solutions generating active oxygen species (AOS) such as the superoxide anion ("O$_2^-$") and hydrogen peroxide (H$_2$O$_2$) which subsequently can react with transition metals generating hydroxyl radicals (OH$^*$) via the Haber-Weiss reaction (Fig. 1.2). Furthermore, ozone reacts with thiol groups, amines and phenolic compounds such as caffeic acid, and extracellular ascorbate which exacerbates the production of OH$^-$ and 'O$_2$ (Kanofsky and Sima, 1995). Therefore, as soon as ozone reaches the cell wall it can undergo reactions with cell wall constituents including phenolic groups, olefinic compounds and amide-proteins that will result in the production of highly toxic AOS (Heath, 1987). In a process referred to as ozonolysis, ozone reacts with lipid molecules, such as those found in the plasma membrane, to generate
Weiss Reaction: $O_3 + OH^- \leftrightarrow O_2^-$

Ozonolysis: $O_3 \leftrightarrow H_2O_2$

Dismutation:

\[ \text{HO}_2^- + H^+ \leftrightarrow \text{HO}_2^* \]

\[ \text{HO}_2^* + \text{HO}_2^- \leftrightarrow H_2O_2 + O_2 \]

\[ \text{HO}_2^- + \text{HO}_2^* + H_2O \leftrightarrow H_2O_2 + OH^- + O_2 \]

Haber-Weiss Reaction:

\[ \text{Fe}^{3+} + \text{O}_2^- \leftrightarrow \text{Fe}^{2+} + O_2 \]

\[ \text{Fe}^{2+} + H_2O_2 \leftrightarrow \text{Fe}^{3+} + OH^* + OH^- \]

Figure 1.2. Presumptive chemical reactions of ozone dissociation in leaf extracellular space. Ozone ($O_3$) is believed to enter the leaf through the stomata and rapidly dissociate, generating superoxide radicals ($O_2^-$). Ozone also reacts with lipid molecules of the plasma membrane generating stoichiometric amounts of $H_2O_2$ and aldehydes. $O_2^-$ is subsequently dismutated to $H_2O_2$ either spontaneously or enzymatically via endogenous superoxide dismutases. The resulting $O_2^-$ and $H_2O_2$ can then react with transition metals or secondary phenolic compounds to generate hydroxyl radicals ($OH^*$). Because plants are incapable of metabolizing $OH^*$, it is extremely toxic, resulting in damage to cellular membranes, proteins, and organelles. The ability of plants to metabolize both $O_2^-$ and $H_2O_2$ functions to reduce the formation of $OH^*$. 
stoichiometric amounts of aldehydes and $\text{H}_2\text{O}_2$ (Fig. 1.2). It has long been believed that ozone exerts its toxicity through the production of these AOS.

Antioxidant Defenses in Plants

Normal cellular metabolism in plants also results in the continuous production of AOS such as superoxide ($'\text{O}_2^-$), hydrogen peroxide ($\text{H}_2\text{O}_2$), and singlet oxygen ($'\text{O}_2$). Under normal conditions, plants have acquired a number of distinct mechanisms to rapidly metabolize these AOS (Scandalios, 1997) and remove them from the different cellular compartments. Examples of nonenzymatic scavengers of AOS utilized by plants include a number of compounds with high reducing potentials such as vitamin C (ascorbic acid), vitamin E, β-carotenes, polyamines and the tripeptide glutathione (Alscher and Hess, 1993; Kangasjärvi et al., 1994; Sandermann Jr., 1996). Glutathione and ascorbic acid work in concert with enzymes such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) to modulate the oxidation state of the cell (Sharma and Davis, 1997). In the ascorbate-glutathione cycle, $\text{H}_2\text{O}_2$ is converted to $\text{H}_2\text{O}$ at the expense of NADPH (Fig. 1.3). In addition, other antioxidant enzymes function to remove AOS including multiple isoforms of superoxide dismutase (SOD) which converts $'\text{O}_2^-$ into $\text{H}_2\text{O}_2$ as well as catalases and peroxidases that further metabolize $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ (Inzé and Van Montagu, 1995; Sandermann Jr., 1996).
Figure 1.3. The ascorbate-glutathione cycle (from Sharma and Davis, 1997). Ascorbate and glutathione are not consumed in this pathway but participate in the cyclic transfer of reducing equivalents, which permits the reduction of $H_2O_2$ to $H_2O$ using electrons derived from NAPH. (GSH) reduced glutathione; (GSSG) oxidized glutathione; (DHA) dehydroascorbate; (MDHA) monodehydroascorbate.
Ozone Induction of Antioxidant Responses

When exposure to ozone or other environmental stress results in excess production of AOS, the scavenging mechanisms of the plant can be overwhelmed. If not efficiently metabolized, these AOS rapidly oxidize lipids, proteins and other cellular organelles disrupting the cellular machinery which can result in a net decrease in photosynthetic rate (Reich and Amundson, 1985) and ultimately lead to the death of the cell and the appearance of necrotic lesions. For these reasons, a number of studies have focused on correlating levels of antioxidant compounds and enzymes in plants with an enhanced tolerance to ozone. These studies have lead to the suggestion that differential tolerance to ozone within a plant species is dependent on the relative amounts of ascorbate, polyamines, glutathione and levels of the glutathione-ascorbate pathway enzymes.

Apoplastic ascorbate has been proposed to be the first line of defense against ozone. Earlier studies have consistently recorded increases in ascorbate levels in several plant species exposed to ozone (Castillo and Greppin, 1988; Eckey-Kaltenbach et al., 1993; Luwe et al., 1993;). More recently, an ozone-sensitive mutant of Arabidopsis thaliana (formerly soz1, currently vtc1) was found to contain only 25% of wild type levels of ascorbic acid due to a defect in ascorbic acid biosynthesis (Conklin et. al., 1996). Furthermore, treatment of vtc1 with exogenous ascorbic acid increased tolerance to ozone, indicating that ascorbic acid plays a major role in providing resistance to oxidative stress.
Conjugated polyamines located in the extracellular leaf space have also been proposed to act as scavengers of free radicals (Bors et al., 1989). Levels of polyamines have been reported to increase in ozone-treated potato, tobacco and barley plants (Bouchereau et al., 1999). In tobacco, ozone-induced putrescine accumulation was reported only in the tolerant cultivar but, in contrast to these results, ozone increased levels of putrescine in ozone-sensitive birch clones rather than tolerant (Langebartels et al., 1991; Tuomainen et al., 1996). As a result, it was suggested that only conjugated forms of polyamines are efficient scavengers. Alternatively, these results may indicate that there are different antioxidant defense mechanisms in herbaceous plants and trees. Furthermore, as predicted by the reactions of the ascorbate-glutathione cycle, ozone has also been shown to affect glutathione metabolism (Hausladen and Alscher, 1993). Examples of such affects include a report in poplar trees where ozone treatment resulted in significant increases in GSH, GSSG and total glutathione levels (Sen Gupta et al., 1991). Another study correlated the ozone tolerance of *Phaseolus vulgaris* with high levels of GSH and GR activity (Guri, 1983). In addition to changes in the levels of GSH and GSSG, ozone has also been reported to result in increased expression of GST in both barley and Arabidopsis (Price et al., 1990; Sharma and Davis, 1994; Conklin and Last, 1995). Transcripts for ascorbate peroxidase, another component of the ascorbate-glutathione pathway, were shown to be elevated in ozone-treated
Arabidopsis plants as well as transcripts for cytosolic Cu/Zn SOD, another antioxidant enzyme (Sharma et al., 1996; Conklin and Last 1995).

Defense Signaling in Plants

To insure their continued productivity, plants must be able to adapt to changing conditions. In order to adapt to environmental change, plants are equipped with mechanisms to alter patterns of gene expression that result in physiological and biochemical changes most favorable for survival. When exposed to an environmental stress the plant must first perceive the stress. Subsequently, a signal must be generated to initiate the downstream protective responses. For example, as described in the previous section, when exposure to ozone results in the production of toxic AOS, the plants must first recognize the oxidative stress then generate a signal to activate the various antioxidant defense mechanisms. Several signal molecules have been identified that are involved in the modulation of plant response to stress in their environment including AOS, salicylic acid (SA), jasmonic acid (JA) and ethylene.

Of the signal molecules identified to date, perhaps the most well-studied in plant-pathogen interactions is SA. SA has been shown to play a role in influencing the oxidative burst, defense responses and subsequent cell death (Mur et al., 1996; Shirasu et al., 1997; Thulke and Conrath, 1998) that ultimately leads to the development of a hypersensitive response (HR) and systemic acquired resistance (Delaney et al., 1994). In addition, SA is also believed to
influence plant response to several abiotic stresses including UV-B (Yalpani et al., 1994). Another important signal molecule, JA, was first reported to increase in response to wounding in soybean by Creelman et al., (1992). McConn et al., (1997) demonstrated that JA was essential for defense response against insect attack by creating a triple mutant in Arabidopsis (fad3-2fad7-2fad8) that fails to accumulate significant amounts of jasmonates. JA has also been studied for its role in pathogen response in a variety of plant species (Blechert et al., 1995; Staswick, et al., 1998).

Because the accumulation of SA (Malamy et al., 1990) and SA-inducible PR proteins (Brederode et al., 1991; Memelink et al., 1990) is not induced by wounding, it has been suggested that the SA-signaling pathway is independent of the JA-signaling pathway. However, as our understanding of the complexity of these signaling pathways has expanded, it has become clear that there are extensive areas of interaction. SA has been demonstrated to be antagonistic to JA by inhibiting both its biosynthesis (Pena-Cortes et al., 1993) and its ability to induce proteinase inhibitor synthesis (Doares et al., 1995). Transgenic tobacco plants overexpressing a gene encoding a Rab-type, small GTP-binding protein contained 6-fold higher levels of cytokinins than control plants and accumulated both JA and SA in response to wounding (Seo et al., 1997). Wild-type plants treated with cytokinins were also able to accumulate SA in response to wounding, suggesting a role for cytokinins in the regulation of both SA and JA. Furthermore, by using the transgenic rgp1 plants, Sano et al., (1996) were able
to show that high levels of JA inhibited the accumulation of SA and its glucoside
derivative. Interestingly, O'Donnell et al., (1998) recently reported the cloning of
a novel wound-responsive gene homologous to a family of glucosyl transferases
that is independent of both JA and ethylene but is induced by exogenous SA. In
addition to antagonistic relationships between SA and JA, evidence for
synergistic interactions was reported by Mur et al., (1996) who demonstrated that
SA "potentiates" the expression of local defense genes that do not directly
respond to SA but become hyper-induced upon pathogen attack or wounding.
Similar results were obtained in studies of the potentiation of \textit{PAL} expression by
SA in parsley cells (Thulke and Conrath, 1998). Molecular evidence for overlap
between these signal transduction pathways is also provided by the identification
of an \textit{as-1} type cis-acting element (Xiang et al., 1996) that is responsive to both
SA and methyl jasmonate (MeJA).

Another common link between SA and JA pathways appears to be
ethylene. Ethylene has long been thought of as a signal molecule that plays a
role in the induction of defense response genes (Ecker and Davis, 1987).
Recent reports reveal that ethylene functions synergistically with both SA and JA.
The JA-induction of \textit{pin} gene expression was shown to require ethylene by using
ethylene deficient tomato plants (O'Donnell et al., 1996). A defensin gene in
Arabidopsis is induced in an SA-independent manner, requiring both ethylene
and JA (Penninckx et al., 1996) while osmotin and \textit{PR-1} were reported to be
hyperactivated by the combination of ethylene and JA. Furthermore, a novel
pathway for induced systemic resistance has been identified in Arabidopsis that is SA-independent but requires components of both JA and ethylene responses (Pieterse et al., 1998). Ethylene has been hypothesized to play a role in disease resistance and susceptibility and had been shown in Arabidopsis to mediate pathogen-induced damage by modulating the spread of cell death (Bent et al., 1992; Greenberg and Ausubel, 1993). The role of SA in programmed cell death has also been clearly defined (Dangl et al., 1996; Rao and Davis 1999).

Recent evidence supports the idea that defense gene expression can be induced through signaling pathways that are independent of SA, JA and perhaps even ethylene. For example, JA-independent wound-induction of the sesquiterpene cyclase gene in tobacco cell suspensions (Rickauer et al., 1997), FAD7 in leaf tissue of Arabidopsis; (Nishiuchi et al., 1997), and accumulation of the phenolic compound, feruloyltyramine, (Pearce et al., 1998) in wounded tomato leaves has been reported.

Ozone as an Abiotic Elicitor

The fact that ozone spontaneously generates AOS in leaf extracellular spaces combined with the fact that the lesions induced by acute ozone exposure resemble those resulting from a HR, led to the hypothesis that there must be significant overlap between the signaling pathways for ozone- and pathogen-induced responses. In fact, recent studies have shown that there is significant overlap in not only the defense related genes that are induced by ozone and
pathogen infection (Sharma and Davis, 1994; Sharma et al., 1996) but also by wounding (Orvär et al., 1997; Koch et al., 1998), UV (Rao et al., 1996), cold, drought, and heavy metal toxicity in various plant species (reviewed in Kangasjarvi et al., 1994; Sharma and Davis, 1997; Sandermann et al., 1998). These studies indicate that there must be significant overlap in the signaling pathways involved in mediating the induction of these defense response genes. Compelling evidence for AOS as the common denominator in these overlapping stress responses comes from the observations that ozone does indeed trigger an oxidative burst, resulting in the de novo generation of AOS in addition to those resulting from the degradation of ozone upon entering the cell (Schraudner et al., 1998; Rao and Davis, 1999). In addition, a variety of environmental stress factors including cold (Prasad, 1996), high light (Karpinski et al., 1999), heavy metals (Richards et al., 1998) mechanical and physical stresses (Orozco-Caredenas and Ryan, 1999) drought (Scandalios, 1997), UV-radiation (Rao et al., 1996; Surplus et al., 1998) as well as pathogens (Doke, 1997; Alvarez et al., 1998) act by stimulating the production of AOS.

In addition to AOS, SA is also thought to be an important component in the overlapping signaling pathways triggered by pathogen infection and ozone exposure leading to activation of HR and SAR (Delaney et al., 1994; Klessig and Malamy, 1994; Shulaev et al., 1995). This idea is supported by convincing evidence obtained using plants constitutively expressing a bacterial salicylate hydroxylase (NahG) gene that prevents any significant accumulation of SA by
converting it into the biologically inactive compound catechol. NahG tobacco and Arabidopsis plants do not express PR-1, undergo a HR or establish SAR in response to pathogen (Gaffney et al., 1993; Delaney et al., 1994). Furthermore, Arabidopsis plants with the NahG transgene were found to be more susceptible to ozone and ozone-treatment failed to induce PR-1 transcripts as well (Sharma et al., 1996). While ozone treatment of wild-type plants has been shown to trigger an effective resistance response to bacterial pathogen, NahG plants failed to exhibit this response.

Interestingly, while ozone-treatment was shown to increase transcript levels for PR-1, GST and PAL in wild-type Arabidopsis, induction of PR-1 and GST was not observed in Nah G plants. However, PAL mRNA levels were the same, if not higher than in ozone-treated wild-type plants. These results indicate that ozone triggers at least two distinct signaling pathways, one that is SA-dependent and associated with the activation of pathogen responses and the other that is independent of SA. Evidence that the SA-independent pathway may involve JA came from work by Orvär et al., (1996) who demonstrated that mechanical wounding or the direct application of JA prior to ozone exposure decreased the amount of visible ozone injury in tobacco plants.

Similar to AOS, SA and JA, ethylene has long been proposed to be a signal involved in mediating ozone responses as well. Numerous plant species have been reported to emit ‘stress ethylene’ in response to ozone exposure. A study of several pairs of ozone-sensitive and ozone-tolerant cultivars of six
different plant species found that of the variables measured, only ethylene emission consistently correlated with ozone-sensitivity (Wellburn and Wellburn, 1996). It has been suggested that the primary foliar injury observed in ozone-sensitive plants is due to the production of stress ethylene which subsequently reacts with ozone to form damaging AOS (Mehlhorn and Wellburn, 1987). Additional evidence for the role of ethylene as a signal modulating ozone responses comes from recent studies that showed that the inhibition of ethylene biosynthesis reduced ozone-induced damage in tomato (Tuomainen et al., 1997). Furthermore, some ozone-induced genes and enzyme activities such as peroxidases, GST, PAL and chitinase are also induced by ethylene (Mehlhorn, 1990; Ecker, 1995). Clearly, it can be concluded that ozone acts as an elicitor of a wide spectrum of plant defense responses which may be modulated by several signal molecules including AOS, SA, JA and ethylene.

**Hybrid Poplar as a Model System**

Although recent advances have begun to sort out plant defenses to ozone and the signal molecules that trigger them, most of these experiments have been performed using model herbaceous plant species such as Arabidopsis and tobacco. In spite of the fact that the physiological responses of forest trees to ozone have been well characterized, very little is know about their defense responses to ozone and other stresses at the molecular level. Of the signal molecules identified in herbaceous plants involved in modulating defense
responses, only ethylene has been studied in some detail in trees (Telewski, 1992; Kargiolaki et al., 1991; Telewski, 1990). Given that the roles of signal molecules in defense responses of herbaceous plants have been found to vary somewhat from one plant species to another (Coquoz et al., 1995; Silverman et al., 1995; Chen et al., 1997) and that tree species have unique characteristics that distinguish them from herbaceous plant species, e.g., longevity and the ability to undergo the process of wood formation, it is likely that trees may have defense response and signaling systems that are distinct from those found in herbaceous plants.

Due to the role of ozone in contributing to forest decline and the ecological impact thereof, it is imperative to study the defense responses induced by ozone and the molecular mechanisms that underly ozone sensitivity and tolerance using a tree system. Because of its economic and ecological importance as well as several technical advantages associated with performing molecular assays on this species, hybrid poplar (*Populus maximowizii* X *Populus trichocarpa*) was chosen for use as a model system for this project. Advantages to developing hybrid poplar as a model system for molecular studies include the small genome size, the extremely fast growth rate, the ease of vegetative propagation, the availability of RFLP and AFLP markers, the newly generated EST sequences available in Genbank (Sterkey et al., 1998), the relative ease of nucleic acid extraction, and the availability of well-developed transformation and regeneration protocols.
Until recently, hybrid poplar was used primarily as a source of pulpwood, grown in high density plantations. Lately, hybrid poplar has been used as a short rotation crop for biomass generation for energy uses (Bowersox and Ward, 1976; Lo and Abrahamson, 1996). Being one of the fastest growing hardwoods in North America, poplars have been a favorite choice for the establishment of wood biomass plantations (Lo and Abrahamson, 1996). The parental poplar *P. trichocarpa*, commonly known as the black cottonwood, is the largest hardwood in the Western United States and is also prevalent in Canada. Another closely related species, *P. deltoides*, is an important component of both Canadian forests and those in the Central United States (Demeritt, 1990).

The specific clones used in this study, NE-245 and NE-388, were previously determined to be ozone-tolerant and ozone-sensitive, respectively, based on visible leaf injury (Wood and Coppolino, 1972). The ozone-sensitive clone NE-388 has also been reported to be highly susceptible to the fungal pathogen *Septoria musiva* (Ostry and McNabb, 1985), particularly when grown on plantations (Moore and Wilson, 1983). This observation provides a preliminary indication that ozone sensitivity in hybrid poplar overlaps with pathogen sensitivity. The fundamental hypothesis of my thesis work is that differences in defense gene expression and/or the signal transduction pathways that activate them contribute to ozone sensitivity in hybrid poplar. This hypothesis was tested using molecular tools to compare stress-induced gene expression and defense
signaling pathways between these two differentially ozone-sensitive hybrid poplar clones.
CHAPTER II

OZONE SENSITIVITY IN HYBRID POPLAR IS CORRELATED WITH THE LACK OF DEFENSE-GENE ACTIVATION

INTRODUCTION

Although the physiological responses of forest trees to ozone have been well characterized, very little is known about the responses at the molecular level. It is believed that when ozone enters the mesophyll through the stomata, where it is rapidly degraded, generating active oxygen species (AOS; Kanofsky and Sima, 1995). The presence of AOS activates defense mechanisms that may operate either by preventing the formation of AOS and/or by scavenging them once they are formed. Glutathione and superoxide dismutase, proposed components of these oxidative defense systems, have been shown to increase upon ozone exposure in poplar (Sen Gupta et al., 1991). Total cellular activities of superoxide dismutase, guaiacol peroxidase and glutathione reductase were shown to increase in birch as a result of ozone exposure (Tuomainen, et al., 1996).
A variety of plant systems have exhibited ozone induction of mRNAs for defense-related genes known to be induced by pathogens and other stresses (reviewed in Kangasjärvi et al., 1994; Sandermann Jr., 1996; Sharma and Davis, 1997). This overlap of induced gene expression is likely due to the fact that many of these stresses produce AOS. AOS trigger many different pathways, some of which require salicylic acid (SA) as a signal molecule. One such pathway is the systemic acquired resistance (SAR) pathway (Lamb and Dixon, 1997; Mehdy et al., 1996) characterized by the induction of pathogenesis-related (PR) proteins. By using cross-reacting antibodies to PR proteins from herbaceous plants, it was demonstrated that ozone induced increased PR protein levels in Norway spruce (Kärenlampi et al., 1994). This induction of PR proteins by ozone is also well documented in several herbaceous plant species (Sandermann Jr., 1996; Sharma and Davis, 1997).

The phenylpropanoid biosynthetic pathway, which produces a number of defense metabolites, is also induced by ozone. Transcript levels for enzymes involved in this pathway, including PAL in birch (Tuomainen et al., 1996), and cinnimoyl alcohol dehydrogenase in Norway spruce (Galliano et al., 1993), were shown to be induced by ozone. The induction of PAL gene expression by ozone has been shown not to require SA in Arabidopsis, suggesting that ozone activates a SA-independent signal transduction pathway as well (Sharma et al., 1996). Further evidence for a second signal transduction pathway in ozone responses has recently been reported. Orvär et al., (1997) demonstrated that
mechanical wounding or the direct application of jasmonic acid (JA, a known mediator of wound responses) prior to ozone exposure decreased the amount of ozone injury in tobacco plants.

To further elucidate the molecular mechanisms of ozone responses in tree species, hybrid poplar (*Populus maximowizii X Populus trichocarpa*) was used as a model forest tree. In this chapter, comparisons of physiological and molecular responses to ozone between an ozone-sensitive (NE-388) and an ozone-tolerant (NE-245) clone will be discussed. To determine if ozone sensitivity is related to specific stress response pathways, expression patterns of marker genes including *PAL* (the first enzyme in the phenylpropanoid biosynthesis pathway), *OMT* (an enzyme involved in lignin formation), *PR-1* (a pathogenesis-related protein characteristic of SA-mediated responses) and *WIN3.7* (a wound-inducible trypsin inhibitor, Bradshaw et al., 1989) were examined. Clear differences in the pattern of expression of these genes in the ozone-tolerant and ozone-sensitive plants were observed. These differences coincided with differences in the physiological responses of the two clones to ozone as well. To determine if the ozone-sensitive plants were also more susceptible to other stresses, wounding and pathogen infiltration experiments were performed. A similar difference in gene expression was observed, suggesting an overlap not only between ozone and SA-mediated pathways, but also between ozone and wound-induced pathways in hybrid poplar.
METHODS AND MATERIALS

Plant Growth

Initial greenwood cuttings of hybrid poplar (Populus maximowizii X Populus trichocarpa) designated as ozone sensitive (clone NE-388), or as ozone tolerant (clone NE-245) based on visible lesion formation in response to a single acute dose of ozone (Wood and Coppolino, 1972), were obtained from E. Pell, Pennsylvania State University. Cuttings were placed in sand, kept under mist until roots were established, then transplanted into 15-cm diameter pots containing Metromix 500 (Hummert, St. Louis, MO) amended as described by Pell et al., (1995). A drip irrigation system delivered approximately 300 ml of water to each cutting daily. All side shoots were pruned so that each plant consisted of a single stem. No pruning was performed for at least one week prior to ozone fumigation or wounding.

Ozone Fumigation

Six weeks following transplantation, cuttings were transferred to growth chambers modified for ozone fumigation and were acclimated for 2-3 days prior to treatment. Growth chamber conditions averaged 25°C,
67% relative humidity, 370 ppm CO₂, with a 14 h photoperiod averaging 200 μmole m⁻² s⁻¹ at the top of the canopy. The cuttings were then fumigated for 6 h per day (0900-1500) for 4 days with either ambient air (<30 ppb ozone) or with ozone at 300 ± 50 ppb. Ozone was generated with an Orec ozone generator (model 03V10-0, Ozone Research and Equipment Corp., Phoenix, AZ) and each treatment was replicated for a total of four chambers. At 3, 6, 12, 24, 30, 54 and 78 h after the start of the fumigation period, the second and fourth fully expanded leaves were collected from eight plants per clone per treatment, frozen in liquid nitrogen, and stored at -80°C until used for RNA isolation.

Gas Exchange Analysis

Gas exchange was measured under controlled conditions after two and four days of fumigation by placing the fourth fully expanded leaf in a 1 L cuvette of a LI-COR 6200 photosynthetic system (LI-COR, Lincoln, NE). Photosynthetic rates and stomatal conductance were measured at 1050 ± 25 μmoles m⁻² s⁻¹ (saturating light) and 225 ± 25 μmoles m⁻² s⁻¹ (low light) using a GE cool-beam PAR lamp (Model 300 PAR 56/2 WFL) as the light source following a 30 min acclimation period. The LI-COR cuvette was flushed with outside air for several minutes and conditions were allowed to stabilize prior to taking measurements. Cuvette conditions averaged 23°C, 350 ppm CO₂ and 45% RH. Measurements were taken between
1300 and 1600 h on four plants per chamber per clone. This experiment was performed using a completely randomized design and analysis of variance (ANOVA) with chamber as the experimental unit and plants as subsamples was used to determine differences in gas exchange due to ozone treatment, clone, and number of days of exposure. SAS (SAS Institute Inc., Cary, NC) was used for all statistical analyses and data presented include means ± SEM. All gas exchange measurements were performed in collaboration with Dr. Amy Scherzer (USDA Forest Service, Delaware, OH)

Wounding Experiments

Mechanical wounding of cuttings was performed by crimping the leaves with pliers. Each leaf from the fourth fully expanded leaf on down was wounded 20 times at 0900 h, and this was repeated at 1100, 1300 and 1500 h every day for 4 consecutive days. At 3, 6, 24, 36, 54 and 78 h after the initiation of wounding, the second and fourth fully expanded leaves were collected, frozen in liquid nitrogen, and stored at -80°C until used for RNA isolation.

Bacterial Infection Experiments

Overnight cultures of Pseudomonas syringae pv. maculicola KD4326 (Wanner et al., 1993) were diluted in 10 mM MgCl₂ to a final OD₆₀₀ of 0.1. The
bacterial suspension was then hand infiltrated into the undersides of the second through the fifth fully expanded leaves, using a plastic 1 ml syringe without a needle. The infiltrated area of the leaf was outlined with marker and collected at 3, 6, 12, or 24 hours after infiltration for RNA analysis. Mock inoculations were performed by infiltrating with 10 mM MgCl₂ alone.

**Gene Probes**

Clones for poplar PAL, OMT and WIN3.7 were kind gifts from O. Douglas (Subramaniam et al., 1993), M. Van Montageau (Dumas et al., 1992) and M. Gordon (Bradshaw et al., 1989), respectively. A probe for PR-1 was generated by reverse transcriptase polymerase chain reaction (PCR) using total RNA isolated from ozone-treated NE-245 plants and degenerate nucleotide primers (5' GCNCARAAYTCNCCNCARGAYTA 3' and 5'CCANACNACYTGNNGTRTARTG 3') corresponding to conserved amino acid sequences AQNSP/QDY and HYTQVVW, respectively. The 310 nucleotide PCR product was cloned into a TA-vector (Invitrogen), sequenced and compared with other known plant PR-1 genes. This analysis demonstrated that the PCR product had approximately 68% identity with other PR-1 genes.
RNA Extraction and Analysis

Total RNA was isolated as described by Parsons et al., 1989. Fifteen µg of total RNA was fractionated on 1.5% formaldehyde agarose gels in MOPS buffer and transferred by capillary blotting onto a Duralon membrane (Stratagene). Filters were UV-crosslinked then prehybridized for one h at 42°C in 6X SSC, 1% SDS, 2X Denhardt’s solution, 25 µg/ml denatured salmon sperm DNA, and 50% formamide. DNA probes were labeled by random priming (RadPrime, BRL) and hybridized at 42°C for 16-18 h. Filters were washed with 2X SSC containing 1% SDS at 55°C for 15 minutes followed by a 30 minute wash in 0.5X SSC + 1% SDS at 65°C. Filters were then exposed to PhosphorImager screens and the signal intensity was quantified using Molecular Dynamics ImageQuant software. The data shown have been corrected for loading differences by quantititating counts obtained by rehybridizing with a 28S ribosomal gene probe from pea (Wanner and Gruissem, 1991). All experiments were performed at least twice with two replicates per test condition. The data shown are from representative experiments.
RESULTS

Lesion Development

To confirm the differential ozone sensitivity of the hybrid poplar clones NE-388 and NE-245, cuttings were exposed to 300 ppb ozone 6 h daily for 5 days. Visible signs of ozone damage first appeared at 12-24 h after the start of the initial 6 h ozone fumigation and peaked at 36 h with little additional injury occurring on subsequent days of exposure (Fig. 2.1). The vast majority of injury was observed on mid-aged and older leaves with only modest amounts of injury occurring in leaves that were not yet fully expanded. The ozone-sensitive clone NE-388 plants displayed large necrotic regions on 30-80% of all leaves, and generally had at least one leaf that was covered with lesions over 50% or more of the leaf area. Ninety percent of the NE-388 plants fumigated with ozone developed some type of injury including stipple as well as large and small necrotic lesions (EPA, 1976). The tolerant clone NE-245 plants developed stipple or flecks, but did not exhibit the extensive regions of necrosis observed in clone NE-388 plants. Only 30% of the clone NE-245 plants developed any lesions. Of those that did, 20% or less of all leaves had visible ozone damage.
Figure 2.1. Comparison of ozone-induced leaf injury observed in the ozone-tolerant clone NE-245 (left) and the ozone-sensitive clone NE-388 (right). Cuttings were grown in the greenhouse for 6 to 8 weeks before being transferred to chambers, where they were allowed to acclimate for 2 to 3 d before treatment with 300 ppb ozone for 6 h daily. The photograph was taken 36 h after the start of the first 6 h of ozone fumigation.
Effects of Ozone on Photosynthesis and Gas Exchange

Prior to fumigation, six-week-old cuttings of the two clones exhibited different patterns of growth and physiology. Clone NE-245 averaged 8.5 cm taller than clone NE-388 (59.5±0.74 vs. 51.1±0.61 cm respectively) and had about 1.5 times as much aboveground biomass (6.54±0.44 vs. 4.36±0.16 g) and 1.8 times as much leaf area (1386±87 vs. 778±30 cm²). Both clones averaged 20 leaves each, resulting in a larger average leaf size for clone NE-245. Light response curves (data not shown) indicated that the photosynthetic capacity of clone NE-388 was significantly (P<0.05) lower than clone NE-245 when measured under saturating light. The two clones, however, demonstrated similar rates of photosynthesis when measured under low light conditions simulating those used during ozone exposure experiments.

After two days of exposure, ozone significantly (P<0.05) reduced photosynthetic rates in both clones (Fig. 2.2.A). Statistical analysis indicated that the absolute values of photosynthesis were not significantly different between the two clones. However, the percent reduction was greater in clone NE-245 as compared to clone NE-388 (72% vs. 56%). Ozone continued to reduce photosynthetic rates throughout the study, and by the fourth day of fumigation, all of the ozone-treated plants had photosynthetic rates that were less than those of the ambient-grown plants and many had negative net photosynthetic rates, indicating the leaf tissue was respiring rather than photosynthesizing (Fig. 2.2.A).
Figure 2.2. Effects of ozone on net photosynthesis rate ($P_n$) and stomatal conductance ($g_s$). Measurements were made on the fourth fully expanded leaves of ozone-tolerant clone NE-245 plants or ozone-sensitive clone NE-388 plants after 2 and 4 days of exposure to ambient air or a 6-h daily treatment with 300 ppb ozone. Bars represent the mean of eight plants ± SE. Results of ANOVA produced the following $P$ values for photosynthesis (ozone = 0.0035, clone = 0.7058, day = 0.0036, ozone x clone = 0.1477, ozone x day = 0.0081, and clone x day = 0.7137) and stomatal conductance (ozone = 0.0150, clone = 0.0906, day = 0.0001, ozone x clone = 0.0002, ozone x day = 0.0001, and clone x day = 0.0026).
Ozone exposure reduced stomatal conductance as well, and the magnitude of the reduction was significantly different (P<0.05) between the clones (Fig. 2.2.B). After two days of exposure to ozone, stomatal conductance of the ozone-sensitive clone NE-388 was reduced by approximately 30% compared to a reduction of 76% in the ozone-tolerant clone NE-245. After 4 days of exposure, this same reduction of 30% was present in clone NE-388, but the difference between ozone and ambient plants for clone NE-245 was only 12%. The change in percent reduction from day 2 to day 4 in clone NE-245 was due to a large reduction in stomatal conductance values in the ambient-grown plants combined with an increase in the stomatal conductance values in the ozone-fumigated plants. This change in ambient-grown NE-245 plants also accounted for the significant ozone-by-day and clone-by-day interactions.

**Ozone-Induced Gene Expression**

Because *PAL* induction is often a useful indicator of a general, coordinate plant defense response, both sensitive and tolerant clones were tested to determine if they differed in their accumulation of *PAL* transcripts in response to ozone. RNA blot hybridization studies demonstrated ozone induced accumulation of *PAL* transcripts in both the sensitive and tolerant clones (Fig. 2.3.A). However, the magnitude of this increase was 3-5 fold greater in clone NE-245 than in clone NE-388. An early, transient induction of *PAL* mRNA was observed in both clones with the highest levels observed at 3 h after the initiation.
Figure 2.3. Comparison of ozone-dependent induction of PAL and OMT mRNA accumulation in hybrid poplar clones NE-245 and NE-388. Total RNA was extracted from the combined second and fourth fully expanded leaves of 6- to 8-week old cuttings that were exposed to either ambient air or 300 ppb ozone for 6 h. The amount of hybridizing radioactivity was quantitated using a phosphor imager and is expressed as relative counts. A) RNA accumulation during the first 24 h after the start of the first 6-h ozone treatment. B) RNA accumulation measured on sequential days after a 6-h ozone treatment.
of ozone exposure. Levels of \textit{PAL} mRNA returned to those seen in the controls by 24 h. Trees that were treated with ozone for four consecutive days also showed a transient induction of \textit{PAL} each day (Fig. 2.3.B). Transcripts for \textit{OMT}, an enzyme involved in lignin biosynthesis, were also induced by ozone. \textit{OMT} mRNA reached the highest levels 12 h after the start of ozone treatment and like \textit{PAL} mRNA, returned to near control levels by 24 h (Fig. 2.3.A). As was observed for \textit{PAL} mRNA induction, \textit{OMT} transcripts were transiently induced each day over a four day treatment period (Fig. 2.3.B).

Previous work has demonstrated that Arabidopsis has ozone-inducible defense mechanisms that provide some protection and that the induction of this defense required a SA-dependent signaling pathway (Sharma et al., 1996). Therefore, \textit{PR-1} was used as a marker gene for a SA-mediated response and accumulation of \textit{PR-1} mRNA in ozone-treated plants was monitored to determine if this pathway was also induced in hybrid poplar. In clone NE-388, very little (3-fold) induction of \textit{PR-1} by ozone was observed (Fig. 2.4.A). However, the tolerant clone NE-245 demonstrated increased levels of \textit{PR-1} transcripts as early as 6 h after the initiation of ozone exposure, with the maximum level (24-fold) being reached after the second day of fumigation. This high level of \textit{PR-1} transcript accumulation was attained on each of the subsequent days of exposure (Fig. 2.4.B).

Recent work has indicated that in tobacco, ozone protection can be induced by wounding and treatment with jasmonates prior to ozone exposure.
Figure 2.4. Comparison of ozone-dependent induction of PR-1 and WIN 3.7 mRNA accumulation in hybrid poplar clones NE-245 and NE-388. Total RNA was extracted from the combined second and fourth fully expanded leaves of 6- to 8-week old cuttings that were exposed to either ambient air or 300 ppb ozone for 6 h. The amount of hybridizing radioactivity was quantitated using a phosphor imager and is expressed as relative counts. A) RNA accumulation during the first 24 h after the start of the first 6-h treatment. B) RNA accumulation measured on sequential days after a 6-h ozone treatment.
(Örvar et al., 1997). To examine the role of JA-mediated gene expression in ozone responses, levels of WIN 3.7 mRNAs in ozone-treated poplar plants were monitored (Fig. 2.4). WIN 3.7 is a wound-inducible gene from poplar that is homologous to the trypsin family of proteinase inhibitors (Bradshaw et al., 1989) that can also be induced by exogenous application of MeJA (Fig. 2.5). In clone NE-245, levels of WIN 3.7 mRNA were induced up to 20-fold by ozone, while in clone NE-388 only a 6-fold induction was observed. Similar to the induction pattern of PAL, WIN 3.7 transcripts were induced maximally by 3 h, then returned to control levels by 24 h (Fig. 2.4.A). This induction was evident every day over four consecutive days of ozone exposure (Fig. 2.4.B).

**Wound- and Pathogen-Induced Gene Expression**

Comparisons of the ozone-induced patterns of defense gene expression in ozone-sensitive and -tolerant clones of hybrid poplar indicated that clone NE-388 has a greatly attenuated response with respect to ozone-induced gene expression. To determine if this defect was specific to ozone treatment, the patterns of gene expression in wounded leaves and in leaves infiltrated with an avirulent *P. syringae* pv. *maculicola* strain were examined. Wounding treatment appeared to induce PAL, OMT and WIN 3.7 expression in both clones (Fig. 2.6). However, the extent of this induction was far greater in the ozone-tolerant clone NE-245 compared to the ozone-sensitive clone NE-388. In NE-245, wounding induced PAL mRNA levels 10-fold above unwounded controls, in contrast to
Figure 2.5. Induction of WIN3.7 mRNA by MeJA. Clone NE-245 plants (6- to 8-week-old cuttings) were sprayed with an aqueous solution containing either 0.1% Triton X-100 (Control) or 0.1% Triton X-100 containing 100 mM MeJA until the leaves were completely wetted. At the indicated times after spraying, the second and fourth fully expanded leaves were harvested. Total RNA was extracted, subjected to RNA-blot hybridization analysis, and the amount of hybridizing radioactivity was quantitated using a phosphor imager. Time points represent the hours after the beginning of the treatments.
Figure 2.6. Comparison of wound-induced accumulation of *PAL*, *OMT*, and *WIN 3.7* mRNAs in hybrid poplar clones NE-245 and NE-388. Total RNA was extracted from the combined second and fourth fully expanded leaves of 6- to 8-week-old cuttings that had been wounded as described in the text. RNA-blot hybridization analysis was performed using a phosphor imager to quantitate the amount of hybridizing radioactivity. Time points represent the hours after the initial wounding event.
ozone, which induced $PAL$ levels by only 3- to 5-fold above ambient air controls (Fig. 2.6). Induction of $PAL$ transcripts occurred as early as 6 h after wounding, peaked at 30 h, and remained elevated throughout the 4 days of wounding. In clone NE-388, wound-induced levels of $PAL$ mRNA were barely detectable above control levels. $OMT$ transcript accumulation was also induced by wounding to a greater extent in clone NE-245 (Fig. 2.6). The highest expression level of $OMT$ mRNA (3- to 5-fold above controls) was not reached until 54 h after the start of the experiment. Expression of the wound- and MeJA-inducible $WIN3.7$ gene increased in both clones (Fig. 2.6). However, clone NE-245 showed an increase of $WIN3.7$ mRNA up to 15-fold greater than the levels seen in clone NE-388 after wounding. $PR-1$, as expected, was not induced in either NE-388 or NE-245 by wounding (data not shown).

To determine if defense-gene expression via a SA-dependent pathway activated by pathogen infection was also reduced in the ozone-sensitive clone NE-NE-388, the bacterial phytopathogen $P. syringae$ pv $maculicola$ KD4326 was used to induce a nonhost HR and the induction of $PR-1$ mRNA accumulation was monitored. Infiltration of bacteria into leaves produced visible lesions by 12 h in both clones. Lesion formation was strictly limited to the site of infiltration, and the timing of lesion appearance was not significantly different between the clones. $PR-1$ transcripts were induced in both clones but, again, the magnitude of this induction was significantly less in the ozone-sensitive clone NE-388 (Fig. 2.7). At
24 h after infiltration, PR-1 transcript levels were induced only 2-fold over mock-inoculated controls in NE-388 compared with a 10-fold induction in NE-245.
Figure 2.7. Comparison of pathogen-induced PR-1 mRNA accumulation in hybrid poplar clones NE-245 and NE-388. Leaves of 8-week-old cuttings were infiltrated with either *P. syringae* pv *maculicola* KD4326 (Psm) in 10 mM MgCl₂ or mock inoculated with 10 mM MgCl₂ (Mock). Total RNA was extracted from the infiltrated area of the second through the fifth fully expanded leaves, subjected to RNA-blot hybridization analysis, and the amount of hybridizing radioactivity was quantitated using a phosphor imager. Time points represent the hours after infiltration.
DISCUSSION

Ozone is known to induce a variety of stress responses in plants at both the physiological and molecular levels (reviewed in Kangasjärvi et al., 1994; Sandermann Jr., 1996; Iqbal et al., 1996; Heath and Taylor, 1997; Sharma and Davis, 1997). However, few studies have connected ozone-induced physiological responses to the underlying changes in gene expression, particularly in woody tree species. In this chapter, direct comparisons were made of both physiological and molecular responses in two hybrid poplar clones that exhibit differential sensitivity to ozone. These initial studies have shown that differences in both physiological changes and gene expression patterns observed in the two clones correlate with their ozone sensitivity.

The phenomenon of ozone causing a decrease in photosynthetic rate and stomatal conductance has been previously reported in hybrid poplar (Sen Gupta et al., 1991; Pell et al., 1992) and other plant species (Darrall, 1989; Dann and Pell, 1989). Comparisons between the ozone-sensitive and ozone-tolerant hybrid poplar clones also show that ozone reduced stomatal conductance and photosynthesis. However, after two days of exposure to ozone the percent reduction in stomatal conductance was significantly greater in the ozone-tolerant
clone compared to the ozone-sensitive clone. Because the stomata regulate the entry of gaseous pollutants into the plant, they may play an important role in determining plant sensitivity to ozone (Iqbal et al., 1996). Although both clones demonstrated an active avoidance response of stomatal closure during exposure to ozone, the ozone-tolerant clone responded more quickly. Thus, the tolerant clone may have excluded more ozone, resulting in less visible damage to the leaves.

After four days of exposure, photosynthesis was further reduced in both clones. However, stomatal conductance in ozone-treated plants actually increased slightly in clone NE-245 from day 2 to day 4 but remained below levels measured in ambient air treated plants. The percent reduction in stomatal conductance in NE-245 decreased from 80% on day 2 to 8.5% on day 4. This is accounted for not only by the increase in stomatal conductance rate in ozone-treated NE-245 plants, but also by a 53% decrease in stomatal conductance in ambient air treated NE-245 plants. This decrease in stomatal conductance in ambient air treated NE-245 is likely related to the change in biomass within the chambers. Clone NE-245 is an extremely fast growing variety and exhibited considerable growth and significant increases in biomass during the 4 day experimental period (data not shown). The increase in biomass was not accompanied by an increase in the rate of watering and may have lead to a shortage of water to the plant, which could cause a decrease in stomatal conductance (Winner et al, 1988). However, even with the decrease in stomatal
conductance observed in the control NE-245 plants, the ozone-induced reduction was greater.

Differences were also observed in defense-related gene expression between the ozone-tolerant and ozone-sensitive clones. A transient 5-fold induction of \textit{PAL} in the ozone-tolerant clone NE-245 was observed, which reached a maximum level at 3 h after treatment. These results are consistent with those reported for ozone-treated herbaceous species (Sharma and Davis, 1994; Eckey-Kaltenbach et al., 1994) and a deciduous tree (Tuomainen et al., 1996). However, the level of ozone-induced \textit{PAL} transcripts in the ozone-sensitive clone NE-388 was 4-fold less compared with the tolerant clone. During the four days of ozone exposure, the levels of ozone-induced \textit{PAL} transcripts in the ozone-sensitive clone remained significantly below the levels attained in the ozone-tolerant clone. Transcripts for \textit{OMT}, a phenylpropanoid biosynthetic enzyme involved in lignin formation, were induced 5-fold by ozone in clone NE-245. This induction was transient, with maximum expression reached at 12 h after treatment. The difference in \textit{PAL} and \textit{OMT} transcript induction kinetics is likely attributable to the fact that \textit{OMT} is active downstream in the phenylpropanoid biosynthetic pathway from \textit{PAL}. As was observed with \textit{PAL} mRNA, induction of \textit{OMT} mRNA in clone NE-388 was only 2-fold above ambient air controls throughout the four day exposure.

The induction of phenylpropanoid biosynthesis by ozone is well documented; thus, the reported induction of \textit{PAL} and \textit{OMT} transcripts by ozone
in hybrid poplar is not surprising. In addition to the ozone-induction of \textit{PAL} and \textit{OMT} transcripts, ozone treatment has been shown to cause increased isoflavonoid and flavonoid biosynthesis in soybean (Keen and Taylor, 1975). Ozone-induced increases in the activities of phenylpropanoid biosynthetic enzymes in pine have also been reported (Rosemann et al., 1991; Wegener et al., 1997). The observation that the phenylpropanoid pathway is induced in a variety of plants by ozone provides correlative evidence that synthesis of phenylpropanoid derivatives may play a protective role during ozone stress. This protective effect may be related to the ability of plant phenolics and flavonoid derivatives to function as antioxidants due to their ability to trap free-radicals (Lewis, 1993). Thus, the increased ozone sensitivity of clone NE-388 may be attributable in part to the lack of sufficient induction of the phenylpropanoid pathway to provide protective levels of these antioxidant compounds.

The results presented in this chapter concerning the induction of \textit{PAL} mRNA differ from those reported by Tuomainen et al. (1996) in which ozone-induced \textit{PAL} transcript levels were the same in both ozone-sensitive and ozone-insensitive birch clones. That study also compared polyamine levels and enzyme activities of superoxide dismutase, peroxidase, and glutathione reductase and found that they reached higher levels in the ozone-sensitive birch clone compared to the ozone-insensitive birch clone. They concluded that the higher levels of putrescine and AOS scavenging enzymes correlated with the appearance of physical damage. The differences in \textit{PAL} induction in ozone-
sensitive and ozone-tolerant hybrid poplar and birch varieties may be related to
distinct mechanisms of lesion formation in these two experimental systems. The
hypothesis that different mechanisms of ozone sensitivity are important in
different pairs of ozone-sensitive and ozone-tolerant plants is consistent with the
results of Wellburn and Wellburn (1996). In their study, it was found that
changes in levels of polyamines, phenols, reduced glutathione, reduced
ascorbate, and total ascorbate in pairs of tolerant or sensitive selections or
cultivars of six different species were not correlated with either tolerance or
sensitivity. Increased ethylene emission was the only parameter measured that
was consistently observed in the ozone-sensitive plants.

Previous work has demonstrated that ozone activates at least two distinct
signaling pathways in Arabidopsis, one of which overlaps with the HR and SAR
activation pathway and is SA dependent (Sharma et al., 1996). To examine the
potential role of this pathway in hybrid poplar, \textit{PR-1} was used as a marker gene.
In clone NE-245, \textit{PR-1} was induced 24 fold over ambient controls, reaching
maximum levels 12 h after the start of ozone treatment. This result is consistent
with the ozone-induced accumulation of \textit{PR-1} transcripts in Arabidopsis reported
by Sharma et al., (1996). Again, a difference between the ozone-tolerant and
ozone-sensitive clones was observed. Induction of \textit{PR-1} in clone NE-388
reached levels only 2-3 fold over ambient air controls, about 10-fold less than the
levels attained in the tolerant clone. Because activation of a SA-dependent
pathway may provide some protection to ozone (Sharma et al., 1996), the
apparent lack of a significant induction of SA-dependent gene expression may have enhanced the ozone sensitivity of clone NE-388. A significant difference in the induction of \textit{PR-1} mRNA accumulation was also observed in clones NE-245 and NE-388 in response to infection with an avirulent bacterial pathogen; \textit{PR-1} transcript levels were 20-fold higher in the ozone-tolerant clone NE-245 than in NE-388 (Fig. 2.7). These results demonstrate that attenuated \textit{PR-1} expression is not specific to ozone exposure and suggests that clone NE-388 may have generally diminished SA-mediated stress responses.

The overlap in the induction of SA-mediated responses by both ozone and pathogens is likely due to the fact that both stresses result in the production of AOS. Increased production of AOS by ozone upon entering the plant could mimic the "oxidative burst" which in plant-pathogen interactions is thought to trigger the signaling events that activate HR and SAR, resulting in disease resistance (reviewed in Mehdy et al., 1996; Lamb and Dixon, 1997). It has therefore been proposed (Sharma et al., 1996) that the necrotic lesions observed in some ozone-treated plants may be due to the activation of the programmed cell death component of HR (reviewed in Dangl et al., 1996; Greenberg, 1997).

The results presented in this chapter on \textit{PR-1} induction indicate that the ozone-sensitive hybrid poplar clone NE-388 is deficient in the SA-mediated response that would normally lead to the formation of these ozone-induced HR-like lesions. Because the sensitive clone NE-388 develops much larger regions of necrosis and the tolerant clone NE-245 develops smaller HR-like lesions, it
could be argued that these lesions develop via two distinct mechanisms. In the sensitive clone NE-388, the ozone-induced lesions may be caused by the toxic effects of AOS that accumulate due to the lack of any induced defense responses. In the ozone-tolerant clone NE-245, ozone may induce lesions by activating a cell death pathway associated with HR. This interpretation is consistent with previous studies in tobacco, in which the ozone-sensitive cultivar Bel-W3 exhibited higher levels of PR gene expression compared with the more tolerant cultivar Bel-B (Ernst et al., 1992; Schraudner et al., 1992). Similar results were obtained in comparisons of tolerant and sensitive ecotypes of Arabidopsis (Sharma and Davis, 1997; I. Aguilar, Y. Sharma, and K. Davis, unpublished data). In Bel-W3 and the highly ozone-sensitive Arabidopsis ecotype, the apparent increased ozone sensitivity may be attributable to an enhanced HR in response to the ozone-induced production of AOS.

Evidence that a second, SA-independent pathway is also activated by ozone, resulting in the induction of PAL transcripts in A. thaliana, was presented by Sharma et al. (1996). The possibility that JA may be involved in ozone-activated signal transduction pathways was addressed by Orvär et al., (1997) who demonstrated that pretreatment of tobacco by mechanical wounding or with JA decreased the amount of ozone injury in tobacco plants. Induction of the wound-induced WIN3.7 gene by MeJA was confirmed, and this gene was subsequently used as a marker for JA-mediated responses. Ozone induced WIN3.7 transcript accumulation in the ozone-tolerant clone NE-245 by 20-fold
over ambient-air-grown controls. This same transcript was only induced 3-fold in
the ozone-sensitive clone NE-388. The induction of WIN3.7 transcripts cannot
be an indirect response to ozone-induced lesion formation because transcript
accumulation was detected at 3 h after the start of ozone treatment, whereas
lesions in clone NE-245 were not visible until between 12-24 h. Furthermore, if
induction of this gene is a by-product of lesion formation, it would be expected to
correlate with the level of injury development. However, in clone NE-388, which
develops the most severe lesions, WIN 3.7 transcript levels were less than 10%
of the levels reached in clone NE-245.

To determine if the reduced levels of defense gene expression in clone
NE-388 were due only to a deficiency in the ability to respond to ozone stress,
wounding experiments were performed. Wounding induced transcript levels of
PAL, OMT and WIN 3.7 in both the sensitive and tolerant clones. However,
wound induction of these transcripts in clone NE-388 was significantly reduced
when compared with that in clone NE-245. Thus, the ozone-sensitive clone
appears to have greatly attenuated ozone- and wound-induced responses,
indicating that these responses may share at least a portion of the same
signaling pathway. A potential link between ozone- and wound-induced
responses is JA. JA is associated with a variety of physiological responses
(reviewed by Sembdner and Parthier, 1993; Reinbothe et. al, 1994; Creelman
and Mullet, 1997), many of which correlate with ozone responses. For example,
exogenous application of JA has been reported to accelerate leaf senescence,
promote stomatal closure, inhibit photosynthetic activity, inhibit Rubisco biosynthesis and induce defense gene expression.

The studies reported in this chapter show that clone NE-245, upon exposure to ozone, displays inhibition of photosynthetic activity, enhanced stomatal closure and induction of both PAL and a proteinase inhibitor gene. Furthermore, studies by Landry and Pell, (1993) on this same clone indicated that ozone caused accelerated leaf senescence and inhibited both Rubisco activity and photosynthesis. The ozone-sensitive clone NE-388 also exhibits reduced photosynthetic activity and stomatal conductance, confirming results by Eckardt et. al., (1991) and Pell et. al., (1992). However, the data reported by Landry and Pell, (1993), Eckardt et. al., (1991) and Pell et al., (1992) did not include any direct comparisons between these two hybrid poplar clones. The data presented in this chapter includes measurements of photosynthetic rate and stomatal conductance that were performed under identical conditions for both clones so that the responses may be compared. These results indicate that the ozone-induced reduction in stomatal conductance in clone NE-388 plants is significantly (P<0.05) less than the reduction measured in clone NE-245 plants. The ozone-induced reduction in photosynthetic rate did not differ significantly between the clones; this may be due to high variability between individuals combined with a small sample size. In addition, when photosynthetic rate was measured under saturating light, the ozone-induced reduction in photosynthetic rate in clone NE-245 plants was significantly greater than the reduction
measured in clone NE-388 plants (data not shown). This may indicate that the greater reduction in photosynthetic rate observed in clone NE-388 compared to clone NE-245, although not statistically significant, may have biological significance. Furthermore, very little senescence was observed in NE-388 due to ozone treatment (data not shown) and a reduced level of proteinase inhibitor gene expression was measured in clone NE-388 compared to clone NE-245. All of these results are consistent with JA having a role mediating some ozone-induced responses in hybrid poplar.

The apparent attenuated JA-mediated responses observed in clone NE-388 may act as an important component of its increased ozone sensitivity by causing a slower rate of stomatal closure. Thus, in clone NE-388, it is possible that more ozone enters the mesophyll and generates more AOS, resulting in more tissue necrosis. The effects of increased ozone entering the mesophyll may be compounded by the lack of SA-inducible antioxidant defense responses. Alternatively, the enhanced ozone sensitivity of clone NE-388 may not be the direct result of the reduced levels of defense-response induction but, rather, may be caused by the increased stomatal conductance and higher levels of AOS production that exceed the antioxidant capacity of the cells. This could result in sufficient cell damage to prevent the defense responses from being activated. This alternative seems less likely given the observation that the timing of lesion development was very similar in clones NE-388 and NE-245 and occurred well after early defense-gene activation was observed. It would be expected that
more rapid and extensive cell damage would be associated with more rapid necrosis. Moreover, the defense responses of clone NE-388 were attenuated not only in response to ozone, but also in response to wounding and pathogen treatments. Thus, it is more likely that the increased ozone sensitivity of NE-388 is related to the reduced activation of defense signaling pathway(s) that are common for all three stresses, e.g. a reduced ability to generate or respond to stress signals such as ethylene, JA, and SA.

Additional study of the differential ozone sensitivity of the hybrid poplar clones NE-245 and NE-388 should prove important in further characterizing the mechanisms of interaction between ethylene-, SA- and JA-activated defense response pathways during exposure to oxidative stress. The current study allows a comparison of the ozone-induced responses in a woody tree species with responses of herbaceous plants such as tobacco and Arabidopsis. In addition, this system will be a valuable tool in defining novel signaling pathways for defense gene induction in trees and the identification of specific genes that may be useful in increasing stress resistance.
CHAPTER III

OZONE SENSITIVITY IN HYBRID POPLAR CORRELATES WITH INSENSITIVITY TO BOTH SALICYLIC ACID AND JASMONIC ACID: THE ROLE OF PROGRAMMED CELL DEATH IN LESION FORMATION

INTRODUCTION

Plants must efficiently adapt to changing conditions to insure their continued productivity. To adapt to environmental change, plants use several distinct regulatory mechanisms to alter the patterns of gene expression that ultimately cause the biochemical and physiological changes most favorable for survival. The ability to rapidly respond to environmental change is especially important in woody plant species which must be able to adapt through changing seasons over a life span that may range up to hundreds of years. Among the conditions to which trees must adapt, ozone is one of the major anthropogenic stresses contributing to forest decline (Schmeiden and Wild, 1995; Johnson and Taylor, 1989).
Recently, molecular tools have been applied to discern the molecular basis of ozone-induced responses in herbaceous plants (reviewed in Kangasjärvi et al., 1994; Sharma and Davis, 1997; Sandermann et al., 1998). These studies have shown that there is an overlap in the signaling pathways and defense-related genes that are induced by ozone and other stresses such as pathogen infection (Sharma et al., 1996), wounding (Orvar et al., 1997; Koch et al., 1998), UV (Rao et al., 1996), cold, drought, and heavy metal toxicity (Kangasjarvi et al., 1994; Sharma and Davis, 1997; Sandermann et al., 1998) in various plant species, including hybrid poplar (Chapter II; Koch et al., 1998).

Several signal molecules, including salicylic acid (SA), jasmonic acid (JA), ethylene, and active oxygen species (AOS) such as superoxide and hydrogen peroxide, have been implicated in the modulation of plant responses to ozone and other stresses. The connection between these stresses most likely involves AOS in primary signaling events that activate multiple signal transduction pathways. When ozone enters the plant via the stomata, it interacts with cellular constituents and water in the mesophyll, leading to the rapid generation of AOS (Kanosfsky and Sima, 1995). Furthermore, recent results in tobacco (Schraudner et al., 1998) and Arabidopsis thaliana (Sharma et al., 1996; Rao and Davis, 1999) indicate that ozone induces an oxidative burst, generating AOS and activating signal transduction pathways that overlap with those triggered during pathogen infection. This ozone-induced production of AOS is potentiated by SA which is required for induction of antioxidant defense pathways and in some
cases, leads to activation of a programmed cell death (PCD) pathway (Rao and Davis, 1999; Aguilar and Davis, unpublished results).

Although it is clear that SA is an important factor in regulating ozone responses in plants, the available data also support the notion that ozone activates a second, SA-independent pathway that is likely to require JA and/or ethylene (Kangasjarvi et al., 1994; Sharma et al., 1996; Rao and Davis, 1999). The induction of *PAL* and *cytAPX* gene expression by ozone has been shown to be SA-independent, suggesting the involvement of a second signal transduction pathway (Sharma and Davis, 1996; Rao and Davis, 1999). Orvar et al. (1997) demonstrated that mechanical wounding or the direct application of JA prior to ozone exposure resulted in a decrease in the amount of ozone injury in tobacco plants. In the previous chapter, it was determined that in hybrid poplar ozone induced accumulation of transcripts encoding *WIN 3.7*, a gene that is both wound-inducible and JA-inducible (Koch et al., 1998). These results clearly implicate JA as an important signal in ozone-induced responses.

To further understand ozone-induced signal transduction pathways in tree species, the studies described in Chapter II used *WIN 3.7* and *PR-1* as marker genes for JA- and SA-mediated defense response pathways and characterized the ozone-induction of these genes in ozone-sensitive (NE-388) and ozone-tolerant (NE-245) hybrid poplar clones (Koch et al., 1998). The findings showed that when treated with 300 ppb ozone, clone NE-388 developed large regions of necrosis on up to 90% of all leaves and had greatly attenuated levels of both JA-
and SA-dependent defense gene expression compared to NE-245. Lesions development on NE-245 was observed on less than 20% of all leaves and the lesions themselves resembled small, HR-like lesions. The attenuated level of induced gene expression was also observed in clone NE-388 in response to either wounding or infiltration with an avirulent *Pseudomonas syringae* strain, indicating a lack of responsiveness to signaling pathway(s) shared by all three stresses. This non-responsiveness could be attributed to either the inability of NE-388 to produce signal molecules such as SA and/or JA or the inability to perceive these signal molecules.

In this chapter, the attenuated ozone response of clone NE-388 is attributed to insensitivity to both SA and JA. In addition, evidence is reported to demonstrate that the SA/JA-insensitive clone NE-388 undergoes a mechanism of lesion formation that is distinct from the ozone-tolerant clone NE-245. The characterization of this hybrid poplar clone as being insensitive to both SA and JA establishes an experimental system based on a woody plant species that can be used to study signal transduction pathways that regulate defense gene expression. Furthermore, this system may provide a unique opportunity to define novel signal transduction pathways that are largely SA- and JA-independent.
MATERIALS AND METHODS

Growth and Treatment of the Plants

Greenwood cuttings of hybrid poplar (*Populus maximowiczii* X *Populus trichocarpa*) were rooted under mist and transplanted as described earlier (Chapter II; Koch et al., 1998). Six weeks following transplantation, cuttings were transferred to growth chambers modified for ozone fumigation and were acclimated for 2-3 days. Ozone treatments were carried out as previously described (Chapter II; Koch et al., 1998) using an Orec ozone generator (model 03V10-0, Ozone Research and Equipment, Phoenix, AZ).

SA and MeJA Treatments

SA was diluted in distilled water containing 0.1% Triton X-100 as a surfactant to concentrations of 2.5, 5.0, 10.0 and 20 mM. A 100-mM stock solution of MeJA was made by first dissolving in dimethylformamide. Dilutions of 50, 100, 200 and 400 μM were subsequently made in distilled water containing 0.1% Triton X-100. Control plants were treated with either 0.1% Triton X-100 or dimethylformamide + 0.1% Triton X-100. Three plants per concentration per experiment were sprayed.
on both the abaxial and abdaxial surfaces until run-off occurred. Twenty-four h
after treatment, the fourth fully expanded leaf from each plant was harvested,
and the three leaves from each concentration were pooled and frozen in liquid
nitrogen for subsequent RNA extraction. The data shown are from a
representative experiment of three independently replicated experiments.

RNA Extraction and Analysis

Total RNA was extracted as described by Parsons et al. (1989). RNA
was fractionated, blotted and hybridized as previously described (Koch et al.,
1998). \textit{WIN 3.7} (Bradshaw et al., 1989), a kind gift from M. Gordon (University of
Washington, Seattle) was used as a marker gene for JA-mediated gene
expression. A poplar partial cDNA for \textit{PR-1} (Koch et al., 1998) was used as a
marker gene for SA-mediated gene expression. Hybridized filters were exposed
to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA) and the signal
intensity was quantified using Molecular Dynamics ImageQuant software
(Molecular Dynamics). The data shown have been corrected for loading
differences by measuring counts obtained by rehybridizing with a 28S ribosomal
gene from pea (Wanner and Gruissem, 1991). All experiments were performed
at least twice with at least two replicates per test condition. The data shown are
from representative experiments.
JA Measurements

Due to the large amounts of tissue required to measure JA, careful consideration was given to experimental design. Based on previous observations that mid-aged leaves (fully expanded leaves 2-10) in both clones respond similarly to ozone with respect to defense gene induction, reduction of stomatal conductance and photosynthetic rate, and the timing of lesion formation (Koch et al., 1998 and unpublished results), fully expanded leaves 2-8 were collected and pooled from individual trees for JA analysis. Tissue was collected from plants treated with either ambient air or 300 ppb ozone, major veins were removed, and tissue was frozen in liquid nitrogen and ground to a homogeneous mixture. JA analysis was performed by suspending frozen aliquots of 4 g of pulverized leaf tissue in 60:40 (vol:vol) acetone/methanol, and homogenizing using a polytron. Leaf tissue was removed by filtration, 2-5 ml of H₂O was added, and a known amount of [¹³C]-labeled JA was added as an internal standard. Acetone and methanol were removed by rotary evaporation, 50 ml of 0.1 M sodium phosphate and 5% NaCl (pH 8.5) was added, and the sample was extracted twice with dichloromethane. The aqueous phase was then acidified to pH 2.0 by the addition of 6N HCl, and extracted once with hexane and once with dichloromethane. JA was partitioned under acidic conditions into dichloromethane, which was collected and evaporated to near dryness. Following the addition of a small volume of 5 mM HCl, the remaining dichloromethane was evaporated. 1-2 ml of 5 mM HCl was added, and the
sample was sonicated, filtered, and loaded onto a C18 Sep-Pak cartridge (Waters Corp., Milford, MA) previously equilibrated with 5 mM HCl. The cartridge was rinsed with 5 mM HCl followed by H2O, and centrifuged to remove residual H2O. The sample was eluted from the cartridge using methanol then subsequently dried, methylated using etherial diazomethane, and analyzed by GC/MS/selected ion monitoring according to Creelman and Mullet (1995). Data shown are representative of a single experiment in which three individual trees were assayed per time point. The entire experiment was duplicated with similar results. Quantitation of JA was performed by Dr. Robert Creelman (Dept. of Biochemistry and Biophysics Crop Biotechnology Center, Texas A & M University).

SA Measurements

Tissue was collected for SA analysis as described above for JA quantification. SA was extracted as described previously (Enyedi et al., 1992) with the following modifications. Leaves were ground in liquid nitrogen with a mortar and pestle and 3-hydroxybenzoic acid in methanol was added at a level of fifty micrograms per gram fresh weight as a recovery measurement spike. Total SA (free plus glucosyl SA) was determined from methanol extracts digested with β-glucosidase (Sigma, St. Louis, MO) as described by Seskar et al. (1998). Dried extraction samples were resuspended in an HPLC mobile phase of 75% 40 mM sodium acetate (pH 3.5)/25% methanol, and filtered through Costar Spin-X
0.2 μm nylon filters (Corning Incorporated, Corning, NY). Fifty microliter samples were injected onto a Waters Nova-Pak C₁₈ 60Å 4 μm Guard-Pak insert column linked to a Waters Nova-Pak C₁₈ 60Å 4 μm column (3.9 x 300 mm) maintained at 40°C. A linear segment gradient of methanol to 40 mM sodium acetate, pH 3.5, was applied at a constant flow rate of 1 ml/min as follows: 25% to 45% methanol over 12 min, 45% to 100% methanol over 6 min, and 100% to 25% methanol in 5 min to re-equilibrate the column. A Waters 490 absorbance detector was used for 3-hydroxybenzoic acid quantitation at 236 nm. In tandem, a Waters 474 scanning fluorescence detector was used for SA quantitation with the gain set to 10, excitation wavelength at 295 nm, and emission wavelength at 405 nm; SA (eluting at 7.4 min) and 3-hydroxybenzoic acid (eluting at 6.7 min) were resolved baseline-to-baseline as monitored by absorbance at 236 nm and identified using authentic standards. Quantitation of SA was performed either at the Northeastern Research Station, USDA Forest Service, Delaware, OH, by Dr. Steve M. Eshita or by Dr. Mira Seskar at the Biotechnology Center for Agriculture and the Environment, Rutgers University.

Data Analyses

Statview software (Abacus Concepts, Inc., 1995) was used for all statistical analyses, and the data presented include means ± SE of at least two independent experiments. All data were subjected to ANOVA, with the exception
of the percent injury after MeJA treatment data, which was subjected to the
nonparametric Kolmogorov-Smirnov test.

**TUNEL Analysis**

Tissue samples were taken from the second through fifth fully expanded
leaves at 1, 3, and 6 h after the start of ozone treatment. Tissue was vacuum
infiltrated for 5 minutes with 4% paraformaldehyde in PBS, then incubated
overnight at 4°C prior to sectioning. TUNEL analysis was performed on frozen
sections using the *In Situ* Cell Death Detection Kit, Fluoroscein (Boehringer
Mannheim, Indianapolis, IN) and propidium iodide (R & D Systems, Minneapolis,
Minnesota) was used as a counterstain. Sections were viewed using a Zeiss
LSM (Thornwood, NY) confocal microscope. Fluoroscein incorporation was
visualized using FITC filter sets and nuclear staining was visualized using PI filter
sets. Two independent experiments were performed and for each sampling time
at least 5 slides containing at least 5 separate sections were screened per clone.
RESULTS

NE-388 is Insensitive to Exogenous SA and MeJA

Previous results demonstrating that ozone-, pathogen-, and wound-induction of both SA- and JA-regulated gene expression is attenuated in clone NE-388 indicate that a deficiency may exist in a signal transduction pathway(s) that is activated by all three stresses. This deficiency could be due to the inability to synthesize SA and JA or by an inability to perceive these signal molecules. To distinguish between these two possibilities, experiments were performed using exogenously applied MeJA and SA (Fig. 3.1, Fig. 3.2). Application of MeJA caused induction of *WIN* 3.7 transcripts in NE-245 (Fig. 3.1.A). At 100 μM MeJA, a modest induction of *WIN3.7* transcripts was detected and at 200 μM MeJA a 10-fold increase in *WIN* 3.7 transcripts was observed. No visible lesions formed, even at concentrations as high as 400 μM MeJA. In contrast, *WIN* 3.7 transcripts were barely detectable in NE-388, even at 400 μM MeJA. Again there were no visible signs of lesion formation.

It has been reported previously that in tobacco, pretreatment with wounding or JA resulted in a reduction of ozone-induced lesion formation (Orvär et al., 1997). In order to further explore the role of JA in ozone tolerance and to confirm
Figure 3.1. Effects of pretreatment with exogenous MeJA on ozone-induced leaf damage and defense gene induction. A) Individual leaves were sprayed with different concentrations of JA in 0.1% Triton X-100 until run-off occurred. The fourth fully expanded leaf from three trees per concentration was harvested 24 h after treatment and pooled for RNA isolation and subsequent Northern blot analysis using WIN3.7 as a probe. B) Plants were treated either 3 h or 24 h prior to ozone fumigation with a solution of 200 μM MeJA in 0.1% Triton X-100 and 24 h after the start of the ozone, plants were assessed for the percent leaf injury. The data for each test condition represent the mean ± SE of 2 to 6 individual plants. Data shown is representative of one of three independently performed experiments, each producing similar results.
that NE-388 is nonresponsive to JA, experiments in which plants were treated
with MeJA prior to ozone exposure were performed. A concentration of 200 μM
MeJA was chosen for use in these experiments because it produced the greatest
extent of WIN 3.7 induction 24 h after application (Fig. 3.1.B). MeJA
pretreatments were performed both 3 and 24 h prior to ozone exposure. A
statistically significant reduction in percent leaf injury (from 19% to 6%, P = 0.01)
was observed in the tolerant clone NE-245 when treated with 200 μM MeJA both
3 h and 24 h prior to ozone treatment (Fig. 3.1.B). A slight increase in percent
leaf injury was recorded for MeJA pretreated NE-388 plants, however, this
increase was not statistically significant (P = 0.20). These results further confirm
that NE-388 does not to respond to JA.

Experiments with exogenously applied SA revealed that NE-388 is also
not able to respond to SA. A 3-fold induction of PR-1 transcripts was observed
at 24 h in the ozone-tolerant clone NE-245 when the plants were treated with 2.5
mM SA (Fig. 3.2). Maximum levels of PR-1 transcript accumulation were
observed at 5 mM SA, while higher concentrations did not cause significant PR-1
induction. The lack of induction at the higher concentrations correlated with the
appearance of SA-induced lesions. Conversely, in the ozone-sensitive clone NE-
388 there was no detectable induction of PR-1 transcripts by treatment with SA
concentrations as high as 20 mM. Interestingly, as was observed with NE-245,
SA-induced lesion formation in NE-388 also occurred at 10 and 20 mM SA.
Figure 3.2. Defense gene induction by SA. Individual leaves were sprayed with different concentrations of SA in 0.1% Triton X-100 until run-off occurred. The fourth fully expanded leaf from three trees per concentration were harvested 24 h after treatment and pooled for RNA isolation and subsequent Northern blot analysis using PR-1 as a probe. Data shown is representative of one of three independently performed experiments. Similar results were obtained in each experiment.
Measurement of Endogenous Levels of SA and JA

The inability to respond to exogenously applied SA and MeJA indicates that the lack of normal defense gene expression in NE-388 is not due to the inability to synthesize these molecules but in the perception of them. To determine if both clones accumulate SA and JA in response to ozone exposure, the levels of these two molecules were measured in both clones that had been kept in ambient air or exposed to 300 ppb ozone. Ozone exposure of both NE-388 and NE-245 increased JA levels by 3.2-fold and 2.7-fold, respectively, compared to control plants (Fig. 3.3). Although an increase in JA was detected within 3 h of ozone exposure, the induction of JA in ozone-treated plants compared to control plants was not statistically significant until 6 h.

Endogenous levels of SA were also determined and both clones were found to have high constitutive levels of free SA compared to herbaceous plant species such as tobacco and *A. thaliana*. The basal levels of free and total SA were 2.4-fold and 1.5-fold higher, respectively, in NE-388 compared to NE-245 (Fig. 3.4). Time course experiments revealed that at 6 h after the start of ozone treatment, a modest increase in free SA levels of 36% for NE-388 and 31% for NE-245 was detected (Fig. 3.4A), but these increases were not found to be statistically significant (P = 0.09 for NE-388 and P = 0.22 for NE-245). Levels of free SA were measured in three independent experiments and in each experiment modest increases were observed at 6 h, ranging from 8% to 56% in clone NE-388 and from 16% to 68% in clone NE-245. In only one experiment...
Figure 3.3. **Ozone-induced JA accumulation.** Data shown represent the mean ± SE of three individual trees from representative experiment. Similar results were obtained in an independently performed duplicate experiment.
Figure 3.4. Accumulation of SA in ozone-treated plants 6 h after onset of treatment. A) Free SA accumulation. Data shown represent the mean ± SE of 6 to 8 trees from three independent experiments. The average values for free SA levels in untreated plants for the three experiments were 3.12 ± 0.33 for NE-388 and 1.31 ± 0.18 for NE-245. ANOVA resulted in the following P values: P ≤ 0.0001 for interaction due to clone, P = 0.2177 for interaction due to treatment in clone NE-245 and P = 0.0891 for interaction due to treatment in clone NE-388.

B) Total SA accumulation. Data represent the mean ± SE for both the free and conjugated SA measured in two independent experiments. The average values for total SA in untreated plants in the two experiments were 145 ± 17 for NE-388 and 99 ± 10 for NE-245. ANOVA resulted in the following P values: P = 0.0002 for interaction due to clone, P = 0.1523 for interaction due to treatment in clone NE-245, and P = 0.1163 for interaction due to treatment in clone NE-388.
was this increase found to be statistically significant for both clones. Total SA was also measured and no statistically significant increase due to ozone exposure was observed at 1, 3, 6 or 12 h (Fig. 3.4.B). Although no significant induction of either free or total SA was found in ozone-treated trees, SA was determined to be present in both clones and in fact, the basal levels of both free (P = < 0.0001) and total (P = 0.0002) SA were significantly higher in NE-388 (1.5- to 2.7-fold). Thus, the lack of response of NE-388 to ozone treatment is not related to a lack of SA production.

TUNEL Analysis of Ozone and Pathogen-Treated Plants

Previous experiments, both in hybrid poplar (Koch et al., 1998) and *A. thaliana* (Rao and Davis, 1999; Aguilar and Davis unpublished results), suggest that ozone-induced lesion formation can occur by two different mechanisms; the activation of PCD or by necrosis. To test this further, DNA fragmentation, an indicator of PCD, was examined using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis. PCD is characteristic of the SA-dependent activation of a hypersensitive response (HR) by pathogens or ozone (Dangl et al., 1996; Greenberg, 1997; Sharma and Davis, 1997). DNA fragmentation was detected in ozone-treated leaf tissue from the tolerant clone NE-245 at both 3 h (Fig. 3.5.C) and 6 h (data not shown) after treatment, as indicated by the incorporation of fluorescein-labeled dUTP. Sections were counterstained with propidium iodide to allow nuclei to be visualized (Fig. 3.5.D).
Figure 3.5. TUNEL assay to detect DNA fragmentation in clone NE-245. Panel C shows incorporation of fluorescein-labeled dUTP in the ozone-treated NE-245 tissue, visualized as bright green spots within the cells. Panel D is the same section as seen in panel C except propidium iodide was used to stain all nuclei present, which appear as bright red spots. When panels C and D are compared, it is clear that most, but not all, of the nuclei in this particular section have undergone DNA fragmentation. Panel E shows fluorescein incorporation in pathogen treated tissue and panel F is the propidium iodide staining of panel E. No evidence of fluorescein incorporation was seen in untreated tissue (panel A), even though numerous nuclei were visible in the propidium iodide stained control tissue (panel B). The white line at the bottom of panel F represents 50 μm.
By comparing the pattern of fluoroscein incorporation (Fig. 3.5.C) with the pattern of propidium iodide stained nuclei, it is clear that the fluoroscein is localized to a subset of nuclei. There is no evidence of fluoroscein incorporation (Fig. 3.5.A) in untreated tissue samples even though numerous nuclei are visible in the propidium iodide stained sample (Fig. 3.5.B). No evidence for DNA fragmentation was found in ozone-treated tissue of clone NE-388 even though numerous propidium iodide-staining nuclei are visible (Fig. 3.6). Sections were taken from both clones from the 2nd and 4th fully expanded leaves from regions near the petiole, which typically develop lesions first.

Given the clear differences in lesion formation in clones NE-388 and NE-245 in response to ozone, we investigated whether pathogen-induced lesion formation was also different in these two clones. Pathogen infiltration resulted in the appearance of DNA fragmentation in clone NE-245 (Fig. 3.5.E), but no DNA fragmentation was detectable in NE-388 (Fig. 3.6.E). Similar to ozone-treated tissue, DNA fragmentation was detected at both 3 h (Fig. 3.5.E) and 6 h (data not shown) after infiltration in the tolerant clone and occurred prior to visible lesion formation.
Figure 3.6. TUNEL assay to detect DNA fragmentation in clone NE-388. Panel C is a section of ozone-treated tissue from clone NE-388 that has been treated with terminal transferase. No bright green nuclei were visible, although numerous stained nuclei were clearly evident after staining with propidium iodide (panel D). Similar results were obtained with pathogen treated tissue (panel E and F) and untreated tissue (panel A and B). The white line at the bottom of panel F represents 50 μm.
DISCUSSION

The attenuated response of both SA- and JA- inducible defense gene expression in clone NE-388 to ozone, phytopathogen infection, and wounding indicates a deficiency in a component of a signaling pathway that is common for all three stresses (Koch et al., 1998). The results presented in this chapter demonstrate that exogenous application of SA and MeJA fails to cause induction of PR-1 and WIN 3.7, respectively, in the ozone-sensitive clone NE-388 even at levels that far exceed endogenous levels. This indicates that NE-388 is deficient in perceiving SA and JA. This conclusion is supported by quantitative analyses demonstrating that clone NE-388 synthesizes at least as much SA and JA as the ozone-tolerant clone NE-245. The levels of JA were not significantly different between the two clones while the levels of SA were significantly higher in NE-388 compared to NE-245. Therefore, because NE-388 produces just as much JA as NE-245 and even more SA than NE-245, the attenuated response of the ozone-sensitive clone NE-388 cannot be attributed to a deficiency in the biosynthesis or increased metabolism of these signal molecules. Taken together, these data strongly suggest that the ozone-sensitive clone NE-388 lacks the ability to efficiently perceive both SA and JA.
When levels of JA were measured in hybrid poplar plants that had been treated with ozone, approximately a 2.5-fold increase at 6 h after the start of treatment was found in both the sensitive and tolerant clones. This is the first report of ozone induction of endogenous levels of JA and provides direct evidence, in conjunction with previous work in hybrid poplar (Chapter II; Koch et al., 1998) and work in tobacco (Orvár et al., 1997), that JA is an important ozone-induced signal molecule. The finding that treatment with MeJA prior to ozone exposure results in a reduction in visible leaf injury in the tolerant clone NE-245 supports similar findings in tobacco (Orvár et al., 1997) and Arabidopsis (Rao and Davis, unpublished results). However, JA pretreatment does not reduce levels of leaf injury in the JA-insensitive clone NE-388. These data provide additional evidence that JA is an important signal molecule involved in modulating ozone responses in a tree system as well.

SA, also known to be an important signal in ozone-induced responses, has been shown to increase 4- to 5-fold after 3 to 6 h of ozone exposure in Arabidopsis (Sharma et al., 1996) with a concomitant increase in conjugated forms of SA over a 24 h period. Results presented in this chapter using ozone-treated hybrid poplar clearly differ from these findings. Only a slight increase in free SA levels (31%-36%) which was not statistically significant was measured, and no increase at all was observed in total SA levels for both clones. However, both free and total SA levels were approximately 2 fold higher in NE-388. This may be due to natural variation between these two clones or may indicate that
NE-388 has aberrant SA metabolism. The average free SA level found for NE-245 was 1.3 ± 0.2 μg/gFW and 3.1 ± 0.3 μg/gFW for NE-388, compared to <50 ng/gFW in tobacco and Arabidopsis (Malamy et al., 1990, Enyedi et al., 1992, Vernooij et al., 1994; Sharma et al., 1996). When comparing basal levels of SA between herbaceous plant species, a great degree of variability also exists. For example, in tobacco a 1.2- to 4-fold increase in the low basal levels of SA is associated with the induction of PR gene expression (Malamy et al., 1990; Ohshima et al., 1990). Yet, the basal levels of tomato, potato, and soybean have been reported to exceed even the elevated levels measured in tobacco that are associated with systemic acquired resistance (SAR, Raskin et al., 1990), as do the levels reported here in hybrid poplar. The levels of both free and total SA in both of the hybrid poplar clones were found to be constitutively higher than the levels measured in tobacco and Arabidopsis that are associated with SAR.

Further variability exists when comparing the role of both SA and JA between different herbaceous plants. For example, unlike tobacco and Arabidopsis, rice shows no change in SA levels in response to pathogen infection (Chen et al., 1997) and in potato, only local increases in SA are observed (Coquoz et al., 1995). However, in both rice and potato there appears to be a correlation between levels of endogenous SA and pathogen resistance in different cultivars (Coquoz et al., 1995; Silverman et al., 1995). In addition, rice shows no increase or changes in JA levels in response to pathogen yet JA-responsive genes are induced (Schweizer et al., 1997).
The requirement for SA to mediate a successful HR and SAR in tobacco and Arabidopsis was established by using transgenic plants expressing salicylate hydroxylase (NahG) which converts SA into biologically inactive catechol (Gaffney et al., 1993; Delaney et al., 1994). In marked contrast, transgenic NahG potato plants with reduced levels of SA did not exhibit increased disease severity when inoculated with the fungal pathogen *Phytophthora infestans*. However, SA is still believed to play an important role in potato because arachidonic acid, the naturally occurring elicitor produced by *P. infestans* that normally elicits SAR, was unable to do so in NahG plants (Yu et al., 1997).

The mechanism of SA signaling in hybrid poplar may be more similar to rice and potato than to tobacco and *A. thaliana*. In tobacco and *A. thaliana*, levels of SA are increased in response to stress until a certain threshold is reached. The elevated SA level, in conjunction with the ability to perceive the SA, subsequently induces changes in gene expression. Similar to rice and potato, SA levels in hybrid poplar are constitutively high. Therefore, it may be that elevation of SA levels is not required to induce a response, but rather, exposure to stress may simply induce an increased ability to perceive the already high levels of SA. In these cases, the ability to perceive SA may be the stress-induced trigger that leads to changes in gene expression. Alternatively, SA may act more indirectly by interacting with other signal molecules induced by environmental stress.
Even though the mechanism of SA in defense response signaling in hybrid poplar is somewhat more comparable to potato and rice than to tobacco and *A. thaliana*, differences do exist between these plant systems as well. When the tolerant clone is treated with SA, induction of *PR-1* gene expression can be observed, yet in potato and rice there is no induction or only local induction of *PR-1* (Coqouz et al., 1995; Silverman et al., 1995). Furthermore, in rice, application of JA induced *PR-1* protein accumulation (Schweizer et al., 1997) whereas *PR-1* transcript accumulation was not detected in hybrid poplar following JA treatment (data not shown). Therefore, although the constitutively high levels of SA in hybrid poplar are more like what is found in potato and rice, actual induction of *PR-1* occurs in a SA-dependent manner similar to *PR-1* induction in tobacco and *A. thaliana*. Even though hybrid poplar shares certain characteristics of SA and JA signaling with some herbaceous plant systems, it does not clearly fit all of the patterns established in any of the well-studied herbaceous plant systems.

Results presented in this chapter distinctly show that DNA fragmentation, an indicator of SA-dependent PCD, occurs in response to ozone in the ozone-tolerant, SA-responsive hybrid poplar clone (NE-245) but not in the ozone-sensitive SA-insensitive clone (NE-388). Identical results were obtained using tissue infiltrated with an avirulent strain of *P. syringae*. In regions of the leaf that were known to have been infiltrated with bacteria, NE-388 did not display DNA fragmentation yet it was readily detectable in NE-245. These data provide
evidence that SA perception is required for the activation of a hypersensitive cell
death pathway and that ozone can in fact induce lesion formation via the
activation of PCD. In addition, the inability of NE-388 to undergo PCD in
response to ozone or pathogen infection, while DNA fragmentation is readily
detectable in NE-245, indicates that the mechanism for lesion formation in NE-
245 in response to either stress not only requires SA, but also is essentially the
same. Thus, ozone-induced lesion formation occurs via two distinct mechanisms
in the two hybrid poplar clones. Cell death in the tolerant clone is caused by the
induction of a PCD pathway associated with a HR, while cell death in the
sensitive clone is likely to be necrosis caused by the lack of induction of
antioxidant defenses to a level that is sufficient to prevent the formation of toxic
ozone-induced AOS intermediates (Koch et al., 1998).

The hypothesis that two distinct mechanisms of lesion formation occur in
hybrid poplar in response to ozone is supported by recent work in Arabidopsis.
Rao and Davis (1999) determined that transgenic NahG plants undergo ozone-
induced toxic cell death due to an inability to maintain the cellular redox state.
This mechanism of lesion formation is distinct from Cvi, an ecotype that
hyperaccumulates SA and has been shown to undergo ozone-induced nuclear
DNA fragmentation (Aguilar and Davis unpublished results). Furthermore,
pretreatment of Cvi with MeJA results in a reduction of ozone-induced leaf injury
(Rao and Davis, unpublished results) similar to what was observed for the ozone-
tolerant hybrid poplar clone. These results indicate that JA may modulate SA-
mediated responses, including PCD. Although both Cvi and NE-245 undergo PCD, the higher induced levels of SA in Cvi compared to more tolerant ecotypes appear to stimulate a runaway hypersensitive response that leads to high levels of lesion formation. Taken together, these results suggest that ozone-sensitivity is determined by a delicate balance of several distinct, interacting signaling pathways. For ozone tolerance, optimal concentrations (and perception) of SA and JA are required to achieve maximal induction of defense responses with minimal induction of the programmed cell death pathway.

Although SA appears to function similarly in ozone induction of a PCD pathway leading to lesion formation in both hybrid poplar clone NE-245 and A. thaliana ecotype Cvi, these plants appear to differ in how the SA signal is transmitted. In Cvi, ozone induces SA levels at least 10-fold, while no ozone-induced elevation in SA levels was detected in the hybrid poplar ozone-tolerant clone even though both undergo SA-dependent DNA fragmentation in response to ozone. These results demonstrate that direct extrapolation from model herbaceous plants to trees is not always possible; in fact if reliable data are to be obtained on signal transduction pathways in a woody plant species, a woody plant system must be used.

In conclusion, the data presented in this chapter clearly shows that not only is the ozone-sensitive hybrid poplar clone (NE-388) insensitive to both SA and JA, but that it also undergoes a mechanism of lesion formation via necrosis that is distinct from the SA-mediated activation of PCD that precedes lesion
formation in the ozone-tolerant clone (NE-245). The SA-mediated activation of
PCD appears to be inhibited by JA, and supports the growing body of literature
indicating significant interaction between these two pathways (Sano et al., 1996;
Seo et al., 1997; Romeis et al., 1999, Shah et al., 1999). This naturally occurring
variant will be useful in further studies aimed at dissecting these interacting
signal transduction pathways involved in defense responses in hybrid poplar and
may prove extremely useful in identifying novel SA/JA-independent signaling
pathways.
CHAPTER IV

INDUCTION OF ETHYLENE AND PUTRESCINE BY OZONE: COMPARISON OF OZONE-SENSITIVE AND OZONE-TOLERANT HYBRID POPLAR CLONES

INTRODUCTION

The phytotoxic effects of ozone have been generally attributed to the ability of ozone to generate lethal active oxygen species (AOS) through reactions with cellular constituents and water in the leaf mesophyll. Significant progress has been made in recent years in understanding the molecular basis for the response of plants to ozone (Kangasjärvi et al., 1994; Schraudner et al., 1996; Pell et al., 1997; Rao et al., 1999). It has now been demonstrated in both tobacco (Schraudner et al., 1998) and Arabidopsis (Rao and Davis, 1999) that ozone induces an oxidative burst, resulting in the generation of AOS in addition to those resulting from the chemical decomposition of ozone. The ozone-induced oxidative burst subsequently activates signal transduction pathways overlapping with those induced by a variety of stresses including pathogen...
infection (Sharma and Davis, 1994; Koch et al., 1998), wounding (Orvär et al., 1997; Koch et al., 1998), UV (Rao et al., 1996), cold, drought, and heavy metal toxicity (Kangasjärvi et al., 1994; Sharma & Davis, 1997; Sandermann Jr. et al., 1998).

In addition to the role of AOS, several other signal molecules including SA, JA, and ethylene (reviewed in Rao et al., 1999) are known to be involved in modulating plant responses to ozone and other stresses. In the previous two chapters, data were presented that correlates ozone-sensivity to an insensitivity to SA and JA in hybrid poplar. As described in the previous chapter and by Rao and Davis (1999), in both hybrid poplar and Arabidopsis, there are two distinct mechanisms of ozone-induced lesion formation. One mechanism involves the activation of a SA-dependent PCD pathway. In the ozone-tolerant hybrid poplar clone (NE-245), activation of this pathway leads to the induction of defense response genes as well as DNA fragmentation which results in the formation of a relatively small number of HR-like lesions (Koch et al., 1998; Chapter III). The ozone-sensitive Arabidopsis ecotype Cvi-O, hyperaccumulates SA in response to ozone resulting in the production of excess AOS via a SA-mediated amplification loop (Rao and Davis, 1999). Consequently, the PCD pathway is also hyperactivated leading to the rapid appearance of extensive areas of lesion formation. The second mechanism of lesion formation occurs independently of SA and appears to be due to toxicity resulting from the accumulation of reactive oxygen species. Lesion formation via this mechanism is exacerbated by the
inability to adequately scavenge these reactive molecules due to a lack of induction of SA-dependent antioxidant defenses. This mechanism of lesion formation is illustrated by Arabidopsis plants carrying the NahG transgene which prevents any significant accumulation of SA (Rao and Davis, 1999), and by the ozone-sensitive hybrid poplar clone NE-388 which has been shown to be insensitive to both SA and JA (Chapter III).

In addition to the role of SA in ozone-induced lesion formation, recent studies have made it clear that JA is of considerable importance as well. Studies on tobacco, Arabidopsis, and hybrid poplar have shown that pretreatment with JA or MeJA reduces the extent of injury due to ozone in some cases (Orvâr et al., 1997; Rao et al., 1999; Chapter III). More detailed analyses in Arabidopsis have indicated that JA may play a role antagonistic to that of SA. Specifically, JA-perception appears to be essential in controlling the ozone-induced oxidative burst by regulating the SA-mediated production of AOS and subsequently preventing hyperactivation of the PCD pathway (Rao and Davis, 1999; Rao et al., 1999).

Current reports from several laboratories have shown that ethylene acts in conjunction with both SA and JA to activate defense response pathways. For example, the JA-induction of pin gene expression was shown to require ethylene (O'Donnell et al., 1996) and a defensin gene was found to be induced in a SA-independent, JA- and ethylene-dependent manner (Penninckx et al., 1996; Penninckx et al., 1998). Although not extensively documented, the finding that
the basal levels of PR-1 are higher in ethylene insensitive mutants is suggestive of interaction between SA- and ethylene- mediated pathways (Lawton et al., 1994; Dong, 1998; Rao, Koch & Davis unpublished results).

Ethylene has also been shown to play a role in mediating pathogen-induced damage in Arabidopsis by modulating the spread of cell death (Bent et al., 1992; Greenberg & Ausubel, 1993) and recent evidence indicates a potential role for ethylene in influencing ozone-induced cell death as well. For example, a strong correlation exists between ozone sensitivity in a variety of plant species and ozone-induced stress ethylene production (Wellburn & Wellburn, 1996). Inhibition of ethylene biosynthesis resulted in a reduction of ozone-induced damage in tomato (Bae et al., 1996; Tuomainen et al., 1997). Furthermore, ethylene is known to induce senescence and premature senescence is a well-documented response to ozone (Pell et al., 1997). The process of senescence, similar to plant responses to ozone and pathogen, involves genetically programmed cell death. Taken together, these data provide strong evidence that ethylene may play a significant role in ozone-induced lesion formation.

Polyamines are small ubiquitous molecules thought to be intimately involved in growth processes and in particular to retard senescence, (Altman, 1989) in marked contrast to the role of ethylene in promoting senescence. Polyamines, like ethylene, have been shown to increase in many plants in response to ozone (reviewed by Bouchereau et al., 1999) and in some plant species there is a correlation between ozone-tolerance and polyamine
production. (Langebartels et al., 1991; Schraudner et al., 1996). Furthermore, it has been demonstrated that feeding polyamines to both tomato and tobacco plants results in reduced levels of ozone-induced visible injury (Ormrod and Beckerson, 1986; Bors et al., 1989). The protectant effect of polyamines has been proposed to stem from not only their anti-senescence characteristics but also directly through the ability of free polyamines to inhibit lipid peroxidation (Schraudner et al., 1996; Borrell et al., 1997) and of conjugated polyamines to act as free radical scavengers (Bors et al., 1989). Alternatively, the ability of polyamines to interact with polyanions such as those in the phosphate residues of cell membranes has lead to the hypothesis that they may function to stabilize the membrane from the damaging AOS (Smith 1990). Interestingly, the biosynthetic pathways for both ethylene and polyamines share a common precursor, S-adenosylmethionine. These pathways appear to have negative regulatory interactions with each other, although this may depend on the available concentration of SAM (Smith, 1990). Specifically, polyamines have been shown to inhibit the ethylene biosynthetic enzymes ACC synthase and ACC oxidase while ethylene is an effective inhibitor of arginine decarboxylase and AdoMet decarboxylase, key enzymes in polyamine biosynthesis (Dumbroff, 1990).

Clearly, the response of plants to ozone involves a complicated myriad of complex signaling pathways involving SA, JA and ethylene. In the previous chapter it was determined that the ozone-sensitive hybrid poplar clone (NE-388)
is insensitive to both SA and JA and does not undergo PCD in response to ozone or pathogen. To determine if potential deficiencies in ethylene contribute to the attenuated levels of defense response gene induction in NE-388, ozone-induced ethylene levels were compared in both clones. Although there is evidence for the influence of SA- and/or JA on regulation of transcripts for ethylene biosynthesis, no direct effect on ethylene emission was observed. Therefore, the SA/JA-insensitivity of NE-388 that leads to attenuated levels of defense gene expression is not due to a deficiency in ethylene production. Furthermore, because of the proposed role of polyamines in ozone tolerance and the proposed interactions between polyamine and ethylene biosynthesis, comparisons of ozone-induced levels of these molecules in both the sensitive and tolerant clones are reported. These direct comparisons indicate that in hybrid poplar polyamine accumulation correlates with the extent of injury and does not appear to play a role in determining ozone tolerance. Finally, the data presented in this chapter shows that in ozone-treated hybrid poplar, the biosynthetic pathways for polyamine and ethylene production are not antagonistic.
METHODS AND MATERIALS

Growth and Treatment of the Plants

Greenwood cuttings of hybrid poplar (*Populus maximowizii* X *Populus trichocarpa*) were rooted under mist and transplanted as described earlier (Koch et al., 1998). Six weeks following transplantation, cuttings were transferred to growth chambers modified for ozone fumigation and were acclimated for 2-3 days. Ozone treatments were carried out as previously described (Koch et al., 1998) using an Orec ozone generator (model 03V10-0, Ozone Research and Equipment, Phoenix, AZ).

Tissue Sampling

Tissue was collected from three individual trees at each timepoint. To obtain adequate quantities of tissue to assay ethylene, polyamine and transcript levels from individual trees, multiple leaves per tree were harvested. Based on previous observations that mid-aged leaves (fully expanded leaves 2-10) in both clones respond similarly to ozone on the basis of defense gene induction, reduction of stomatal conductance and photosynthetic rate, as well as lesion formation (Koch et al., 1998 and unpublished results), leaves number 2-8 were
used. Leaf 2 was immediately frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\) until used for RNA extraction. Leaves 3, 5, 6, and 7 were used to analyze ethylene emission and leaves 2 and 8 were used for polyamine analysis.

**Analysis of Transcript Levels for Ethylene Biosynthesis**

Total RNA was extracted as described by Parsons et al. (1989). RNA was fractionated, blotted and hybridized as previously described (Koch et al., 1998). *PE-ACS1* and *PE-ACS2*, poplar cDNAs for ACC synthase were used as probes as well as *PE-AC01*, a poplar cDNA for ACC oxidase. The cDNAs for these genes were kind gifts from Nobuyoshi Nakajima, Hikaru Saji (National Institute for Environmental Studies, Japan), and Izumi Yasutani (Toyota Co., Japan). *PE-ACS1*, *PE-ACS2*, and *PE-AC01* were all previously identified as being ozone-induced. High stringency hybridization conditions (Chapter II; Koch et al., 1998) coupled with a 68.2% homology at the amino acid level between *PE-ACS1* and *PE-ACS2* minimized any cross-hybridization. Hybridized filters were exposed to PhosphorImager screens (Molecular Dynamics, Sunnyvale, CA) and the signal intensity was quantified using Molecular Dynamics ImageQuant software (Molecular Dynamics). The data shown have been corrected for loading differences by quantitating counts obtained by rehybridizing with a 28S ribosomal gene from pea (Wanner & Gruissem, 1991). All experiments were performed at least twice with at least two replicates per test condition. The data shown are from representative experiments.
Analysis of Ethylene

Ethylene emission was measured on detached leaves by placing fully expanded leaves 3, 5, 6, and 7 with their petioles intact into a 1.1 L sealed container. At each timepoint, leaves were harvested from trees that were treated with 150 ppb ozone, 300 ppb ozone, or ambient air. Ambient air treated tissue was a control for any ethylene accumulation due to wounding during tissue collection. Once sealed, the containers were placed in the ambient air treatment chamber for 1 h. One ml aliquots of gas were removed from the container and analyzed by injection into a Packard 436 gas chromatograph (Packard, Downers Grove, IL) at 150°C. Ethylene was separated on 80-100 mesh alumina (Coast Engineering, Redondo Beach, CA) columns (50 x 0.6 cm), with a nitrogen carrier gas at 60 ml min⁻¹ and a flame ionization detector.

Analysis of Polyamines

At each sampling time, fully expanded leaves number 2 and 8 were collected from three to six individual trees per treatment. The leaves were wiped clean and leaf discs were punched using a paper puncher avoiding any major veins. The samples (approximately 200 mg) were then placed in individual pre-weighed microfuge tubes containing 1 ml of 5% perchloric acid (PCA). The paper punchers were cleaned in between samples with alcohol. The tubes were kept on ice sample collection and then stored at -20°C until they were ready to be shipped to the USDA Forest Service Laboratory in Durham, N. H. to be
processed by Dr. Rakesh Minocha. The samples were weighed, frozen and thawed (3X) as described in Minocha et al. (1994), and centrifuged at 14,000 rpm in a microfuge for 10 min. For quantitation of polyamines, heptanediamine was added as an internal standard to aliquots of the above extracts prior to dansylation. One hundred µl of the extract were dansylated according to the procedure described in Minocha et al. (1990). Dansylated polyamines were separated by reversed phase HPLC (Perkin-Elmer Corp., Norwalk, CT) using a gradient of acetonitrile and heptanesulfonate, and quantified by a fluorescence detector (Minocha et al., 1990).

**Statistical Analysis**

For each experiment the effects of ozone and sampling time were analyzed for each clone. Data were subjected to analysis of variance (ANOVA) using StatView software (Abacus Concepts, Inc., 1995). Each data point represents N = 3-5 individual plants and significance was accepted at the P≤0.05 level.
**RESULTS**

**Ethylene Biosynthesis**

To determine if JA and/or SA are involved in regulating the transcription of either ACC synthase or ACC oxidase, Northern blot analysis was performed to compare the level of expression of these transcripts in both the ozone-sensitive, JA-, SA-insensitive hybrid poplar clone NE-388 and the ozone-tolerant, JA-, SA-responsive clone NE-245. In Figure 4.1.A and 4.1.B, Northern blots of total RNA isolated from trees treated with 300 ppb ozone were hybridized using cDNAs for both *PE-ACS1* and *PE-ACS2*, two different members of the poplar ACC synthase family. Both of these transcripts are induced in both clones and there is very little difference in the expression levels between the two clones. However, the timing of induction of *PE-ACS1* and *PE-ACS2* does differ somewhat between the clones (Fig. 4.1. A & 4.1.B). At only 1 h after the start of ozone treatment clone NE-245 shows a 4-fold induction of *PE-ACS1* transcripts, while in NE-388 *PE-ACS1* levels are still near ambient levels at the same timepoint. Not until 3 h after treatment do *PE-ACS1* transcripts increase above ambient levels in NE-388. A rapid return to ambient levels of *PE-ACS1* transcripts is observed in both clones at 6 h. Peak levels of induction of *PE-ACS1* transcripts...
Figure 4.1. Ozone-induction of transcripts for ACC-synthase and ACC-oxidase. Fully expanded leaf number two was harvested and pooled from three individual plants at the indicated time points, total RNA extracted and subjected to RNA blot hybridization using either A) PE-ACS1 hybrid poplar ACC synthase, B) PE-ACS2 hybrid poplar ACC synthase, or C) PE-ACO1 hybrid poplar ACC oxidase as probes.
ACS2 are reached at 3 h after the start of ozone treatment for NE-245 and remain elevated, not returning to ambient levels until 24 h (data not shown). In clone NE-388, PE-ACS2 levels do not peak until 6 h but they rapidly return to ambient levels by 12 h.

Analysis of the ozone-induction of PE-AC01, a cDNA for poplar ACC oxidase, reveals that the pattern of expression of this gene is in marked contrast to the expression patterns of both PE-ACS1 and PE-ACS2. PE-AC01 is induced by ozone in both clones, reaching maximal levels of induction 3 h after the start of ozone treatment (Fig. 4.1.C). However, the magnitude of the induction is significantly different when comparing the two clones. In the ozone-tolerant clone NE-245, PE-AC01 is induced by 5-fold at 3 h, while in the ozone-sensitive clone NE-388 only a 1.5-fold induction is observed. Levels of PE-AC01 return to ambient levels in NE-388 by 12 h, but in NE-245 levels do not completely return to ambient levels until 24 h (data not shown). A similar pattern of PE-AC01 transcript accumulation was observed in experiments performed using 150 ppb ozone, however the magnitude of induction in both clones was greater when plants were treated with 300 ppb (data not shown). In contrast, transcripts of PE-ACS1 and PE-ACS2 were barely detectable in both clones treated with only 150 ppb ozone (data not shown).
Analysis of Ethylene Emission

To determine if the attenuated level of *PE-AC01* expression or the delayed induction of *PE-ACS1* and *PE-ACS2* expression observed in the ozone-sensitive clone NE-388 has any effect on the actual ozone-induced emission of ethylene, we compared the ozone-induced levels of ethylene in both clones treated with 150 ppb and 300 ppb (Fig. 4.2). Both clones exhibited a rapid increase in ethylene emission in response to either 150 or 300 ppb, returning to near ambient levels by 27 h or 24 h, respectively, after the start of ozone treatment. Tissue from trees treated with 300 ppb ozone reached a peak level of ethylene emission at 3 h, while tissue treated with 150 ppb ozone did not have elevated ethylene levels until 6 h after the start of treatment. In addition, the peak level of ozone-induced ethylene emission was about 3-fold higher in plants treated with 300 ppb. Interestingly, at both 150 ppb and 300 ppb ozone, peak levels of ethylene emission were higher in the ozone-sensitive clone NE-388. At 150 ppb ozone, ethylene emission in NE-388 was 45% higher than levels reached in NE-245 and at 300 ppb levels of ethylene were 30% higher in NE-388 compared to NE-245. Lesion development in both clones correlated with ethylene emission; at 300 ppb visible lesions appeared at 3 h and at 150 ppb lesions were not observed until 6 h. Both clones developed lesions at both concentrations of ozone in a manner similar to previous reports (Chapter II; Koch et al., 1998) with the tolerant clone developing only small,
Figure 4.2. Ozone-induced ethylene emission. A) Ethylene production by plants treated with 150 ppb ozone or ambient air. Six hours after the start of treatment, levels of ethylene have significantly increased over ambient controls in both NE-388 (P=.04) and NE-245 (P=.002). B) Ethylene production by plants treated with 300 ppb ozone or ambient air. Three hours after the start of treatment, levels of ethylene have significantly increased over ambient controls in both NE-388 (P=<.0001) and NE-245 (P=.002). Each data point represents the mean ± SE of at least three individuals. Data shown are representative of one of three replicate experiments.
HR-like lesions on 9-20% of all leaves while NE-388 developed larger regions of necrosis on up to 80% of all leaves. The number of leaves with 50% or greater of the leaf surface area containing a lesion was less in NE-388 plants treated with 150 ppb compared to 300 ppb.

Quantitation of Polyamines

Putrescine, spermidine and spermine were assayed in both clones treated with ambient air or 150 ppb ozone. Only putrescine showed any reproducible significant increase in response to ozone (Fig. 4.3). Putrescine levels in both clones were elevated above ambient controls by 6 h and reached peak levels at 10 h past the onset of ozone treatment, returning to near ambient levels at 24 h. Putrescine levels were 40% higher in the ozone-sensitive clone NE-388 compared to the ozone-tolerant clone at 10 h.
Figure 4.3. Accumulation of putrescine in response to treatment with 150 ppb ozone or ambient air. Fully expanded leaves number two and eight were harvested from three to five individual trees per time point. Data shown represent the mean ± SE of these individuals. Significant increases in putrescine levels in ozone-treated plants compared to ambient controls is observed at 10 h after the start of treatment in both NE-388 (P=0.003) and NE-245 (P=0.04). Similar results were obtained in an independently performed duplicate experiment.
DISCUSSION

Although the physiological responses of forest trees to ozone have been well characterized, little is known about the underlying molecular mechanisms that trigger these responses. To address this, hybrid poplar was developed as a model system as described in the previous chapters, comparing the molecular responses of an ozone-sensitive clone (NE-388) to those of an ozone-tolerant clone (NE-245). These studies demonstrated that there is a correlation between ozone sensitivity and a lack of defense gene induction in response to not only ozone but pathogen inoculation and wounding as well (Koch et al., 1998). The attenuated response of NE-388 to stress was shown in Chapter III to be caused by an insensitivity to the signal molecules SA and JA. Furthermore, the study of these two clones led to the finding that ozone causes lesion formation via two distinct mechanisms.

In this chapter, the possibility that the insensitivity of NE-388 may be due to a deficiency in ethylene production was examined. Several previous studies have provided evidence that ethylene interacts with both SA (Lawton et al., 1994; Dong, 1998) and JA (Penninckx et al., 1994; Penninckx et al., 1998) to mediate activation of defense response genes. Furthermore, comparisons of ethylene
biosynthesis in the two hybrid poplar clones can determine if SA and/or JA plays a role in the regulation of ethylene biosynthesis.

The data comparing ozone-induced levels of transcripts for the ethylene biosynthetic enzymes ACC synthase and ACC oxidase demonstrated differences in the regulation of transcripts for these two enzymes. First of all, both PE-ACS1 and PE-ACS2 transcripts were induced to similar levels in both clones but for both transcripts there was a slight delay in the timing of induction when comparing NE-388 to NE-245. PE-AC01 was also induced in both clones, but the magnitude of the induction was greater in NE-245 compared to NE-388. A 5-fold induction of PE-AC01 transcripts was observed in NE-245, while only a 2-fold induction was found in NE-388. The attenuated induction of PE-AC01 in NE-388 is similar to differences previously reported in Chapter II in the activation of PR-1, WIN 3.7, OMT and PAL in this clone, which is attributable to an insensitivity to SA and JA (Koch et al., 1998; Chapter III). This indicates that SA and/or JA may also in part, regulate the induction of PE-AC01 transcripts.

The patterns of ozone-induced expression we report are similar to previous reports on the ozone induction of transcripts for both ACC synthase and ACC oxidase. In potato and Arabidopsis, levels of ozone-induced ACC synthase transcripts are detectable between 30 minutes and 1 h after the start of treatment (Schlagnhaufer et al., 1995; Vahala et al., 1998), and in tomato ACC synthase transcripts were detected at 2 h (Tuomainen et al., 1997). However, in tomato ACC oxidase transcripts are clearly induced prior to ACC synthase while the data
presented in this chapter show that in ozone-treated hybrid poplar, transcript levels for both enzymes are induced at the same time.

To determine if the attenuated induction of $PE-AC01$ and the delayed induction of $PE-ACS1$ and $PE-ACS2$ in NE-388 effected ethylene biosynthesis, ethylene emissions in response to ozone were measured in both clones. Increased levels of ethylene are reported for both clones at both 150 ppb and 300 ppb ozone. Treatments with 150 ppb ozone resulted in peak ethylene production 6 h after the start of the experiment in both clones, while 300 ppb ozone resulted in a peak of ethylene emission at 3 h. Ethylene emission was dose-dependent; the highest levels achieved in plants treated with 150 ppb ozone were 3-fold lower than the peak values measured when 300 ppb ozone was used. Interestingly, at both concentrations of ozone the sensitive clone had higher levels of ethylene emission compared to the tolerant clone. This is in accordance with several other studies that have concluded that high levels of ethylene emission correlate with ozone sensitivity (Langebartels 1991; Mehlorn and Wellburn, 1987)

These data indicate that the delayed induction of $PE-ACS1$ and $PE-ACS2$ in concert with the attenuated induction of $PE-AC01$ does not result in a delay or reduction in ethylene emission in the ozone-sensitive clone NE-388. This finding is contradictory to a study reported by Tuomainen et al., (1997) that demonstrated in tomato plants that the inhibition of either ACC synthase or ACC oxidase reduced both ethylene emission and ozone-induced lesion formation in
tomato plants. A possible explanation for this is that ethylene biosynthesis is regulated post-transcriptionally and that these transcripts are expressed in excess so that even the attenuated levels of ACO-1 are sufficient to produce substantial amounts of ethylene. Evidence for post-transcriptional regulation of both ACC synthase (Felix et al. 1994; Spanu et al. 1994) and ACC oxidase (Kim et al., 1997) has been reported by several groups. Another possibility is that hybrid poplar may have other ozone-inducible isozymes of ACC synthase and ACC oxidase that were not detected in the present study that also contribute to ethylene biosynthesis in response to ozone.

We conclude that the JA/SA-insensitive phenotype of the ozone-sensitive hybrid poplar clone NE-388 cannot be attributed to a deficiency in ethylene production. In fact, it is possible that the attenuated levels of defense gene expression that are observed in NE-388 are due to low levels of induction by ethylene and if ethylene were deficient perhaps there would be a total lack of detectable defense gene induction. It may be that maximal induction requires SA and/or JA in addition to ethylene, but similar to the defensin gene in A. thaliana (Penninckx et al., 1996; Penninckx et al., 1998), ethylene alone induces minimal amounts of expression. Furthermore, although JA and SA may play a role in the regulation of PE-AC01 transcript levels, the production of ethylene is not negatively effected in the SA-, JA-insensitive clone NE-388 indicating that these signals do not regulate ozone-induced ethylene production. Alternatively, if SA and/or JA are required for ethylene biosynthesis it must be via a pathway that
is distinct from the SA/JA signaling pathways that are deficient in NE-388 resulting in attenuated defense gene activation.

Ethylene shares a common precursor with polyamines, S-adenosylmethionine. Interestingly, both ethylene and polyamines are elevated in response to stress (Bouchereau et al., 1999) and both have been correlated with ozone sensitivity. In several plant species, a metabolic switch based on regulation of these compounds has been proposed; when the precursor is driven to synthesis of one, synthesis of the other is inhibited. This hypothesis has been supported by findings that ethylene can inhibit enzymes involved in polyamine biosynthesis and polyamines can inhibit enzymes involved in ethylene biosynthesis. In addition to their biosynthetic pathways being antagonistic, their physiological roles within the plant also appear to be antagonistic. For example, ethylene promotes senescence while polyamines inhibit it. Conjugated polyamines have been shown to be free radical scavengers, while ethylene has been postulated to react chemically with ozone to form AOS (Elstner et al., 1985). Feeding polyamines to tobacco and tomato prior to ozone exposure reduces visible lesion formation (Ormrod and Beckerson, 1986; Bors et al., 1989) while ethylene appears to promote ozone-induced lesion formation (Tuomainen 1997).

To investigate the relationship between ethylene emission and polyamine formation in response to ozone in a tree species, polyamine production in both NE-388 and NE-245 was measured. Only putrescine levels were increased in
response to ozone. Increases in putrescine were observed in both clones, although NE-388 accumulated 40% more putrescine per gram of fresh weight than NE-245. This indicates that putrescine production occurs independently of SA and JA, or at least via a pathway distinct from the SA/JA pathway deficient in NE-388. Peak levels of putrescine accumulation were observed 10 h after the start of ozone treatment, 4 h after peak ethylene emission was measured.

These data indicate that in hybrid poplar polyamine biosynthetic pathways are not antagonistic to ethylene production but instead the two seem to be synthesized sequentially. Although our data contradict the metabolic switch theory of ethylene and polyamine synthesis there have been a significant number of reports in other plant systems that do not support this theory as well. For example, tomato plants subjected to ammonium toxicity and potassium deficiency accumulated putrescine and this was accompanied by increased ethylene synthesis (Corey and Barker, 1989). In fact, it may be that the metabolic switch hypothesis describes developmental regulation of ethylene and polyamines and different regulatory pathways are activated in response to stress. Alternatively, clone NE-388 in addition to being insensitive to JA and SA may also be deficient in the ability to perceive ethylene as well. An insensitivity to ethylene could result in the inability to suppress polyamine biosynthesis. Furthermore, ethylene insensitivity could contribute to the differential patterns of expression of PE-ACS1, PE-ACS2 and PE-ACO1 reported in NE-245 compared to NE-388. Several examples of autoregulation of ethylene through influence on
both ACC synthase and ACC oxidase transcription and activity have been previously reported in several different plant species (Woodson et al., 1992; Kim and Yang, 1994; Lasserre et al., 1996; Barry et al., 1996; Kim et al., 1997).

In tobacco, the ozone tolerant cultivar Bel B accumulated putrescine 10 h after ozone treatment, similar to the hybrid poplar clones (Langebartels et al., 1991). However, the ozone-sensitive tobacco cultivar Bel W3 accumulated putrescine to a lessor extent than Bel B and levels of putrescine did not begin to increase until 24 h after ozone treatment. These data are in contrast to what we have observed in hybrid poplar. In this case, both the sensitive and tolerant clones have elevated levels of putrescine by 10 h after ozone treatment and the levels measured in the sensitive clone were slightly higher than in the tolerant clone. Putrescine accumulation was reported in an ozone-sensitive birch clone while little to no accumulation was reported in the tolerant clone (Tuomainen et al., 1996) In this case, the authors concluded that the accumulation of high levels of putrescine in the sensitive birch clone correlated with the onset of visible lesion formation and was not connected with putrescine playing a role in protection from ozone but rather was the result of the extensive ozone damage (Tuomainen et al., 1996). This appears to be the case in the sensitive tobacco cultivar Bel W3 and in both hybrid poplar clones as well.

In conclusion, ethylene and putrescine levels are induced in both a sensitive and tolerant hybrid poplar clone in response to ozone. Due to the fact that the ozone-sensitive clone is insensitive to SA and JA it appears that these
signal molecules do not play a major role in the regulation of ethylene or polyamine biosynthesis, (unless it is through a pathway distinct from the pathway leading to attenuated defense gene activation in NE-388.) However, the attenuated induction of PE-AC01 and delayed induction of PE-ACS1 and PE-ACS2 in the SA/JA-insensitive clone NE-388 indicates that SA and/or JA may influence the transcriptional regulation of these enzymes without negatively influencing ethylene production. Finally, ethylene and polyamines are synthesized sequentially in hybrid poplar treated with ozone and the elevated levels in both clones appear to correlate with lesion development.
CHAPTER V

CONCLUSION

The studies presented in "The Molecular Basis of Ozone Sensitivity in Hybrid Poplar" distinctly demonstrate that there are differences in defense responses, gene expression, and signal transduction pathways that correlate with sensitivity to ozone. Specifically, the ozone-sensitive hybrid poplar clone NE-388 displays an attenuated level of defense gene expression in response to ozone exposure, inoculation with phytopathogenic bacteria, and insect chewing mimicked through mechanical wounding. This attenuated response, based on studies described in Chapters II and III, is clearly due to an insensitivity to both SA and JA. Accumulation of SA and JA as well as emission of ethylene in response to ozone treatment was similar in both clones. The responsiveness of NE-388 to ethylene is undetermined as of yet, but the ozone-sensitivity and insensitivity to both SA and JA cannot be attributed to a deficiency in ethylene production. The inability of NE-388 to adequately perceive SA and JA leads to not only attenuated levels of defense gene induction but also appears to be involved in diminishing
physiological responses to ozone as well, including inhibition of stomatal closure and reduction in photosynthetic rate.

In addition, the molecular analysis of ozone sensitivity in hybrid poplar clones NE-245 and NE-388 presented here has provided direct evidence supporting the hypothesis that ozone mimics a HR by demonstrating that ozone activates a SA-dependent PCD pathway identical to that activated by a pathogen-induced HR. Furthermore, these studies have shown that ozone causes lesion formation via two distinct pathways, one which involves the activation of PCD and one which occurs in the absence of PCD and is likely due to the toxicity of AOS. These findings are supported by work done in Arabidopsis that has shown that an ozone-sensitive ecotype hyperaccumulates SA in response to ozone, resulting in an amplified HR (Rao and Davis, 1999). Arabidopsis NahG plants, which fail to accumulate SA, develop lesions in response to ozone independent of PCD similar to NE-388 via the accumulation of toxic AOS exacerbated by the lack of SA-mediated antioxidant defenses (Fig. 5.1).

The results described in the previous chapters have also provided valuable insight into the potential ways in which different signal transduction pathways interact. The finding that JA pretreatment reduces the subsequent amount of ozone-induced damage only in NE-245, the clone that was shown to develop lesions through the activation of PCD, indicates that JA inhibits this SA-dependent pathway. Recent results in Arabidopsis support this hypothesis. The
Figure 5.1. Proposed model for the two mechanisms of ozone-induced lesion formation.
ozone-sensitive ecotype Cvi-O was found to have a reduced ability to perceive JA, but pretreatment with high concentrations of MeJA resulted in attenuated ozone-induced oxidative burst and SA accumulation. Cell death was also completely abolished (Rao and Davis, unpublished data). However, similar to observations in NE-388, MeJA pretreatment failed to decrease ozone-induced lesion formation in NahG plants.

Although many of the findings reported here in hybrid poplar are similar to findings in herbaceous plant species, differences were found as well. Variations have been reported within the herbaceous plant species in regard to levels of SA (Malamy et al., 1990; Ohshima et al., 1990; Raskin et al., 1990) and also even within a single plant, as illustrated by rice (Silverman et al., 1995). Differences among herbaceous plant species have also been reported at the level of gene induction mediated by both SA (Yu et al., 1997) and JA (Schweizer et al., 1997). As reported in Chapter III, SA signaling in hybrid poplar shares some characteristics with herbaceous plant species. However, the requirement for SA sensitivity to induce PR-1 expression, coupled with high basal levels of SA that are not accompanied by any additional stress-induced increases in free or total SA levels is unique to poplar, among all species studied to date. Furthermore, the results described in Chapter IV on polyamine accumulation provide evidence that polyamines may function differently in regard to ozone sensitivity in tree species compared to herbaceous plants. In hybrid poplar, like birch (Tuomainen et al., 1996), accumulation of polyamines is correlated with the extent of injury.
development. Conversely, in tobacco an early peak of polyamine production is detected without the prior occurrence of visible injury and correlates with ozone tolerance (Langebartels et al., 1991).

These differences demonstrate clearly that in order to obtain reliable data relevant to how specific genes and metabolites are regulated in a tree species, the studies must be done in a tree species. The characterization of the differences between the ozone-sensitive and ozone-tolerant hybrid poplar clones has established an ideal model system for the continued study of defense gene expression in tree species as well as the signal transduction pathways that activate them. In particular, this system will be especially useful in detecting defense genes induced via novel SA/JA-independent pathways. For example, in Chapters II and III, it was established that WIN 3.7 is induced by both MeJA and wounding in the ozone-tolerant clone NE-245. However, in the ozone-sensitive clone NE-388, the MeJA- and wound-induction of WIN 3.7 is attenuated. Remarkably, recent evidence has been obtained indicating that WIN 3.7, or a cross-hybridizing member of the WIN 3 family, is induced by pathogen-infiltration of NE-388 (Fig. 5.2). As shown in Fig. 5.2, expression of PR-1 under the same conditions remains attenuated when compared to NE-245, indicating that WIN 3.7 under these conditions is being activated independently of SA and/or JA.

Another potential use for this model system likely to significantly contribute to scientific knowledge of signal transduction pathways in trees, is to determine the exact cause of SA/JA insensitivity in NE-388. The data presented in this work
Figure 5.2. Comparison of pathogen-induced PR-1 mRNA accumulation in hybrid poplar clones NE-245 and NE-388. Leaves of 8-week-old cuttings were infiltrated with either *P. syringae* pv *maculicola* KD4326 (Psm) in 10 mM MgCl₂ or mock inoculated with 10 mM MgCl₂ (Mock). Total RNA was extracted from the infiltrated area of the second through the fifth fully expanded leaves, subjected to RNA-blot hybridization analysis, and the amount of hybridizing radioactivity was quantitated using a phosphor imager. Time points represent the hours after infiltration.
has shown that SA, JA, and ethylene are all synthesized similarly in both NE-388 and NE-245 in response to ozone. Therefore, the deficiency resulting in the attenuated responses of NE-388 must be downstream in the signal transduction pathway. It is not known whether the difference between these two clones is due to a single gene or multiple genes. However, reasonable hypotheses can be developed and tested. For example, recent research in other laboratories has shown that genes with defense-related activities such as lipoxygenase (Rouster et al., 1997) and glutathione S-transferase (Xiang et al., 1996) contain promoter elements responsive to signals such as auxin, SA, MeJA, and hydrogen peroxide. Furthermore, MAP kinases have been identified that are both SA- and JA-inducible (Bögre et al., 1997; Zhang and Klessig, 1997; Romeis et al., 1999) and protein dephosphorylation has been shown to play a role in mediating SA-induction of PR genes in tobacco (Conrath et al., 1997). Based on these results, a plausible hypothesis for the insensitivity of NE-388 and the resulting attenuated defense gene expression is that NE-388 may be lacking either a kinase or phosphatase required to activate a subset of transcription factor or a subset of transcription factors required for SA- and JA-mediated defense gene expression.

Certainly, many hypotheses for the deficiency in NE-388 can be formulated and tested, but in conclusion the work presented in "The Molecular Basis of Ozone Sensitivity in Hybrid Poplar" clearly contribute a sound basis for the understanding and continued study of the mechanisms involved in stress perception, signal activation, and the subsequent expression of defense genes.
that results in the physiological and biochemical changes most favorable for survival of hybrid poplar.
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