THE EFFECTS OF WOUNDING RESPONSE AND CYTOSOLIC ASCORBATE PEROXIDASE LEVELS ON OZONE SUSCEPTIBILITY IN OZONE-SENSITIVE TOBACCO CULTIVAR

by

Björn Lárus Örvar

B.Sc., University of Iceland, 1984

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES

Department of Plant Science

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April 1997

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of PLANT SCIENCE

The University of British Columbia
Vancouver, Canada

Date April 29th 1997

DE-6 (2/88)

ABSTRACT

The study of signal transduction in plants in recent years has revealed that many different chemical and environmental signals can elicit similar physiological responses. We know little about how a potential ozone stress-signal is transduced into a protective response in plant cells, and within the context of this complex response, it is uncertain which components are most important for reducing cellular damage during ozone exposure. The general objective of this study was to use different tobacco genotypes to investigate the interplay between two stresses, i.e. ozone and wounding, that are both thought to involve redox perturbations in the challenged tissue. Transgenic technology was also employed to create a modified antioxidant defence system in a hypersensitive tobacco genotype, to see whether this would alter its ability to tolerate ozone stress. Both wounding and treatment with methyl jasmonate (MeJA) prior to exposure of the tissue to high concentrations of ozone (250 to 500 ppb) produced a dramatic decrease in ozone injury. A systemic pattern of increased ozone tolerance developed within 3 to 6 hr after wounding and also after local application of jasmonic acid. Ozone treatment of transgenic tobacco plants (line called NahG) expressing bacterial salicylate hydroxylase, showed that the inability of these plants to accumulate salicylic acid, which has been shown to inhibit jasmonate-mediated responses, was also accompanied by increased ozone tolerance. Expression of mRNA encoding the antioxidant enzyme ascorbate peroxidase (APX) was upregulated by ozone challenge, by wounding and by MeJA exposure within 3 to 4 hr, while levels of carbonic anhydrase mRNA were simultaneously depressed following ozone exposure and MeJA treatment.

In order to investigate the effects of a modified antioxidant defence system on ozone tolerance in tobacco, a cDNA for a cytosolic APX was first cloned from tobacco and subsequently used to make transgenic tobacco plants with altered expression of cytosolic APX. The expression of an antisense construct, comprising about 45% of the 3' coding region of the cytosolic APX, significantly reduced both the endogeneous APX mRNA

levels and the cytosolic APX catalytic activity in these plants, apparently without affecting the chloroplastic APX activity. These transgenic plants displayed a significantly higher level of ozone injury following very high ozone exposure, and an even more drastic difference in injury levels, following lower, prolonged exposures. In contrast, transgenic plants overexpressing APX displayed a level of ozone injury following ozone exposure similar to that seen in control plants. To my knowledge, the data presented in this thesis provide the first substantial evidence for common signalling mechanisms linking plant responses to ozone with their responses to other environmental stresses. Furthermore, the data presented in this thesis provide the first substantial evidence for the importance of cytosolic APX in ozone defence in tobacco.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

Chapter 1

Chapter 2

Chapter 3

ABBREVIATIONS

AMV	avian myeloproliferative virus
APX	ascorbate peroxidase
ARE	antiperoxidative element
Bel	Beltsville
bp	base pair
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
CHS	chalcone synthase
DAG	diacylglycerol
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddH_2O	double distilled water
DIG	digoxigenin
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphate
DPI	diphenylene iodonium
DTT	1,4-dithioerythritol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetra acetic acid
EGTA	ethylene glycol-bis(B-aminoethyl ether)
GST	glutathione S-transferase
GUS	glucuronidase
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPRNI	human placental ribonuclease inhibitor
HSE	heat-shock elements
IP_3	inositol triphosphate (IP_3)
JA	jasmonic acid
KCl	potassium chloride
kDa	kilodalton
LB	Luria Broth
LOX	lipoxygenase
MAPK	mitogen-activated protein kinase
MeJA	methyl jasmonate

MOPS	3-(N-morpholino)propane-sulfonic acid
mRNA	messenger ribonucleic acid
µE	µEinsteins
NADH	nicotinamide adenine dinucleotide
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor kappa B
Nos	nopaline synthase
PAL	phenylalanine-ammonia-lyase
pCMPSA	*p*-chloromercuriphenylsulphonic acid
PCR	polymerase chain reaction
Pin	proteinase inhibitor
PIP_2	phosphoinositides
PLC	plasmalemma-associated phospholipase C
ppb	parts per billion
PPO	polyphenol oxidase
RbcS	ribulose-1,5-bisphosphate carboxylase/oxygenase
PR	pathogenesis-related proteins
RACE	rapid amplification of cDNA ends
RH	relative humidity
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SA	salicylic acid
SAR	systemic aquired resistance
S.D.	standard deviation
SDS	sodium dodecyl sulfate
soz	sensitive to ozone
Taq	Thermus aquaticus
TMV	tobacco mosaic virus
U	Units
UTR	3' end untranslated sequences
UV	ultra-violet
V	volts

ACKNOWLEDGEMENTS

I would like to thank my research supervisor, Dr. Brian E. Ellis, for all his help and positive guidance throughout this study, and for his tremendous, supportive interest in my research work, the members of my committee, Dr. Ben Sutton, Dr. Carl Douglas and especially Dr. Victor C. Runeckles, who provided me with an excellent feed-back on my work, as well as assisting me in the set-up of the ozone exposure system. I would also like to thank all the wonderful people I had the privilege to work with in Dr. Ellis's multi-cultural lab, the support staff in Plant Science, Robyne, Ashley, Sean and Derek, as well as Peter Garnett and my good friends, Dr. Joan McPherson, Mesfin Tesfaye, Murali Srinivasan and Marcus Samuel for their interesting, creative dialogues, and Dr. Naza Azizbekova for all her trust and help. A special gratitude goes to my joyful friend and lab-bench companion for almost four years, Stefanie Butland, for her patient scoring of all those plants and for the many entertaining social gatherings she has organized, and to Nanna Ingvadottir for the security she has provided for me, my spouse Unnur and my son Thorsteinn here in Canada.

I would like to pay a tribute to my native roots, my wonderful family in Iceland that has always showed its interest in my work, in spite of often long and painful separation. Specifically, I would like to thank my parents, Bjorn and Hanna Marta, and my in-laws, Thorsteinn and my intelligent mother-in law and friend Asgerdur - who has earned herself a better-than-doctoral degree in human relationship and kindness - for their emotional and financial support and for their unlimited, devoted dedication to the well being of me and my family. Finally, a special appreciation goes to my best friends ever, Unnur and Thorsteinn, for their strong and radiant spirit and for providing me with such an affectious environment to live in.

Chapter 1

General Introduction

One of the most widespread tropospheric phytotoxic air pollutants is ozone (Krupa *et al.* 1993; Krupa and Manning 1988). It forms an important constituent of photochemical smog pollution (Demerjian 1986; Langebartels *et al.* 1991) and is the most toxic component of the oxidizing air pollutants (Menzel 1976). The high ambient ozone concentrations observed in industrialized areas in Europe and North America frequently go beyond levels that are toxic to plants. Peak ozone levels in major urbanized areas in North America can sometimes exceed 400 ppb (1 hr daily maximum), and in the most affected areas the maximum 3-month mean of 8 hr daily maxima can be 130 ppb (McCurdy 1994). In rural areas subjected to urban influence the 1 hr daily maximum can reach 160 ppb (McCurdy 1994).

Ozone is considered to be partly responsible for the forest decline phenomena seen in North America and Europe and experimental evidence has shown that ambient ozone concentrations can affect growth and yield of some sensitive crop species and cultivars in these two regions (Brown *et al.* 1995; Becker *et al.* 1990; Hewitt *et al.* 1990; Salter and Hewitt 1992). It is estimated that ozone alone causes more damage to crops than all other air pollutants combined, particularly in areas that account for a large proportion of the world's grain production (Bowler *et al* 1992; Heagle, 1989; Chameides *et al.* 1994). For example, it is estimated that ozone, alone or in combination with other air pollutants, is responsible for approximately 90% of the crop-loss in the United States that is caused by air pollution (Tingey *et al.* 1994). In the United Kingdom it is predicted that crop losses due to ozone exposure are somewhere between 5 and 15% (Smith *et al.* 1995). In the *National Crop Loss Assessment Network* study (NCLAN), open-top field exposure chambers and typical agronomic practices were used to develop an ozone

exposure-response relationship for crop yield (Tingey *et al.* 1994). Based on these studies it was predicted that approximately 57% of crops would exhibit 10 % yield reduction at ozone concentration below 50 ppb (7 hr (09:00 - 16:00) seasonal mean).

While numerous controlled exposure studies have provided evidence that vegetation exposed to increased ozone suffers loss in growth rate and yield, it has been difficult to determine whether, and how much, ozone affects plant growth and yield in a natural environment (Krupa and Manning 1988). The significant temporal and spatial variability in ozone concentration is one problem, since its concentration varies with altitude, latitude, season and hour of day (Runeckles and Krupa 1994; Manning and Krupa 1992). Exposure of plants to ozone can therefore be inconsistent in time and geographical location (Manning and Krupa 1992; Runeckles and Krupa 1994). This may, in part, underlie the apparently complex relationship between ozone levels and plant injury or crop yield (Chameides *et al.* 1994; Krupa *et al.* 1993; Rawlings and Cure 1985). Another factor is the interaction of ozone effects with various biotic and abiotic influences that can also limit plant growth (Krupa and Manning 1988). For example, temperature, soil moisture, light intensity and air movement all affect plant responses to ozone (Manning and Krupa 1992).

1.1 ORIGIN OF TROPOSPHERIC OZONE

The levels of tropospheric (ground level) ozone (O_3) are influenced by complex photochemical reactions that are especially prominent in urbanized areas, resulting in frequent episodes of air pollution, accompanied by high levels of ozone (Chameides and Lodge 1992). These episodes are usually associated with warm-weather months of the year and with high solar radiation (McCurdy 1994). The elucidation of the critical chemical reactions and pathways involved in photochemical smog pollution has been the subject of intensive research and debate (Demerjian 1986) but the most common chemical reactions that occur in the polluted atmosphere are:

(1) $NO_2 + UV \rightarrow NO + O$ ($UV < 420$ nm)

(2) $O + O_2 \rightarrow O_3$

(3) $NO + O_3 \rightarrow NO_2 + O_2$

Because reaction (3) consumes the ozone molecule formed in reaction (2) and regenerates NO_2, the steady-state ozone concentration is low, and as long as [NO] is > [NO_2] it stays very low. These three reactions, therefore, cannot by themselves explain the buildup of tropospheric ozone since they form a cycle in dynamic equilibrium. In a polluted atmosphere, however, the ozone buildup occurs over a period of hours and is accompanied by an increase in NO_2. This increase requires the presence of hydroxyl radicals (OH·) (Demerjian 1986), which can be produced in polluted air by photolysis of aldehydes (RCHO) and nitrous acid (HONO), or by photolysis of ozone itself (Chameides and Lodge 1992; Demerjian 1986). The hydroxyl radicals can subsequently oxidize carbon monoxide (CO) or volatile hydrocarbons such as methane (CH_4) to ultimately generate a peroxy radical ($HO_2^{\bullet}$). In the presence of nitric oxide (NO), the peroxy radical participates in a chain-propagating sequence which converts NO to nitrogen dioxide (NO_2) while simultaneously forming another hydroxyl radical that can initiate further oxidation of volatile hydrocarbons or CO (Chameides and Lodge 1992; Demerjian 1986; Runeckles and Krupa 1994). This leaves very little of the NO to react with ozone (reaction (3) above) and results in ozone-buildup to large concentrations as a result of photolysis of NO_2 (reaction (1) and (2) above).

These atmospheric reaction sequences are profoundly complicated by reactions with a variety of volatile organic compounds (VOC) introduced through human activity (largely automotive transportation and industry)(Demerjian 1986), and there can be hundreds of different VOC's in a polluted troposphere (Chameides and Lodge 1992). Hydrocarbon emission from trees (e.g. isoprene and α-pinene), in both urban and rural areas, can also make a significant contribution to VOC levels in the atmosphere

(Chameides and Lodge 1992). The basic feature of the photochemical reaction cycle involving these VOC's is free radical attack on the VOC that can eventually result in the generation of different peroxy radical species (such as $HO_2\cdot$, $RO_2\cdot$ and $R'O_2\cdot$, where R' represents a chain with one less C atom). These peroxy radical species react with NO, converting it to NO_2 with simultaneous formation of hydroxy radical (see above)(Demerjian 1986). While the importance of hydrocarbons and NO_x as ozone precursors is well known, the relationship between atmospheric precursor levels and ozone episodes is still not fully established (Chameides and Lodge 1992). It has been predicted, however, that exposure of crops to phytotoxic levels of ozone may triple by the year 2025 if rising anthropogenic NO_x emissions ($NO + NO_2$), are not reduced (Chameides *et al.* 1994).

1.2 OZONE AND OXIDATIVE STRESS IN PLANT TISSUES

Ozone enters the plant via the stomata and diffuses through the inner air spaces of the mesophyll layer, finally reaching the cell wall and the plasmalemma (Salter and Hewitt 1992). In this environment, ozone has the potential to interact with water, and its solutes, as well as with the plasmalemma or other cellular components. The fate of ozone once it has reached the interior of the leaf tissue remains the source of considerable speculation. For example, Laisk *et al.* (1989) have suggested that the apoplastic space and the plasmalemma are the major sinks for ozone, and that reactions involving ozone in the apoplastic space and the plasmalemma can largely prevent further penetration of the ozone itself into the cell. According to this hypothesis, ozone reacting directly with intracellular components, such as macromolecules (proteins, lipids, DNA), would be a relatively unimportant factor. It has also been calculated that the major part of any ozone which reaches the plasmalemma is broken down inside the plasmalemma, damaging the membrane in the process. According to these estimates, only a small fraction of ozone (or its derivatives) enters the cytosol (Luwe *et al.* 1993). *In vivo* experiments have,

however, demonstrated effects of ozone exposure on intracellular enzymes, although there is no direct evidence to date suggesting that these effects are caused directly by ozone itself (Runeckles and Chevone 1992; Morgan and Wenzel 1985).

Ozone is a hydrophilic molecule which makes it less likely to partition into a hydrophobic environment such as the lipid bilayer of the plasmalemma (Heath 1988). Despite this, Pauls and Thompson (1980) detected increased lipid peroxidation in plant membranes following ozone exposure, as measured by increases in the levels of malondialdehyde (MDA) in isolated microsomes from young pea cotyledons (Pauls and Thompson 1980). MDA is an end-product of step-by-step degradation of lipids (Salter and Hewitt 1992) and its production is commonly used as an indicator of non-specific lipid peroxidation (Runeckles and Chevone 1992). This study did not address whether the increased lipid peroxidation observed was initiated by aqueous decay of ozone and generation of reactive products, or by more direct involvement of ozone in the lipid degradation. In spite of a large research effort over the past few decades, the chemistry of ozone decomposition remains uncertain, as are the biochemical mechanisms of ozone injury to plants (Kanofsky and Sima 1995). For example, ozone can directly oxidize many compounds *in vitro* , whereas in biological systems free radical formation always appears to be involved (Morgan and Wenzel 1985). Nevertheless, it is generally accepted that ozone exposure initially results in the production of reactive oxygen species (ROS), causing oxidative stress, once it has penetrated into the plant tissues (Grimes *et al.* 1983; Hoigne and Bader 1975; Kangasjarvi *et al.* 1994) and that these ROS can initiate a complex cascade of events leading to small- or large-scale tissue injury.

1.2.1 Oxidative stress and properties of reactive oxygen species

Oxidative stress has been defined as an increase in the prooxidant/antioxidant ratio inside the cell, leading to potential damage (Sies 1991). According to this definition, a simple loss in antioxidants (see section 1.4) or an increase in oxidative challenge

(prooxidants), may not necessarily result in oxidative stress, as long as the cell is able to cope with the change in the prooxidant/antioxidant ratio (Sies 1991).

Reactive oxygen species (ROS) are free oxygen radicals (oxyradicals) and their reactive oxygen intermediate molecules (Feher *et al.* 1987). These include the hydroxyl radical (OH·), hydroperoxyl radical (HO_2·) and superoxide anion radical (O_2^-), and the oxygen intermediates hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$)(Cadenas 1995; Feher *et al.* 1987). Free radicals are molecules that contain unpaired electrons in their outermost orbitals and due to the natural tendency for electron pairing, unpaired electrons are forced to "look for a partner". These radicals are, therefore, extremely reactive and short-lived although their stability varies substantially (Cadenas 1995; Feher *et al.* 1987). Ground-state molecular oxygen (O_2), however, is a so-called biradical, containing two unpaired electrons with a parallel spin which causes it to be a relatively weak oxidant (Feher *et al.* 1987). ROS can be generated through the successive additions of electrons to the ground-state oxygen (O_2^-, H_2O_2 and OH·), or by energy-transfer reactions ($^1O_2$)(Cadenas 1995). The O_2^- radical is generated by one-electron transfer. The reactivity of O_2^- is limited but its prooxidant feature lies in its ability to be transformed into other reactive oxygen intermediates such as HO_2· and H_2O_2 (see section 1.2.2 and 1.4.2)(Cadenas 1995; Feher *et al.* 1987). HO_2· (a protonated O_2^-) is a stronger prooxidant than O_2^- and can freely cross biological membranes, in contrast to O_2^- (Cadenas 1995; Feher *et al.* 1987). H_2O_2 is not a free radical and it displays a moderate reactivity (Cadenas 1995). As H_2O_2 can cross biological membranes and since it is required for the formation of OH· radicals (see section 1.2.2), it is an important prooxidant (Cadenas 1995). OH· radicals are extremely reactive with a half-life of only 10^{-9} seconds (Sies 1991; Feher *et al.* 1987).

Molecular oxygen can also absorb energy (energy-transfer) to form $^1O_2$. In this process one of the unpaired electrons in ground-state oxygen is transferred to a higher

energy orbital and its spin is inverted (Cadenas 1995; Feher *et al.* 1987). This ROS has a half-life of 10^{-6} seconds (Sies 1991).

1.2.2 Ozone reactions in aqueous solutions

Ozone reactions with aqueous solutions are quite complex and far from being completely understood (Heath 1988). Ozone has been reported to produce substantial quantities of $^1O_2$ upon reaction with aqueous extracts of intercellular fluid from leaves of *Sedum album* (Kanofsky and Sima 1995), and the production of H_2O_2, as well as O_2^- and OH· radicals, have been reported in rat cell cultures following ozone exposure (Morgan and Wenzel 1985). The observation that H_2O_2 scavengers can protect antioxidant enzyme activity (catalase) against ozone-imposed inhibition *in vitro* , has led to the suggestion that H_2O_2 formation may result from ozone exposure (Whiteside and Hassan 1988). H_2O_2 can, in turn, be converted to the OH· radical by the *Haber-Weiss* reaction, in the presence of a metal ion, such as Fe^{2+} (Asada 1992; Salter and Hewitt 1992):

$$H_2O_2 + O_2^- \rightarrow OH^- + O_2 + OH\cdot$$

The OH· radical is believed to be the major ROS responsible for modifications of macromolecules and cellular damage in living cells (Mehdy 1994). Under non-physiological conditions in aqueous solutions, in the presence of hydroxide ions (OH-), ozone can decompose and generate OH· radical (Hoigne and Bader 1975). On the other hand, although many investigations involving ozone stress have focused on the importance of O_2^- and H_2O_2 in early events leading to ozone induced cellular injury, evidence for their participation is both conflicting and indirect (Runeckles and Chevone 1992; Grimes *et al.* 1983). Grimes *et al.* (1983) demonstrated that neither O_2^- nor H_2O_2 is a major intermediate in OH· radical production *in vitro*, indicating that ozone or OH· radicals should be considered the primary toxic species in ozone injury (Grimes *et al.*

1983). Recently, Runeckles and Vaartnou (1997) have obtained special evidence for the formation of O_2^- *in situ* in leaves exposed to low levels of ozone.

1.2.3 Ozone reactions with organic compounds

One of the hypothesized modes of action by which ozone may cause plant injury, is its reaction with endogenous hydrocarbons (Salter and Hewitt 1992). According to this hypothesis, aqueous decay of ozone is unlikely to play a major role in initiating damage to the plasmalemma because of the low rate constant for OH· radical production in aqueous solutions and the relatively low physiological pH (low concentration of OH-) in the mesophyll tissue (Salter and Hewitt 1992). Instead, *ozonolysis* takes place where ozone directly attacks unsaturated hydrocarbons in the presence of water, as the ozone diffuses through the mesophyll layer or after its absorption by the plasmalemma (Menzel 1976; Salter and Hewitt 1992). Ozone reacts with the carbon-carbon double bond in polyunsaturated fatty acids (in the plasmalemma)or other hydrocarbons, such as isoprenes (available in extracellular air space and the mesophyll), to form *ozonides* :

$$O_3 + R - HC = CH - R' \rightarrow R - CH(O_3)CH - R'$$

ozonide

These ozonides decompose rapidly to form a carbonyl compound and a so-called *Criegee diradical* or carbonyloxide (Becker *et al.* 1990; Salter and Hewitt 1992; Runeckles and Chevone 1992):

$$R - CH(O_3)CH - R' \rightarrow R - HC = O + R' - HC = O^+ - O^-$$

Criegee diradical

In an aqueous or water-saturated gaseous environment like the mesophyll, the Criegee diradical can react with water to form a second carbonyl product and H_2O_2:

$$R' - HC = O^+ - O^- + H_2O \rightarrow R' - HC = O + H_2O_2$$

In the Haber-Weiss reaction (see above), H_2O_2 can then give rise to OH· radicals which, in turn, can initiate lipid autoxidation (adapted from Salter and Hewitt 1992; Menzel 1976):

$$R_1H + OH\cdot \rightarrow R_1\cdot + H_2O \quad (1)$$

$$R_1\cdot + O_2 \rightarrow RO_2\cdot \quad (2)$$

$$RO_2\cdot + R_2H \rightarrow RO_2H + R_2\cdot \quad (3)$$

where R_1H is an unsaturated fatty acid, $R_1\cdot$ is an alkyl free radical, $RO_2\cdot$ is a peroxy free radical and RO_2H a relative stable fatty acid hydroperoxide. The hydrogen abstraction occuring in reaction (3) propagates this autoxidation (Menzel 1976).

Becker *et al.* (1990) demonstrated, using an *in vitro* system, that ozone can react with a number of different alkenes to produce H_2O_2. They also showed that the presence of water vapour significantly increased the H_2O_2 yields, indicating the direct reaction of water vapor with the Criegee diradical (Becker *et al.* 1990). In contrast to these results, however, Hewitt *et al.* (1990) reported that H_2O_2 is not accumulated as a major ROS in plants following ozone exposure. In their study, the isoprene-emitting California poppy was exposed daily to ozone (120 ppb/10 hr), from germination until harvesting (days 15 - 18). No H_2O_2 was detected in these plants under the experimental conditions used, whereas different organic hydroperoxides (ROOH) accumulated during this period, presumably as a result of ozone initially attacking the carbon-carbon double bond in alkenes (isoprene and other biogenic alkenes). These results were substantiated by Wellburn and Wellburn (1996), who observed a positive correlation between ethylene production and ozone injury in tobacco. Following ozone exposure, the ozone-sensitive genotype, Bel W3, produced significantly higher amounts of ethylene than the more ozone-tolerant cultivar, Bel B (Mehlhorn *et al.* 1991; Wellburn and Wellburn 1996). Mehlhorn *et al.* (1991) also reported that by inhibiting ethylene production, using an

ethylene biosynthesis inhibitor aminoethoxyvinylglycine (AVG), ozone injury in pea plants was reduced. These results could be explained by the hypothesis that harmful free radicals are formed, perhaps in the mesophyll, in a direct reaction between ethylene and ozone (Mehlhorn *et al.* 1990). In fact, it was shown that free-radicals (unidentified) were formed following ozone exposure in intact leaves, but inhibition of ethylene biosynthesis had no effect on this free radical formation (Mehlhorn *et al.* 1990). Thus the correlation between high ethylene production and increased ozone injury is more complex and cannot simply be attributed to a direct reaction between these two species.

In summary, investigating the biochemistry of ozone stress on leaf tissue has turned out to be a difficult task and the mechanisms underlying ozone-catalyzed tissue damage are far from clear. Some of the ROS are highly reactive and short-lived, making them difficult to quantify. They can react with a wide range of compounds and cellular components, giving rise to a complicated mixture of secondary intermediates, some of which are themselves radicals. In addition, the plant cell can activate both enzymatic and non-enzymatic antioxidant systems that can, presumably, scavenge some of these ROS at early stages in ozone stress and therefore mask the generation of ROS during ozone exposure. While the increased H_2O_2 scavenging capability induced in plant tissue by ozone stress (see section 1.4.4 for more detail) might suggest that H_2O_2 accumulates during ozone exposure, there is still no direct evidence of H_2O_2 accumulation in plant tissue following ozone exposure.

1.3 THE EFFECTS OF OZONE EXPOSURE ON PHYSIOLOGICAL PROCESSES IN PLANTS

Whole plants, organs or individual cells can be affected by ozone exposure (Manning and Krupa 1992). The effects of ozone on physiological processes in plants can vary significantly, depending on ozone concentration, duration of exposure, environmental conditions, and the species or cultivar involved (Chameides *et al.* 1994).

Ozone injury symptoms can range from subtle biochemical changes in cell metabolism to visible damage such as chlorosis and necrotic lesions (Manning and Krupa 1992). According to Heath (1988), plant injury due to ozone exposure can be broadly categorized in the following way:

(1) *Acute stress.* Cell and tissue death resulting in a reduction in the total mass of photosynthetically active tissue; no recovery of productivity or repair of tissue damage is possible.

(2) *Chronic stress.* Some localized cell death accompanied by establishment of a new stable homeostasis that reduces the plant's ability to achieve its potential for productivity in a given environmental condition.

Acute stress results from exposure of plants to high ozone levels for short time periods, while *chronic stress* results from frequent exposure to lower levels of ozone for short periods (Heath 1988). In addition to these categories another level of ozone-induced injury, called *accelerated senescence*, closely resembles natural aging processes. *Accelerated senescence* is often difficult to distinguish from normal aging patterns (Heath 1988).

Acute stress can lead to complete disruption of normal metabolic processes in the cell, to the point where adaptation (new homeostasis) cannot stabilize the disruption and cell death is unavoidable (Heath 1988). The visible evidence for such disruptions is the appearance of tissue necrosis, often in large areas called *flecks* (Krupa and Manning 1988). *Flecking* can be uni- or bifacial, but in broad-leaved dicotyledonous plants, symptoms of ozone injury appear first in the adaxial palisade mesophyll (Manning and Krupa 1992; Krupa and Manning 1988). A *fleck*, therefore, represents a locus at which a large number of palisade cells have collapsed and died (Krupa and Manning 1988).

Successful adaptation to ozone exposure results in a new pattern of metabolic activity (*chronic stress*) which may lead to lower net productivity, since it is not optimized

for the existing environment (Heath 1988). Under *chronic stress,* visible symptoms may include tiny punctuate necrotic spots (called *stippling*), where only a few palisade cells have collapsed and died, pigmentation (accumulation of phenolic pigments) and chlorosis (Krupa and Manning 1988). This adaptation can sometimes be quantified when it affects measurable metabolic processes (see below). However, it should be emphasized that there is not a complete agreement in terminology in ozone stress research. For example, stippling can be regarded as a manifestation of an *acute stress* symptom by one researcher or a *chronic stress* symptom by another (Heath 1988; Krupa and Manning 1988).

1.3.1 Ozone and photosynthesis

Exposure to elevated levels of ozone often results in irreversible inhibition of photosynthesis (Runeckles and Krupa 1994; Darrall 1989). While measurements of changes in photosynthesis cannot be used as a specific early diagnosis of ozone injury, it is apparent that ozone exposure can affect photosynthetic rate (Darrall 1989). In short-term fumigation (1 hr), inhibition of photosynthesis has been reported at 100 ppb ozone in alfalfa, while tobacco exposed to 400 ppb for 90 minutes showed 80% reduction in photosynthesis (Darrall 1989). Ozone exposure also affects photosynthetic pigment concentrations in leaves; in wheat grown in relatively high ozone (120 ppb/8 hr) for five weeks, both total chlorophyll and carotenoid levels were reduced (Rao *et al.* 1995). Ozone also induced premature reduction in levels of both the RLSU (rubisco large subunit) and the RSSU (rubisco small subunit) in wheat under the same conditions (Rao *et al.* 1995).

1.3.2 Ozone and respiration

In general, normal respiration is a function of the mitochondria where progressive oxidation of intermediates in the tricarboxylic acid cycle ultimately leads to the release of ATP into the mitochondrial matrix (Runeckles and Chevone 1992). Ozone has been found to stimulate respiration at concentrations higher than 150 ppb, in both the presence

and absence of visible injury (Darrall 1989). It is, however, difficult to differentiate between direct effects of ozone on respiration and indirect effects resulting from leaf injury (Runeckles and Chevone 1992), since the increased demand for injury repair could also influence the rate of maintenance respiration (Darrall 1989).

1.3.3 Ozone and membrane alterations and ion fluxes

The plasmalemma serves to contain the cell contents and to mediate communication with the environment (Menzel 1976). An important feature of cell membranes is their semipermeability (Runeckles and Chevone 1992) and it appears that ozone exposure can change the "leakiness" of the plasmalemma and can also inhibit pumps and transporter channels in the membrane (Heath 1988). The leakiness could be explained by an increased proportion of the membrane lipids adopting a crystalline state following ozone exposure, a change that has been related to premature senescence in plants (Pauls and Thompson 1980). Changes in membrane permeability or in the function of membrane pumps and transporter channels could affect ion fluxes across the membrane, and there is evidence that ozone can affect the ionic homeostasis of the cell (Castillo and Heath 1990).

One of the ions involved in plant cell homeostasis is Ca^{+2}. Ca^{+2} is a very important regulator of many intracellular processes, acting alone or binding to receptors or intracellular messengers (see section 1.5.1)(Trewavas and Gilroy 1991). Although they exist in a high calcium environment, plant cells maintain a very low level of free Ca^{+2} in the cytosol. Uncontrolled changes in the cytosolic concentration of free Ca^{+2} can therefore have serious consequences for the regulation of various metabolic pathways in the cell (Castillo and Heath 1990; Heath 1988). There is strong evidence that ozone treatment increases the Ca^{+2} leakiness of the membrane, causing an increase in Ca^{+2} influx into the plant cell (Price *et al.* 1996; Heath 1988). An increase in Ca^{+2} influx has been measured in epidermal cells from barley exposed to ozone (Price *et al.* 1996), and Castillo and Heath (1990) showed that ozone can also affect the Ca^{+2} balance by impeding Ca^{+2}

transporters (for Ca^{+2} efflux) located in the plasmalemma. A specific role for Ca^{+2} in oxidative stress is further discussed in section 1.5.1.

1.4 OZONE AND ANTIOXIDANT SCAVENGING IN PLANT TISSUE

Although ozone exposure can have serious consequences for plant metabolism, plant cells are not simply "passive", non-responsive victims of ozone stress. Among the changes in plant metabolism induced by ozone are some that serve to mitigate the effects of the associated oxidative stress. These mechanisms have presumably evolved as part of the plant's need to control potential damage from ROS arising from metabolic and environmental sources, such as low levels of ozone in the troposphere, independent of human activity.

In a normal cell, a number of different metabolic events continually gives rise to ROS that have to be scavenged or detoxified before they disrupt the cellular integrity or function (Foyer *et al.* 1994). ROS are produced in many diverse cellular reactions, but photo-oxidation in chloroplasts, where the primary ROS is O_2^-, is perhaps the largest source of ROS in plant tissues (Foyer *et al.* 1994; Asada 1992). The reason is that large amounts of oxygen are produced in the immediate vicinity of powerful oxidation-reduction systems in the chloroplasts (Dalton 1995). Oxidation generates O_2^- via direct donation of an electron to oxygen from reduced ferredoxin in the photosynthetic electron transport chain in illuminated chloroplasts (Foyer *et al.* 1994; Kangasjarvi *et al.* 1994; Asada 1992). Photorespiration is another metabolic process contributing to oxidative stress in plants. In chloroplasts, part of the pool of ribulose bisphosphate is oxidized, leading ultimately to the release of glycolic acid. The glycolic acid moves into peroxisomes where it is oxidized, giving rise to H_2O_2 and glyoxylic acid (Salisbury and Ross 1992). Under favorable conditions, oxidative damage caused by endogenous ROS is minimized through the action of sophisticated enzymatic and non-enzymatic ROS-scavenging mechanisms that

are able to maintain the oxidative stress within manageable, non-destructive limits (Foyer *et al.* 1994).

As mentioned earlier (section 1.2), ozone exposure is basically an oxidative stress. A number of recent investigations have detected rapid induction of various biochemical pathways in plants following ozone exposure (Kangasjarvi *et al.* 1994). These responses include changes in gene expression and concentrations of various metabolites, often directly or indirectly related to an increase in ROS in the plant tissue.

1.4.1 Ozone and non-enzymatic reactive oxygen species scavenging

Pauls and Thompson (1980) reported that isolated microsomes from pea cotyledons sustained more oxidative damage if treated with ozone in the absence of cytosol than if treated in its presence. These results indicate that plants possess a constitutive defence system that can minimize the deleterious effects of oxidative stress such as those following ozone exposure.

Ascorbate, one of the major soluble antioxidants in plants, (Smirnoff and Pallanca 1996; Foyer *et al.* 1994) is present at high concentrations in the chloroplasts, the cytosol and the apoplast (Foyer *et al.* 1994). After ozone has entered the intercellular air space, radical scavenging by ascorbate in the apoplast would help protect the plasmalemma. The double bonds of the unsaturated fatty acids of the plasmalemma can be attacked by ozone or its derivatives (Luwe *et al.* 1993), but ozone reacts more rapidly with ascorbate than with unsaturated fatty acids (Luwe *et al.* 1993). In fact, it has been suggested that ascorbate could act as a sink for ozone in the apoplast (Runeckles and Chevone 1992) and calculations made by Chameides (1989) indicate that a major portion of the ozone diffusing through the leaf can react with ascorbate in the apoplast. Apoplastic ascorbate could, therefore, limit the amount of ozone that can reach the plasmalemma. Also, ascorbate reacts rapidly with $OH\cdot$, O_2^- and $HO_2\cdot$ radicals, and would be very effective in scavenging these radicals from any solution (Foyer *et al.* 1994; Heath 1988). Furthermore, ascorbate is required for the regeneration of α-tocopherol, another powerful

antioxidant (Foyer *et al.* 1994). In contrast to ascorbate, the lipophillic α-tocopherol cannot easily react with O_2^- although it reacts rapidly with $HO_2\cdot$ (Heath 1988).

In tobacco, total ascorbate levels increased significantly following two short (8 hr) episodes of ozone exposure (Wellburn and Wellburn 1996). Examples of increased ascorbate levels following oxidative stress are, however, few, and the increase involved is relatively small (Smirnoff and Pallanca 1996). Luwe *et al.* (1993) showed that in spinach, ascorbate was exported into the apoplast during ozone fumigation, and that this export was discontinued if ozone fumigation was terminated. Ascorbate in the apoplast was, apparently, only able to consume a fraction of the ozone entering the leaves, and the oxidized ascorbate (dehydroascorbate) could not be reduced efficiently in the apoplast (Luwe *et al.* 1993). The importance of ascorbate in defence against ozone was, however, best demonstrated by Conklin *et al.* (1996). They isolated an ozone hypersensitive *Arabidopsis thaliana* mutant (*soz1*) which is deficient in ascorbate (only 30% of wild type levels). These two traits i.e. ozone hypersensitivity and ascorbate deficiency co-segregated in genetic crosses and the phenotype could be reversed by exogenous feeding with ascorbate (Conklin *et al.* 1996). Runeckles and Vaartnou (1997) showed that the formation of O_2^- *in situ* in leaves exposed to ozone, could be reduced if the leaves were infiltrated with ascorbate prior to ozone exposure.

Reduced glutathione (γ-glutamyl-cysteinyl glycine (GSH)) is another antioxidant that can protect thiol groups of enzymes and detoxify ROS such as $^1O_2$ and $HO_2\cdot$ (Foyer *et al.* 1994; Kanofsky and Sima 1995). It is also important in the regeneration of reduced ascorbate (dealt with in more detail in section 1.4.2) and has the potential to directly scavenge ozone within the cell (Runeckles and Chevone 1992). Increases in total glutathione levels in plants following ozone exposure have been reported (Runeckles and Chevone 1992; Heath 1988), but the picture is not consistent. While GSH increased significantly in the tobacco cultivar Samsun following ozone exposure, GSH levels in the cultivars Bel B and Bel W3 did not (Wellburn and Wellburn 1996) and

in tomato, glutathione levels declined following ozone exposure (Kirtikara and Talbot 1996).

Ozone exposure (120 ppb/2 hr for two days) induces accumulation of polyamines in plants (Wellburn and Wellburn 1996) and it has been shown that polyamines administered to the roots of the ozone-sensitive tobacco cultivar, Bel-W3, prior to ozone exposure reduces ozone injury significantly (Bors *et al.* 1989). Polyamine metabolism seems to be involved in conditioning plant tolerance against ozone (Langebartels *et al.* 1991). For example, the activity of a key enzyme in polyamine synthesis, arginine decarboxylase, increased rapidly in the more ozone tolerant tobacco cultivar Bel B in response to ozone exposure, whereas the increase was much slower in Bel W3 (Langebartels *et al.* 1991). The endogenous levels of polyamines (both conjugated and free putrescine) also increased more rapidly in Bel B than in Bel W3 in response to ozone (Langebartels *et al.* 1991). It is not clear how polyamines provide protection against ozone stress. The conjugated form (conjugated predominantly with hydroxycinnamic acid) has been argued to serve, to some degree, as a radical scavenger in tobacco (Bors *et al.* 1989) but free polyamines may also protect plants from ozone injury by inhibiting lipid peroxidation, without being effective radical scavengers (Bors *et al.* 1989). For example, polyamines could chelate metal ions in the plasmalemma, where the presence of metal ion (e.g. Fe^{+2}) catalyzes the formation of $OH\cdot$ radicals (see section 1.2.2) (Bors *et al.* 1989; Kangasjarvi *et al.* 1994).

In general, there are no consistent differences in changes in the pools of various antioxidants between ozone-sensitive and ozone-tolerant cultivars (Wellburn and Wellburn 1996). In a given case, however, it may be possible to deduce why certain cultivars are sensitive or tolerant, if we assume that different cultivars may rely on different pools of antioxidants (Wellburn and Wellburn 1996). Gross tissue analysis of leaves for potential scavengers of ozone has its limitations, however, since pools of compounds like ascorbate and glutathione are very likely compartmentalized (Runeckles and Chevone

1992). This is a limitation that would also apply to enzyme activity measurements in leaves (see 1.4.2).

1.4.2 Ozone and enzymatic reactive oxygen species scavenging

The ability of plants to enzymatically scavenge ROS depends largely on induction of superoxide dismutases (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and catalases (Foyer *et al.* 1994; Kangasjarvi *et al.* 1994). In addition to these established antioxidant enzymes, others, including glutathione S-transferase (GST), guaiacol peroxidase and glutathione peroxidase, may also be significant for ROS scavenging in plants (Conklin and Last 1995). Together, these enzymes provide the plant with a very efficient ROS scavenging mechanism that is able to adapt to adverse oxidative stress conditions (Foyer *et al.* 1994).

Different types of SOD are located in chloroplasts, mitochondria and the cytosol, and catalyze the following reaction (Bowler *et al.* 1992):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

This is an important reaction since O_2^- is generally more toxic than H_2O_2 and since O_2^- can also react with H_2O_2, in the presence of a metal ion (Haber-Weiss reaction), to form the highly reactive OH· radical (see section 1.2.2)(Asada 1992; Salter and Hewitt 1992). Ozone exposure can induce increases in total SOD activity in plants (Chanway and Runeckles 1984; Rao *et al.* 1995; Kirtikara and Talbot 1996). In *Arabidopsis thaliana,* ozone exposure induced increased mRNA levels for the cytosolic SOD-isoform (Cu/Zn-SOD) but reduced the mRNA levels for the chloroplastic isoform (Fe-SOD) (Conklin and Last 1995).

One product of the SOD-catalyzed reaction is H_2O_2, which is important to scavenge since it participates in the Haber-Weiss reaction (Van Camp *et al.* 1994). The plant can utilize both catalases and APX for the destruction of H_2O_2 and their activity is

closely associated with SOD activity (Van Camp *et al.* 1994; Sen Gupta *et al.* 1993). Catalase is located in peroxisomes and catalyzes the following reaction:

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

Ozone treatment has been reported to induce increases in catalase in plants (Willekens *et al.* 1994). The importance of catalase for ROS scavenging in the cytosol and chloroplasts might be limited , however, because of its exclusive compartmentalization in peroxisomes and its low affinity for H_2O_2 (Foyer *et al.* 1994; Asada 1992).

APX participates in the so-called ascorbate-glutathione cycle which is located in both chloroplasts and the cytosol (Kangasjarvi *et al.* 1994). The reactions of the ascorbate-glutathione cycle are summarized in Figure 1.1.

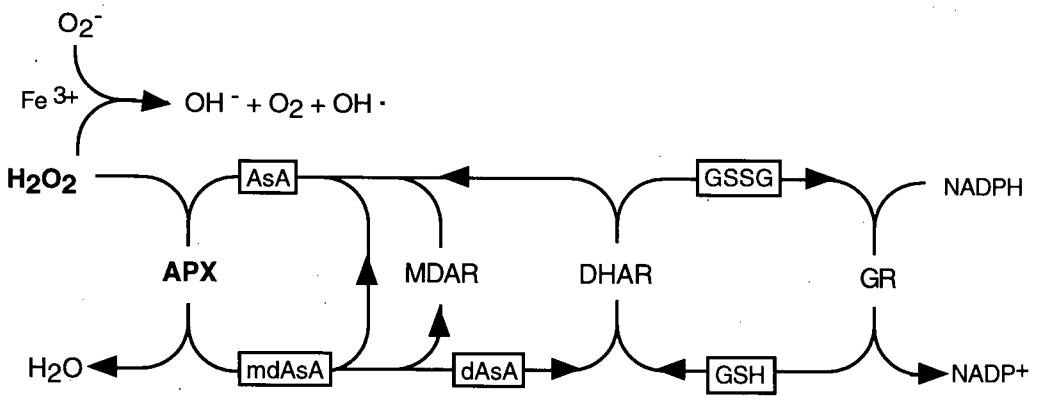


Figure 1.1 Diagrammatic presentation of the role of ascorbate peroxidase in the ascorbate-glutathione pathway in hydrogen peroxide scavenging (adapted from Foyer et al. 1994).

Abbrevations: APX; ascorbate peroxidase, AsA; reduced ascorbic acid, mdAsA; monodehydroascorbate, dAsA; dehydroascorbate, MDHAR; monodehydroascorbate reductase, DHAR; dehydroascorbate reductase, GSH; reduced glutathione, GSSG; oxidized glutathione, GR; glutathione reductase.

The first step in this cycle is the reduction of H_2O_2 to H_2O, catalyzed by APX using ascorbate as the electron donor (Mittler and Zilinskas 1991b):

$$2 \text{ ascorbate} + H_2O_2 \rightarrow 2 \text{ monodehydroascorbate} + 2 H_2O$$

Under non-stressful circumstances, more than 90% of the total ascorbate pool exists in the reduced form and the ascorbate-glutathione cycle has the capacity to maintain this pool primarily in the reduced form (Smirnoff and Pallanca 1996). Regeneration of reduced ascorbate is accomplished by two routes. Monodehydroascorbate can be directly reduced by monodehydroascorbate reductase using NAD(P)H as an electron donor (Foyer *et al.* 1994; Hossain *et al.* 1984). Alternatively, monodehydroascorbate can disproportionate to ascorbate and dehydroascorbate (Foyer *et al.* 1994). Dehydroascorbate is unstable at physiological pH (Smirnoff and Pallanca 1996) but it is reduced to ascorbate by dehydroascorbate reductase, using GSH as an electron donor. The resulting GSSH (oxidized glutathione) is reduced to GSH by glutathione reductase in an NAD(P)H dependent reaction (Foyer *et al.* 1994; Kangasjarvi *et al.* 1994). This pathway is considered to be especially important in chloroplasts which do not contain catalase but do generate H_2O_2 (Smirnoff and Pallanca 1996).

Ozone exposure is known to affect the expression of antioxidant enzymes. For example, both APX and GR activity and mRNA transcript levels were enhanced following ozone exposure in number of different plant species (Conklin and Last 1995; Rao *et al.* 1995; Mehlhorn 1990; Tanaka *et al.* 1988). These observations do not, however, necessarily reflect changes in expression of individual isoforms (Rao *et al.* 1995).

Glutathione S-transferase (GST) mRNA transcript levels are increased dramatically and very rapidly following ozone exposure (Conklin and Last 1995; Sharma and Davis 1994). GST detoxifies the by-products of lipid peroxidation by conjugation of glutathione with alkyl free radicals (Pickett and Lu 1989).

In summary, the increases in activities and gene expression of antioxidant enzymes following ozone exposure strongly suggest that regulation of the expression of

these enzymes provides the plant with additional defences against oxidative stress (Foyer *et al.* 1994).

Ozone treatment can also affect enzymes of the phenylpropanoid pathway. Increases in activity and mRNA transcript levels of phenylalanine-ammonium lyase (PAL), chalcone synthase (CHS) and p-coumarate:CoA ligase (4CL) following ozone exposure have been observed in parsley and tobacco (Bahl *et al.* 1995; Eckey-Kaltenbach *et al.* 1994). This pathway may play a significant role in ozone defence since it synthesizes many compounds (e.g. flavonoids; hydroxycinnamyl amides or esters) that could be potentially protective against ROS generated during ozone exposure (Kangasjarvi *et al.* 1994).

How ozone affects plant gene expression is not known. Direct alteration of DNA-binding sites in DNA-binding proteins (transcription factors) by ozone or its derivatives may influence mRNA transcription, although it seems unlikely that this would produce specific response patterns (Steinberg *et al.* 1990). Events further upstream in well established signal transduction pathways could also be responsible for ozone effects on gene expression. These signal transduction pathways are already an integrated part of a sophisticated defence system in plants, and several studies suggest that ROS can mediate some of the common responses observed under different environmental stresses.

1.5 SIGNAL TRANSDUCTION AND ENVIRONMENTAL STRESS

The sedentary nature of all plants necessitates an accurate sensing of fluctuating, often unfavourable, environmental conditions (Trewavas and Gilroy 1991). The dynamic aspects of this process include phenomena such as discrimination between self and non-self, and the determination of symbiotic and pathogenic relationships (Hahlbrock *et al.* 1995), followed by a coordinated response that optimizes plant survival, growth and development (Trewavas and Gilroy 1991). In this process a complex cascade of *stimulus-transmittance-response* couplings takes place: an extracellular signal (stimulus) is

recognized by the plant and converted into intracellular signals that are transmitted (signal transduction) and subsequently translated into an array of biochemical changes (response) in target cells (Hahlbrock *et al.* 1995; Bowler and Chua 1994). The extracellular stimulus can take the form of abiotic or biotic factors. The signal transduction is fast, sensitive and specific, and appears to be modulated by counteracting negative- and positive-controlling elements (Bowler and Chua 1994).

In contrast to animal systems, signal transduction pathways in plants are poorly understood (Cote and Crain 1994), although intensive research is now producing information about such signal transduction pathways (Bowler and Chua 1994), especially in plant-pathogen interactions. Signal transduction pathways following other environmental stresses, such as wounding, have also been extensively studied, and an understanding of signaling following low-temperature stress is emerging (Monroy and Dhindsa 1995).

It appears that ROS and oxidative stress play an important part in signaling pathways in plants responding to these different environmental stresses. These pathways, therefore, might provide us with a better understanding of how plants respond to a sudden increase in oxidative stress, such as that following ozone exposure (see section 1.2). Since the emphasis in stress signal transduction research in plants in recent years has been on plant-pathogen relationships and mechanical injury, such as insect feeding, the literature review below is drawn primarily from these studies.

1.5.1 Oxidative burst

One of the earliest processes observed in plants in response to pathogens or elicitors is an *oxidative burst*, a sudden release of ROS in the plant tissue (Mehdy 1994; Legendre *et al.* 1993). In plant-pathogen interactions, the increase is usually fast and transient and the predominant ROS are O_2^-, H_2O_2 and $OH\cdot$ (Mehdy 1994). For example, in cultured soybean cells, H_2O_2 levels increased in less than five minutes after fungal elicitor addition (Apostol *et al.* 1989). It is generally accepted that elicitors of the

oxidative burst are pathogen-derived macromolecules or host cell-wall fragments released during pathogen invasion (Yahraus *et al.* 1995).

In plant-bacterial interactions, the oxidative burst can be divided into two distinct phases; an early, short-lived burst (Phase I) and a delayed, long-lived burst (Phase II). Phase I is relatively non-specific and occurs immediately after the addition of either a compatible or incompatible pathogen (see section 1.5.2) (Baker and Orlandi 1995). Phase II occurs 1.5 to 3 hr after inoculation and, in contrast to the Phase I oxidative burst, appears to be specific to incompatible interactions in several plant species, including tobacco (Baker and Orlandi 1995).

Mechanical pressure has also been shown to induce rapidly (in a few minutes) an oxidative burst in cell suspension culture (Yahraus *et al.* 1995). It has been suggested that mechanically-derived signals resulting from degradation, puncture, or deformation of cell walls and plasmalemma could be a more universal sensor for pathogen invasion than species-specific interactions, although little information is available concerning the physical pressure associated with pathogen penetration (Yahraus *et al.* 1995). The rapid generation of ROS has also been observed following wounding in *Zinnia elegans* (Olson and Varner 1993), UV-irradiation in tobacco (Green and Fluhr 1995) and chilling stress in maize seedlings (Prasad *et al.* 1994).

Model for the generation of oxidative burst in plant-pathogen interaction and the role of Ca^{+2} influx

The process leading to the oxidative burst in plant-pathogen interactions may closely resemble the so called *respiratory burst* that phagocytes utilize to kill invading bacteria (Baker and Orlandi 1995). For example, ROS generation in several plant species, as in animal cells, appears to involve Ca^{+2} influx and phospholipase and protein kinase activation (Hahlbrock *et al.* 1995; Chandra and Low 1995; Legendre *et al.* 1993; Mehdy 1994). The key event in the generation of the oxidative burst appears to be the mobilization of Ca^{+2} (Mehdy 1994) and one of the most rapid responses to elicitors

reported so far is the Ca^{+2} influx across the plasmalemma (Nurnberger *et al.* 1994). As pointed out previously (see section 1.3.3), plant cells avoid the potentially toxic effects of Ca^{+2} by maintaining a very low level of free Ca^{+2} in the cytoplasm, three to four orders of magnitude lower than in other cellular compartments (Bush 1993; Heath 1988). Ca^{+2} passes into the cytoplasm from organelles or the apoplast via special membrane-spanning protein channels driven by a strong electrochemical gradient (Tester 1990; Heath 1988). The intracellular Ca^{+2} is constantly extruded back into cellular organelles (e.g. vacuoles and endoplasmic reticulum) or into the apoplast, against this strong electrochemical gradient, by ATP-dependent membrane-bound transporter proteins (Bush 1993; Castillo and Heath 1990; Heath 1988).

The specific role for Ca^{+2} in generation of the oxidative burst remains to be fully elucidated but it is possible to describe a hypothetical model using both elements that are known to exist in various plant cells (Bush 1993) and pathways that have been established in animal cells (Cote and Crain 1994). The first step in this simplified model is the binding of an elicitor (stimulus) to a plasmalemma-bound receptor that is coupled with a GTP-binding protein (G-protein). The binding of elicitor to the receptor stimulates the exchange of GTP for GDP in the α-subunit of the heterotrimeric G-protein, which leads to dissociation of the α subunit from the remaining two subunits (β and γ). In this model the α subunit (now coupled with GTP) activates a plasmalemma-associated phospholipase C (PLC) that hydrolyzes phosphoinositides (e.g. PIP_2; phospholipids with inositol phosphate) in the plasmalemma to yield inositol triphosphate (IP_3) and diacylglycerol (DAG). The IP_3 triggers opening of Ca^{+2} channels in the plasmalemma, which leads to Ca^{+2} influx and a sudden increase in intracellular Ca^{+2} concentrations. This increase activates Ca^{+2}-dependent protein kinase (CDPK), which can phosphorylate NAD(P)H oxidase (or NADH oxidase) located in the plasmalemma. This modification activates the NAD(P)H oxidase which rapidly reduces O_2 to O_2^-, using NAD(P)H as the electron donor. Part of this O_2^- can be converted to H_2O_2 (see section 1.2.1), generating an

oxidative burst (Cote and Crain 1994; Mehdy 1994; Bush 1993; Legendre *et al.* 1993; Trewavas and Gilroy 1991).

This model describes a transient activation of Ca^{+2} channels located in the plasmalemma but it should be emphazised that IP_3 could also affect Ca^{+2} channels in membranes of subcellular compartments that can store Ca^{+2}, such as the endoplasmic reticulum and vacuoles (Cote and Crain 1994; Trewavas and Gilroy 1991).

Evidence supporting the model for generation of oxidative burst

All the known elements of Ca^{+2}-dependent signal transduction in animal systems have been found in plants (Trewavas and Gilroy 1991), although they have not yet all been observed in any one cell (Bush 1993). Evidence for these elements will be discussed in the sequence in which they act during signal transduction. Several receptors for elicitors have now been localized to the plasmalemma (Mehdy 1994). For example, parsley membranes contain a high affinity binding site for an oligopeptide (13 amino acids) that is responsible for the oxidative burst elicitor capability of a fungal glycoprotein elicitor (Nurnberger *et al.* 1994). The binding site has all the characteristics for a receptor since the binding of the ligand (oligopeptide) is reversible, saturable and highly specific (Nurnberger *et al.* 1994).

G-proteins have highly conserved structures (Wilkie and Yokoyama 1994) and DNA sequence information from mammalian G-proteins has been successfully used to clone G-protein α subunits from *Arabidopsis*, tomato and rice (Ishikawa *et al.* 1995; Ma *et al.* 1991; Ma *et al.* 1990). Legendre *et al.* (1993) showed that administration of mastoparan (a fourteen-residue oligopeptide from wasp venom that directly activates G-proteins) initiated an oxidative burst in soybean cultures in the absence of elicitors. The presumed effector of the dissociated α subunit is phospholipase C (Legendre *et al.* 1992) which hydrolyzes PIP_2 to IP_3 and DAG (Cote and Crain 1994). The presence of both phospholipase C and enzymes that synthesize PIP_2 has been demonstrated in plants (Cote and Crain 1994; Tate *et al.* 1989) and a cDNA encoding plasmalemma-

associated phospholipase C in soybean has been cloned (Shi *et al.* 1995). PIP_2 and IP_3 have also been measured in plant cells (Cote and Crain 1994) and Legendre *et al.* (1993) showed that IP_3 levels in cultured soybean cells increased after only one minute following elicitor or mastoparan treatment. They also showed that the oxidative burst following elicitor treatment can be inhibited by pretreating the culture with an inhibitor for PLC (neomycin sulfate) (Legendre *et al.* 1993).

In parsley cell suspension cultures, a transient Ca^{+2} influx was observed within two to five min following elicitation and this increase was followed by an immediate increase in H_2O_2 (Nurnberger *et al.* 1994). The importance of Ca^{+2} channels for the oxidative burst in plant-pathogen interactions has been demonstrated by Baker *et al.* (1993). They showed that the Ca^{+2} channel-blocker La^{+3} inhibited oxidative burst in tobacco cell suspension following elicitor treatment. Another study showed that adding EGTA (a chelator of Ca^{+2}) to suspension-cultured spruce cells significantly reduced the oxidative burst, demonstrating the importance of an extracellular source for Ca^{+2} (Schwacke and Hager 1992).

Ca^{+2} influx has not been associated only with plant-pathogen interactions; many stimuli can initiate this influx. For example, cold-shock and mechanical stress induce Ca^{+2} influx (Monroy and Dhindsa; Bush 1993) suggesting that Ca^{+2} influx might be a general response to environmental stress.

Protein phosphorylation has been demonstrated to regulate diverse cellular processes in plants and a number of studies indicate that phosphorylation changes are important in plant defence signaling pathways (Chandra and Low 1995). Ca^{+2} -dependent protein kinases have been identified in plants and a few of them have been cloned (Abo-El-Saad and Wu 1995). There is also evidence for protein kinase involvement in generation of the oxidative burst, since staurosporine and K252a (protein kinase inhibitors) can block the oxidative burst in elicitor-treated soybean and spruce cell cultures (Tenhaken *et al.* 1995; Schwacke and Hager 1992).

No direct evidence is available for the presence of an NAD(P)H oxidase participating in the oxidative burst in plants (Baker and Orlandi 1995), but antibodies raised against subunits of a human (neutrophil) NAD(P)H oxidase complex cross-reacted with proteins with the same molecular weight from plants (Dwyer *et al.* 1996). Furthermore, a specific inhibitor of the human NAD(P)H oxidase is also able to inhibit the oxidative burst in soybean cell suspension cultures (Dwyer *et al.* 1996). There is, however, some doubt about the potential role of NAD(P)H oxidase in the oxidative burst. In elicitor-treated cell cultures of French bean, the rapid oxidative burst was not accompanied by enhanced activation of membrane-bound NAD(P)H oxidase (Bolwell 1996). Instead, it appeared that cell-wall peroxidases were involved, since the oxidative burst was blocked by cyanide, which is a known peroxidase inhibitor (Bolwell 1996).

The phosphorylation state of the cell is in a dynamic equilibrium, meaning that proteins are continuously being phosphorylated by protein kinases and dephosphorylated by phosphoprotein phosphatases (Barford 1991). There is evidence that both protein kinases and phosphatases are involved in the oxidative burst, adding to the complexity of pathways leading to it (Chandra and Low 1995). In fact, phosphatase inhibitors (*calyculin A* and *okadaic acid*) can induce the oxidative burst in soybean cell suspension cultures in the absence of an elicitor (Chandra and Low 1995). Vera-Estrella *et al.* (1994) showed that elicitor-treatment of tomato cell suspension cultures, or addition of the G-protein activator, mastoparan, increased a membrane-bound NADH oxidase activity. However, in contrast to the result from Chandra and Low (1994), the elicitor-induced increase in NADH oxidase activity was almost completely abolished when okadaic acid was included in the culture (Vera-Estrella *et al.* 1994). These investigations also showed that elicitor treatment of tomato cell suspension cultures, or addition of mastoparan, led to a decrease in membrane-bound APX activity. Furthermore, this reduction could be abolished if okadaic acid was included. This indicates that the elicitor-induced increase in H_2O_2 could be due partly to inhibition of an H_2O_2-scavenging

mechanism (Vera-Estrella *et al.* 1994). In this model APX would be constitutively active (i.e. phosphorylated) but becomes inactive following dephosphorylation. In general, these results suggest that phosphatases become activated during plant-pathogen interaction, and that G-proteins can activate both protein kinases and phosphatases.

In summary, there are a number of different studies that suggest the participation of G-proteins, PLC, protein kinases, phosphatases and Ca^{+2} channels in both plant-pathogen interactions and environmental stress responses, but it is apparent that the *stimulus-transmittance-response* coupling is very complicated. For instance, this simple linear model does not readily explain the mechanism for two distinct phases in ROS production following plant-bacteria interaction (see section 1.5.1). It appears, however, that ROS can mediate diverse cellular processes, perhaps through multiple pathways. This could be manifested in changes in gene transcription and the metabolic state of the cell, which, ultimately, could affect its survival. This will be dealt with in the following section.

1.5.2 Oxidative burst and hypersensitive response (HR)

Attempted infection by a pathogen can induce a hypersensitive response (HR), a phenomenon which is central to natural disease resistance in plants (Hahlbrock *et al.* 1995). The pathogen is recognized by the host, which then quickly responds to the invasion by initiating a complex defensive strategy. A number of physiological, molecular and biochemical events are concomitant with the onset of HR (Hahlbrock *et al.* 1995; Malamy and Klessig 1992). These include cross-linking of cell wall proteins, induction of cellular protectant genes, and ultimately, hypersensitive cell death. Furthermore, HR is frequently accompanied by induction of systemic acquired resistance (SAR) (Tenhaken *et al.* 1995; Baker and Orlandi 1995; Prasad *et al.* 1994; Malamy and Klessig 1992).

Plant pathogens that evoke HR are called avirulent, or incompatible, and the plant host is said to be resistant. The interaction between an incompatible pathogen and the plant host depends on "gene-for-gene"- matching between an *avr* (avirulent) gene in the

pathogen (which might encode an elicitor) and a corresponding *R* (resistance) gene in the host (which might encode a receptor) (Staskawicz *et al.* 1995). If either the *avr* gene or the *R* gene is missing in this interaction, the pathogen, which is now called virulent, or compatible, can spread throughout the plant, causing damage or even death of the host (Malamy and Klessig 1992).

The mechanism of HR is not fully understood but recent evidence indicates that H_2O_2 production and the oxidative burst play a central role in its orchestration (Bolwell 1996; Hahlbrock *et al.* 1995; Levine *et al.* 1994).

Hypersensitive response and cell wall cross-linking

Reactive oxygen species generation following pathogen invasion can directly modify cell wall chemistry (Baker and Orlandi 1995). For example, elicitor treatment caused a rapid, H_2O_2-dependent, cross-linking of cell wall structural proteins in soybean cell suspension culture (Bradley *et al.* 1992). This rapid (less than two minutes) cross-linking was also observed in tissues immediately surrounding incisions in wounded plants (Bradley *et al.* 1992). The elicitor-induced cross-linking of cell wall proteins is thought to harden the cell wall against attacks by hydrolytic enzymes secreted by the pathogen. This could slow the pathogen ingression and give the plant cell sufficient time to activate transcription-dependent defences (Tenhaken *et al.* 1995).

Hypersensitive response and gene regulation

One of the proposed roles for the oxidative burst and ROS in environmental stress responses is regulation of gene transcription (Mehdy 1994). A large number of genes in plants respond to pathogen elicitation, and these changes in gene transcription coincide with the onset of HR (Hahlbrock *et al.* 1995). The genes involved include those participating in phenylpropanoid metabolism, as well as genes encoding proteinase inhibitors (Pin), pathogenesis-related proteins (PR), or defence genes such as GST, which metabolizes the products of membrane lipid peroxidation (Hahlbrock *et al.* 1995; Levine *et al.* 1994; Malamy and Klessig 1992).

The complex nature of gene regulation coinciding with HR was clearly demonstrated by Levine *et al.* (1994). They showed that soybean cell cultures treated with either H_2O_2 or a fungal elicitor accumulated GST transcripts, whereas addition of H_2O_2 traps (such as dimethylthiourea or catalase) to the culture inhibited the GST induction (Levine *et al.* 1994). The GST accumulation was blocked if the cell culture was treated with either the NADPH oxidase inhibitor diphenylene iodonium (DPI) or protein kinase inhibitors (staurosporine and K252a), treatments that simultaneously blocked the oxidative burst (Levine *et al.* 1994). The same study showed that PAL and chalcone synthase (CHS), two enzymes of the phenylpropanoid pathway, were strongly induced by the fungal elicitor, but only weakly induced by H_2O_2. Furthermore, staurosporine and DPI had no effect on elicitor-induced expression of CHS. These results indicate that elicitor-induced gene expression is mediated by at least two pathways, one that involves the oxidative burst (H_2O_2), and another that is independent of the oxidative burst (Levine *et al.* 1994).

Similar conclusions can be drawn from a study by Green and Fluhr (1995), who investigated the importance of ROS in the UV-B induction of PR-1 in tobacco leaves. When tobacco leaves were treated with antioxidants (that scavenge ROS), the UV-B induced accumulation of PR-1 was inhibited, whereas an ROS generator could directly induce PR-1 expression (Green and Fluhr 1995). UV-irradiation can induce salicylic acid (SA) accumulation in one day (Yalpani *et al.* 1994) and Green and Fluhr (1995) showed that PR-1 was induced by SA. This SA-induction was unaffected by simultaneous addition of antioxidants. Earlier, Ward *et al.* (1991) had shown that both pathogen inoculation (TMV) and SA treatment coordinately induced elevated transcript levels for ten different PR-proteins, including PR-1. It has also been demonstrated that SA levels of TMV-treated leaves increased dramatically in TMV-resistant tobacco cultivars, whereas no SA increase was observed in susceptible cultivars (Malamy and Klessig 1992). These results are consistent with the concept that at least two different pathways,

presumably both activated in HR, can induce PR-1 accumulation. One of these is dependent on ROS, and the other, on SA.

The potential role for endogenous SA in plant defence responses has been a matter of considerable debate, however. Recently, Chen *et al.* (1993) isolated from tobacco a cDNA encoding a SA-binding catalase that is inhibited by SA (*in vitro* and in crude extracts). They showed that SA treatment of detached leaves increased H_2O_2 levels by 50 to 60% and that injecting leaves with H_2O_2 induced the expression of PR-1 (Chen *et al.* 1993). According to these results, the role of SA as a signal for gene transcription appears to be mediated through H_2O_2 which may act as a second messenger on the route to PR-1 induction (Chen *et al.* 1993). They also constructed a transgenic tobacco plant expressing antisense RNA for this catalase gene, and preliminary results showed that this plant constitutively expressed the PR-1 genes (Chen *et al.* 1995). These results seem, however, to be inconsistent with the ROS-independent induction of PR-1 reported by Green and Fluhr (1995). Yong-Mei *et al.* (1995) also showed that neither SA feeding nor avirulent pathogen inoculation reduced catalase activity in tobacco plants, and that H_2O_2 was, in fact, only a weak inducer of PR-1 (Yong-Mei *et al.* 1995).

It is still uncertain how increased oxidative stress can affect gene transcription in plants, but the function of H_2O_2 as a second messenger for gene transcription in mammalian cells is better established (Levine *et al.* 1994). NF-κB is a transcription factor which is sequestered in the cytosol of many different mammalian cell types (Schreck *et al.* 1991). In its non-DNA-binding form it is composed of three subunits, but upon activation, one subunit, IκB, is released. The remaining dimer is translocated into the nucleus where it activates a number of different defence genes (Schreck *et al.* 1991). In mammalian cells, H_2O_2 is able to mediate the activation of NF-κB (i.e. the release of the IκB subunit) (Schreck *et al.* 1991) and it has been demonstrated that the direct target of H_2O_2 is a G protein (called $p21^{ras}$) which, in turn, mediates activation of a mitogen-activated protein

kinase (MAPK). This indicates that protein phosphorylation is contributing to the activation of NF-κB (Lander *et al.* 1995). Although MAPKs have been cloned from several species (Seo *et al.* 1995) homologues for NF-κB have not been found in plants. Therefore, NF-κB may have no relevance at all to MAPKs in plants.

Hypersensitive response and hypersensitive cell death

The first microscopically visible sign of successful HR in plants is the formation of localized necrotic lesions in the host (Bolwell 1996; Hahlbrock *et al.* 1995). These lesions are the manifestation of hypersensitive cell death, or rapid collapse and death in challenged host cells (Tenhaken *et al.* 1995). This response, together with cell wall cross-linking and changes in gene expression, are thought to limit the spread of the pathogen by trapping it in and around the lesion, thus preventing a systemic infection in the plant (Tenhaken *et al.* 1995).

H_2O_2 from the oxidative burst is both necessary and sufficient to trigger hypersensitive cell death (Tenhaken *et al.* 1995). Blocking the oxidative burst by using a NADPH oxidase inhibitor, a protein kinase inhibitor, or a catalase, reduces pathogen induced hypersensitive cell death in soybean cell culture (Levine *et al.* 1994). A several-fold higher concentration of H_2O_2 is, however, required to induce cell death than to induce defence gene expression (Levine *et al.* 1994), and unlike induction of defence gene transcription following HR, hypersensitive cell death appears to be dependent on H_2O_2 threshold values (Tenhaken *et al.* 1995). For example, Levine *et al.* (1994) showed that 4 - 6 mM H_2O_2 was needed to induce cell death in soybean cell cultures, while transcripts of GST (defence gene) accumulated in response to addition of only 1 - 2 mM H_2O_2. Levine *et al.* (1994) speculate that H_2O_2 can function as a mobile intercellular alarm signal, diffusing from infected cells (with high H_2O_2 levels and undergoing hypersensitive cell death) to adjacent cells. This, in turn, activates cellular protectant genes (but not hypersensitive cell death) in these neighbouring cells, since H_2O_2 has not reached the threshold levels required to trigger hypersensitive cell death.

Hypersensitive response and systemic acquired resistance (SAR)

Once HR has been activated, the plant is often more capable of resisting subsequent pathogen attack. This phenomenon, which is called systemic acquired resistance (SAR), is a long-lasting, broad-spectrum systemic resistance that has been demonstrated in many plant species, infected by incompatible and sometimes, compatible pathogens (Ryals *et al.* 1994). In an experiment carried out by Ward *et al.* (1991) one leaf (primary) on a tobacco plant was inoculated with TMV and the effects of a second TMV inoculation on a distal (secondary) leaf were measured. The primary leaf developed the characteristic HR (hypersensitive cell death) but the secondary leaf, which was inoculated seven days later with TMV, developed smaller and many fewer necrotic lesions than secondary leaves on plants that had not been inoculated earlier with TMV (Ward *et al.* 1991).

The mechanism for SAR is far from being understood but experimental evidence suggests that SA plays an important role in SAR (Ryals *et al.* 1994). Endogenous SA levels increase dramatically in the leaf following avirulent pathogen infection (Malamy and Klessig 1992), as well as in distal, non-inoculated leaves (Malamy and Klessig 1992), and localized application of SA can increase TMV resistance in the treated tobacco leaves within three days (Ward *et al.* 1991). SA treatment, however, cannot lead to a systemic increase in SA in tobacco nor can it activate SAR (Horvath and Chua 1994). The importance of SA accumulation for SAR was demonstrated by Gaffney *et al.* (1993). They produced a transgenic tobacco plant (called NahG) expressing the bacterial gene for salicylate hydroxylase, an enzyme which catalyzes decarboxylative hydroxylation of SA, generating catechol. The NahG plants did not accumulate SA following pathogen infection and they did not display induction of SAR (Gaffney *et al.* 1993). This study did not resolve the question whether SA is the systemic signal necessary for SAR, however.

Rasmussen *et al.* (1991) showed in cucumber that the signal for the systemic increase in SA exits the inoculated leaf before any significant increase in SA is detected in

the phloem exudates of the inoculated leaf. This suggests that SA is not the translocated SAR signal. Similar conclusions can be drawn from another study where the NahG plant, and its parental genotype (Xanthi), were used in reciprocal grafting experiments. When a Xanthi rootstock leaf was inoculated with TMV, the NahG-grafted scion leaves did not develop SAR when challenged seven days later with TMV (Vernooij *et al.* 1994). In contrast, when the rootstock was from NahG but the scion from Xanthi, SAR was manifested in the Xanthi scion, to the same extent as that observed in Xanthi/Xanthi grafted control plants (Vernooij *et al.* 1994). The systemic expression of PR-1 mRNA was also observed in Xanthi scions on NahG rootstocks. Interestingly, despite the SAR development in the Xanthi scion/NahG rootstock plants the NahG rootstock showed no significant increase in SA levels (Vernooij *et al.* 1994).

These results can be compared to a recent study where transgenic tobacco plants with suppressed levels of PAL were used in similar grafting experiments. These plants accumulate less SA following TMV inoculation (Pallas *et al.* 1996), not surprisingly, since PAL is essential for SA synthesis (Lee *et al.* 1995). Reciprocal grafts between wild-type and PAL-suppressed plants showed that PAL-suppressed rootstocks were able to establish SAR in wild-type scion but not *vice versa* (Pallas *et al.* 1996).

The conclusions drawn from these two studies are that SA is most likely not the translocated signal necessary for SAR in tobacco, but that SA accumulation, based at least partly on newly synthezised SA, is required in the distal leaf for the establisment of a resistant state (Pallas *et al.* 1996; Vernooij *et al.* 1994). These results, however, cannot exclude the possibility that only very low SA build-up in the primary inoculated leaf is needed for SA to become an effective mobile signal for systemic protection.

Shulaev *et al.* (1995) have shown that a substantial part of the SA that accumulates in distal leaves of tobacco plants following TMV inoculation is transported from the inoculated leaf, and that detaching the inoculated leaf before it starts to accumulate

SA, blocks systemic SA increase (Shulaev *et al.* 1995). These results demonstrate that the role of SA in SAR is far from being completely understood.

1.5.3 Jasmonate biosynthesis pathway and stress signaling

In recent years jasmonates (jasmonic acid and related molecules) have attracted increased attention from plant researchers. Jasmonic acid (JA) and its volatile methyl ester, methyl jasmonate (MeJA) have been detected in every plant species, healthy or stressed, investigated so far (Gross and Parthier 1994), and recent evidence suggests that jasmonates may play an important role in signaling normal developmental events (Staswick 1993). It is, however, their presumed role in the wounding response and plant defence that has created the most interest.

Physiological and biochemical effects of jasmonates

Jasmonates affect a variety of physiological and biochemical processes in plants. For example, jasmonates inhibit seedling stem growth, seed germination, chlorophyll formation and photosynthesis (Sembdner and Parthier 1993) and can induce fruit ripening, chlorophyll degradation, respiration, leaf senescence and ethylene biosynthesis (Sembdner and Parthier 1993; Saniewski *et al.* 1987). Jasmonates promote stomatal closure in some plant species (Sembdner and Parthier 1993) or have no effects on their opening in others (Creelman and Mullet 1995; Horton 1991).

Jasmonate biosynthesis pathway

The jasmonate biosynthetic pathway in plants has been elucidated (Sembdner and Parthier 1993). It starts with the peroxidation of linolenic acid by lipoxygenase (LOX) with subsequent conversion of the fatty acid to jasmonate by the action of allene oxide synthase (AOS), allene oxide cyclase (AOC), and NADPH-reductase, followed by three steps of B-oxidation (Fig. 1.2) (Sembdner and Parthier 1993; Farmer and Ryan, 1992).

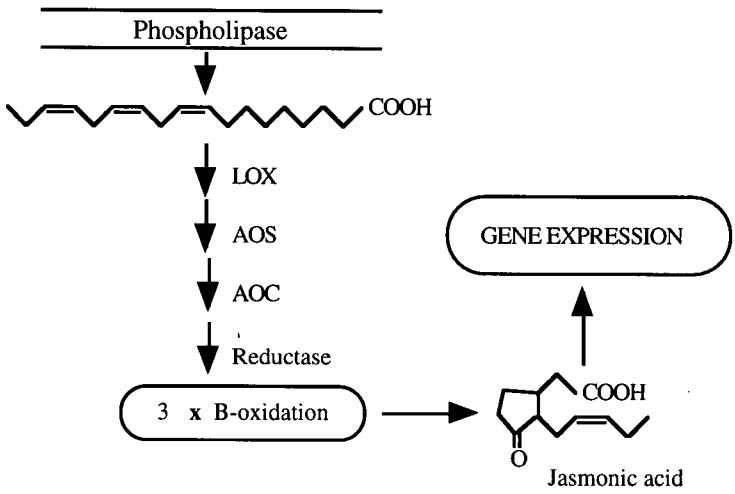


Figure 1.2 Diagrammatic presentation of the biosynthesis of jasmonic acid, starting with the release of linolenic acid by lipase.

The three step B-oxidation presumably takes place in microbodies (adapted from Creelman and Mullet 1995; Sembdner and Parthier 1993; Staswick 1993).

Abbrevations: LOX; lipoxygenase, AOS; allene oxide synthase, AOC; allene oxide cyclase, Reductase; NADPH-reductase.

In addition to these confirmed steps, it has been proposed that the linolenic acid itself is released from plasma membranes by phospholipase action (Creelman and Mullet 1995). It has also been proposed that two parallel jasmonate biosynthetic pathways may exist, one starting in the cytosol and one in the chloroplast, but that they converge to a single pathway in the peroxisomes where the final steps (B-oxidation) take place (Creelman and Mullet 1995). For example, both chloroplastic and cytosolic isoforms of LOX have been cloned from *Arabidopsis thaliana* (Melan *et al.* 1993; Song *et al.* 1993). The presence of AOS and AOC enzyme activities in chloroplasts, and the cloning of a putative chloroplastic isoform of LOX in *Arabidopsis thaliana* and AOS in flax, support further the chloroplastic location of the jasmonate biosynthetic pathway (Creelman and Mullet 1995; Bell and Mullet 1993).

Jasmonates and gene expression

The link between changes in intracellular concentrations of jasmonates and down-stream responses is not well defined but a number of studies have shown that exogenously supplied jasmonates are potent inducers of an array of stress-related genes, including LOX, PAL, proteinase inhibitors I and II (Pin) and polyphenol oxidase (PPO)(Constabel *et al.* 1995; Melan *et al.* 1993; Bell and Mullet 1993; Sembdner and Parthier 1993; Gundlach *et al.* 1992; Farmer and Ryan 1990). At the same time, elevated levels of JA can down-regulate other genes, such as those encoding proteins required for photosynthesis e.g. ribulose bisphosphate carboxylase/oxygenase small subunit (RbcS)(Reinbothe *et al.* 1994). The jasmonate control over gene expression is complicated but can involve transcriptional control, post-transcriptional modification and translational control (Reinbothe *et al.* 1994).

Activation of the jasmonate biosynthesis pathway

Many of the genes induced by jasmonates are associated with plant defence in one way or another (Staswick 1993). This reflects earlier observations that wounding can induce both accumulation of jasmonates and expression of jasmonate-inducible genes (Farmer and Ryan 1990). JA increase has been detected in wounded tobacco leaves as early as thirty minutes after injury (Baldwin *et al.* 1994), and Blechert *et al.* (1995) showed that caterpillar feeding on pea leaves induced a transient JA accumulation in less than fifteen minutes with a peak after forty-five minutes. LOX activity also increased following wounding in *Arabidopsis* (Bell and Mullet 1993). Furthermore, transgenic *Arabidopsis* expressing a chloroplastic LOX transgene that co-suppressed an endogenous chloroplastic LOX gene, also failed to accumulate JA in wounded tissue (Bell *et al.* 1995).

Pathogens also appear to be also capable of inducing jasmonates in different plant species (Blechert *et al.* 1995; Gundlach *et al.* 1992). For example, yeast cell wall elicitor induced JA accumulation within five to fifteen minutes in suspension cell cultures of

the grass *Agrostis tenuis* (Blechert *et al.* 1995). JA accumulation was dependent on protein phosphorylation since it was completely blocked by the protein kinase inhibitor staurosporine (Blechert *et al.* 1995). Seo *et al.* (1995) reported that a transgenic tobacco plant with suppressed levels of MAPK, failed to accumulate jasmonates in response to wounding. These studies demonstrate the importance of protein phosphorylation for JA accumulation in response to wounding or pathogens.

Wounding and systemic response

Localized wounding not only initiates responses in the traumatized leaf but also in distal leaves (Schaller and Ryan 1996). For example, wounding can induce JA and PinII accumulation in distal parts of the plant (Pena-Cortes *et al.* 1988; Baldwin *et al.* 1994) and local application of jasmonate can induce systemic accumulation of PinI (Farmer *et al.* 1992). The translocated signal(s) responsible for the wound-induced systemic response have not yet been identified, but a small mobile peptide (eighteen amino acids), called *systemin*, has been implicated in the systemic response in tomato (Schaller and Ryan 1996; Pearce *et al.* 1991). When applied to cut stems or leaf wounds, systemin can induce both JA accumulation and PinI and II gene expression in distal leaves of tomato (Schaller and Ryan 1996; Doares *et al.* 1995).

Systemin is thus a leading candidate for the translocated signal in wound-induced systemic response, at least in some *Solanaceae* species (Schaller and Ryan 1996). It has been suggested that systemin is translocated through the phloem to distal parts of the plant following wounding, where it stimulates receptors in target cells that activate the jasmonate biosynthesis pathway (Schaller and Ryan 1996; Doares *et al.* 1995). When radiolabeled systemin was applied to a wound site in a tomato leaf, it was transported out of the leaf through the phloem within three hr (Pearce *et al.* 1991). Transgenic tomato plants expressing antisense RNA for prosystemin (systemin precursor), showed strong suppression in systemic wound induction of PinI and II proteins (McGurl *et al.* 1992), consistent with a requirement for systemin as the mobile signal. On the other hand,

McGurl *et al.* (1992) also showed that systemin mRNA is induced in all parts of the plant following localized wounding. Systemic accumulation of systemin may not necessarily be the direct result of systemin export from the wounded leaf, but could be caused by another unknown mobile signal or even jasmonate itself (Baldwin *et al.* 1994; Enyedi *et al.* 1992).

Electrical signals have also been implicated in systemic wound-response. Wildon *et al.* (1992) showed that the wound-induced systemic increase in Pin activity and PinII mRNA levels in tomato seedlings correlated with a systemic electrical signal (action potential) and was not affected by inhibition of phloem translocation. The systemic response (measured as an increase in Pin activity) was detected even when the wounded leaf was excised five minutes after wounding, consistent with the greater speed of the electrical signal relative to a phloem-translocated signal (Wildon *et al.* 1992). No systemic increase in Pin-activity was detected when the wounded leaf was excised before the electrical signal had exited the leaf (Wildon *et al.* 1992). In a similar study carried out by Pena-Cortes *et al.* (1995), an electrical current (10V) was used as a stimulus instead of mechanical wounding. The results showed that localized electrical current stimulation can also cause a systemic increase in PinII mRNA in tomato plants (Pena-Cortes *et al.* 1995). They propose that mechanical wounding may actually generate an electrical signal that is propagated throughout the plant, and that this signal may also elicit the release of active systemin (Pena-Cortes *et al.* 1995).

In contrast to the slower phloem-translocated signal, the speed of the electrical signal exiting wounded leaves could explain the very fast systemic accumulation of mRNA's observed in wounded plants. For example, Pena-Cortes *et al.* (1988) demonstrated that mRNA for PinII becomes detectable systemically within twenty minutes following wounding. An even faster systemic response was detected in tobacco by Seo *et al.* (1995), who detected accumulation of mRNA for MAPK within 10 min in leaves

adjacent to the wounded leaf. It is also possible that multiple systemic signals are involved, with some acting more rapidly than others.

1.5.4 Wounding and hypersensitive response

The extent of overlap or cross-talk between transduction pathways initiated by different effectors, such as wounding or pathogens, is not well defined. For example, pathogens induce accumulation of both the acidic and basic isoforms of PR-proteins in tobacco, whereas wounding induces only the basic isoforms (Sano and Ohashi 1995). Furthermore, pathogens can activate the JA biosynthesis pathway whereas wounding cannot activate SA-accumulation (Sano and Ohashi 1995).

Recent studies suggest that SA is involved in cross-talk between the JA biosynthesis pathway and the SA-mediated pathway activated in plant-pathogen interaction. In tomato plants, Pena-Cortes *et al.* (1993) showed that SA can block JA accumulation following wounding, as well as wound-induced expression of PinII. A second site of interaction is also likely, since SA has been shown to inhibit JA-induced gene expression, presumably by acting at some point between JA accumulation and the transcriptional activation of PinI and PinII (Doares *et al.* 1995). This same study showed that SA can block both the elicitor-induced expression of these genes and the systemin-induced systemic accumulation of PinI and PinII in tomato plants (Doares *et al.* 1995). These observations lead to the suggestions that the SA accumulation observed in HR may, among other things, inhibit both the JA biosynthesis pathway and JA-controlled downstream effects.

The cross-talk between the JA biosynthesis pathway and the SA-mediated pathway also appears to involve a direct impact of JA on the SA pathway. Seo *et al.* (1995) showed that transgenic tobacco plants defective in the JA biosynthesis pathway (co-suppressed MAPK gene expression; see earlier) accumulated SA following wounding, in contrast to wild type tobacco where wounding does not result in SA accumulation (Seo *et al.* 1995). Furthermore, these transgenic plants accumulated acidic PR-protein

transcripts following wounding, instead of basic PR-protein transcripts (Seo *et al.* 1995). Similarly, transgenic tobacco plants in which expression of a small G-protein gene is suppressed, were found to accumulate SA and acidic PR-protein transcripts following wounding but not PinII (Sano *et al.* 1994). These G-protein transgenic plants, however, responded normally to MeJA treatment and accumulated PinII (Sano *et al.* 1994). This pattern suggests that some intermediate or product of the JA biosynthesis pathway normally suppresses SA accumulation following wounding. If this suppression is released (such as in the MAPK- or small G-protein-suppressed transgenic plants), the resulting phenotype displays a "typical" SA-induced response to stress. Based on these results, Sano and Ohashi (1995) have proposed a model for the relationship between the wound-induced pathway and the HR following pathogen attack. According to this model, the wound signal pathway that activates JA biosynthesis is downstream from the HR signal that activates both the JA- and SA-pathways (Sano and Ohashi 1995).

Trauma resulting from attack by pathogens and pests induces an array of responses, presumably through a combination of different signal transduction pathways. Wounding activates a JA-dependent pathway that leads to increased ethylene synthesis and to changes in transcription rates for numerous genes, as represented by the induction of PinI and PinII. This pathway is also activated by pathogen attack. Plant-pathogen interaction, on the other hand, activates another pathway which is dependent on SA and also regulates gene expression, controlling, for example, the induction of PAL and PR-1. This pathway is also responsible for the establishment of SAR in systemic leaves. In contrast to pathogens, wounding is unable to activate the pathogen-induced SA-mediated pathway, indicating that the pathogen stimulus lies either upstream from the wound-stimulus or activates two parallel pathways, one responsible for JA biosynthesis, the other responsible for SA accumulation. This might explain why pathogen infection can induce, to some extent, a similar response to wounding, including JA accumulation and

ethylene synthesis (Paradies *et al.* 1980), and similarities in gene expression, whereas the wounding response does not include the full spectrum of a pathogen response. It is also consistent with the observation that neither wounded- nor ethylene-treated tobacco leaves aquired higher resistance to subsequent TMV inoculation (Brederode *et al.* 1991). These proposed pathways also have the potential to communicate (cross-talk) and affect each other in a complex manner. An example of this cross-talk is the potentially antagonistic effects of SA and JA on gene expression. However, since SA and JA accumulation induced by stress does not seem to be synchronized (Seo *et al.* 1995), the effects of such cross-talk are most likely given to be time-dependent.

1.6 PROBLEM STATEMENTS AND THESIS OBJECTIVES

The study of signal transduction in plants in recent years has revealed that many different chemical and environmental signals can elicit the same physiological response in plants (Trewavas and Gilroy 1991). It is even possible that different stress signals, such as those associated with cold and pathogens, can converge into a single, or common, signal transduction mechanism (Prasad *et al.* 1994). It is also possible that single environmental stresses, such as pathogen invasion (see earlier), can activate more than one defensive pathway. Eckey-Kaltenbach *et al.* (1994) have reported that ozone stress can function as such a *cross-inducer.*

Although a number of studies have described the physiological responses of plants to ozone stress, there are many aspects of ozone-induced stress that need further clarification. First, we know little about how aa ozone stress-signal is transduced into a potentially protective response in plant cells. Second, within the context of this complex response, it is difficult to determine which components are most important for reducing cellular damage. Many aspects of ozone-induced responses in plants, however, are similar to pathogen-induced response (Kangasjarvi *et al.* 1994; Eckey-Kaltenbach *et al.* 1994). This includes similarities in the patterns of altered gene expression (Kangasjarvi *et*

al. 1994; Eckey-Kaltenbach *et al.* 1994), and the development of HR-like necrotic spots in ozone exposed tissue. There are also documented examples of a so-called *cross-protection*, where application of one stress can reduce the damage caused by another, subsequent stress. For example, Brennan and Leone (1969) showed that inoculating tobacco plants with TMV prior to ozone exposure improved their ozone tolerance.

It is generally accepted that the plant tissue response to ozone is initiated by the accumulation of ROS (Heath *et al.* 1988; Grimes *et al.* 1983; Hoigne and Bader 1975), and a similar increase in ROS has been observed during the oxidative burst associated with pathogen attack, and with wounding (Mehdy 1994; Olson and Varner 1993). In fact, Heath (1988) and Kangasjarvi *et al.* (1994) have argued that the initial events of ozone injury elicit a general wounding response in plant tissue. For example, both wounding and ozone exposure induce a rapid increase in ethylene biosynthesis and both stresses induce a similar array of genes (Kangasjarvi *et al.* 1994; Langebartels *et al.* 1991). Therefore, it could be predicted that these two stimuli might share all or part of the subsequent signal transduction mechanisms.

The general objective of this study was to investigate the interplay between two stresses that are thought to involve redox perturbations in the challenged tissue. Specifically, I wished to determine whether one stress treatment, wounding, could modify the responses of tobacco to subsequent ozone stress, and whether a modified antioxidant defence system would alter the ability of tobacco to respond to ozone stress. These predictions are based on the hypothesis that ozone defence (i.e. decreased susceptibility to ozone injury) might be induced through activation of a wound-signaling pathway, and that the downstream responses affected by this pathway are aimed at reducing oxidative stress in the plant tissue. In this response, cytosolic ascorbate peroxidase, as the first member of the ascorbate-glutathione pathway, is likely to play an important role in reducing ozone injury. The working model is summarized in Figure 1.3.

According to this model, ozone stress can activate the JA-biosynthesis pathway which, in turn, induces an antioxidative response characterized by increased accumulation of cytosolic APX and down-regulation of metabolic genes that might contribute to oxidative stress. As suggested by other studies, SA has the potential to interfere with such a defence response activated by JA. This model is useful and testable, but it is not intended to exclude the possibility that ozone stress might also activate other pathways which could contribute to activation of ozone defence mechanisms.

Specific objectives of the study are:

(i) to investigate the ability of the wounding response to modify the tissue response to ozone stress in tobacco (Chapter 2)

(ii) to examine, at the gene expression level, whether wounding and ozone stress both induce responses aimed at reducing oxidative stress in the plant tissue, by up-regulating antioxidant genes and down-regulating basic metabolic genes in tobacco (Chapter 2)

(iii) to clone and characterize the cytosolic antioxidant enzyme ascorbate peroxidase in tobacco (Chapter 3)

(iv) to use transgenic technology to examine the significance of modified levels of ascorbate peroxidase to ozone tolerance in tobacco (Chapter 4)

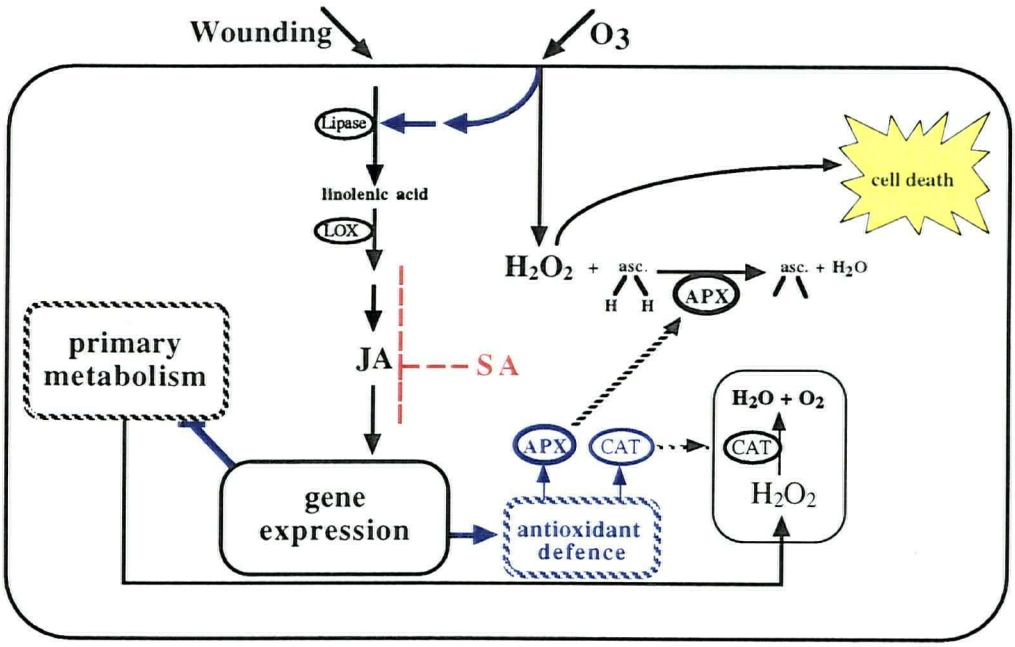


Figure 1.3 Diagrammatic presentation of the metabolic model used in this study showing the proposed relationship between ozone stress, wound-signaling and antioxidative response.

Abbrevations: APX; ascorbate peroxidase, LOX; lipoxygenase, CAT; catalase, JA; jasmonates, SA; salicylic acid, asc; ascorbate. Blue shading; signalling proposed in this study, black shading; pathways suggested by various researchers. The interaction between SA and the jasmonate-biosynthesis pathway is shown as hatched lines.

Chapter 2

Pre-Activating Wounding Response in Tobacco Prior to High Level Ozone Exposure Prevents Necrotic Injury

2.1 INTRODUCTION

The specific transduction mechanisms by which ozone exposure generates changes in plant metabolism and gene expression are unknown but it is generally accepted that the response to ozone is initiated by the accumulation of reactive oxygen species (ROS) in the plant tissue (Grimes *et al.* 1983; Hoigne and Bader 1975). This resembles the rise in intracellular oxidative stress detected in plant tissues exposed to other stresses, including pathogen attack and wounding (Mehdy 1994; Olson and Varner 1993). Heath (1988) and Kangasjarvi *et al.* (1994), in fact, have argued that the initial events in ozone stress represent a wounding response, with an early characteristic rise in ethylene biosynthesis and a similar pattern of gene induction. This suggests that these different environmental challenges may be linked to induced cellular responses by common signalling mechanisms.

As discussed in the previous chapter, wounding of plant tissues has been proposed to activate phospholipid lipases, directly or indirectly. Their action on membrane systems releases linolenic acid, which is subsequently converted to jasmonic acid (JA) (Farmer and Ryan 1992). Jasmonates are potent inducers of an array of stress-related genes but also down-regulators of other genes (Reinbothe *et al.* 1994). The link between jasmonates and these down-stream responses, however, is not well defined, nor is the extent of cross-talk between the jasmonate transduction pathway(s) and pathways initiated by other stimuli. For example, ozone treatment has been shown to increase salicylic acid (SA) levels in tobacco and SA appears to modulate the activity of jasmonates in a complex fashion (Leon *et al.* 1995; Yalpani *et al.* 1994). Both JA synthesis and JA-induced gene expression have been reported to be inhibited by salicylates in tomato (Doares *et al.* 1995; Pena-Cortes *et al.* 1993).

If both wounding and ozone challenge induce an oxidative stress in plant tissues, it follows that these two stimuli might share all or part of the subsequent signal transduction pathways, and that jasmonates and salicylates could play a role in this

signal transduction. It could also be predicted that both of these stresses would induce elevated antioxidant responses, manifested in the induction of antioxidant genes and down-regulation of primary metabolic genes. To test this hypothesis, the ability of wounding to influence the response of tobacco leaves to a subsequent ozone challenge was examined. The involvement of jasmonates and salicylate in the ozone response pathway was explored as was the expression of two antioxidant genes (*APX* and *Sa-Cat*) and one primary metabolic gene (carbonic anhydrase) in response to ozone exposure and to wounding.

2.2 MATERIALS AND METHODS

2.2.1 Plant growth conditions

Plants of the *Nicotiana tabacum* genotypes Bel-W3 and Bel-C (courtesy of Dr. Verne G Sisson, USDA-ARS-SAA Tobacco Research Laboratory), and Xanthi nc and the transgenic line Xanthi nc NahG-10 (courtesy of Dr. John Ryals, Agricultural Biotechnology Research Unit, CIBA-GEIGY Corp.), were grown from seeds in sterilized soil (50% Metro Mix 290, 50% soil with 3.5 kg m^{-3} Osmocote 14-14-14 controlled release fertilizer (Grace Sierra)), in controlled environment growth chambers with 25ºC/20ºC (day/night) under 16 hr photoperiod (120 - 150 $\mu E\ m^{-2}\ s^{-1}$, 6:00 AM - 10:00 PM) and RH 60% +/- 5%. Plants were watered daily with tap water.

2.2.2 Ozone exposure and injury scoring

All plants were exposed to ozone in week 6 or 7 (Bel-W3 and Bel-C) or 8 or 9 (nc and NahG), in growth chambers with 25ºC/20ºC (day/night) under 16 hr photoperiod (100 - 120 $\mu E\ m^{-2}\ s^{-1}$) and RH 60% +/- 5%. Ozone was generated from air with a DELZONE ZO-300 Ozone Generating Sterilizer (DEL Industries) and monitored with a Dasibi 1003-AH ozone analyzer (Dasibi Environmental Corp). Ozone exposure regimes used were either *massive ozone exposure* (MOE), 500ppb/8 hr for one day (1:00 PM - 9:00 PM), or *acute ozone exposure* (AOE), 250 ppb/4 hr, applied for three successive days (12:00

PM - 4:00 PM) to the sensitive tobacco genotype Bel-W3 and less sensitive genotype Bel-C, or for five days to the more ozone-resistant tobacco genotype Xanthi nc and Xanthi nc NahG10. Exposure levels varied no more than +/- 10% over the course of the treatment. Two days after exposure was ended, the level of leaf blade necrosis in all experiments was scored visually on a % area basis using a subjective scale of 0%, 1 - 5%, 6 - 10%, 11 - 15% etc.. The scorer had no prior knowledge of the experimental design or treatment. This scoring technique was evaluated by comparing it with two other scoring techniques (digital images-scoring and leaf area meter-scoring) (see Appendix). These 3 techniques gave similar results. Proportions were subjected to arcsin transformation and two-tail t - tests were used to test differences.

2.2.3 Wounding treatment

One half of leaf #4 or #5 from the top (leaf #1 was the first leaf > 1cm long) in *N. tabacum* Bel-W3 was wounded about 30 times (or as indicated) with a sterile scalpel without cutting main veins. The wounds (2 - 5 mm long) were regularly distributed at ca. 1 cm intervals (Figure 2.1). This was repeated on four plants for each treatment, and each experiment was repeated four or five times. For RNA isolation, both leaf-halves (leaf 10 - 12 cm long) were wounded 20 times. Each sample (time-point) was pooled from leaves of four plants and the experiment was repeated once.

2.2.4 Jasmonate treatment

Plants were sealed in a 8L Nalgene desiccator with either 10µl or 100µl MeJA (25mM in 95% ethanol; Bedoukian Research Inc.) or 10µl or 100µl 95% ethanol as a control treatment. Solutions were applied to a cotton ball inside the container, not touching the plant. After 24 hr treatment (9:00 AM - 9:00 AM), or 1 hr treatment (7:00 AM - 8:00 AM) plants were removed and left on the greenhouse bench for 3 hr (or 6 hr) prior to ozone exposure. To investigate the role of jasmonates in local protection, leaves (15 - 20 cm long) from 8-week old *N. tabacum* Bel-C plants, were excised close to the stem and placed in Hoagland medium (see Appendix) in 35 ml test-tubes containing different concentrations of JA, 18 hr prior to ozone exposure. The temperature was lowered from

25ºC to 20ºC to reduce the transpiration rate. To investigate the systemic response, JA (≥ 90%; Apex Organics, England) was dissolved in lanolin to 150 µM and then applied in 10 to 12 separate dots (ca. 0.25 cm^2/dot) on the adaxial surface on one leaf-half per plant (leaf #4 or #5 from the top; leaf #1 the first leaf > 1cm long) 18 hr prior to ozone exposure. Control plants received lanolin only. For RNA isolation for Northern blot analysis (see section 2.2.8) leaves (10 - 12 cm long or 14 - 16 cm long) from 6 - to 7 - week old *N. tabacum* Bel-W3 were used. Each sample (time-point) was pooled from leaves from four plants and the experiments were repeated once.

2.2.5 Stomatal conductance

Stomatal conductance was measured in both leaf-halves of wounded leaves (avoiding wounds) in plants of similar size and age as those used in the ozone-treatment experiments, with a Li-1600 Steady State Porometer Model Li-1600 (Li-Cor, Inc.), at different time intervals following wounding. The experiment was carried out twice using one plant each time (measuring four wounded leaves), and two times using four plants each time (measuring one wounded leaf per plant). To measure stomatal conductance following MeJA exposure, two leaves each from four MeJA-treated plants and four control plants were tested prior to 24 hr exposure in 8 L Nalgene containers (100 µl 95% ethanol or 100 µl MeJA) and at different time intervals following the exposure. This experiment was repeated but with two MeJA-treated plants and two control plants.

2.2.6 Restriction enzyme-digest, gel-purification and cloning

All restriction enzyme digestions were carried out as recommended by the manufacturer (Pharmacia). DNA fragments were separated on a 1% agarose gel (in TAE buffer)(GIBCO-BRL), stained with ethidium bromide, viewed under a UV-light using a UV-Transilluminator (Fotodyne Inc.) and photographed using Polaroid film or an IS-500 Digital Imaging System (Alpha Innotech Corporation). Fragments were purified from the gel using the GENECLEAN II Kit (BIO 101 Inc.) as described by the manufacturer. All ligation reactions were carried out according to the T4 DNA ligase manufacturer's (BRL)

instructions, using a 3:1 molar ratio of insert to vector (total 100 - 200 ng DNA), 2.5 units of T4 DNA ligase in 20 µl reaction volume for 1 hr at room temperature (RT). Recombinant plasmids were introduced into *E. coli* DH5α using heat-shock transformation. The DNA was added to 100 µl of competent *E.coli* DH5α cells, the mixture incubated for 30 min on ice, heat-shocked for 45 sec at 42°C and subsequently incubated in 1 ml LB (10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 gr NaCl in 1 L) at 37°C for 1.5 hr with gentle shaking. Aliquots were plated out on LB plates (LB medium containing 0.7% agar, 100 mg L^{-1} ampicillin and 40 µg ml^{-1} X-gal; 5-bromo-4-chloro-3-indoyl-B-D-galactopyranoside) for selection of positive transformants. To prepare competent cells, *E. coli* DH5α cells were grown in LB to $O.D._{600\ nm}$ 0.45 - 0.55, kept on ice for 10 min, and centrifuged at 5000g in a Sorvall RC-5B centrifuge (Du Pont Instruments) for 10 min at 4°C. The pellet was resuspended in Solution 1 (30 mM potassium acetate, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15% glycerol, pH 5.8), incubated on ice for 5 min, centrifuged, resuspended Solution 2 (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$ and 15% glycerol, pH 6.5) and stored at -80°C. For mini-preparations of plasmids from transformants, 1 ml of overnight culture (LB including 100 mg L^{-1} ampicillin) of a single colony was centrifuged in an Eppendorf tube for 25 sec at 16000g, resuspended in 200 µl STET (8% sucroes, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-Cl, pH 8.0), plus 20 µl lysozyme solution (20mg ml^{-1}, SIGMA), the mixture shaken gently for 5 min at RT and the tube placed in boiling water for 60 sec. The mixture was then immediately centrifuged for 10 min at 16000g and the supernatant transfered to a new tube. The DNA was precipitated with ice-cold isopropanol and the pellet washed with 70% ethanol before resuspension in ddH_2O (Holmes and Quigley 1981).

2.2.7 Single-stranded cDNA synthesis

For single-stranded cDNA synthesis, total RNA was prepared as follows (Kay *et al.* 1987): 5 - 10 g leaf tissue was ground in liquid nitrogen and 7 ml extraction buffer (25 mM Tris-HCl, 25 mM EDTA, 75 mM NaCl and 1% SDS, pH 8.0) was added to the

powder with further grinding. Phenol (5 ml) was added to the mix and, after thorough grinding, poured into a 35 ml Oak Ridge tube and mixed well with 5 ml chloroform. The emulsion was centrifuged at 6 800g for 10 min and the aqueous layer removed to a new tube. Chloroform (10 ml) was added, the mixture shaken a few times and centrifuged again (6 800g for 10 min). The aqueous layer was removed to a new tube and the nucleic acids (RNA/DNA) precipitated overnight with sodium acetate (0.1 M) and 2 volumes of 95% ethanol. Following centrifugation (7700g for 15 min), the pellet was rinsed with cold 70% ethanol, resuspended in 6 ml 0.1 M sodium acetate (pH 5.0) and an equal volumes of 5 M LiCl added to the mixture. This mixture (2.5 M LiCl specifically precipitates RNA) was left overnight at -20°C, thawed on ice and centrifuged at 7700g for 30 min. The RNA pellet was rinsed with cold 2 M LiCl, centrifuged (7700g for 15 min), resuspended again in 0.1 M sodium acetate (pH 5.0) and precipitated overnight in 0.1 M NaCl and 2.5 volumes of 95% ethanol. After centrifugation (8700g for 15 min), the pellet was finally resuspended in 2 ml TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). mRNA was isolated from total RNA (1 mg) using the PolyATract mRNA Isolation System (Promega) exactly according to the manufacturer's instructions. This system uses biotin-oligo(dT) primers to hybridize to the 3' poly(A) tail of mRNA's. These hybrids are captured in a magnetic field by streptavidin coupled to paramagnetic particles. The mRNA is eluted from the biotin-oligo(dT)-streptavidin-paramagnetic particles by the addition of ddH_2O.

Single-stranded cDNA synthesis was conducted as follows: 2 µg mRNA was denatured at 70°C for 5 min and added to the reverse transcription mixture consisting of 6 µl 5X cDNA buffer (250 mM Tris-HCl, pH 8.3, 250 mM KCl, 50 mM $MgCl_2$, 5 mM DTT, 5 mM EDTA, 50 µg ml^{-1} bovine serum albumin (BSA)), 1 µg $oligo(dT)_{12-18}$, 25 Units human placental ribonuclease inhibitor (HPRNI, BRL), 0.5 mM dNTP's, 4 mM sodium pyrophosphate and 40 units AMV reverse transcriptase (Pharmacia) in a 30 µl reaction. This mixture was incubated for 1.5 hr at 42°C and the reaction stopped with 30 µl of "stop" solution (0.1 M NaCl, 40 mM EDTA). After RNA hydrolysis (using NaOH), the

cDNA was precipitated overnight at -20°C in ammonium acetate (2 M) and 2 volumes of 95% ethanol, and centrifuged for 20 min at 16000g. The pellet was washed in 50 µl 2 M ammonium acetate plus 100 µl of 70% ethanol, centrifuged for 20 min at 16000g, washed with 70% ethanol and resuspended in ddH_2O. A GeneQuant RNA/DNA Calculator (Pharmacia) was used for all nucleic acid quantifications.

2.2.8 Northern analysis

For all Northern blot analyses, total RNA was used. Total RNA was prepared using TRIzol Reagent (GIBCO-BRL) according to the manufacturer's instructions, with the following modifications. The leaf tissue was ground in liquid nitrogen and approximately 200 mg powder added to 1.25 ml Trizol Reagent in a 2.0 ml screw-cap tube. After thorough mixing the solution was incubated for 10 - 15 min at room temperature (RT), and then 250 µl chloroform was added to the solution. The pellet was resuspended in 15 - 20 µl autoclaved ddH_2O. RNA, 10 µg, was resolved on a formaldehyde/agarose gel (1%) and transferred to a nylon membrane (Zeta-probe; Bio-Rad) using capillary transfer in 10X SSC (87.65 g NaCL, 44.1 g sodium citrate, pH 7.5, in 1 L). The filter was prehybridized in 50% formamide, 5% SDS, 0.5 M NaH_2PO_4, pH 7.2, 1 mM EDTA and 1 mg ml^{-1} BSA for 2 - 4 hr at 42°C, and hybridized overnight in the same solution but with approximately [a-^{32}P] dATP-labelled DNA probe (10^6 cpm ml^{-1} hybridization solution). The membrane was washed under high stringency conditions at 55°C: twice in 2X SSPE/0.3% SDS (SSPE; 174 g NaCl, 27.6 g $NaH_2PO_4 \cdot H_2O$, 7.4 g EDTA, pH7.4) for 30 min, once in 1X SSPE/0.5% SDS and once in 0.3X SSPE/1.0% SDS (Davis *et al.* 1994).

Radioactive probes were prepared with [α-^{32}P] dATP (10 µCi/µl, 3000Ci/mmol; ICN Biomedical) using a random priming kit (GIBCO-BRL) according to the manufacturer's instructions. The labeling reaction was incubated at RT for 2 - 4 hr. The probe was purified on a Sephadex G-50 spin column in a 1 cc syringe equlibrated with STE (TE with 0.1 M NaCl) and centrifuged in a clinical centrifuge (3 000 rpm for 3 min) (Sambrook *et al.* 1989). Purified probe (1 µl) was counted using a liquid scintilliation counter (Beckman LS

5000TA). Autoradiography was performed using Kodak X-OMAT AR film at -70°C for 2 to 4 days with one intensifying screen. For comparison of RNA loading and correction, all autoradiographs and ethidium bromide-stained RNA gels were converted to digital images using the IS-500 Digital Imaging System and quantified using NIH Image (Version 1.57) software.

2.2.9 cDNA probes

APX mRNA probe:

A 1040 bp cytosolic APX cDNA from *Nicotiana tabacum* Xanthi was used as a probe (Chapter 3 describes the cloning of this cDNA).

SA-Cat mRNA probe

A partial SA-Cat cDNA was amplified with polymerase chain reaction (PCR) to give an 834 bp fragment, using single stranded cDNA synthesized from RNA extracted from leaves of 6 to 7 week old *Nicotiana tabacum* Xanthi from plants exposed to 450 µE m^{-2} s^{-1} light. The primers 5'-CCGGAATTC$_{EcoRI}$AAGGTTATGGTGTTCACGC-3' (SACAT1) and 5'-GCTCTAGA$_{XbaI}$GTGAGACGAGAAGCGACC-3' (SACAT2) were selected based on the published cDNA sequence (Chen *et al.* 1993). Both primers were synthesized using an Oligo 1000M DNA Synthesizer (Beckman). The primers were resuspended in 100 µl 30% NH_4OH, vortexed vigorously for 15 sec with 1ml n-butanol, centrifuged for 1 min at 10 000g, resuspended in 100 µl ddH_2O and re-extracted with n-butanol (Sawadogo and Van Dyke 1991). The PCR conditions were as follows: 20 ng single-stranded cDNA template, 1 mM $MgCl_2$, 2.5 mM dNTP's, 60 pmol primer SACAT1, 60 pmol primer SACAT2, 2.5 U Taq DNA polymerase (Appligene) and 2.5 µl 10x Taq DNA polymerase buffer (Appligene) in a 25 µl reaction, overlaid with 30 µl mineral oil. The temperature profile was as follows: 4 min at 94°C (1 cycle)/ 1 min at 94°C, 0.5 min at 50°C, 1 min at 72°C (3 cycles)/ 1 min at 94°C, 0.5 min at 65°C, 1 min at 72°C (30 cycles), followed by 4 min at 72°C, using a Techne PHC-3 thermocycler (Mandel Scientific Company Ltd).

CA mRNA probe

Published CA cDNA sequences from pea and spinach (Majeau and Coleman 1991; Fawsett *et al.* 1990), were used to design two degenerate primers, 5'-GGCTCTAGAGCIGCIAT(A/C/T)GA(A/G)TA(C/T)GC-3' (CA1)and 5'-CGGAATTCCA(A/G)ICCCAIA(A/G)(C/T)C(A/G)AA-3' (CA2). Both primers were synthesized at N.A.P.S. (Nucleic Acid Protein Service, Biotechnology Laboratory, University of British Columbia), using an ABI 394 oligo synthesizer (Applied Biosystems Inc.). These primers were used in PCR amplification of a 516 bp partial cDNA using single stranded cDNA synthesized from RNA from *N. tabacum* Xanthi leaves. The PCR conditions were as follows: 20 ng single-stranded cDNA template, 1 mM $MgCl_2$, 2.5 mM dNTP's, 50 pmol primer CA1, 50 pmol primer CA2, 5 U Taq DNA polymerase (Appligene) and 2.5 µl 10x Taq DNA polymerase buffer (Appligene) in a 25 µl reaction, overlaid with 30 µl mineral oil. The temperature profile was as follows: 10 min at 94°C(1 cycle)/, 1.5 min at 94°C, 1 min at 55°C, 1 min at 72°C (30 cycles) followed by 5 min at 72°C.

2.2.10 DNA sequencing and comparison

The identity of the SA-Cat cDNA clone was confirmed with automated DNA sequencing using an Applied Biosystems 373A DNA sequencer with Applied Biosystem AmpliTaq DyeDeoxy terminator sequencing. This sequencing was carried out by N.A.P.S.. The identity of the CA-cDNA clone was confirmed using the T7 Sequencing kit as described by the manufacturer (Pharmacia). [^{35}S]dATP was obtained from New England Nuclear (NEN) and the sequencing reaction run on a 6% acrylamide gel according to standard procedures (Sambrook *et al.* 1989). Gels were mounted on Whatman 3MM filter paper, covered with plastic wrap, and vacuum-dried for 1 hr at 80°C. All DNA and amino acid sequences were analyzed and compared using the PCGene sequence analysis software (IntelliGenetics, Inc.), NCBI web site (National Center for Biotechnology Information; http://www2.ncbi.nlm.nih.gov/cgi-bin/genbank) and the BLAST algorithm program (Altschul *et al.* 1990).

2.3 RESULTS

2.3.1 Ozone injury symptomology

Two levels of ozone treatment were used in these experiments, together with four tobacco genotypes. Since different tobacco genotypes display markedly different susceptibilities to ozone injury, the intensity and timing of appearance of injury symptoms following ozone challenge varied, as noted below. Treatments designated as *massive ozone exposure* (MOE) for intact tobacco plants (500ppb/8 hr for 1 day) caused bifacial necrotic zones (*flecks*) on the sensitive tobacco genotypes Bel-W3 and Bel-C within 6 hr to 24 hr after the start of the exposure. Smaller unifacial necrotic spots (1 - 2 mm in diameter)(*stippling*) also appeared on parts of the leaf outside the bifacial zones one to two days after the end of ozone exposure. The irregularly shaped bifacial necrotic zones ranged from <1 cm in diameter to contiguous damaged areas that covered almost the entire leaf. *Acute ozone exposure* (AOE) (250 ppb/4 hr, applied for three days to the sensitive tobacco genotypes, or for five days to the more ozone-tolerant tobacco genotype Xanthi nc., generated typical unifacial necrotic spots (1 - 2 mm in diameter) within two to three days (Bel-W3 and Bel-C) or three to four days (Xanthi nc). Ozone injury symptoms were normally scored 48 hr after the end of ozone exposure, expressing necrotic lesion area per leaf as a percentage of total leaf surface area.

2.3.2 The effect of wounding on expression of ozone injury symptoms

In order to determine the effect of the wounding response on the response to ozone, mechanically wounded leaves of Bel-W3 were challenged with *MOE* and the pattern of responses in both local (wounded) and distal leaves was investigated. Leaf-halves injured 3 hr prior to *MOE* developed much less necrosis than did the non-wounded halves of the same leaves (Figure 2.1). When leaves were wounded 6 hr prior to *MOE*, however, protection against ozone damage extended throughout the wounded leaf (Figures 2.1, 2.2 and 2.3a) and to other regions of the plant (Figure 2.3b), indicating that a

long-range systemic response had occurred within a 3 to 6 hr period following the wounding stimulus.

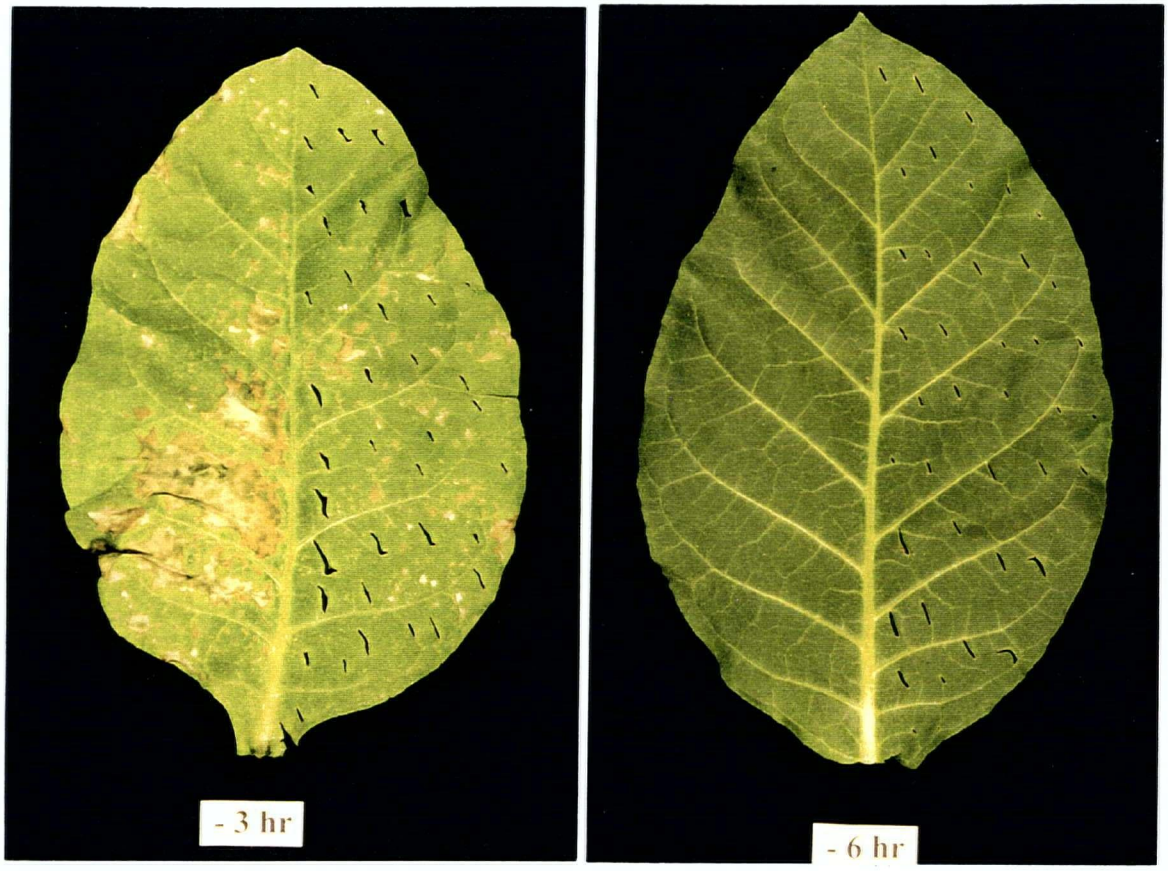


Figure 2.1 Typical response seen in leaves of Bel-W3 plants wounded prior to ozone exposure.

Excised leaves from two different plants, wounded 3 hr (left) or 6 hr (right), prior to *MOE*, and photographed two days after exposure. Each leaf was wounded (the right half of the leaf) by scalpel puncture (30 times).

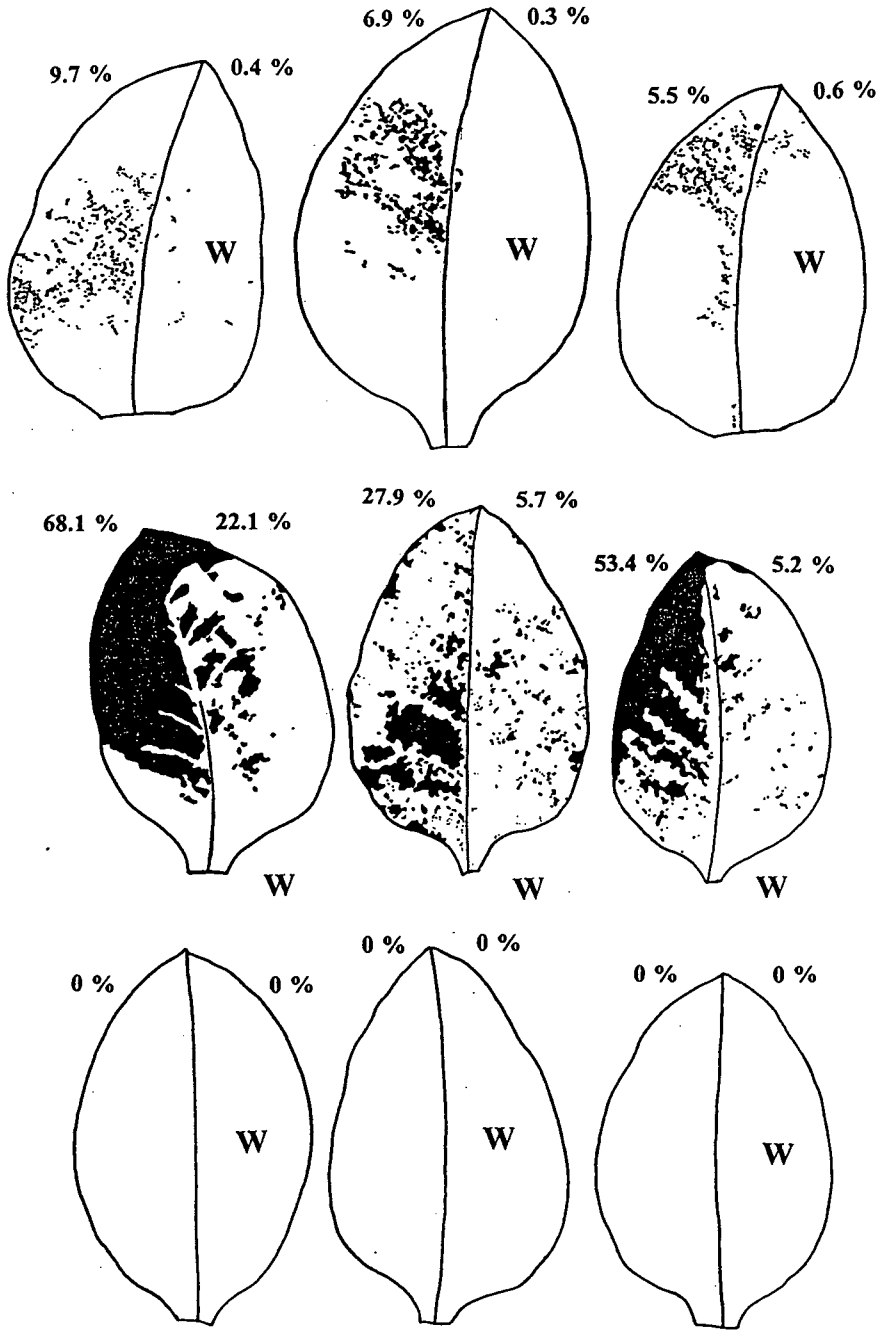


Figure 2.2 Visible necrotic zones observed in wounded leaves of Bel-W3 plants following ozone exposure.

Leaves were wounded at 3 hr (row 1 and 2) and 6 hr (row 3) prior to ozone exposure and injury scored two days later (traced on clear plastic transparency). Leaves in row 1 were exposed to *AOE*. Leaves in rows 2 and 3 were exposed to *MOE*. Each leaf was wounded by scalpel puncture (30 - 40 times) on one half (labeled W). Leaf number 2 in row 2 is the leaf seen on the left in Figure 2.1 and leaf number 1 in row 3 is the leaf seen on the right in Figure 2.1. Also shown is the degree of necrosis in each leaf-half, estimated by image analysis, and expressed as % of total leaf-half area.

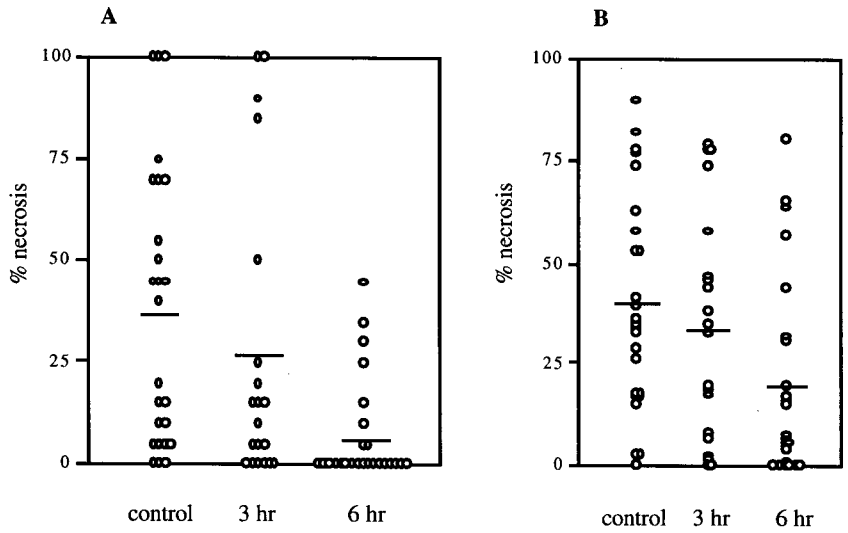


Figure 2.3 Visible necrosis, following ozone exposure, of Bel-W3 plants wounded prior to MOE.

The results from four or five independent experiments are plotted.
(A) Relative degree of necrosis in non-wounded leaf-halves from both wounded and non-wounded leaves. Necrosis was recorded as % necrotic area/leaf-half. Wounding took place either 3 hr (n = 20 plants) or 6 hr (n = 25 plants) prior to ozone exposure. Control leaves were not wounded (n = 25 plants). Only leaves of a similar developmental stage were included in the analysis. Each data point represents one leaf from one plant. The calculated mean % necrosis/leaf-half is indicated as a horizontal line. The differences between means for control and 6 hr prior wounding, and for 3 hr and 6 hr prior wounding, were significant ($P < 0.01$, Students t - test).
(B) Average % necrosis in wounded and non-wounded plants seen in (A), with wounded leaves omitted from the analysis. Each data point represents the observed level of necrosis in one plant, recorded as average % necrotic area/leaf. Total number of leaves scored per plant was four or five. The calculated mean necrosis/leaf is indicated as a horizontal line. The differences between means for control and 6 hr prior wounding and for 3 hr and 6 hr prior wounding were significant at $P < 0.01$ and $P < 0.05$, respectively (Students t - test).

2.3.3 The effect of jasmonates on ozone tolerance in tobacco

Jasmonates have been proposed to serve as downstream components of signal pathways in the wounding response (Creelman *et al.*, 1992; Farmer and Ryan 1992). Bel-W3 tobacco plants exposed to gaseous MeJA for 24 hr prior to challenge with *AOE*, showed dramatically lower levels of ozone injury than control plants receiving no MeJA treatment (Figures 2.4 and 2.5b). Furthermore, as with tobacco plants wounded 6 hr

before *MOE*, plants exposed for 1 hr to MeJA (100µl) 6 hr prior to *MOE* showed lower levels of ozone injury than control plants (Figure 2.5b).

Figure 2.4 Effect of gaseous MeJA exposure on development of ozone injury symptoms on individual leaves in plants subsequently receiving *AOE*.

Leaves (from position five below the first leaf > 1 cm in length) were excised and photographed two days after the last day of ozone exposure. The leaf on the left is from a MeJA-treated plant (100 µl) and on the right from a control plant.

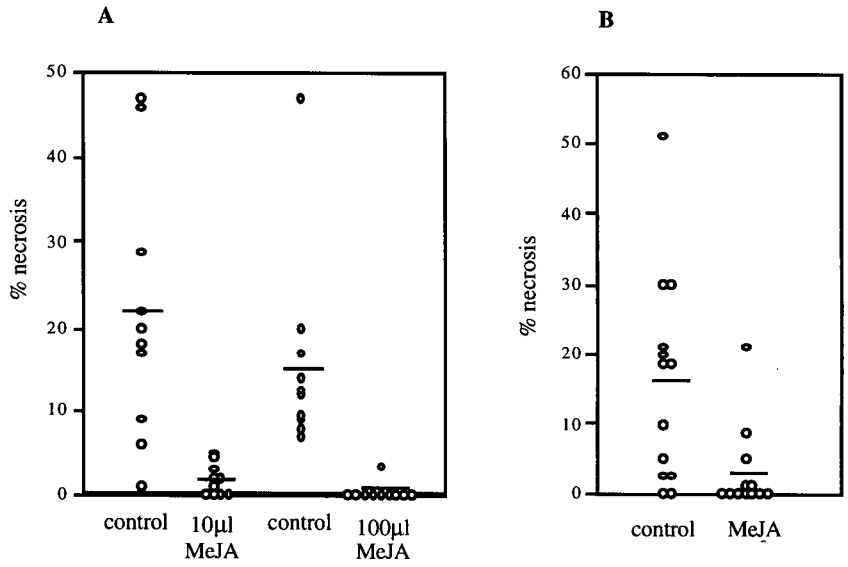


Figure 2.5 Visible necrosis, following ozone exposure (AOE) in leaves of Bel-W3 plants pre-exposed to MeJA

The results from three independent treatments are plotted. Each data point represents the observed level of necrosis in one plant, recorded as average % necrotic area/leaf.
(A) Necrosis induced in plants pre-exposed to MeJA for 24 hr. Treatments consisted of two different levels of gaseous MeJA (10 µl or 100 µl). Total number of leaves scored per plant was five or six.
(B) Necrosis induced in plants pre-exposed for 1 hr to MeJA (100 µl), 6 hrs. before ozone exposure. Total number of leaves scored per plant was four or five.
The calculated mean necrosis/leaf is indicated as a horizontal line. The differences between means for control and MeJA treatments were significant at $P < 0.01$ (Students t - test).

When excised leaves from Bel-C plants were transpiration-fed with JA at concentrations as low as 1 µM prior to challenge with *MOE*, a similar pattern of suppression of ozone injury was observed (Table 2.1).

Application of lanolin paste containing 150 µM JA on a single Bel-W3 leaf-half 18 hr prior to *MOE* produced a dramatic reduction in ozone injury in both halves of the treated leaf (Figure 2.6). A degree of long-range systemic protection was also observed, indicating that a signal moves from the JA-treated tissue to other parts of the plant, analogous to the wounding-induced systemic response.

Table 2.1 Ozone injury (necrosis) induced by MOE in excised Bel-C tobacco leaves following JA transpiration feeding

JA - treatment (μM)	Number of leaves with ≥15% necrosis[a]	Average % necrosis per leaf
0	13	21.9
1	5	14.4
10	3	6.1
100	1	1.1

[a]For each treatment n = 18 leaves.

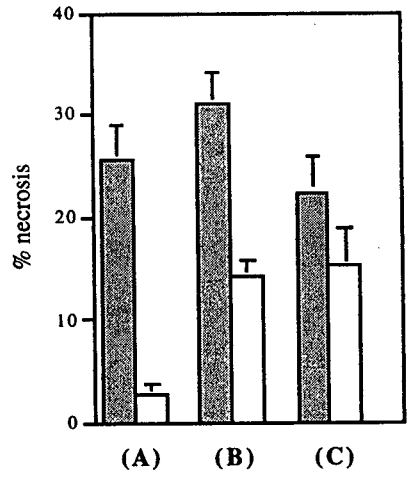


Figure 2.6 Impact of localized JA treatment on induction of ozone injury by MOE in Bel-W3 plants.

In each plant, one leaf-half was treated with either lanolin paste or lanolin paste containing 150 μM JA, 18 hr prior to ozone exposure. Levels of necrosis were recorded as mean % necrotic area/leaf or leaf-half. The results from three independent experiments are plotted as means +/- SD (n = four plants). Shaded bars represent data from treatment with lanolin paste; open bars represent data from treatments with lanolin plus JA.
(A) Mean level of necrosis in treated leaf-halves ($P < 0.01$; Students t - test).
(B) Mean level of necrosis in non-treated halves of treated leaves ($P < 0.01$; Students t - test).
(C) Average level of necrosis in treated plants, omitting treated leaf-halves. Total number of leaves scored per plant is four ($P < 0.05$; Students t - test).

2.3.4 The effect of wounding and jasmonate-treatment on stomatal conductance

To investigate whether the differences in ozone injury observed following wounding or jasmonate treatment could be attributed to differences in stomatal opening, the stomatal conductance was measured in leaves that had been wounded or treated with MeJA. The results from four experiments investigating the effect of wounding on stomatal conductance in Bel-W3, are shown in Figure 2.7. Three of them were carried out in a growth chamber and one in a greenhouse. In two experiments, four leaves on a single plant were measured (Figure 2.7a and b) and in the other two experiments (Figure 2.7c and d) four leaves on four different plants were measured. No significant difference in stomatal conductance was observed between wounded and non-wounded leaf-halves, except after 2 hr and 3 hr in the experiment conducted in greenhouse light (Figure 2.7c). It is, therefore, not likely that differences in stomatal opening could account for the observed differences between wounded and non-wounded leaf halves in responses to ozone stress.

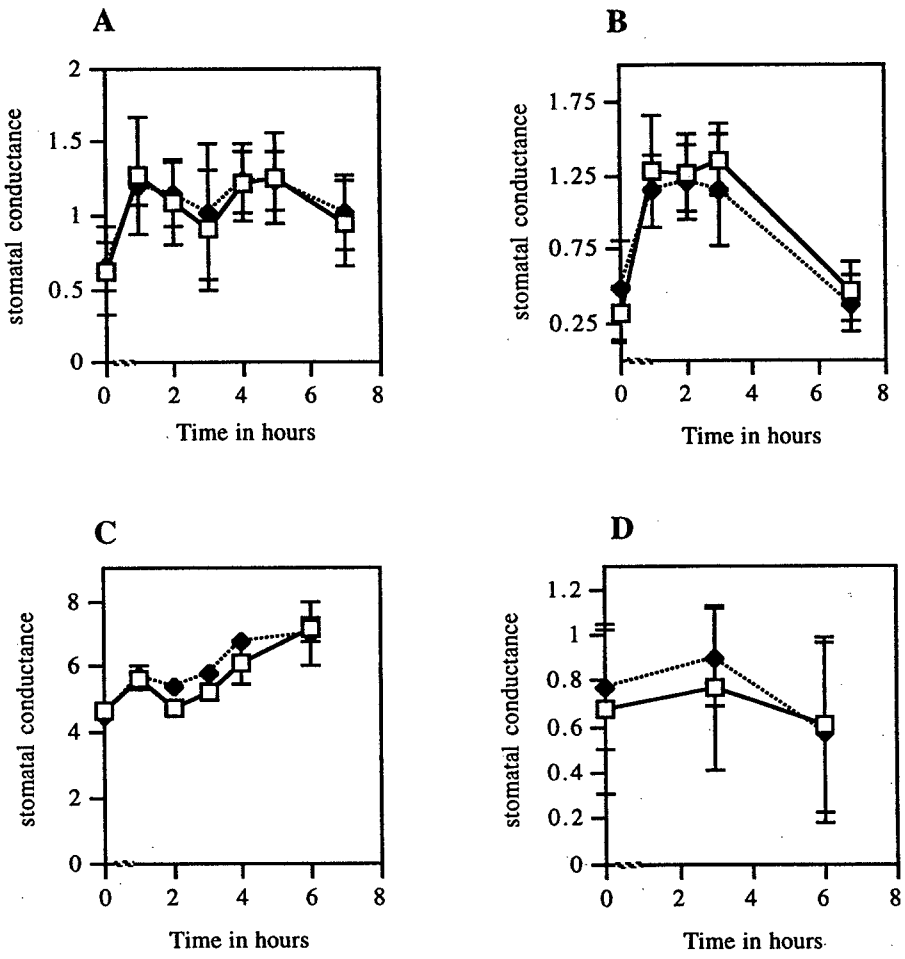


Figure 2.7 The effects of wounding on stomatal conductance ($\mu g\ m^{-2}\ s^{-1}$) in Bel-W3 leaves.

Open squares represent data from wounded leaf-halves; Filled squares represent data from non-wounded leaf-halves in wounded leaves. Stomatal conductance just prior to wounding is represented by "0 hr"; other data points show stomatal conductance following wounding.
(a) Stomatal conductance in four leaves on a single plant (leaf #1 > 8 cm), measured under continuous light (50 - 60 $\mu E\ m^{-2}\ s^{-1}$) and plotted as means +/- SD. Leaf-halves were wounded 50 times at 12:00 noon.
(b) Stomatal conductance in four leaves on a single plant (leaf #1 > 8 cm), measured under continuous light (50 - 60 $\mu E\ m^{-2}\ s^{-1}$) and plotted as means +/- SD. Leaf-halves were wounded 30 times at 9:00 AM.
(c) Stomatal conductance using a single leaf (10 - 14 cm long) from four different plants and plotted as means +/- SD. This experiment was carried out in a greenhouse (bright light but no direct sunlight) and the leaf-halves were wounded 30 times at 10:00 AM.
(d) Stomatal conductance using a single leaf (10 - 14 cm long) from four different plants, measured under continuous light (50 - 60 $\mu E\ m^{-2}\ s^{-1}$) and plotted as means +/- SD. Leaf-halves were wounded 30 times at 1:00 PM.

The results from two experiments investigating the effect of MeJA treatment on stomatal conductance in Bel-W3 are shown in Figure 2.8. As with wounding, MeJA exposure did not appear to affect stomatal conductance in Bel-W3, when compared to the control treatment. This is in agreement with results from Creelman and Mullet (1995) and Horton (1991) who showed that MeJA treatment did not affect stomatal opening. Therefore, the striking difference in degree of ozone injury symptoms following MeJA vapor exposure was not correlated with differences in stomatal behaviour.

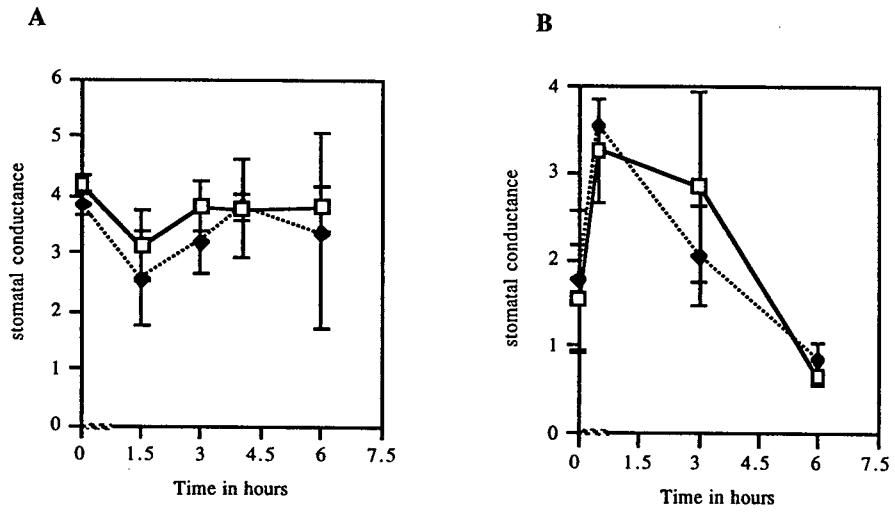


Figure 2.8 The effects of MeJA treatment on stomatal conductance ($\mu g\ m^{-2}\ s^{-1}$) of Bel-W3 leaves.

After 24 hr treatment (10:00 AM - 10:00 AM; see section 2.2.5) plants were kept in the greenhouse (bright light but no direct sunlight) and measured and plotted as means +/- SD. Open squares represent data from MeJA treated plants; filled squares represent data from control plants. Stomatal conductance just prior to the 24 hr treatment is represented by "0 hr"; other data points show stomatal conductance following MeJA treatment. The results from two independent experiments are shown.
(A) Each data point represents the average stomatal conductance of two leaves, from two different plants (leaf #1 > 8 cm). This experiment was carried out in June.
(B) Each data point represents the average stomatal conductance in two leaves from four different plants. This experiment was carried out in January.

2.3.5 Salicylic acid and ozone tolerance

Transgenic tobacco plants expressing a bacterial salicylate hydroxylase gene (*Nah*G) have been shown to be unable to accumulate SA, or to display SAR, following

pathogen inoculation (Gaffney *et al.* 1993). To examine the interaction between SA-signalling and the ozone response in tobacco, both transgenic NahG-10 tobacco plants and the parental non-transgenic genotype (Xanthi nc.) were challenged with AOE for five days and monitored for ozone injury. Under these conditions, necrotic symptoms first appeared in both genotypes after three days of exposure, but the NahG-10 plants suffered much less ozone injury than did the non-transgenic parental plants (Figure 2.9).

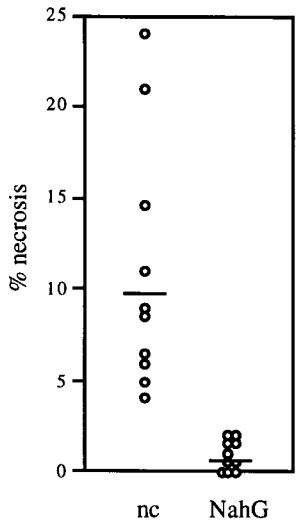


Figure 2.9 Necrosis induced by *AOE* in *N. tabacum* Xanthi nc (nc) and *N. tabacum* Xanthi NahG-10 (NahG).

Levels of necrosis were recorded as mean % necrotic area/leaf. The results from three independent experiments are plotted. Total number of leaves scored per plant was 10 - 13. The calculated mean level of necrosis/leaf is indicated as a horizontal line. The differences between means for nc and NahG were significant at $P < 0.01$ (Students t - test).

2.3.6 Expression of ascorbate peroxidase, salicylic acid-catalase and carbonic anhydrase following ozone exposure, wounding and methyl jasmonate-treatment

Ascorbate peroxidase and SA-Cat mRNA levels were assessed following wounding, ozone exposure or MeJA treatment to gain some insight into antioxidant responses that might involve modulation of gene transcription. Both enzymes belong to the array of antioxidant functions plants use to reduce the levels of damaging ROS. APX is capable of reducing hydrogen peroxide to water, using ascorbate as the electron donor,

whereas SA-Cat can directly decompose hydrogen peroxide to molecular oxygen and water.

Northern blot analysis showed increases in both APX and SA-Cat mRNA levels in the leaves of genotype Bel-W3 by 6 hr after initiation of exposure to 250 ppb ozone for 6 hr (Figures 2.10A and 2.10B). This level of ozone exposure did not produce any visible necrotic symptoms in non-harvested plants scored 48 hr after exposure.

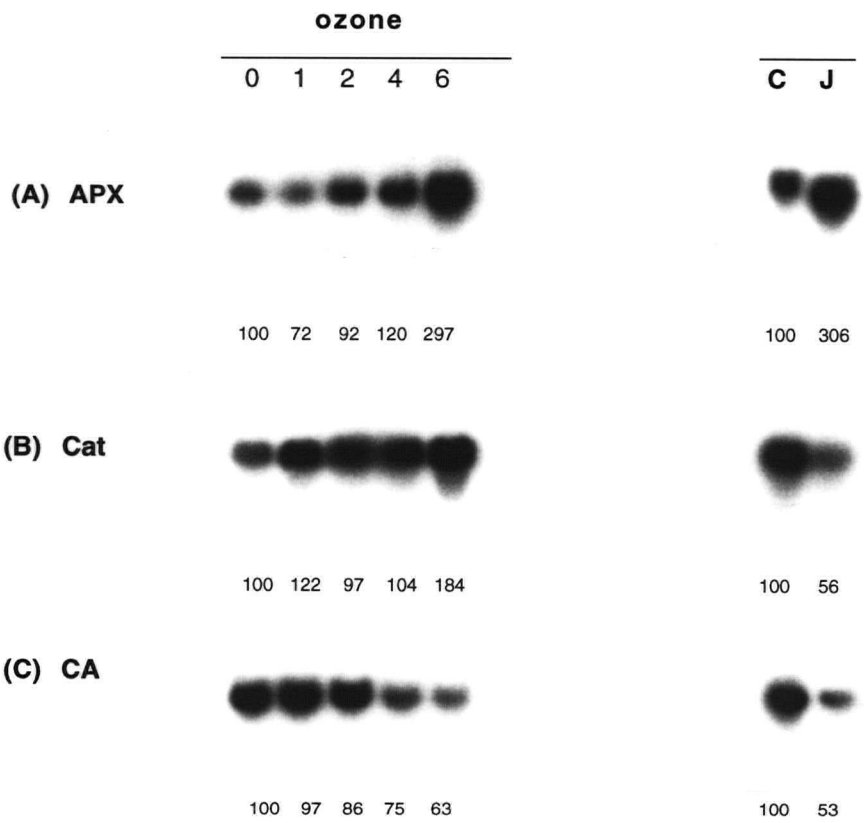


Figure 2.10 Ozone- and MeJA-regulated expression of APX, SA-Cat and CA genes.

Northern blot analysis of 10 µg of total RNA isolated from leaves of 6 - to 7 - week old *N. tabacum* Bel-W3. Each sample was pooled from leaves of four plants. For ozone treated plants (250 ppb; 0 hr, 1 hr, 2 hr, 4 hr or 6 hr treatment) RNA was isolated from leaves 9 - 12 cm long. For MeJA ("J"; control: "C") treated plants RNA was isolated from leaves 14 - 16 cm long, 3 hr after 24 hr treatment with 100µl of 25mM MeJA. Relative density (corrected for loading) is shown under each lane as a percentage of density in the first lane. cDNA probes used were as follows (A) ascorbate peroxidase (APX), (B) SA-binding catalase (Cat) and (C) carbonic anhydrase (CA). Each experiment was repeated twice with similar results.

However, the pattern of expression of these same mRNA species in leaves exposed to MeJA or in leaves mechanically wounded, differed from that induced by ozone treatment. While APX mRNA levels had increased following 24 hr of MeJA vapor exposure, SA-Cat mRNA levels were depressed (Figures 2.10A and 2.10B). Similarly, a slight increase in mRNA levels of APX and a strong decrease in mRNA levels of SA-Cat were detected at 3 hr following wounding (Figures 2.11A and 2.11B). This expression pattern was also observed following 1 hr MeJA treatment although the SA-Cat mRNA decrease was much less (Figures 2.11A and 2.11B). However, the changes in APX- and SA-Cat mRNA expression following both wounding and 1 hr MeJA treatment appeared to be transient.

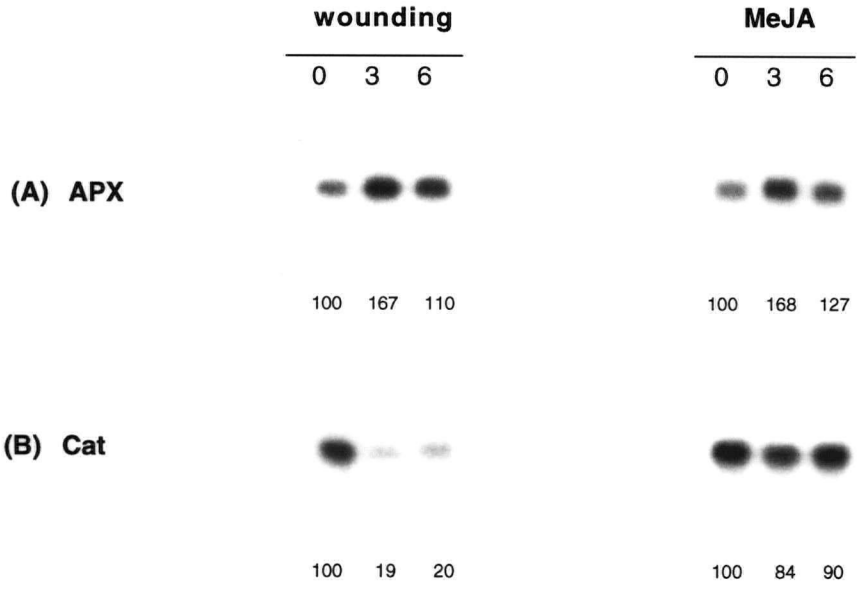


Figure 2.11 Wound- and MeJA-regulated expression of APX and SA-Cat genes.

Northern blot analysis of 10 μg of total RNA isolated from leaves (10 - 12 cm long) of 6 - to 7 - week old *N. tabacum* Bel-W3. Each sample was pooled from leaves of four plants. For wounded plants RNA was isolated 0, 3 or 6 hrs after mechanical wounding and for MeJA treatment RNA was isolated 3 (3) or 6 hr (6) following 1 hr exposure to 100 μl of 25mM MeJA. For control (0) RNA was isolated 6 hr following 1 hr exposure to 100 μl of 95% ethanol. Relative density (corrected for loading) is shown under each lane as a percentage of density in the first lane. cDNA probes used were as follows (A) ascorbate peroxidase (APX) and (B) SA-binding catalase (SA-Cat). Each experiment was repeated twice with similar results.

Carbonic anhydrase, which catalyzes the reversible hydration of CO_2 in support of photosynthetic carbon fixation by Rubisco (Sültenmeyer *et al.* 1993), represents a housekeeping function central to primary metabolism. CA mRNA levels declined moderately (at least 50%) during the course of a 6 hr exposure of tobacco leaves to ozone (Figures 2.10c). Exposure to a MeJA concentration (100 µl for 24 hr) that provides strong protection against ozone injury severely depressed CA expression (Figures 2.10c).

2.4 DISCUSSION

Marked genetic differences exist in susceptibility to ozone injury both between and within plant species, indicating that the innate ability to deal with oxidative injury has a range of capacities. Shorter-term physiological responses may also modify susceptibility to oxidative stress, since virus-infected tobacco plants have been reported to be more resistant to ozone injury than are healthy plants (Brennan and Leone 1969; Yalpani *et al.* 1994). The toxicity of ozone to living cells results, at least in part, from its ability to generate secondary ROS (Mehlhorn *et al.* 1990; Grimes *et al.* 1983; Hoigne and Bader 1975). In the mammalian immune system, phagocytes have been proposed to exploit this reactivity of ROS to kill bacterial invaders (Morel *et al.* 1991), and an analogous process has been proposed to form part of the earliest responses of plant tissues to pathogen attack or loss of tissue integrity (Apostol *et al.* 1989). The obvious parallels between ozone reactivity and induction of a rapid "oxidative burst" in plant cells following pathogen attack (Mehdy 1994) or through tissue trauma (Olson and Varner 1993; Yahraus *et al.* 1995), suggest that these stimuli might trigger similar patterns of downstream responses in plant tissues, and there is some evidence for this in the literature. For example, both wounding and ozone exposure induce expression of defence related genes, such as *SOD*, *LOX* and *PAL* (Bell and Mullet 1993; Lois and

Hahlbrock 1992; Maccarrone *et al.* 1992; Perl-Treves and Galun 1991; Sharma and Davis 1994).

The parallels in gene expression between ozone-induced and wound-induced responses in plants, and the increased oxidative stress common to both stimuli, prompted me to examine the possibility that wounding might influence the ability of plant tissues to withstand a subsequent challenge from high levels of ozone. My data show that the response induced in less than 3 hr in tobacco plants by wounding strongly modifies the level of tissue injury resulting from a subsequent exposure of the wounded plants to either massive or acute levels of ozone. In addition, both mechanical wounding and jasmonate application are able to rapidly activate similar local defence responses that provide effective protection against ozone injury, and systemic transmission of this protection develops within 3 to 6 hr following wounding.

The nature of the signal transduction pathways linking oxidative stress stimuli to downstream responses has not been determined. However, metabolites of the jasmonate-biosynthesis pathway, such as jasmonic acid, have been implicated as second messengers in wound-induced accumulation of proteinase inhibitor proteins in tomato (Graham *et al.* 1985), and systemin-induced accumulation of mRNAs coding for polyphenol oxidase in tomato (Constabel *et al.* 1995). The transient jasmonate accumulation induced by wounding can be relatively rapid (< 30 min.) (Albrecht *et al.* 1993; Baldwin *et al.* 1994; Blechert *et al.* 1995), as is the increase in LOX expression observed following exposure of soybean leaves to ozone (Maccarrone *et al.* 1992). These metabolic changes thus occur quickly enough to enable octadecanoids to serve as signal transduction intermediaries linking both ozone stress and wounding to downstream responses.

Ozone is presumed to damage plant cells through generation of ROS which modify proteins, nucleic acids and membranes, as well as through perturbation of the general redox status of the cell. While both wounding and metabolites of the jasmonate-

biosynthesis pathway are known to modulate systemically the transcription of a number of genes in plants, it is not clear what role, if any, those gene products identified to date might play in suppressing ozone-induced necrosis. It thus seems likely that wounding and these metabolites must activate an even wider array of downstream protective functions than those already characterized. Among the candidates for such protective functions would be elevated expression of genes encoding enzymes involved in scavenging ROS (e.g. *APX*, *SOD*, catalase). The present data shows that mRNA levels for APX are markedly increased within a few hr of ozone exposure or after 24 hr MeJA treatment. APX mRNA levels also appeared to increase slightly within 3 hr following wounding or jasmonate treatment. MeJA severely depressed CA expression but suppression of primary metabolic functions following ozone exposure, such as synthesis of the photosynthetic proteins ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (RbcS) and chlorophyll a/b-binding protein (Cab) has previously been reported (Conklin and Last 1995), and Pena-Cortes *et al.* (1988) also observed that wounding suppressed mRNA levels for RbcS. The downstream responses of tobacco plants to ozone treatment and their responses to methyl jasmonate are not completely related, however, since expression of the SA-Cat gene is stimulated by ozone exposure but strongly suppressed by jasmonates and wounding (Figures 2.10 and 2.11). Signal transduction pathways in metazoan organisms often appear to operate as matrices in which cross-talk and redundant signalling between effectors ensures an appropriate dynamic response to different environmental stimuli. It would thus not be surprising if ozone challenge modulated gene expression both through a jasmonate signalling pathway and through other, as yet undefined, mechanisms. However, the Northern blot analysis precented in this study is unable to confirm that ozone is activating the wound-inducable jasmonate-biosynthesis pathway. Therefore, it should be emphasized that it is likely that ozone stress and wounding activate two separate pathways, independent of each other.

Among other anti-oxidative agents, SA has the potential to directly modulate oxidative damage control, since it has recently been shown to be a potent *in vitro* inhibitor of both SA-Cat (Chen *et al.* 1993) and APX (Durner and Klessig 1995). It has also been suggested that the interaction between SA and catalase and/or ascorbate peroxidase can give rise to a SA free radical that could initiate lipid peroxidation that would ultimately result in plant cell death (Hammond-Kosack and Jones 1996). However, SA is also important in systemic signalling in plants, even though the exact role it plays remains unresolved. The use of NahG transgenic tobacco plants that are unable to accumulate SA has suggested that SA itself is not the transmissable signal, but these studies also show that the ability to generate elevated levels of SA is essential for expression of a systemic response in the distal tissue (Vernooij *et al.* 1994). There is also potential for interaction between SA and the metabolites of the jasmonate-biosynthesis pathway, since SA has been found to interdict both the downstream action of jasmonates as well as jasmonate synthesis (Doares *et al.* 1995; Pena-Cortes *et al.* 1993). Precisely how these metabolites are involved in signalling remains to be determined but the antagonistic interplay between SA and the jasmonates is emphasized by a recent report of transgenic tobacco plants in which expression of a MAP kinase gene had been suppressed (Seo *et al.* 1995). These plants were found to be blocked in wound-induced signalling, and they failed to accumulate jasmonates in response to wounding. They did, however, accumulate SA, in contrast to control plants which accumulated jasmonates but not salicylate. In the context of linkages between oxidative stress, MAP kinases and octadecanoid signalling, the recent observation that MAP kinase can be directly activated by ROS (Guyton *et al.* 1996) is particularly intriguing. SA accumulation in plants could thus potentially increase ozone injury either by inhibiting ROS-scavenging enzymes (such as APX) and lipid peroxidation through a SA free radical, by interfering with the octadecanoid pathway, or both. The results presented in this chapter are consistent with such predictions, since the

presence of the NahG function in tobacco plants was found to render them distinctly more resistant to ozone injury than non-transgenic parent plants.

The present results are, however, in striking contrast to a recent report showing that *Arabidopsis thaliana* plants expressing the NahG function are *more* sensitive to ozone injury than is the parental wild type (Sharma *et al.* 1996). It is not clear whether this difference reflects the existence of species-specific transduction pathway architectures, differences in response thresholds, or variation in mechanisms for dealing with oxidative stress. However, it is interesting to note that, among plant species surveyed, *Arabidopsis* has been found to be relatively insensitive to exogenous jasmonates (Avdiushko *et al.* 1995). I have also observed in preliminary experiments that, in contrast to tobacco, application of methyl jasmonate to *A. thaliana* plants for 24 hr has little, if any, effect in alleviating necrotic injury caused by subsequent ozone exposure. Furthermore, two hours methyl jasmonate treatment, four hours prior to ozone treatment, rendered the *A. thaliana* plants more susceptible to ozone exposure (see Appendix). These results are in sharp contrast to the response of Bel-W3 tobacco plants to similar treatment.

To my knowledge, the data presented in this chapter provide the first substantial evidence for common signalling mechanisms linking plant responses to ozone with their responses to other environmental stresses. Although the levels of ozone used to challenge plants in these experiments exceed those observed in today's environment, they are probably still relevant. Indeed, it has been suggested that ozone stress is not a new problem for land plants, which may have originally evolved in an environment marked by high atmospheric ozone levels (Runeckles and Krupa 1994). The existence of common stress signalling pathways and defence responses could therefore reflect an ancient evolutionary solution to this oxidative challenge.

Chapter 3

Cloning and characterization of a cDNA encoding cytosolic ascorbate peroxidase in tobacco

3.1 INTRODUCTION

In the previous chapter it was shown that ozone exposure induces accumulation of ascorbate peroxidase (APX, E.C. 1.11.1.11) mRNA in tobacco within a few hours. This observation is consistent with earlier data showing that APX activity is higher in ozone-exposed plants than in non-exposed plants (Mehlhorn *et al.* 1987). APX is found in higher plants, algae and some cyanobacteria (Asada 1992). It has been found in every angiosperm surveyed so far (Asada 1992) and is the major peroxidase in leaf tissue (Amako *et al.* 1994). APX is responsive to different abiotic stresses that can generate reactive oxygen species (ROS) (Foyer *et al.* 1994). For example, drought and heat-shock induce higher APX mRNA levels, as do salt stress and abscisic acid treatment (Lopez *et al.* 1996; Mittler and Zilinskas 1994). APX participates in the ascorbate-glutathione cycle in plants, where it catalyzes the reduction of hydrogen peroxide, using ascorbic acid as an electron donor, in the following reaction (see section 1.4.2 for detailed discussion):

2 ascorbate + H_2O_2 -> 2 monodehydroascorbate + 2 H_2O

This reaction is thought to take place primarily in the cytosol and in the chloroplast. Four different isoforms of APX, with different subcellular localizations, have been identified in plants: thylakoid APX, stromal APX, microbody APX and cytosolic APX (Chen and Asada 1989; Mittler and Zilinskas 1991b; Miyake *et al.* 1993; Yamaguchi *et al.* 1996). The cytosolic APX from pea shoots was the first to be characterized and have its cDNA cloned (Mittler and Zilinskas 1991a). This isoform is a heme-containing homodimer (Mittler and Zilinskas 1991b) found in both photosynthetic and non-photosynthetic tissues (Miyake *et al.* 1993). It differs from its chloroplastic counterpart in several ways. For example, the cytosolic APX has a broader pH-range and a higher stability in the absence of ascorbate. Furthermore, antibodies raised to the cytosolic isoform in peas do not cross-react with the

chloroplastic isoform (Mittler and Zilinskas 1991b). In contrast, however, antibodies raised to the spinach chloroplastic isoform can cross-react with the cytosolic isoform from *Arabidopsis thaliana* (Kubo *et al.* 1992). The pea cytosolic *APX* gene has nine introns, one of which is located in the 5' untranslated region, two putative plant heat-shock elements (HSE) and a reversed antiperoxidative element (ARE), which is responsible for the hydrogen peroxide-dependent response of rat glutathione-S-transferase (Rushmore *et al.* 1991). The pea genome appears to have only one copy of the cytosolic gene (Mittler and Zilinskas, 1992), as is also the case in *Arabidopsis thaliana* (Kubo *et al.* 1992).

It has been suggested that APX plays an important role in ozone defence in plants (Foyer *et al.* 1994) and an increase in APX enzyme activity has been correlated with increased tolerance to ozone (Batini *et al.* 1995; Mehlhorn *et al.* 1987). However, a causal relationship between the changes in APX activity and the severity of ozone symptoms following ozone exposure remains to be established. As part of the examination of the involvement of cytosolic APX in tolerance of tobacco to ozone stress, the specific objective of this study was to clone and characterize for the first time, a cDNA encoding cytosolic APX in tobacco, and to compare its sequence to other known APX's from higher plant species.

3.2 MATERIALS AND METHODS

3.2.1 Restriction enzyme-digest, cloning and transformation

As decribed in section 2.2.6

3.2.2 Polymerase chain reaction using degenerate primers

Single-stranded cDNA (prepared as described in section 2.2.5) was use as a template for all PCR amplifications. Single-stranded cDNA for *N. tabacum* Xanthi was prepared using RNA extracted from ozone-exposed plants (200 ppb/8 hr for 2 days). Single-stranded cDNA for *A. thaliana* was prepared using RNA extracted from plants 3

weeks old grown at 300 μE $m^{-2}s^{-1}$ light in the absence of ozone. Two degenerate primers were designed based on a comparison of the APX cDNA sequences from *P. sativum* and *A. thaliana* (Mittler and Zilinskas 1991a; Kubo *et al.* 1992) and synthesized at N.A.P.S. The 5' end primer had the sequence 5'gggaattc$_{EcoRI}$CGCIGAC/TGCIACIAAA/GGG (named APFOR) and the 3' end primer had the sequence 5'gctctaga$_{XbaI}$GCTGIGCC/TTCIGCA/GTAA/GTC (named APBAK).

The PCR conditions were as follows: 20 ng single-stranded cDNA template, 1 mM or 1.5 mM $MgCl_2$, 2.5 mM dNTP's, 120 pmol of each primer, 2.5 units of Taq DNA polymerase (Appligene) and 10 μl 10X Taq DNA polymerase buffer (Appligene) in a 100 μl reaction, overlaid with 50 μl mineral oil. The temperature profile was: 1 min at 94°C, 1 min at 42°C (or 45°C), 1 min at 72°C (3 cycles)/, 1 min at 94°C, 1 min at 60°C, 1 min at 72°C (30 cycles) followed by 5 min at 72°C. For reamplification, the reaction volume was scaled down to 25 μl.

3.2.3 Rapid amplification of cDNA ends (RACE) for 5' end cloning

Single-stranded cDNA RACE was prepared using the protocol for PCR-directed cDNA libraries from Gurr and McPherson (1991) with the following modifications: 3 μg total RNA was used instead of 1 μg, and spermidine, sodium pyrophosphate and the oligo(dT_{17}) primer were excluded from the first-strand synthesis reaction. Instead of the oligo(dT_{17}) primer, a specific primer with the sequence 5'gctctagagc$_{EcoRI}$AAGATGAGGGGATTGGTAGTC, was used for the reverse transcription. This primer (called AP5END) was based on the partial tobacco APX cDNA sequence determined using the degenerate primers APFOR/APBAK (see section 4.3.1). As a 5' end the primer 5'AAGGAATT$_{EcoRI}$ -oligo(dC_{14})(called CEND), was used. Both primers were synthesized at N.A.P.S. The oligo-dG tailing was performed as described by Gurr and McPherson (1991) using terminal transferase (Boehringer-Mannheim), except that the dG-homopolymerization reaction was 30 min. The PCR conditions were as follows: 5 μl of the 20 μl first-strand cDNA synthesis

reaction, 1 mM $MgCl_2$, 2.5 mM dNTP's, 200 pmol CEND, 50 pmol AP5END, 1.5 units of Taq DNA polymerase (Appligene) and 5 µl 10x Taq DNA polymerase buffer (Appligene) in a 50 µl reaction, overlaid with 25 µl mineral oil. The temperature profile was: 4 min at 94°C(1 cycle)/, 0.5 min at 94°C, 2 min at 50°C, 2 min at 72°C (1 cycle)/, 0.5 min at 94°C, 0.5 min at 60°C, 2 min at 72°C (30 cycles) followed by 4 min at 72°C. The PCR products were separated on a 1% agarose gel in TAE buffer. A smear of DNA between 0.4 kb and 1.8 kb was cut and purified from the gel using the GENECLEAN II Kit prior to cloning (see section 2.2.5). Transformed colonies were blotted onto Hybond-N membranes (Amersham), according to manufacturer's instructions and membranes were hybridized with a DIG-labelled APX fragment (338 bp APX clone from the APFOR/APBAK amplification, see 3.2.2), using the DNA Labelling and Detection Kit (Boehringer Mannheim) according to manufacturer's instructions. Positive clones were then selected for further plasmid analysis (see section 2.2.5).

3.2.4 PCR for 3' end cloning

The 3' end of APX was amplified using single-stranded cDNA from ozone-exposed *N. tabacum* Xanthi (see 3.3.1) as a template. The 5' end primer,

5'gctctagagc$_{XbaI}$TTTCACCCTGGTAGAGAGGAC (called AP3END)

was a specific primer based on the partial tobacco APX sequence acquired using RACE (3.2.3). The 3' end primer was an oligo(dT) primer,

5'catgtcgtccaggccgctctggacaaaatatgaattc$_{EcoRI}$ T_{24} (called TGUY).

Both primers were synthesized at N.A.P.S.. The PCR conditions were as follows: 20 ng single-stranded cDNA template, 1 mM $MgCl_2$, 2.5 mM dNTP's, 100 pmol TGUY, 80 pmol AP3END, 2.5 units of Taq DNA polymerase (Appligene) and 10 µl 10x Taq DNA polymerase buffer (Appligene) in a 100 µl reaction, overlaid with 50 µl mineral oil. The temperature profile was as follows: 4 min at 94°C(1 cycle)/ 0.5 min at 94°C, 0.5 min at 58°C, 1 min at 72°C (3 cycles)/ 0.5 min at 94°C, 0.5 min at 37°C, 1 min at 72°C (3 cycles)/ 0.5 min at 94°C, 0.5 min at 60°C, 1 min at 72°C (30 cycles) followed by 4 min at 72°C.

3.2.5 PCR for full-length cDNA cloning

A full-length APX cDNA was amplified using single-stranded cDNA from ozone-exposed *N. tabacum* Xanthi as a template (see 3.3.1). The 5' end primer, synthesized at N.A.P.S., was a specific primer representing the 5' end of the partial sequence acquired using RACE (see 3.2.3). The sequence was 5'gctctagagc$_{XbaI}$TCTCTTTATAGGGTTTAACG (called AP5DNA).
The 3' end primer was TGUY. The PCR conditions and temperature profile were the same as in the PCR for 3' end cloning (described in 3.2.3), except that the first 3 cycles had a 50°C annealing temperature and the 72°C polymerization time was 2 min.

3.2.6 DNA sequencing and comparison

The identities of the cDNA clones from the PCR reactions using the degenerate primers (APFOR/APBAK) were confirmed using the T7 Sequencing kit as described by the manufacturer (Pharmacia). The identities of other cDNA clones were confirmed with automated DNA sequencing using an Applied Biosystems 373A DNA sequencer. All DNA and amino acid sequences were analyzed and compared using the PCGene sequence analysis software (IntelliGenetics, Inc.), NCBI web site (National Center for Biotechnology Information; http://www2.ncbi.nlm.nih.gov/cgi-bin/genbank) and the BLAST algorithm program (Altschul *et al.* 1990). For a more detailed description see section 2.2.8.

3.3 RESULTS

3.3.1 Partial cDNA cloning

As a first step towards cloning a cytosolic APX cDNA from *N.tabacum* Xanthi, cDNA sequences for cytosolic APX from *P. sativum* (Mittler and Zilinskas 1991a) and *A. thaliana* (Kubo *et al.* 1992) (the only available APX DNA sequences at the time) were compared. Homologous regions with low codon degeneracy were selected for primer design. The amino acid sequences PDATKG (residues 131-136) and DYAEAH (residues

235-240) where selected for preparation of degenerate primers for PCR amplification. The 5' end primer, APFOR, had 256-fold degeneracy and the 3' end primer, APBAK, had 128-fold degeneracy (Table 3.1). These primers were first tested on single-stranded

Table 3.1 Characteristics of degenerate primers for PCR-amplification of APX fragment from tobacco cDNA

Primer	a.a. seq.[a]	Deg.[b]	N.t. sequence including RE-site (5' -> 3')[c]	Tm° C[d]
"APFOR"	P D A T K G	256	cggaattccgCIGAC/TGCIACIAAA/GGG	41 - 53
"APBAK"	D Y A E A H	128	gctctagagaTGIGCC/TTCIGCA/GTAA/GTC	50 - 56

[a] The amino acid sequence selected for designing degenerate primers
[b] Primer degeneracy
[c] Lower case represent restriction site for *Eco*RI (APFOR) or *Xba*I (APBAK) plus extra bases
[d] Melting point of the primer, excluding restriction sites, or the temperature at which <50% of the primers are hybridized to the template

cDNA from *A. thaliana* Columbia, using 45°C annealing temperature during the first 3 cycles. A single band, corresponding approximately to the expected size of 338 bp, was amplified (Figure 3.1A, Lane 1). When the same PCR conditions were applied using a single-stranded cDNA template from *N. tabacum* Xanthi leaves, the products appeared to be non-specific. However, when the annealing temperature during the first three cycles of the PCR was lowered to 42°C, a strong background smear appeared along with a faint band of the expected size (Figure 3.1B, Lane 2). This faint band was gel-purified and re-amplified, using the same reaction conditions, to facilitate cloning (Figure 3.1C, Lanes 1 - 4). No attempt was made to further optimize the PCR conditions. Two clones, ap338a and ap338b, from two separate re-amplication reactions were partially sequenced (Figure 3.2). Alignment of these two sequences showed one bp mismatch.

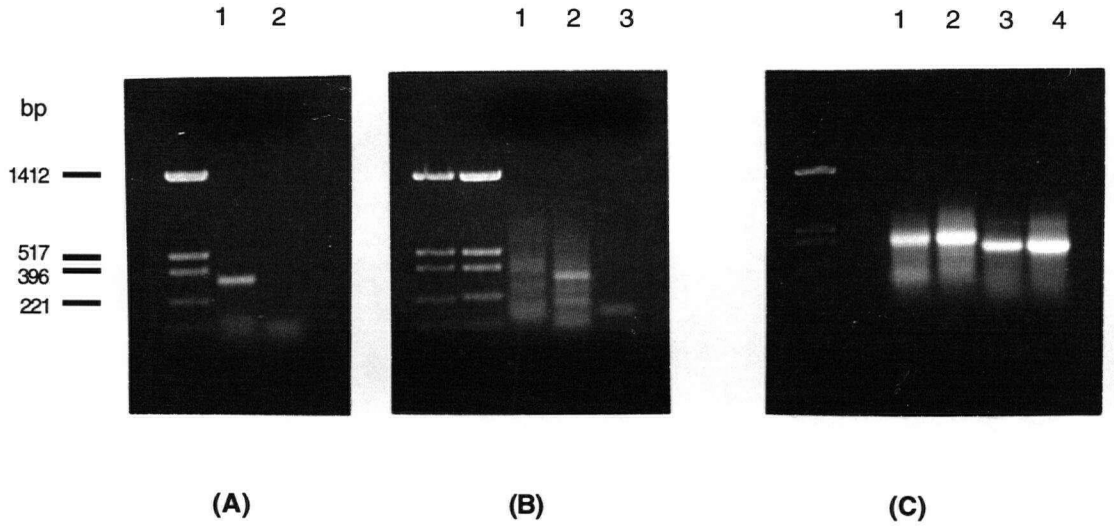


Figure 3.1 Agarose gel analysis of 338 bp PCR amplification product using the degenerate primers APFOR and APBAK

The products were separated on a 1.0 % agarose gel, stained with ethidium bromide and photographed under UV-light. The size markers (on the left) are from *Hinf*I-digested pUC13.
(A) Lane **1**: *A. thaliana* single-stranded cDNA as a template using the primers APFOR and APBAK; Lane **2**: Same reaction conditions as in Lane **1** but without template.
(B) Lane **1**: *N. tabacum* Xanthi single-stranded cDNA as a template using the primers APFOR and TGUY; Lane **2**: *N. tabacum* Xanthi single-stranded cDNA as a template using the primers APFOR and APBAK; Lane **3**:*N. tabacum* Xanthi single-stranded cDNA as a template using the primer TGUY.
(C) Lane **1 - 4**: Re-amplification of the 338 bp gel-purified band seen in Lane **2** in (B), repeated three times.

5′G G T T C T G A C A A C T T G A G A G A T G T G T T T G T G
A A G C A A A T G G G C C T A T C T G A T A A G G A T A T T G
T T G C A C T C T C T G G T G G C C A T A C C T T G G G A A G
G T G C C A C A A G G A A C G T T C T G G T T T T G A G G G A
C C T T G G A C T A C C A A T C C C C T C A T C T T T G A C A
A C T C A T A C T C T A C G G A A C T T T T G A G T G G G G A
G A A A G A A A G G G C T T T T G C A G T T G C C C T T C A G A C
A A G G C T C T C C T C T C T G A T C C T G C T T T C C G C C
C C C T T G T T G A G A A A T A T G C T G C G G A 3′

Figure 3.2 Partial DNA sequence of the clone ap338a.

Total number of bases sequenced is 272.

One of the clones was compared with DNA sequences in GenBank using PCGene (Aug. 1993 edition). The sequence comparison revealed high nucleotide identity with cytosolic APX mRNA from *P. sativum* (78.9%) and *A. thaliana* (75.9%).

3.3.2 Full-length cDNA cloning

A schematic diagram of the next steps in the cloning strategy is shown in Figure 3.3. The protocol described by Gurr and McPherson (1991) was used as a second step towards cloning a full-length cDNA for APX from tobacco. This protocol, for PCR-amplification of size-fractionated cDNA's for cDNA library preparation, with slight modifications, was used as a RACE technique to amplify and clone the 5' end of the APX cDNA.

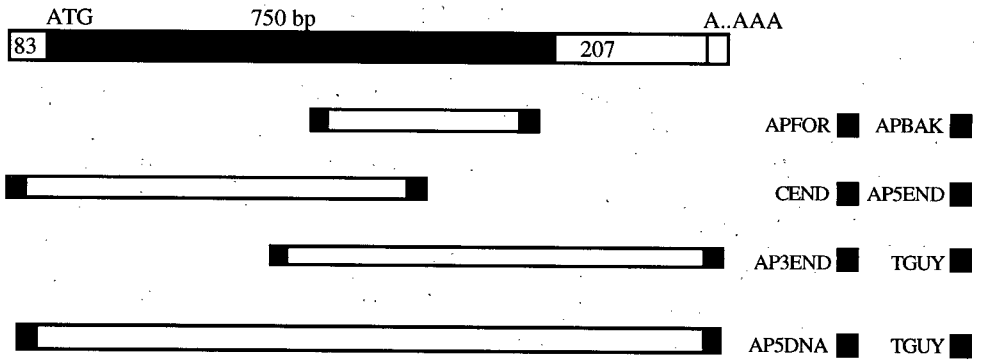


Figure 3.3 Diagram showing the PCR-strategy used for cloning a full-length APX from tobacco.

The first PCR included the degenerate primers APFOR/APBAK. This was followed by PCR using the primer set CEND/AP5END, AP3END/TGUY and AP5DNA/TGUY, respectively.

A specific 3' end primer (AP5END), based on the partial APX sequence (Figure 3.2), and a poly(C) primer (CEND), were used to amplify a 5' end G-tailed cDNA made from the same RNA that had been extracted from ozone-exposed tobacco leaves (see 3.2.2). This reaction gave a strong smear of amplification products with a detectable band around 0.45 kb. The smear between 0.4 kb and 1.8 kb was purified from the gel,

subcloned and screened by colony hybridization with the 338 bp fragment (clone ap338a) (see 3.3.1). The insert of the largest positive clone, s47 (with a 0.6 kb insert), was then sequenced. s47 included 83 bp of the 5' untranslated end of the cDNA and 535 bp of coding sequence that showed 100% identity with the 338 bp fragment in the overlapping region (Figure 3.3). Based on the s47 sequence, two primers, AP3END and AP5DNA, were designed. AP3END, along with a poly-T primer (TGUY) was used to amplify the 3' end of the cDNA (Figure 3.4A). The APCDNA primer, -53 bp to the start codon, was used with TGUY to amplify the complete cDNA (Figure 3.4B).

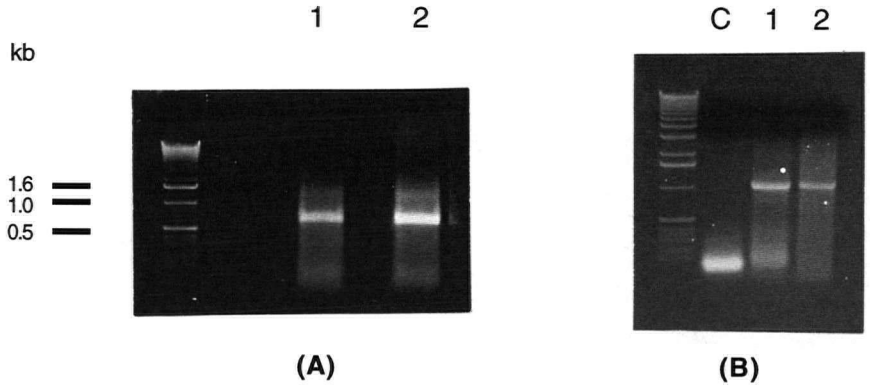


Figure 3.4 Agarose gel analysis of PCR amplification products using the primers AP3END, AP5DNA and TGUY.

The products were separated on a 1.0 % agarose gel, stained with ethidium bromide and photographed under UV-light. The size markers (on the left) are from a 1 kb ladder.
(A) Lane **1 - 2**: *N. tabacum* single-stranded cDNA as a template using the primers AP3END and TGUY, repeated once.
(B) Lane **1**: *N. tabacum* single-stranded cDNA as a template using the primers APCDNA and TGUY; Lane **2**: Same reaction condition used as in Lane **1** except that four times more template was used (80 ng). Lane **C**: Same reaction conditions as in Lane **1** except with no template.

Four clones from three separate AP3END/TGUY amplifications (3e331, 3e341, 3e342, 3e51) and two clones from two separate AP5DNA/TGUY amplifications (ap12, ap21) were sequenced from both ends. These clones, in addition to two clones from CEND/AP5END amplification (s47 and s19), were used for comparison to construct the cDNA sequence for APX from tobacco. However, the sequence between -81 and -53,

was based solely on the s47 clone. Four of the nine clones (3e331, 3e351, S19, ap21) were identical in sequence except for one bp difference between clones ap21 and 3e331 in the 3' untranslated region and between clones s19 and ap21 in the coding sequence. One explanation for these differences could be a bp mismatch introduced during the PCR, either during the initial PCR on the cDNA template or during the DNA sequencing reaction. This is highly likely, since in both cases, only one out of three clones had a different base in this position. No difference was found in the 5' untranslated leader sequence of two clones (s47 and ap21) sequenced in this region.

The cDNA insert of clone ap21 consisted of 1040 bp containing 83 bp of 5' untranslated sequence, a 750 bp open reading frame and 207 bp of 3' untranslated sequence (Figure 3.5). The nucleotide sequence in the vicinity of the putative translation initiation site (UGCUAUGGG) did not reflect the plant consensus sequence (AACAAUGGC) but was exactly the same as the corresponding sequence in pea cytosolic APX cDNA (Mittler and Zilinskas 1992). The in-frame stop codon (-57 to -55) found in the 5' untranslated regions indicates that a correct open reading frame (750 bp) has been selected. A putative polyadenylation signal (Joshi 1987) was located 27 bases upstream from the start of the poly(A) tail.

The open reading frame could encode a protein of 250 amino acids (Figure 3.6) with a predicted molecular mass of 27.388 kDa. No putative transit peptide could be identified in the coding region. Using the BLAST algorithm (Altshul *et al.* 1990), the amino acid identity with known cytosolic APX's was as high as 95.2% for mRNA from *Capsicum annuum* (Table 3.2). Furthermore, the numbers of amino acids in these known cytosolic APX's are from 249 to 251 (Table 3.2). The carboxyl-end of the deduced amino acid sequence had especially high homology with other known cytosolic APX's where only three of the last fifty amino acids were not either identical or conservative substitutions.

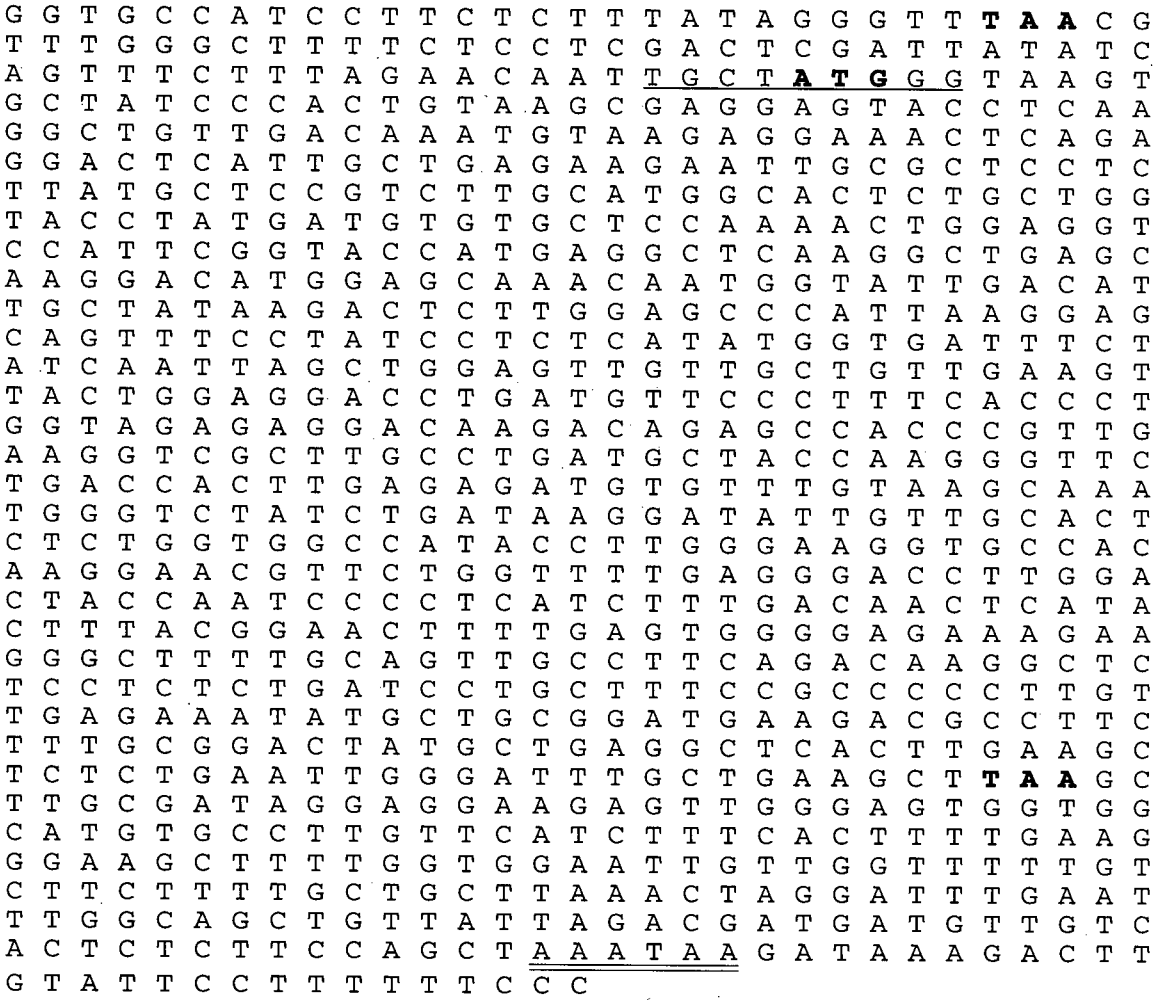


Figure 3.5 Nucleotide sequence of the APX cDNA (clone ap21) from tobacco.

Total length of the cDNA is1040 bp, consisting of 83 bp 5' untranslated sequence, an open reading frame of 750 bp, and an 3' un-translated of 207 bp. A putative translation initiation site is underlined (TGCTATGGG) with the start and stop codon bold-faced, and the putative polyadenylation sequence (AAATAA) double underlined.

M G K C Y P T V S E E Y L K A V D K C K R K L R G L I A E K N
C A P L M L R L A W H S A G T Y D V C S K T G G P F G T M R L
K A E Q G H G A N N G I D I A I R L L E P I K E Q F P I L S Y
G D F Y Q L A G V V A V E V T G G P D V P F H P G R E D K T E
P P V E G R L P D A T K G S D H L R D V F V K Q M G L S D K D
I V A L S G G H T L G R C H K E R S G F E G P W T T N P L I F
D N S Y F T E L L S G E K E G L L Q L P S D K A L L S D P A F
R P L V E K Y A A D E D A F F A D Y A E A H L K L S E L G F A
E A

Figure 3.6 Translated amino acid sequence of an APX (clone ap21) from tobacco.

An open reading frame of 750 bp was used for the translation of the DNA sequence in Figure 3.5 into an amino acid sequence.

Table 3.2 Comparison of the tobacco APX (clone ap21) amino acid sequence with known cytosolic APX sequences.

Name	% identity with *N. tabacum*	Number of amino acids	GenBank accession number[a]
Capsicum annuum	95.2	250	X81376
Daucus carota	88.0	250	Z17398
Zea mays	86.8	250	Z34934
Glycine max	84.4	249	U56634
Pisum sativum	84.4	251	X62077
Arabidopsis thaliana	83.6	249	X59600
Spinacia oleracea	83.2	250	L20864
Raphanus sativus	82.0	250	X78452
Oryza sativa	81.2	249	D45423

[a] The NCBI web site for aquiring these sequences is: http://www2.ncbi.nlm.nih.gov/cgi-bin/genbank.

3.3.3 *APX* copy number in *N. tabacum* Xanthi

The AP3END/TGUY PCR amplification gave the opportunity to compare the 3' end untranslated sequences (UTR) from separate PCR's. This region of the cDNA is considered to be the most variable. A total of six clones was sequenced in this region and three of them, ap21, 3e31 and 3e51, showed 100% homology with each other (as described in section 3.3.2). The other three clones differed markedly in the 3' end UTR identity, both when compared to each other, and also when compared with ap21, 3e31 and 3e51. The differences involved both length and base sequence (see Table 3.3). For example, the distance between the stop codon (UAA) and the first base in the poly(A) signal varied from 150 bases to 172 bases. Although the distance was very close to 172 bp in clone 3e41 (171 bp) the DNA sequence identity was no more than 92%, indicating this was a different transcript. These results indicate that these six different clones represent at least four different transcripts of *APX* genes. It is not clear whether they are different alleles of the same gene or if they represent four different genes.

Table 3.3 Comparison of the 3' end un-translated sequences in 6 different clones from PCR using the AP3END/TGUY-primer set (see section 3.2.4).

clone	5' UAA.....AAAUAA 3'[a]	Type[b]
ap21	172	1
3e31	172	1
3e51	172	1
ap12	158	2
3e41	171	3
3e42	150	4

[a] Number of bases between UAA and AAAUAA
[b] Type of transcripts; same number means 100% identity

3.4 DISCUSSION

This is the first report of an APX cDNA sequence cloned from tobacco (*N. tabacum* Xanthi). In addition to the cytosolic APX cDNA sequences of *P. sativum* L (Mittler and Zilinskas 1992) and *A. thaliana* Columbia (Kubo *et al.* 1992), cDNA sequences for APX from at least seven other species have now been reported (see Table 3.2). These nine sequences have 81% to 95% amino acid identity with tobacco and the proteins range in size from 249 to 251 amino acids (Table 3.2). The most similar gene to tobacco (95.2% identity) is *C. annuum*, which is also a member of the Solanaceae. Only 12 amino acids (out of 250) differ between the *APX* genes from these two species. No attempt was made to determine whether clone ap21 was a full-length cDNA but the size of the cDNA corresponds closely to the mRNA transcript size on Northern blots, when this cDNA was used as a probe (see Figure 2.10). Recently, another APX cDNA from tobacco was cloned (unpublished, GenBank accession number D85912). This cDNA is 1055 bp and has an open reading frame encoding 250 amino acids. The amino acid identity with the tobacco ap21 clone (Figure 3.6) is 99.2% (2 amino acid differences) and the nucleotide identity 93.65%. The distance between the stop codon and the poly-A signal (as compared in Table 3.3) is 158 bp, the same distance as observed in the clone ap12 (Table 3.3). In fact, this cDNA has 100% DNA identity with the 3' end of ap12.

The cytosolic APX from pea shoots was the first to be cloned and characterized from plants (Mittler and Zilinskas 1992). The pea cDNA was cloned using polyclonal antibodies raised against purified cytosolic APX from pea (Mittler and Zilinskas 1991b). Until recently, no APX isoforms other than the cytosolic one had been cloned. However, the cloning of a cDNA for a thylakoid-bound APX from pumpkin has recently been reported (Yamaguchi *et al.* 1996). This cDNA has an open reading frame encoding 421 amino acids, including a putative transit peptide to chloroplasts (N-terminal) and a C-terminal peptide containing a hydrophobic thylakoid membrane-binding domain (Yamaguchi *et al.* 1996). Stromal and thylakoid-bound APX cDNA's have also been cloned recently from

spinach (Ishikawa *et al.* 1996). The thylakoid-bound cDNA and the stromal cDNA encode 415 and 365 amino acids, respectively (Ishikawa *et al.* 1996). These two cDNA's are 100% identical, differing only in the presence (thylakoid) or absence (stromal) of the C-terminal peptide containing the membrane-binding domain (Ishikawa *et al.* 1996). The two chloroplastic isoforms from spinach show very high amino acid similarity with pumpkin thylakoid cDNA, except in the putative chloroplastic transit peptide region (see Figure 3.7). Stromal- and thylakoid-bound APX cDNA's have also been cloned recently from *A. thaliana* (GenBank accession number X98925 and X98926, respectively). These sequences also show high amino acid similarity with the previously cloned chloroplastic isoforms; when compared to each other, the stromal isoforms from pumpkin, spinach and *A. thaliana* show identity between 72 and 76%.

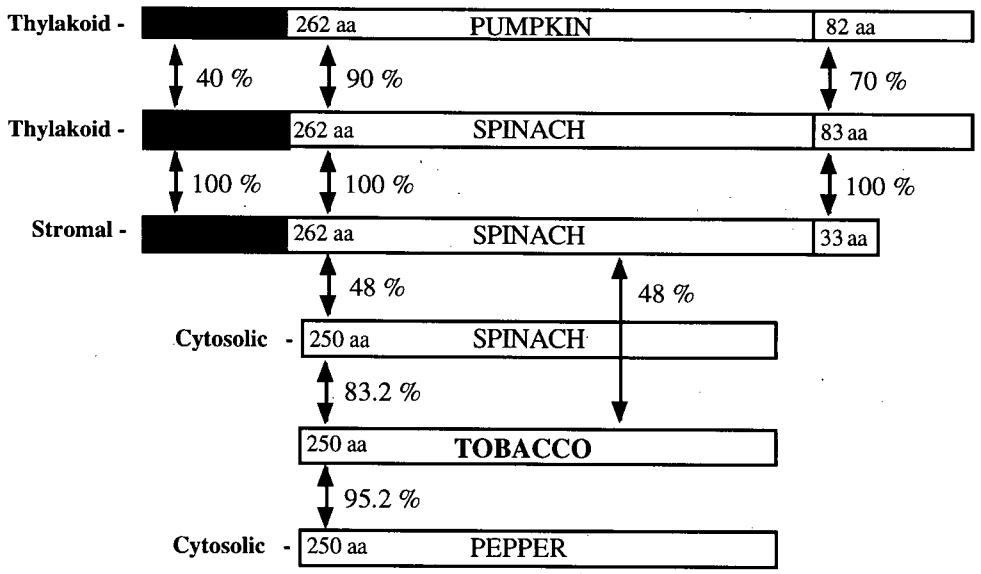


Figure 3.7 Comparison of the amino acid sequence of tobacco APX (clone ap21) with cytosolic-, thylakoid- and stromal-isoforms from three different species

The amino acid identity between different regions in these isoforms is shown as a percentage (%). Shaded areas represent a chloroplastic transit peptide that is cleaved during localization to the chloroplast. The C-terminal peptide in pumpkin and spinach thylakoid-isoforms (82 and 83 amino acids, respectively) contain a putative hydrophobic thylakoid membrane binding domain

When the cDNA (clone ap21) from tobacco is compared to the chloroplastic isoforms a much lower degree of identity is observed. For example, comparison of the deduced amino acid sequence of the thylakoid cDNA from spinach to clone ap21 revealed 48% identity, in addition to very different numbers of amino acids (Figure 3.7). In addition to the three isoforms mentioned above (i.e. cytosolic, stromal and thylakoid) a glyoxysomal membrane-bound APX cDNA has been cloned from *Gossypium hirsutum* (cotton) (Bunkelmann and Trelease 1996). This cDNA codes for 291 amino acids and has 74% identity with the tobacco clone ap21 (Bunkelmann and Trelease 1996). Interestingly, the first 247 amino acids in this cotton cDNA share 88.3% identity with an as-yet-unpublished cDNA from *A. thaliana* (GenBank accession No. X98003). This new *A. thaliana* cDNA codes for 290 amino acids, and the last 43 amino acids, which are absent in the *A. thaliana* cytosolic isoform, share 65% identity with the cotton cDNA. These 43 amino acids include a putative membrane-spanning region (Bunkelmann and Trelease 1996).

In conclusion, based on analysis of the nine reported APX cDNA's and clone ap21 from tobacco (Table 3.2), the tobacco cDNA described here represents an APX cytosolic isoform. This is based on the following observations:

(1) High similarity between clone ap21 and other published cytosolic APX isoforms.
(2) Similar number of amino acids (249 - 251) between clone ap21 and other published cytosolic APX isoforms.
(3) Absence of putative chloroplastic transit peptide sequences from clone ap21.
(4) Relatively low similarity, in both amino acid sequence and number, between clone ap21 and the chloroplastic APX isoforms that have so far been characterized.
(5) Very high amino acid sequence similarity of all published chloroplastic APX isoforms, especially in the region that is comparable to the cytosolic APX isoform.

Chapter 4

Ozone Susceptibility in Transgenic Tobacco Expressing Sense and Antisense RNA for Cytosolic Ascorbate Peroxidase

4.1. INTRODUCTION

The isolation of endogenous plant genes provides the opportunity to genetically engineer the expression of these genes by using transgenic technology. Analyzing transgenic plants that have been engineered to overexpress genes which participate in antioxidant scavenging could give some insight into the mechanism of oxidative stress defence. Alternatively, the endogenous expression could be decreased by expressing antisense constructs although the potential of antisense technology has not yet been extensively exploited in oxidative stress research (Foyer *et al.* 1994).

A number of attempts have been made to modulate ozone tolerance in transgenic plants by overexpressing antioxidant enzymes. Broadbent *et al.* (1995) showed (using chlorophyll fluorescence measurements for leaf injury) that overexpression of pea glutathione reductase (GR) simultaneously in both chloroplasts and mitochondria of transgenic tobacco enhanced the plant's ozone tolerance, although overexpression in the cytosol or chloroplast alone, did not. Overexpressing Mn-superoxide dismutase (SOD) in tobacco chloroplasts increased ozone tolerance in transgenic plants exposed to low levels of ozone (Van Camp *et al.* 1994), whereas overexpression of Cu/Zn SOD in chloroplasts generated no increase in ozone tolerance in transgenic tobacco exposed to higher ozone levels (Pitcher *et al.* 1991).

The effects of overexpressing ascorbate peroxidase (APX) on ozone tolerance in plants have not yet been published, but a recent preliminary report indicates that overexpression of pea APX in tobacco, in both cytosol and chloroplasts, made those plants less susceptible to oxidative injury caused by the superoxide-generating herbicide, paraquat (Webb and Allen 1996). Gene expression studies indicate that APX might play a significant role in ozone defence, in concert with other enzymes of the

ascorbate-glutathione cycle (see section 1.4.2), and increases in APX activity prior to ozone exposure have been correlated with increased tolerance to ozone (Mehlhorn 1990). For example, it has been shown that APX activity is lower in the ozone-sensitive tobacco genotype Bel-W3, than in the more tolerant genotype *Havana*, and also that the APX activity in Bel-W3 increased much less following ozone exposure than in *Havana* (Batini *et al.* 1995). In contrast, SOD levels in these two different genotypes were identical (Batini *et al.* 1995). However, no direct evidence exists to suggest that APX, or other catalysts in the ascorbate-glutathione pathway, are essential for effective ozone defence (Kangasjärvi *et al.* 1994). Although positive correlations between the levels of antioxidant enzymes and ozone tolerance have been described in different plants, such correlations do not allow critical assessment of the relative importance of these individual antioxidant enzymes in scavenging ozone-generated ROS (Conklin and Last 1995). However, if ozone exposure increases the oxidative stress level inside the plant cells, it could be predicted that decreasing the level of an antioxidant enzyme, such as APX, might increase the extent of ozone injury by restricting the capacity of the plant to respond to the increased stress. Furthermore, it could be predicted that an increase in the antioxidant enzyme level (APX) could increase this capacity to respond to oxidative stress, if this particular antioxidant enzyme (APX) is a limiting factor in dealing with increased oxidative stress.

As part of the examination of the involvement of cytosolic APX in tolerance of tobacco to ozone stress, the cDNA for a cytosolic APX in tobacco was cloned and characterized (see chapter 3). This cDNA was then used to both increase and decrease the expression of cytosolic APX in the ozone-sensitive tobacco genotype Bel-W3, using transgenic technology. Ultimately, these transgenic plants, expressing either sense or antisense constructs, were evaluated for their responses to ozone stress.

4.2 MATERIALS AND METHODS

4.2.1 Restriction enzyme-digest, gel electropohoresis and gel-purification

These were performed as described in Chapter 2.

4.2.2 Ligation and transformation

These were performed as described in Chapter 2, except that for *Agrobacterium tumefaciens* transformation, binary vectors were transferred into *A. tumefaciens* strain LBA 4404 using the modified freeze-thaw transformation method (Chen *et al.* 1994). Positive transformants were selected on LB plates with kanamycin (100 mg L^{-1}) and rifampicin (100 mg L^{-1}) and binary vectors isolated for verification (Chen *et al.* 1994).

4.2.3 Sense- and antisense-APX binary vectors

Three different binary vectors, called pBα338, pBGα338 and pB35AP21, were made, based on the binary vector pBI121 (Clontech). pBα338 and pBGα338 had a 338 bp APX gene fragment (see section 3.3.1) placed in antisense orientation directly downstream of the 35S cauliflower mosaic virus promoter (35S CaMV) or the GUS coding sequence, respectively, whereas pB35AP21 had a full-length APX cDNA, including its 3' UTR (see chapter 3), downstream of the 35SCaMV promoter in sense orientation. A schematic diagram of these binary vectors is presented in Figure 4.1. Antisense orientation was confirmed by automated DNA sequencing (see section 2.2.10).

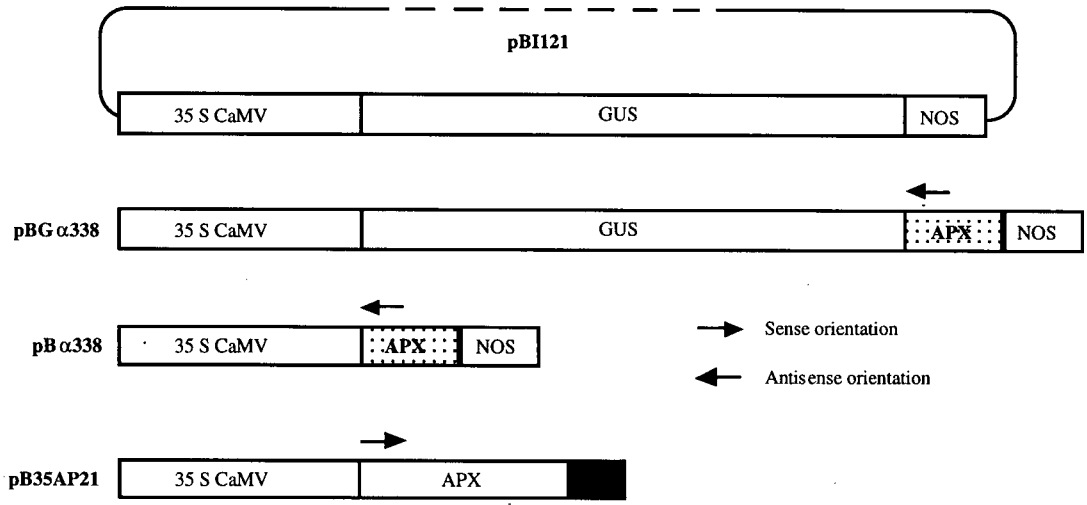


Figure 4.1 Schematic diagram depicting the binary vectors used for the study.

The GαAP338 antisense gene construct (in binary vector pBGα338) releases a 2.47 kb transcript; the αAP338 antisense gene construct (in pB α338) releases a 0.6 kb transcript and the 35AP21 sense gene construct (in pB35AP21) releases a 1.04 kb transcript. APX-antisense region is represented by dotted areas. pBI121 was used as a control.

Preparation of pBα338 binary vector (αAP338 antisense gene construct)

The diagram of the cloning strategy for this construct is shown in Figure 4.2. A 0.9 kb HindIII/XbaI fragment from pBI121, containing the 35S CaMV promoter, was subcloned into the *Hind*III/*Xba*I site in the pUC18 polylinker. This recombinant plasmid, called pUC35S, was digested with *Sma*I, gel-purified, and digested with *Xba*I. This reaction was extracted with phenol/chloroform and precipitated with 95% ethanol according to Sambrook *et al.* (1989). The blunt-ended linearized pUC35S had, therefore, an *Xba*I site at the 3' end of the 35S CaMV promoter. Next, a 338 bp APX fragment was amplified by PCR from tobacco with the degenerate primers

CGGAATTC$_{EcoRI}$CGCIGAC/TGCIACIAAA/GGG and

GCTCTAGA$_{XbaI}$GCTGIGCC/TTCIGCA/GTAA/GTC (see section 4.2.2 for more detailed description)

The amplified fragment was gel-purified, digested with *Eco*RI and *Xba*I, and subcloned between the *Eco*RI and *Xba*I sites of the pUC18 polylinker to make a recombinant plasmid called pUC338. The 338 bp fragment was released from pUC338 with *EcoR*I and *Xba*I digestion, gel-purified and ligated to the *Xba*I site in the linearized pUC35S. Since the *Xba*I site in the 338 bp fragment was the 3' end of this APX fragment it was anticipated that the 338 bp fragment would be ligated in antisense orientation, directly down-stream of the 35S CaMV promoter. This ligation reaction was carried out at 26°C for 1 hr followed by 16 hr at 16°C and a total of 1μg DNA was used. The reaction products were separated on an agarose gel and the fragment of expected size (around 3.8 kb) was gel-purified. The 5' overhang *Eco*RI site was then blunt-ended by filling the 5' overhang with Klenow DNA polymerase according to Sambrook *et al.* (1989) and a blunt-end ligation carried out according to instructions of the manufacturer (BRL), to make the recombinant plasmid p35Sα338. After transformation, positive clones were verified with restriction digest analysis, where an *Eco*RI/*Xba*I-digest, a *Xba*I/*Hind*III-digest, and a *Sac*I/*Hind*III-digest should give a 338 bp-, 850 bp- and 1.2 kb fragment, respectively. Finally, the pUC35Sα338 was digested with *Sac*I/*Hind*III and the 1.2 kb fragment harbouring the 35SCaMV-α338 construct was gel-purified and substituted for the *Sac*I/*Hind*III-fragment in the pBI121 vector to give the binary vector pBα338 (Figure 4.2).

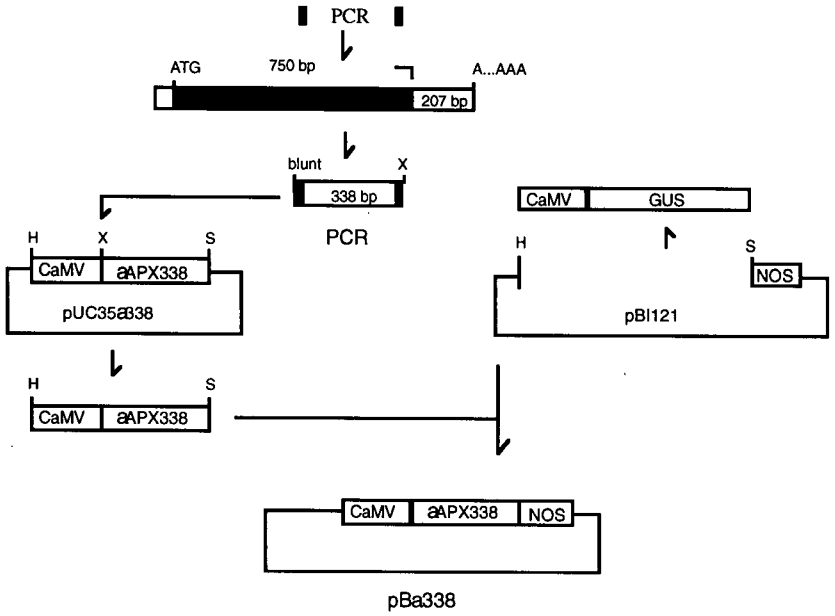


Figure 4.2 Schematic diagram showing the construction of the pBα338 binary vector.

The degenerate primers APFOR and APBAK used for the initial PCR-amplification of cytosolic APX sequence (chapter 3) are represented by filled boxes, the grey box is the APX coding sequence and the A...AAA is the beginning of the poly-A addition. **CaMV** is the cauliflower mosaic virus promoter in pBI121, **H** is *Hind*III, **X** is *Xba*I and **S** is *Sac*I.

pBGα338 binary vector (GαAP338 antisense gene construct)

The diagram of the cloning strategy for this construct is shown in Figure 4.3. pBI121 was digested with *Hind*III/*Eco*RI and the 3.0 kb fragment containing the 35SCaMV-GUS-nos construct was gel-purified. This fragment was cloned between the *Hind*III/*Eco*RI sites in pUC18, to make the recombinant plasmid p35GUSnos, and subsequently linearized with *Sac*I. The 3' overhang *Sac*I site was blunt-ended with T4 DNA polymerase (has exonuclease activity)(Pharmacia) using the following conditions: 2 µg linearized p35GUSnos, 100 µM dNTP's, 2.5 µg BSA, 9 units of T4 DNA polymerase and 5 µl 10x T4 DNA polymerase buffer (Pharmacia) in a 50 µl reaction, incubated for 20 min. at 11°C. This reaction was ethanol-precipitated (Sambrook *et al.* 1989),

resuspended in dH_2O and dephosphorylated with calf-intestinal alkaline phosphatase (CIP) as described by the manufacturer (Pharmacia). The 338 bp APX fragment was released from pUC338 with *Eco*RI/*Xba*I digestion, gel-purified, and then blunt-ended by filling in the 5' overhang with Klenow DNA polymerase according to Sambrook *et al.* (1989). This fragment was then ligated with the blunt-ended, linearized p35GUSnos vector using two-step ligation procedure as follows: the ligation reaction was first carried out at 26°C for 1 hr according to the conditions described in section 2.2.6, except using a 1.5:1 molar ratio of insert to vector in 10 µl reaction volume. 5 µl of this reaction was diluted 20-fold, 7.5 units T4 DNA ligase added (BRL) and the ligation reaction continued overnight at 26°C. Half of this reaction volume (i.e. 50 µl) was used for transformation. This ligation reaction creates a new *Eco*RI site between the *Sac*I site, blunt-ended with T4 DNA polymerase (Pharmacia), and the *Eco*RI site blunt-ended with Klenow DNA polymerase. This new restriction site was used to screen for clones that had the 338 bp APX fragment in antisense orientation to the 35S CaMV promoter. All antisense clones digested with *Eco*RI and *Xba*I should give 3 fragments of 3.8 kb, 2.34 kb and a 260 bp. The new recombinant plasmid, pGUSα338, was linearized with *Eco*RI, gel-purified, blunt-ended with Klenow DNA polymerase (Sambrook *et al.* 1989), digested with *Hind*III and the 3.1 kb fragment (35SCaMV-GUS-α338) was gel-purified. pBI121 was digested with *Sac*I, gel-purified, blunt-ended with T4 DNA polymerase (Pharmacia) and the reaction was ethanol-precipitated (Sambrook *et al.* 1989). The linearized pBI121 was then digested with *Hind*III and the 10 kb vector fragment was gel-purified. This fragment was ligated with the 3.1 kb blunt-end/*Hind*III 35SCaMV-GUS-α338-fragment using the two-step ligation procedure. Positive clones were screened using *Eco*RI/*Hind*III digestion with antisense clones predicted to give three fragments with sizes of 10 kb (vector), 3.1 kb (insert) and 260 bp. The new binary vector was called pBGα338.

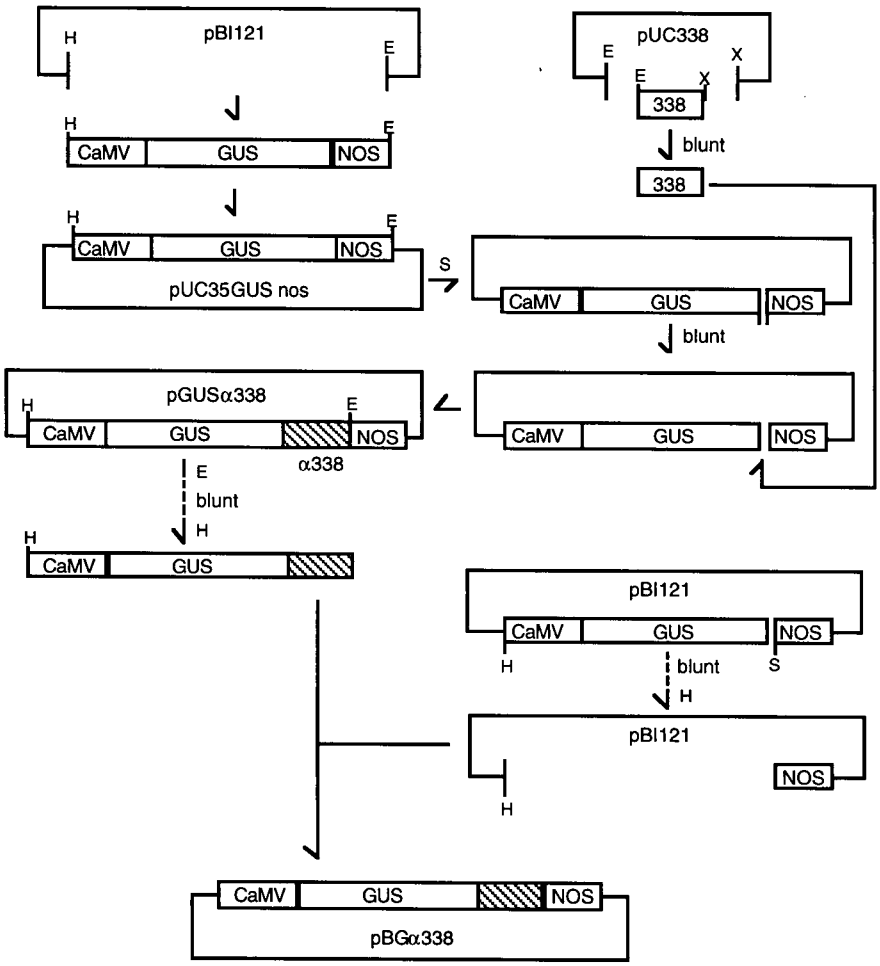


Figure 4.3 Schematic diagram showing the construction of the pBGα338 binary vector.

CaMV is the cauliflower mosaic virus promoter in pBI121, **E** is *EcoR*I, **H** is *Hind*III, **X** is *Xba*I, **S** is *Sac*I and **blunt** means blunt-end reaction. Hatched area is the antisense APX region.

pB35AP21 binary vector (35AP21 sense gene construct)

The diagram of the cloning strategy for this construct is shown in Figure 4.4. The recombinant plasmid pAP21, which has a full-length cDNA for cytosolic APX cloned into the *Eco*RI/*Xba*I sites in pUC18, was digested with *Eco*RI and *Xba*I and the 1040 bp fragment gel-purified. This cDNA insert in pAP21 contains 83 bp of 5′ untranslated

sequence, a 750 bp open reading frame and 207 bp of 3' untranslated sequence (see Figure 3.3 in chapter 3). The 1040 bp fragment was substituted for the *Eco*RI/*Xba*I-fragment in the pBI121 vector to give the binary vector pB35AP21 (Figure 4.4). After *A. tumefaciens* transformation, positive clones were verified with restriction digest analysis, where *Eco*RI/*Kpn*I-digests and *Hind*III-digests should give, in addition to the vector fragment, two fragments of 800 bp and 1.65 kb, respectively.

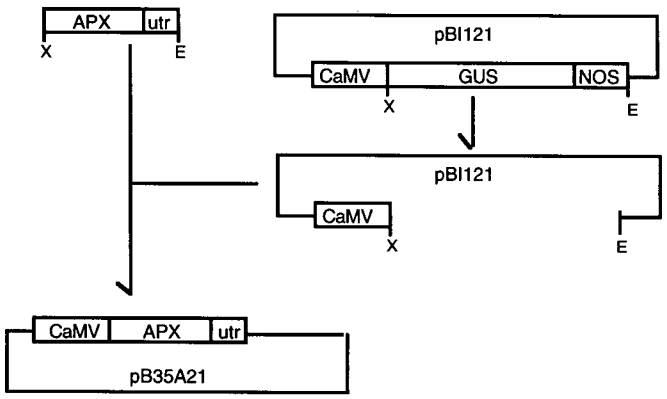


Figure 4.4 Schematic diagram showing the construction of the pB35AP21 binary vector.

CaMV is the cauliflower mosaic virus promoter in pBI121, **E** is *EcoR*I and **X** is *Xba*I. **APX** is the clone ap21 (chapter 3) and **utr** is the untranslated 3' end from that clone.

4.2.4 Plant transformation and growth conditions

Four different *A. tumefaciens* strains, each harbouring pB35AP21, pBα338, pBGα338 or pBI121, were used for plant transformation. The T-DNA's were introduced into *N. tabacum* Bel-W3 by the leaf-disc method according to Horsch *et al.* (1988) using leaves (6 - 10 cm long) from five week old plants. The four different *A. tumefaciens* strains were plated on fresh LB plates (including streptomycin, 100 mg L^{-1}; kanamycin, 50 mg L^{-1}) and incubated for two days at 28°C. Fresh colonies were inoculated in 2 ml of MS (including streptomycin, 100 mg L^{-1}; kanamycin, 50 mg L^{-1}) and grown overnight at 28°C.

The overnight culture was then diluted 10-fold with MS (see Appendix). The leaf-discs were sterilized in 0.6% sodium hypochlorite for 15 min, rinsed three times in sterile distilled water and incubated with the 10-fold diluted *A. tumefaciens* culture for 5 min. After four days on MS plates (see Appendix), leaf-discs were washed for 2 to 3 min in liquid MS selection medium supplemented with the following antibiotics: carbenicillin, 200 mg L^{-1}; kanamycin, 100 mg L^{-1}; vancomycin, 500 mg L^{-1}; blotted on Whatman paper and transferred to MS selection plates (MS, carbenicillin, 200 mg L^{-1}; kanamycin, 100 mg L^{-1}; cefotaxime, 180 mg L^{-1}) and incubated at 26°C under 16 hr photoperiod (70 $\mu E\ m^{-2}\ s^{-1}$). After 25 days, shoots were transferred to Root Induction Medium (including carbenicillin, 200 mg L^{-1}; kanamycin, 50 mg L^{-1}; see Appendix). After 20 to 25 days, regenerated plantlets where transplanted into sterilized soil (50% Metro Mix 290, 50% soil with Osmocote 14-14-14), grown in controlled environment growth chambers with 25°C/20°C (day/night) under 16 hr photoperiod (100 $\mu E\ m^{-2}\ s^{-1}$), RH 60% +/- 10%, and analyzed (APX enzyme assay; see section 4.2.5) 8 to 10 weeks after transplantation. Seeds from independent transgenic lines (T_0), sterilized in 70% ethanol for 1 min. and rinsed three times in sterile dH_2O, were germinated under continuous light (60 $\mu E\ m^{-2}\ s^{-1}$) on MS plates with 100 mg L^{-1} kanamycin. Kan-resistant seedlings (T_1 progeny) were transplanted into soil after 16 to 18 days and transferred to growth chambers under conditions described above. Seeds from untransformed tobacco were germinated in soil. Plants were watered daily without any nutrient supplement. Except for the initial screening of primary transformants (T_0), the T_1 progeny of self-pollinated primary transformants (T_0) were used in all experiments involving transgenic plants.

4.2.5 Ascorbate peroxidase assay

For each APX enzyme assay, either one or two leaves from each of three plants were pooled per sample and ground in liquid nitrogen before protein extraction. However, for screening independent transgenic lines (T_0) only one leaf from each plant was used.

Proteins were extracted in 50 mM HEPES (pH 7.0), 0.1 mM EDTA, 1% (v/v) Triton X-100 including either 0 mM, 1 mM or 10 mM ascorbate (Sen Gupta *et al.* 1993), and quantified according to Bradford (1976). Enzyme activity was determined as described by Sen Gupta *et al.* (1993), except that 0.5 mM ascorbate was used in the reaction solution which was preincubated for 30 sec before measuring the rate of ascorbate oxidation over a 20 sec period, using a UV-visible recording spectrophotometer (Shimadzu). For APX inhibition assay, 0.5 mM *p*-chloromercuriphenylsulphonic acid (*p*-CMPSA) was used as described (Mittler and Zilinskas 1992).

4.2.6 RNA preparation and analysis

Total RNA was prepared from leaves, and Northern blot analysis was performed, as described in Chapter 2 (section 2.2.8). For every RNA extraction one leaf from each of four plants was pooled per sample. However, for screening independent transgenic lines (T1), after the APX assay screening (see 4.2.5), only one leaf from each plant was used.

4.2.7 Ozone exposure and leaf injury scoring

The transgenic plants, six to seven weeks old, were exposed to ozone in a growth chamber under the conditions described in Chapter 2 (see section 2.2.2). The level of necrosis was scored visually as described in section 2.2.2.

4.2.8 Paraquat treatment

Leaves from four to five week-old plants were used to make small leaf-discs (0.75 cm^2) and one leaf-disc was placed into each well in a 24-well Nunclon multidisk (Delta). Row 1 and 3 in each plate contained 0.01% Tween 20 (in dH_2O) and row 2 and 4 contained paraquat (1,1'-dimethyl-4,4'-bipyridylium, 1 µM; SIGMA) in 0.01% Tween 20 solution. Each plate contained leaf-discs from one transgenic line, and for each transgenic line two leaves (6 - 7 cm long) were collected from each of nine plants. Each transgenic line was represented by four plates. Plates were incubated for 1 hr in the dark at 25°C, then kept for 5 hr at 350 µE m^{-2} s^{-1} light. After light-incubation, the plates were kept for 18

hr in the dark at 25°C until scored. After treatment, pigment density for each leaf-disc was assessed by capturing digital images of the disc using the IS-500 Digital Imaging System, and analyzing these using NIH Image Version 1.57 software. The differences in density between control and paraquat treatment in different transgenic lines were quantified, and the proportions were subjected to arcsin transformation. The Students t - test was used to test for significant differences.

4.3 RESULTS

4.3.1 Developmental regulation of APX mRNA levels

Massive (500ppb/8 hr for one day) (MOE) and *acute* (250 ppb/8 hr for six days)(AOE) ozone exposure regimes were used to test the ozone tolerance of transformed and untransformed Bel-W3 plants (see section 2.3.1). In earlier experiments (Chapter 2) using either MOE or AOE, it was noticed that younger leaves usually showed less necrotic injury than recently fully matured leaves. This difference in necrotic injury between leaves of different developmental age appeared to be also reflected in the APX mRNA levels in Bel-W3, since young leaves had higher APX mRNA levels (Lane 1 in Fig. 4.5), compared to recently fully matured leaves (Lane 4 in Figure 4.5). A similar interaction of leaf age and chloroplastic Cu/Zn SOD expression with ozone injury in tobacco was reported by Van Camp *et al.* (1994).

Figure 4.5 Northern blot analysis of developmental regulation of APX mRNA levels in *N. tabacum* Bel-W3.

Total RNA (10 μg) was separated in a 1% formaldehyde/agarose gel (seen on the left) and probed with APX cDNA probe (seen on the right) as described in chapter 2. Numbers represent leaf number from top to bottom. Leaf #1 (Lane 1) is the first leaf > 4 cm in length harvested from seven week-old plants grown at 100 μE m^{-2} s^{-1}.

4.3.2 Characterization of antisense-*APX* primary transformants (T_0)

The two antisense APX gene constructs (αAP338 and GαAP338) used for transformation included approximately 45% (338 bp) of the APX coding sequence from *N. tabacum* Xanthi. This fragment is located towards the 3' end of the APX coding sequence (Figure 4.2). In Table 4.1, the number of potentially transgenic shoots finally establishing roots in root induction medium is shown. Rooting was 92% for shoots transformed with pBI121, 59% for pBα338 and 64% for pBGα338.

Table 4.1 Percentage of rooting in primary transformants transformed with different constructs.

Constructs used for transformation	Total number of shoots transferred to rooting medium	Total number of shoots forming roots (%)	Total number transferred to soil
pBI121	13	12 (92)	7
pBα338	54	32 (59)	24
pBGα338	81	52 (64)	41

Growth and development of the antisense transgenic lines did not differ perceptibly from those of transgenic lines transformed with the pBI121, or from those of untransformed Bel-W3 plants, except that under high light intensity (300 $\mu E\ m^{-2}\ s^{-1}$) antisense seedlings developed depressions on their leaf tips. These pits, which contained almost brittle tissue, were not as visible, or were absent in control lines grown under the same conditions.

mRNA levels and ascorbate peroxidase activity

A total of 65 primary antisense transformants were transfered to soil (see Table 4.1). Of these, only 27 was screened for APX enzyme activity. Based on the results from the APX enzyme activity assay, 15 of these were further analyzed using Northern blot hybridization. Most of them were primary transformants that appeared to have considerably lower APX enzyme activity than the control lines (expressing GUS) used for comparison. Of these 15 transformants, 13 were found to express the antisense gene. Several patterns of antisense and endogenous APX mRNA levels were observed in these transgenic lines. Lines WαAP22 and WαAP29 (both with αAP338 gene construct) showed both high antisense gene expression levels (0.6 kb transcript) and a considerable reduction in the endogenous APX mRNA levels (Fig. 4.6). Lines WGαAP25 and WGαAP26 (both with GαAP338 gene construct) were found to have markedly decreased levels of endogenous APX mRNA, even though the levels of the antisense RNA (2.47 kb transcript) were very low. Line WαAP4 (αAP338 gene construct), by contrast, expressed high levels of the antisense gene but, apparently, had normal endogenous APX mRNA levels (Fig. 4.6). These five lines showing different patterns in antisense transgene and endogenous APX expression were all tested for ozone tolerance. Several lines were used as controls in these experiments, including the untransformed Bel-W3 parent, lines WG3 and WG9 (transformed with pBI121 and expressing the GUS gene) and one antisense line, WαAP3, that did not express the antisense gene in detectable amounts.

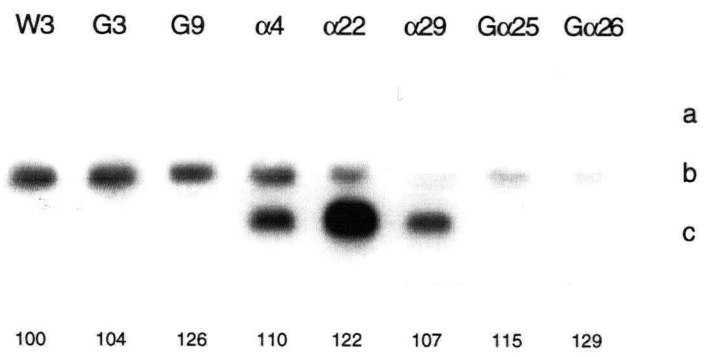


Figure 4.6 Northern blot analysis of antisense-APX transgenic tobacco lines used in this study.

Total RNA (10 μg) was fractionated in a 1% formaldehyde/agarose gel and probed with an APX cDNA probe. The antisense lines are labelled α4 (WαAP4), α22 (WαAP22), α29 (WαAP29), Gα25 (WGαAP25), and Gα26 (WGαAP26). The control lines are labelled G3 (WG3), G9 (WG9) and the un-transformed Bel - W3 labelled W3. RNA was isolated from young leaves (5 - 7 cm in length) harvested from seven week old plants. a; indicates the position of the 2.47 kb antisense transcript, b; indicates the position of the endogenous APX transcript (about 1.1 kb), c; indicates the position of the 0.6 kb antisense transcript. Relative RNA loading is shown under each lane as a percentage of loading in the first lane.

For measurements of APX enzyme activity, young leaves (7 - 9 cm long) were sampled on each plant and extracted in the presence of 1 mM ascorbate. The antisense lines WαAP22, WGαAP25, WGαAP26 and WαAP29 were found to contain 45% - 55% less APX activity than the control lines WG3, WG9 and untransformed Bel-W3 (Fig. 4.7). This reduction in APX enzyme activity was consistent with the decrease in the endogeneous APX mRNA levels seen in these plants (Fig. 4.6, lanes 5 to 8). The antisense line WαAP4, which showed no detectable reduction in the endogenous APX mRNA levels, had APX activity levels similar to those observed in the control lines (Fig.4.7).

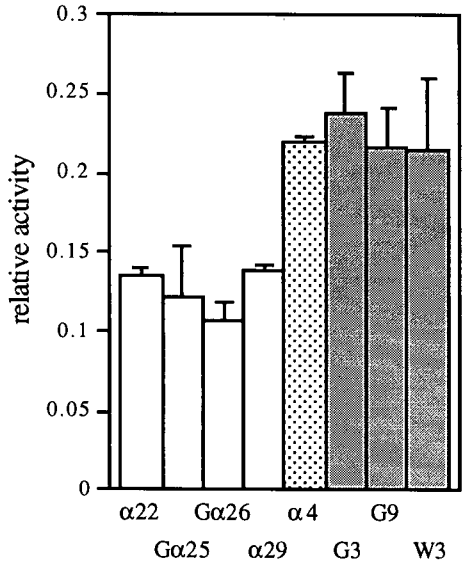


Figure 4.7 The APX activity in transformed and un-transformed plants.

APX enzyme activity was measured in young leaves (7 - 9 cm in length) harvested from seven week old plants. The antisense lines are labelled α4 (WαAP4), α22 (WαAP22), α29 (WαAP29), Gα25 (WGαAP25), and Gα26 (WGαAP26). The control lines are labelled G3 (WG3), G9 (WG9) and the un-transformed Bel - W3 labelled W3. Only the transgenic lines represented by open bars, have reduced endogeneous APX mRNA levels. The data presented for each line are the mean and S.D. of two individual experiments.

The APX enzyme activity assay used in this study is not specific for cytosolic APX, since it also measures oxidation of ascorbate caused by other isoforms of APX, by peroxidases such as the guaiacol peroxidases (GPX), and by non-enzymatic elements (Amako *et al.* 1994; Mittler and Zilinskas 1992). It has been shown that the cytosolic APX isoforms from many different plant species are resistant to ascorbate depletion, whereas the chloroplastic isoforms are readily inactivated in the absence of ascorbate (Amako *et al.* 1994; Mittler and Zilinskas 1991b). Therefore, APX activity was measured in leaves of control plants (WG3) using different concentrations of ascorbate in the extraction buffer. Whereas 0 mM and 1 mM ascorbate gave similar APX activity, use of 10 mM ascorbate increased the APX activity (Figure 4.8A). Since the chloroplastic APX isoforms are very labile in the absence of ascorbate in the extraction buffer, it is conceivable that this

increase largely represents chloroplastic activity. Furthermore, it can be concluded that the APX activity values presented in Figure 4.7 (where 1 mM ascorbate was used) are probably not of chloroplastic origin, since 1 mM and 0 mM ascorbate concentrations gave similar APX activity (Figure 4.8A).

Both the cytosolic and the chloroplastic APX isoforms can be inactivated by *p*-chloromercuribenzoate or *p*-chloromercuriphenylsulphonic acid (*p*-CMPSA), which do not affect GPX activity or non-enzymatic ascorbate oxidation (Mittler and Zilinskas 1992). To evaluate whether only APX was contributing to the activity observed using 0 mM ascorbate, the 0 mM extract was also incubated (for 10 min) with the APX inhibitor *p*-CMPSA (0.5 mM), as described by Mittler and Zilinskas (1992).

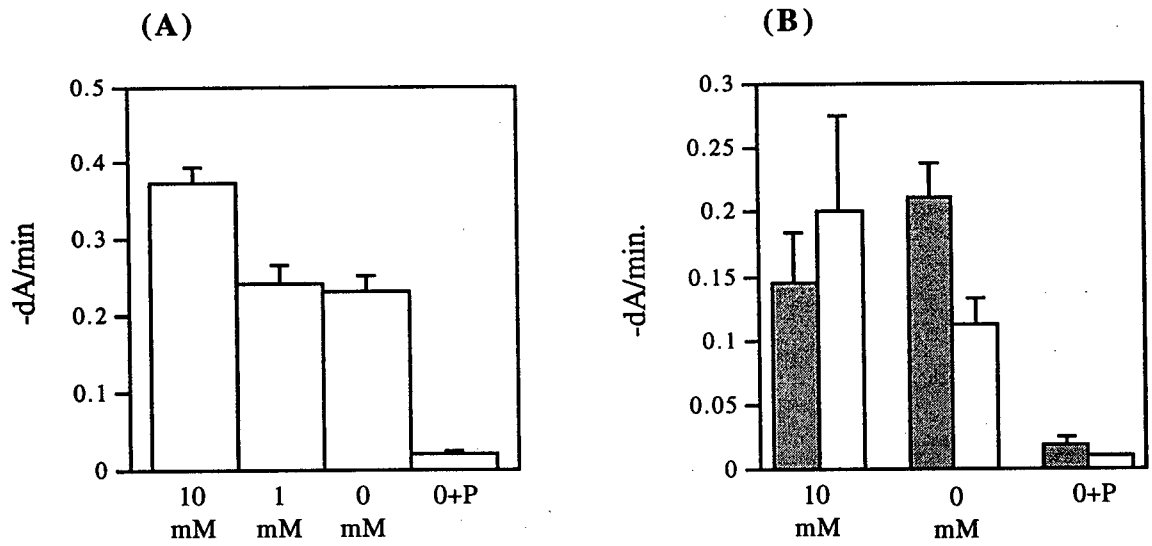


Figure 4.8 Analysis of APX activity in tobacco using different concentrations of ascorbate in the extraction buffer.

APX enzyme activity was measured in leaves (10 - 14 cm in length) harvested from seven week old plants grown at 100 $\mu E\ m^{-2}\ s^{-1}$. The leaf tissue was extracted in 10 mM, 1 mM or no ascorbate. 0+P is 0 mM ascorbate + *p*-chloromercuribenzoate (0.5 mM). All samples were incubated for 10 min at RT before the assay. The data presented for each treatment are the mean and S.D. of two individual experiments.
(A) The effects of different ascorbate concentration in extraction buffer on APX activity in WG3.
(B) Analysis of the APX activity in WG3 (shaded bars) and WαAP29 (open bars) (see text for further explanations).

As presented in Figure 4.8A, it appears that approximately 10 - 15% of the activity observed using 0 mM (or 1 mM) ascorbate, represents non-APX oxidation of ascorbate. This could be both non-enzymatic activity as well as GPX activity. This proportion is similar to that reported by Mittler and Zilinskas (1992) in peas. In Figure 4.8B the APX activity is analysed further in WG3 and WGαAP29 using different ascorbate concentrations in the extraction buffer, as well as *p*-CMPSA. To assess the contribution of chloroplastic APX isoforms in total APX activity (measured using 10 mM ascorbate), the APX activity measured using 0 mM ascorbate in the extraction buffer, and the non-APX activity, measured using the 0 mM extract incubated with *p*-CMPSA, were substracted from the total APX activity. The difference is, presumably, the contribution of the chloroplastic APX isoforms to total APX activity, and is presented in Figure 4.8B as "10 mM" activity. The non-APX activity (represented in Figure 4.8B as "0 + P") was also substracted from the APX activity measured at 0 mM. This APX activity (presented in Figure 4.8B as "0 mM") is the non-labile, non-chloroplastic APX activity. Based on the results presented in Figure 4.8B, it appears that the antisense suppression is not affecting the chloroplastic APX isoforms since the "10 mM" activity appears to be at least as high in WαAP29 as in WG3.

Massive ozone exposure and ozone symptoms in antisense transformants

To test the ability of the antisense lines to tolerate high ozone levels, all plants were exposed to *MOE* after three days incubation in 300 $\mu E\ m^{-2}\ s^{-1}$ light. Figure 4.9 shows the results from three independent experiments in which one control line and four antisense lines were treated simultaneously with *MOE*. In each experiment, the ozone injury level (% necrosis) in the control GUS-expressing line (WG3), and the antisense line (WαAP25), was compared with the injury level in a different antisense line.

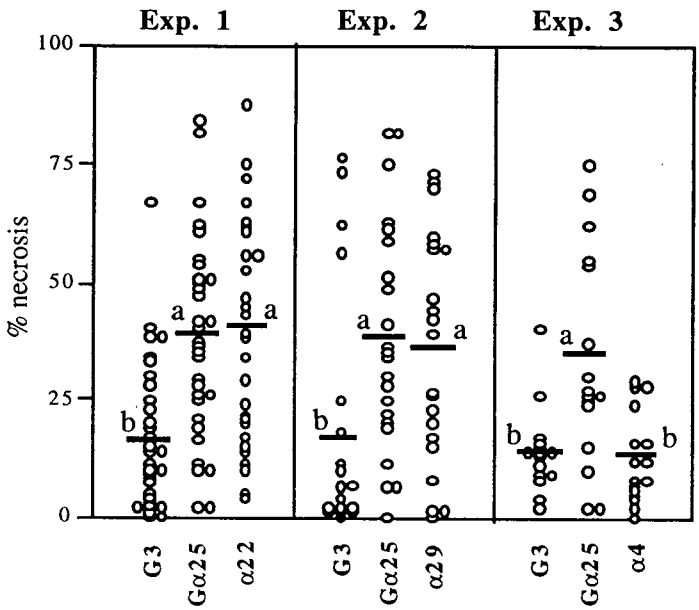


Figure 4.9 Ozone-induced necrosis in control and antisense lines following *MOE*.

Each data point represents the observed level of necrosis in one plant, recorded as average % necrotic area/leaf. The results from three independent experiments are plotted, each repeated three to four times. Four or five leaves per plant were scored. A horizontal line indicates the calculated mean level of necrosis/leaf.
Exp. 1 Comparison between WG3 (G3), WGαAP25 (Gα25) and WαAP22 (α22). The differences in means between WG3, and WGαAP25 and between WG3 and WαAP22, were significant at $P < 0.01$ (Student's t - test).
Exp. 2 Comparison between WG3 (G3), WGαAP25 (Gα25) and WαAP29 (α29). The difference in means between WG3, and WGαAP25 and WαAP29, respectively, was significant at $P < 0.01$ (Student's t - test)
Exp. 3 Comparison between WG3 (G3), WGαAP25 (Gα25) and WαAP4 (α4). Means with different letters are significant at $P < 0.01$ (Student's t - test).

Three antisense lines, WGαAP25, WαAP22 and WαAP29, displayed a significant increase in ozone injury compared to the WG3 control line ($P < 0.01$). However, when the antisense line WαAP4 was compared with WG3 and WGαAP25, there was no significant difference in ozone injury between WαAP4 and WG3 (Fig. 4.9). In Figure 4.10, the means for ozone injury in WG3 (control) and WGαAP25 are compared, using the data from all *MOE* experiments carried out where these lines were exposed simultaneously.

Under *MOE* conditions, the extent of ozone injury was consistently about 2.3 - fold higher in WGαAP25 than in WG3. This is similar to the differences observed between WαAP22 and WG3, and between WαAP29 and WG3 (Fig. 4.9).

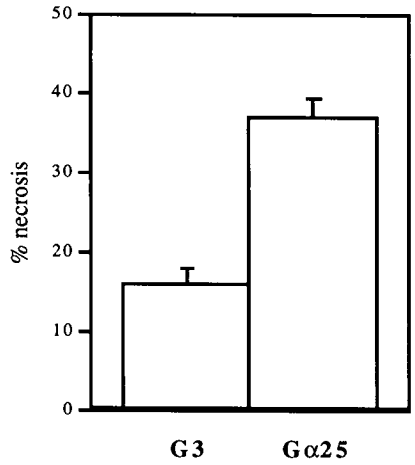


Figure 4.10 Differences in ozone-induced necrosis between WG3 and WGαAP25 following *MOE*

The average % necrotic area/leaf is compared for all experiments (n = 12 experiments) where WG3 (G3) and WGαAP25 (Gα25) were simultaneously exposed to *MOE*. In this figure % necrosis is the mean +/- S.D of the average % necrosis for each line from individual experiments. Four or five leaves per plant were scored in each experiment.

The level of ozone injury induced in line WG3 was compared with that observed in an antisense line, WαAP3, that did not express the antisense APX construct, and with untransformed Bel-W3. No significant difference in ozone injury was observed among these three lines (Figure 4.11a). Finally, WG3 was also compared with another GUS-expressing control line, WG9, which showed levels of APX enzyme activity similar to WG3 (Figure 4.7). No significant difference in ozone injury was obtained when these two control lines where compared (Figure 4.11b).

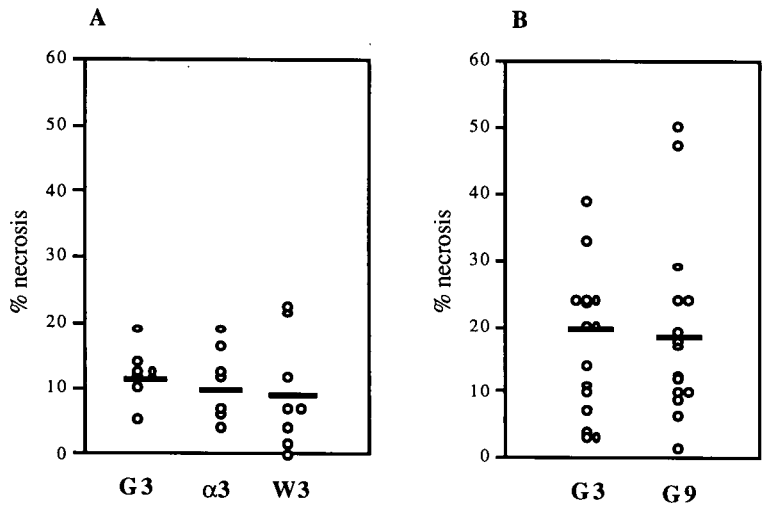


Figure 4.11 Ozone-induced necrosis in different control lines.

Each data point represents the observed level of necrosis in one plant, recorded as average % necrotic area/leaf. Four or five leaves per plant were scored. The results from two independent experiments are plotted together. A horizontal line indicates the calculated mean level of necrosis/leaf.
(A) Plants were exposed to 3 days of 350 ppb/4 hr/day ozone. The difference in means between these lines was not significant (Student's t - test). The lines compared are: the GUS-expressing line WG3 (G3), an antisense line WαAP3 not expressing the antisense gene (α3) and the non-transformed Bel - W3 (W3).
(B) Plants were exposed to *MOE* .

Acute ozone exposure and ozone symptoms in antisense plants

Lines WG3 and WαAP22 were exposed to *AOE* for six days and their levels of ozone injury assessed. Under *AOE* conditions, these two lines differed dramatically in their responses, with approximately 6-fold more necrotic injury in WαAP22 than in WG3 (Fig. 4.12).

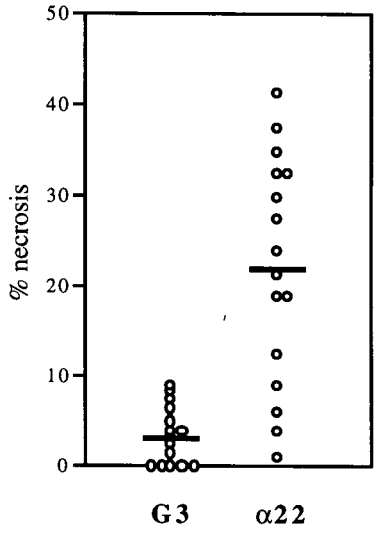


Figure 4.12 Ozone-induced necrosis in lines WG3 and WαAP22 following *AOE*

Each data point represents the observed level of necrosis in one plant, recorded as average % necrotic area/leaf. The results from two independent experiments are plotted together. Four or five leaves per plant were scored. A horizontal line indicates the calculated mean level of necrosis/leaf. The difference in means between WG3 (G3) and WαAP22 (α22) was significant at $P < 0.01$ (Student's t - test).

Transgene copy number

No attempt was made to determine the number of transgene insertions in these transgenic plants using Southern hybridization. However, as described in Materials and Methods, seeds from primary transformants were always germinated on kanamycin plates before being transplanted to soil 16 to 18 days later. This kanamycin screening was used to eliminate those T_1 seedlings that did not express the NPTII gene. Hypothetically, one insert in T_0 should give the phenotypic ratio of three kanamycin-resistant to one sensitive progeny since the NPTII gene behaves like a dominant allele, whereas two insertions should give a fifteen to one ratio. The scores from this kanamycin screening suggested that the primary transformant lines (T_0) tested had from one to more than two inserts (Table 4.2). It should be emphasized that these estimations can only give an approximate idea of the number of antisense genes being expressed in each primary

transformant line, since transformation can occur where only the selectable marker gene, but not the "passenger"-gene (i.e. antisense gene), has been incorporated into the genome.

Table 4.2 Estimations of copy numbers in different primary transformant lines (T_0), based on kanamycin-resistance in T_1 progeny.

Transformant line	kan sensitive/total	Kan^S : Kan^R	probable number of inserts
WG3	20/447	1 : 22.4	2
WG8	27/139	1 : 5.1	1 or 2
WG9	105/471	1 : 4.5	1 or 2
WαAP3	27/97	1: 3.6	1
WαAP4	27/116	1 : 4.3	1 or 2
WαAP22	14/376	1 : 26.9	2
WαAP29	7/361	1 : 52.4	2 or more
WGαAP25	41/773	1 : 18.9	2
WGαAP32	22/82	1 : 3.72	1

4.3.3 Characterization of sense *APX* primary transformants (T_0)

The ap21 clone, containing 83 bp of 5' untranslated sequence, a 750 bp open reading frame and 207 bp of 3' untranslated sequence (see section 3.3.2), was used to prepare a gene construct capable of overexpressing cytosolic APX. The cDNA was substituted for the GUS coding sequence and the NOS 3' untranslated sequence located directly 3' to the 35S CaMV promoter of pBI121 (see Figure 4.4). Growth and development of the sense transgenic lines did not differ from those of transgenic lines

transformed with pBI121 (see section 4.3.2), or from those of untransformed Bel-W3 plants.

mRNA levels and APX activity

A total of 28 primary transformants was initially screened with Northern blot analysis using the ap21 clone as a probe. Since endogenous APX mRNA levels are higher in plants grown under high light than under low light (see Appendix), an attempt was made to lower the endogenous levels before RNA isolation and thus create a differential pattern that would aid in searching for the constitutive, 35SCaMV-driven expression of the ap21 transgene. To do this, the plants were kept in the dark for five days before harvesting one leaf from each plant for total RNA isolation. The transgenic plants WOX11,14, 17 and 28 had significantly higher APX mRNA levels than other transgenic plants (Figure 4.13A and B).

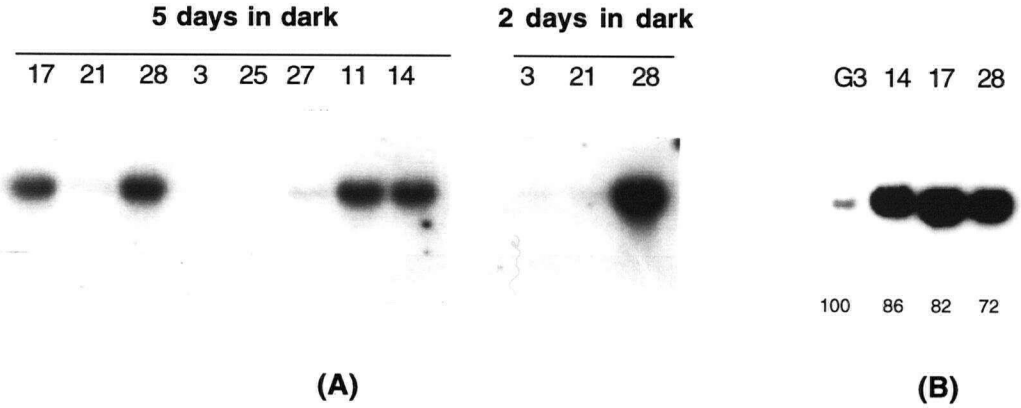


Figure 4.13 Northern blot analysis used for screening for high expression of sense-APX in transgenic tobacco lines

Total RNA (10 μg) was fractionated in a 1% formaldehyde/agarose gel and probed with an APX cDNA probe. The transgenic lines are labelled G3 (WG3; control), 3 (WOX3), 11 (WOX11), 14 (WOX14), 17 (WOX17), 21 (WOX21), 27 (WOX27) and 28 (WOX28).
(A) An example of Northern blot analysis used for screening primary transformants with high expression of sense-APX mRNA, twelve weeks after transplanting to soil. Plants were kept for two or five days in dark before harvesting. RNA was isolated as described in Materials and Methods.
(B) Northern blot analysis of the transgenic lines selected for further analysis. Total RNA isolated from leaves (10 - 12 cm long) of 6 - to 7 - week old plants. Each sample was pooled from leaves of four plants. Relative RNA loading for (B) is shown under each lane as a percentage of loading in first lane.

This difference was also obvious in leaves harvested after two days in the dark (Figure 4.13A). Based on this initial screening, APX activity was measured in the T2 progeny of self-pollinated primary transformants. Figure 4.14 shows that the transgenic lines WOX14, 17 and 28 had approximately 50 - 70% higher APX activity compared to the activity in the control lines WG3. This increase in APX enzyme activity was consistent with the increase in APX mRNA levels observed in these plants after five days incubation in dark (Fig. 4.13B).

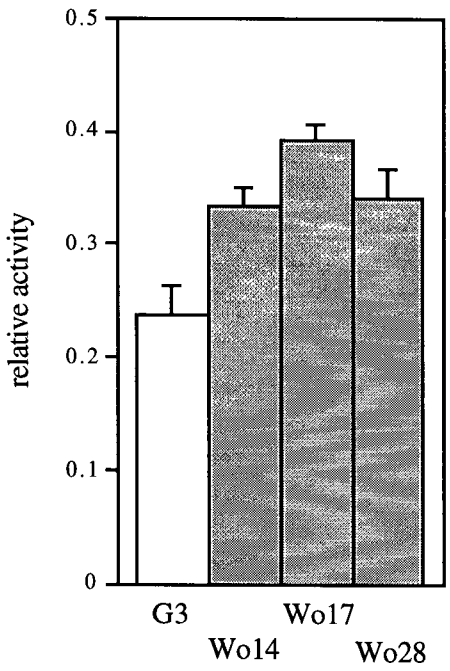


Figure 4.14 The APX activity in transformed and un-transformed plants.

APX enzyme activity was measured in young leaves (7 - 9 cm in length) harvested from seven week old plants grown at 300 $\mu E\ m^{-2}\ s^{-1}$. The sense lines are labelled Wo14 (WOX14), Wo17 (WOX17) and Wo28 (WOX28). The control line is labelled G3 (WG3). The data presented for each line are the mean and S.D. of two individual experiments.

Massive ozone exposure and ozone symptoms in sense transformants

The transgenic lines WOX17 and WOX28 and the control line WG3 were exposed to *MOE*. Figure 4.15 shows the results from five independent experiments in which WOX28 and BW3 were treated simultaneously with *MOE* and the ozone injury levels (% necrosis) were compared, and one independent experiments in which WOX17 was also included. No significant differences in ozone injury levels were observed among these lines ($P > 0.05$).

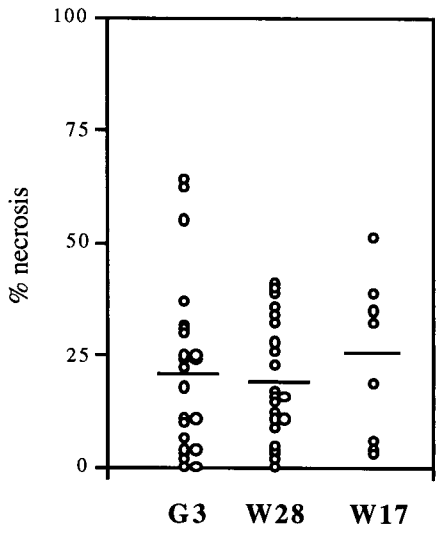


Figure 4.15 Differences in ozone-induced necrosis among WG3, WOX17 and WOX28 following *MOE*

The average % necrotic area/leaf is compared for all experiments (five) where WG3 (G3) and WOX28 (W28) were simultaneously exposed to *MOE*. Also included is WOX17 (W17) which was exposed once with WOX28 and WG3. In this figure % necrosis is the mean +/- S.D of the average % necrosis for each line from individual experiments. Four or five leaves per plant were scored in each experiment. The difference in means between WG3 (G3) and WOX28 (W28) was not significant ($P > 0.05$; Student's t - test).

AOE and ozone symptoms in sense transformants

Lines WG3 and WOX28 were exposed to *AOE* for six days and their levels of ozone injury assessed. Figure 4.16 shows the results from three independent experiments in which WOX28 and WG3 were treated simultaneously with *AOE* and the

ozone injury levels (% necrosis) were compared. No significant difference in ozone injury levels was observed between these lines ($P > 0.05$).

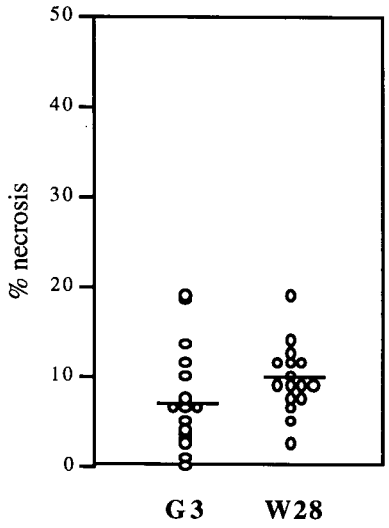


Figure 4.16 Ozone-induced necrosis in lines WG3 and WOX28 following *AOE*

Each data point represents the observed level of necrosis in one plant, recorded as average % necrotic area/leaf. The results from three independent experiments are plotted together. Four or five leaves per plant were scored. A horizontal line indicates the calculated mean level of necrosis/leaf. Plants were grown at 100 $\mu E\ m^{-2}\ s^{-1}$ before *AOE*. The difference in means between WG3 (G3) and WOX28 (W28) was not significant ($P > 0.05$; Student's t - test).

4.3.4 Paraquat tolerance in sense and antisense transformants

Five different transgenic lines were treated with paraquat and their tolerance to this superoxide-generating herbicide was compared. One control line (WG3), one overexpressing line (WOX28) and three antisense lines (WαAP4, WGαAP25 and WαAP29) were used. For each line, leaf-discs were treated with either 1 μM paraquat (in 0.01% Tween 20), or with 0.01% Tween 20 and the average difference in pigment density (digital images) between these different treatments was compared. The results showed that the paraquat treatment reduced the pigment density approximately 25 - 30%

in all of these lines (Figure 4.17). No significant difference in paraquat tolerance, based on pigment density, was observed between the different transgenic lines.

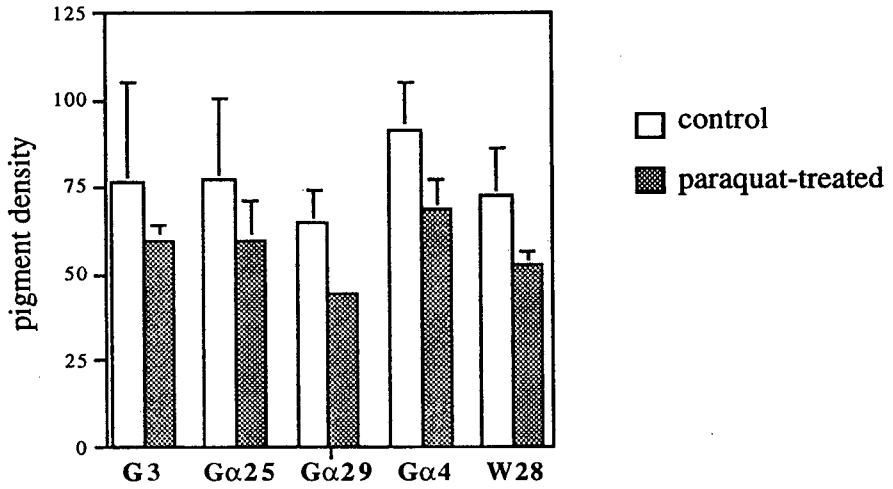


Figure 4.17 Paraquat-induced pigment loss in leaf discs from transformed and un-transformed plants.

For paraquat treatment see Methods and Materials. The control line is labelled G3 (WG3), the antisense lines are labelled Gα4 (WαAP4), Gα25 (WGαAP25) and Gα29 (WαAP29), and the sense line is labelled W28 (WOX18). The data presented for each line are the mean and S.D. of four individual experiments.

4.4 DISCUSSION

In order to assess the contribution of cytosolic APX enzyme activity to the capacity of tobacco plants to deal with ozone stress, *N. tabacum* was transformed with two different antisense constructs that target expression of cytosolic APX mRNA, or with one sense gene overexpressing full-length cytosolic APX mRNA. Four different antisense lines, three expressing a 0.6 kb APX antisense RNA, and one expressing a 2.47 kb chimeric GUS/antisense APX RNA, were selected for a detailed analysis of their response to both *MOE* and *AOE*. Three of these lines, WαAP22, WαAP29 and WGαAP25, showed a considerable reduction in endogeneous APX mRNA levels as well as APX

catalytic activity, while the fourth, WαAP4, showed neither. When exposed to *MOE*, lines WαAP22, WαAP29 and WGαAP25 suffered substantially more ozone injury than the control line WG3, whereas the level of ozone injury in WαAP4 was indistinguishable from that seen in WG3 (Figure 4.9). Under *AOE* conditions the difference in injury level between the control line (WG3) and the antisense line (WαAP22) was even more dramatic (6-fold) (Figure 4.12).

The correlation between APX mRNA levels, enzyme activity and ozone injury strongly suggests that cytosolic APX is an important component in ozone tolerance in tobacco, and that a reduction in cytosolic APX capacity interferes directly with effective oxidative defence. These results also suggest that it is the level of cytosolic APX that critically limits the scavenging capacity of the ascorbate-glutathione pathway in tobacco, since the three antisense lines showing decreased tolerance to ozone exposure appear to have retained at least 40% of the cytosolic APX activity observed in control lines. These results are consistent with the results from Batini *et al.* (1995) who showed that, following ozone exposure, APX activity increased less in Bel-W3 than it did in a more ozone-tolerant genotype (Havana).

As discussed earlier (see section 3.4), at least four different isoforms of APX, with different subcellular localizations, have now been cloned in plants; one cytosolic, two chloroplastic and one in microbodies. So far, only cytosolic isoforms have been cloned from tobacco, but based on the difference in APX activity using either 10 mM or 0 mM ascorbate in the extraction buffer (Figure 4.8A), it appears that tobacco also has at least one chloroplastic isoform. Therefore, it is reasonable to ask if the antisense cytosolic APX constructs used in this study also suppress other isoforms of APX. Numerous Northern blot analyses using the cytosolic APX cDNA as a probe and a high stringency washing, consistently detected only one hybridizing band (approximately 1.2kb). Cytosolic cDNA's from *Arabidopsis*, spinach and pumpkin have similar size to tobacco cytosolic

cDNA (chapter 3) but cDNA's for non-cytosolic isoforms from these plant species are at least 0.3 to 0.45 kb larger than their cytosolic cDNA counterparts (see Figure 3.7 in Chapter 3) and the DNA identity between them and their cytosolic counterparts is between 57% and 64% (between 59% and 68% if the 338 bp region used in the antisense constructs is used for the comparison). Furthermore, using stringent washing, the tobacco APX cDNA probe did not hybridize to *Raphanus sativus* RNA, although the DNA identity between tobacco APX cDNA and *R. sativus* APX cDNA is 82% (see table 3.2). However, these results may not be relevant to *in vivo* conditions for the antisense suppression. Even though tobacco APX cDNA does not hybridize to non-cytosolic APX isoforms in Northern blot analysis, the antisense transcript could conceivably interfere *in vivo* with these same non-cytosolic transcripts. The enzyme assay used in these experiments was not specific for the cytosolic APX. For example, a considerable proportion (approximately 20%) of the APX activity measured in pea shoot extracts has been attributed to heat-stable, non-enzymatic processes (Mittler and Zilinskas 1991b), and other peroxidases capable of using ascorbate as an electron donor for the reduction of hydrogen peroxide might also contribute to the apparent overall APX activity. In addition, since about 80% of the total cellular APX activity is located in the chloroplasts (Sen Gupta *et al.* 1993), rupture of these organelles during the extraction process will contribute to the measured APX activity. These background contributions to cytosolic APX activity levels would make it difficult, for example, to evaluate the true impact of the antisense constructs at the enzyme level. Therefore, an attempt was made to distinguish between some of those different activities by using different ascorbate concentrations in the extraction buffer, as well as an APX inhibitor. Based on a comparison of APX enzyme assay results obtained using 0 mM and 10 mM ascorbate concentrations, respectively, it is unlikely that chloroplastic APX expression was suppressed in the transgenic lines,

since the antisense line tested (WαAP29) appeared to have levels of chloroplastic activity at least similar to those seen in the control line (WG3; see Figure 4.8B).

The difference in ozone injury between antisense and control lines was much more pronounced if the plants were challenged with *AOE* instead of *MOE*. Both the ozone concentration and the length of exposure could be factors in this difference. APX activity has been reported to be directly inhibited by very high levels of ozone (500 ppb) (Tanaka *et al.* 1985). While it is difficult to quantify the impact of such inhibition *in vivo*, it could potentially negate the role of APX in the overall oxidative stress management process under high ozone conditions. Also, *MOE* was applied to the plants for only 8 hr, and APX mRNA only starts to accumulate in tobacco leaves after 4 to 6 hr exposure to 250 ppb ozone (see section 2.3.6). If the kinetics of APX mRNA accumulation are similar, or less, during 500 ppb ozone exposure, the impact of antisense inhibition would probably be reduced within the *MOE* time-frame. In this scenario, the control plants would have less of an advantage over the antisense plants under *MOE* conditions than they would during *AOE*.

It has been hypothesized that stress tolerance can be improved by increasing the endogenous antioxidant capacity of plants; for example, through transgenic technology (Foyer *et al.* 1994). This hypothesis is based on the observations that following stress, oxidative damage to membranes is incurred, and ROS levels increase, as do levels of different antioxidant enzymes (Foyer *et al.* 1994). An attempt was therefore made to overexpress cytosolic APX in *N. tabacum* using a gene construct for full-length cytosolic APX mRNA. One transgenic line (WOX28), with approximately 50% higher APX activity than the control line (WG3), was selected for further studies. However, under neither *MOE* nor *AOE* conditions did WOX28 show a significant increase in ozone tolerance when compared to WG3. These results might suggest that the antioxidant capacity of WOX28 had not been increased. However, increasing APX activity alone during

oxidative stress may be insufficient to strengthen the overall oxidative stress defence mechanism since it could merely deplete the pool of reduced ascorbate necessary for the reduction of hydrogen peroxide. Conklin *et al.* (1996) showed that a high level of reduced ascorbate was very important for efficient ozone defence in *Arabidopsis*. They isolated an ozone-sensitive mutant (*soz1*) of *Arabidopsis*, that accumulated only 30% of the normal ascorbate concentration. Feeding these mutant plants with ascorbate prior to ozone exposure restored the ozone tolerance. This result underlines the importance of ascorbate levels for defence against ozone stress, and emphasizes that regeneration of ascorbate is a critical component of the ROS scavenging system (Rao *et al.* 1995; Foyer *et al.* 1995). Severe oxidative stress results in rapid loss of ascorbate and there is little evidence that ascorbate biosynthesis is enhanced in response to an increase in ROS levels (Smirnoff and Pallanca 1996). The fact that APX is rapidly inactivated by H_2O_2 when the ascorbate concentration is low adds to the impact of ascorbate depletion (Miyake and Asada 1996). Therefore, effective defence against prolonged oxidative stress necessitates faster regeneration of ascorbate (Foyer *et al.* 1995) and, perhaps, manipulation of more than one antioxidant gene. This hypothesis is supported by the results of Pitcher *et al.* (1991) which showed that overexpressing a single antioxidant gene, Cu/Zn SOD, generated no increase in ozone tolerance in transgenic tobacco exposed to ozone, probably because the extremely high SOD activity exhausted the ability of the H_2O_2 -scavenging system to respond to the SOD-associated H_2O_2 generation (Sen Gupta *et al.* 1993). The absence of increased ozone tolerance in WOX28 overexpressing one antioxidant enzyme (APX), could also be a manifestation of this problem.

No significant difference in paraquat damage was observed between the sense, antisense or GUS-expressing transgenic plants in this study, but the relationship between APX activity and paraquat treatment seems to be complicated. For example,

Mittler and Zilinskas (1992) reported that pea leaves sprayed with a 1 μM solution of paraquat had higher cytosolic APX activity than did control leaves, whereas 10 μM paraquat spraying caused a reduction in the cytosolic activity. The relationship between paraquat tolerance and ozone tolerance is also unclear. Shaaltiel *et al.* (1988) assessed the paraquat tolerance of four ozone-tolerant *N. tabacum* genotypes and found that only one genotype (Florida) showed both high ozone- and paraquat tolerance. Kirtikara and Talbot (1996) demonstrated that while ozone exposure increased APX activity more than three-fold in tomato, paraquat treatment did not affect APX activity. They also showed that glutathione levels and GR activity increased following paraquat treatment, while in ozone-treated plants glutathione levels declined and GR activity did not change from that seen in control plants. Finally, Aono *et al.* (1991) reported that transgenic tobacco plants overexpressing glutathione reductase exhibited higher tolerance to paraquat than did control plants, but they were no more resistant to foliar ozone damage.

These results suggest that the mechanism of ozone injury may be significantly different from that of paraquat injury, and that, as a result, paraquat and ozone have different effects on metabolite accumulation and enzyme activity (Kirtikara and Talbot 1996; Aono *et al.* 1991). Nevertheless, a correlation between high total APX activity and paraquat resistance has been reported in *Conyza bonariensis* (Shaaltiel and Gressel 1986) and a preliminary report from Webb and Allen (1996) indicates that overexpression of pea cytosolic APX in tobacco, in either cytosol or chloroplasts, significantly increased the tolerance of paraquat. However, it is difficult to compare these results directly with this study since Webb and Allen (1996) used a different tobacco genotype (Xanthi), and measured differences in electrolyte permeability, instead of pigment density. It may well be that the analysis of paraquat resistance using pigment density is less accurate than measuring electrolyte permeability or chlorophyll content. Paraquat forms a free radical upon accepting an electron from photosystem I in chloroplasts and is recycled back to

paraquat by reacting with O_2 (Shaaltiel *et al.* 1988). In this process, the O_2^- generated can undergo dismutation, catalyzed by SOD (see section 1.4.2), to form H_2O_2 and O_2 (Shaaltiel *et al.* 1988). Since Webb and Allen (1996) used transgenic plants expressing cytosolic APX in the cytosol (and exhibiting a significant increase in paraquat tolerance) it is possible that ROS either escape the chloroplasts to be scavenged in the cytoplasm, and/or that paraquat can form free radicals in the cytosol, as well as in the chloroplasts.

It is clear that oxidative stress management in plants works via complex metabolic adjustments, since ROS can serve both as necessary players in signal transduction (see section 1.5), and as a destructive force under adverse environmental situations. Enhancing oxidative stress defences in plants through genetic engineering without compromising other desired qualities could thus be a challenging task.

Chapter 5

General Discussion

The general aim of this study was to investigate the interplay between two stresses that are both thought to involve increased oxidative stress in the challenged plant tissue. Specifically, the objectives were to determine whether wounding could modify the responses of tobacco to subsequent ozone stress, and whether modifications of the ascorbate-glutathione pathway would affect the sensitivity of tobacco to ozone stress. These predictions were based on the hypothesis that ozone defence could be induced through activation of a wound-signalling pathway, and that the downstream responses affected by this pathway were aimed, at least in part, at reducing oxidative stress in the plant tissue. The hypothetical metabolic model introduced originally in Chapter 1 is reproduced below (Figure 5.1).

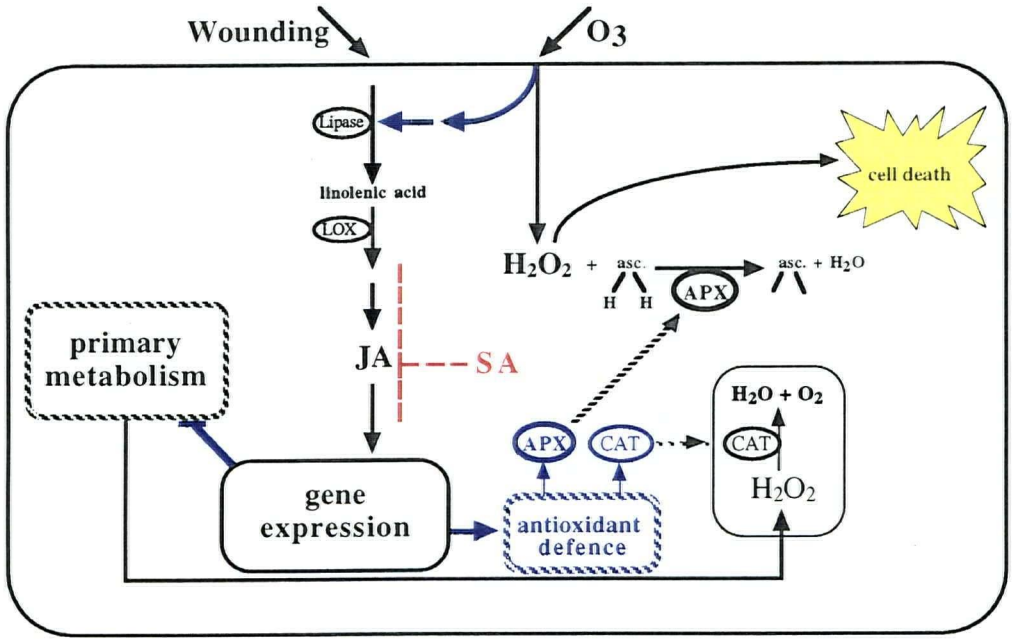


Figure 5.1 Diagrammatic presentation of the metabolic model used in this study showing the proposed relationship between ozone stress, wound-signaling and antioxidative response.

Abbrevations: APX; ascorbate peroxidase, LOX; lipoxygenase, CAT; catalase, JA; jasmonates, SA; salicylic acid, asc; ascorbate. Blue shading; signalling proposed in this study, black shading; pathways suggested by various researchers.

According to this model, ozone stress activates the jasmonate biosynthesis pathway. This in turn induces an antioxidative response characterized by, among other things, increased accumulation of cytosolic APX and down-regulation of metabolic genes that might contribute to oxidative stress. Salicylic acid, another signal molecule, has the potential to interfere with such a defence response activated by jasmonates, either by inhibiting the jasmonate biosynthesis pathway or blocking the downstream jasmonate-controlled events, or both. Such interference by salicylate would be predicted to result in increased ozone susceptibility. This model was not intended to be comprehensive, and does not, for instance, address the possibility that ozone stress might also activate other signalling pathways which could contribute to activation of ozone defence mechanisms, or that antioxidative stress measures other than upregulation of APX could be involved.

5.1 Ozone tolerance is induced through the wound-signalling pathway

Heath (1988) and Kangasjarvi *et al.* (1994) have argued that ozone exposure might activate a general wounding response, characterized by a rapid increase in ethylene biosynthesis and induction of similar genes. Both wounding and treatment with jasmonates prior to exposure of the tissue to ozone produced a dramatic decrease in ozone injury. A systemic pattern of increased ozone tolerance developed within three to six hours following wounding and also after local application of jasmonates. It appears that wound-signalling can activate a defence mechanism(s) that is capable of minimizing the harmful effects of a sudden increase in oxidative stress such as that following ozone exposure. Expression studies showed that mRNA encoding APX was upregulated within few hours by ozone challenge, by wounding and by MeJA exposure, while mRNA levels for the primary metabolic enzyme carbonic anhydrase were simultaneously depressed following ozone exposure and MeJA treatment. These results indicate, as shown in the Figure 5.1, that both ozone exposure and wounding induce an antioxidative response.

However, the expression studies also showed that SA-Cat mRNA levels are upregulated following ozone exposure but down-regulated following wounding or MeJA treatment. This difference suggests that ozone challenge might modulate gene expression both through a wound-signalling pathway and through other, as yet undefined, mechanisms.

One prediction from this scenario is that if ozone exposure activates the wound-signalling pathway, ozone-induced responses (such as alterations in gene expression) should also be accompanied by accumulation of jasmonates and, presumably, in a similar time-frame as that seen following wounding. In following up the present studies, this hypothesis could be tested by measuring jasmonate levels in plants exposed to ozone. At the same time, it would be desirable to determine whether catalytic activities of enzymes involved in the biosynthesis of jasmonates, such as lipoxygenase, allene oxide synthase and allene oxide cyclase (see Figure 1.2), are induced following ozone exposure in a time-frame consistent with any observed jasmonate accumulation. Activity assays are available for all these enzymes.

When two stimuli appear to involve a common response, it is important to establish where the stimulus pathways converge. In the simple model presented in Figure 5.1, both wounding and ozone exposure are presumed to generate ROS, including H_2O_2, and it could be envisaged that this rise in ROS levels is the common trigger that activates the jasmonate biosynthetic pathway. How this activation might be accomplished is unknown, but one major regulatory mechanism for enzyme activity and protein function in plant defence signalling is the reversible phosphorylation of proteins (Chandra and Low 1995). Blechert *et al.* (1995) showed that JA accumulation in plant cell suspension culture after fungal cell wall elicitation was dependent on protein phosphorylation, since it was completely blocked by a protein kinase inhibitor. Seo *et al.* (1995) recently demonstrated that mRNA transcription levels for a 42.9 kDa MAP kinase in tobacco increase in less than ten minutes following wounding. Seo *et al.* (1995) also generated a transgenic tobacco plant overexpressing a cDNA for the 42.9 kDa MAP

kinase. However, this transgenic plant (TMPIS-1) was unable to accumulate MAP kinase transcripts following wounding, apparently due to co-suppression of the endogenous gene(s). It also failed to accumulate jasmonates in response to wounding. If ozone exposure and wounding both activate the wound-signalling pathway, there are several predictions that could be tested with respect to the MAP kinase. For example, it could be predicted that ozone exposure would also induce the accumulation of this MAP kinase mRNA transcript. One also would predict that if ozone exposure induces accumulation of jasmonates through the wound-signalling pathway, the TMPIS-1 transgenic line also should not accumulate jasmonates following ozone exposure. Furthermore, if the jasmonate biosynthesis pathway is the primary pathway responsible for induction of antioxidant responses following ozone exposure, one might predict that ozone exposure (or wounding) would be unable to induce in TMPIS-1 the accumulation of mRNA transcripts that are known to accumulate following ozone exposure (e.g. APX). Finally, if activation of the jasmonate-biosynthesis pathway is critical to the creation of ozone tolerance, one would predict that TMPIS-1 would prove to be more susceptible to ozone than its parental genotype.

Recent studies suggest that salicylic acid (SA) can inhibit both the jasmonate biosynthesis pathway and jasmonate-controlled downstream effects (Pena-Cortes *et al.* 1993), and the metabolic model presented in Figure 5.1 predicts that SA, which accumulates in tobacco following ozone exposure (Yalpani *et al.* 1994), will eventually block the jasmonate-induced ozone defence if the ozone exposure lasts long enough. This prediction was based on the hypothesis that, if both ozone and wounding induce jasmonate accumulation in a similar time-frame, jasmonate could execute its antioxidant defensive role before SA could block its action, since SA accumulation following ozone occurs more slowly than jasmonate accumulation following wounding (Yalpani *et al.* 1994). However, once SA reaches an appropriate level during ozone exposure, its antagonistic effects (JA inhibition) will dominate the plant response to ozone. This "take-

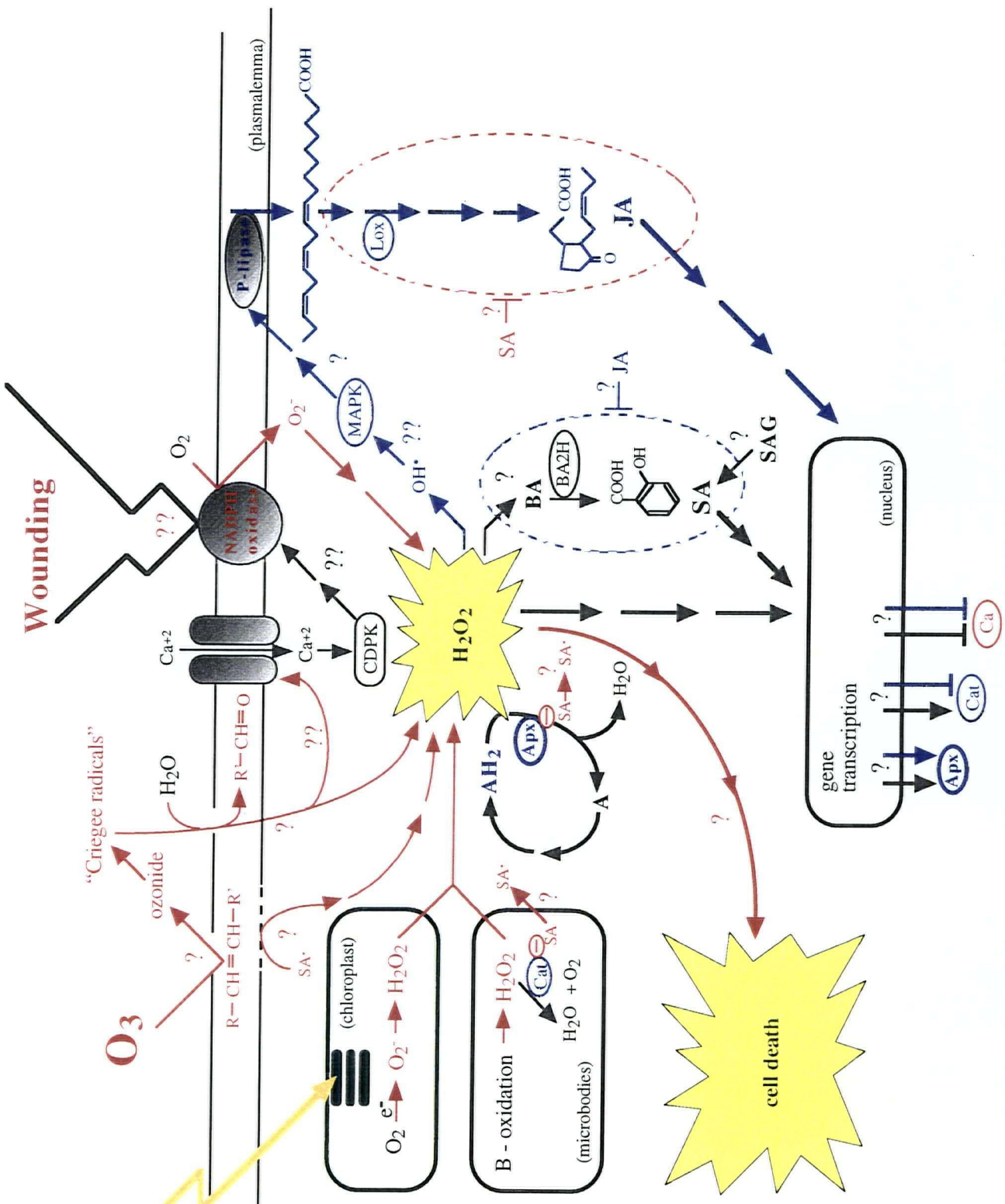
over" by SA could result in metabolic changes that lead to cell-death (necrosis), a process that may be analogous to the so-called programmed cell death observed in plants that are mounting a hypersensitive response to pathogen attack. It follows that if the SA accumulation induced by ozone exposure could be blocked, the plant should be less susceptible to ozone injury. The availability of transgenic plants (NahG) unable to accumulate SA following pathogen attack (Gaffney *et al.* 1993) allowed this prediction to be tested. The data obtained in my study confirm that NahG tobacco plants are much less susceptible to ozone injury than is the parental genotype. While this result is consistent with my working model, it cannot be concluded that this decrease in ozone susceptibility was a direct result of blocking the jasmonate signalling pathway. To examine that question more closely, it would again be useful to monitor jasmonate accumulation in these plants. If SA can inhibit jasmonate accumulation, and if both jasmonate and SA accumulate in tobacco following ozone exposure (but within different time-frames), the model predicts that we should be able to demonstrate higher, or more prolonged, jasmonate accumulation in the NahG plants following ozone exposure, than in the parental genotype, since the NahG plant cannot accumulate SA.

The cross-talk between the jasmonate biosynthesis pathway and the SA-mediated pathway (characteristically induced by pathogens) also appears to involve a direct impact of jasmonates on the SA-mediated pathway since the TMPIS-1 transgenic plant generated by Seo *et al.* (1995) accumulated SA following wounding, in contrast to the parental genotype. Furthermore, TMPIS-1 accumulated acidic PR-protein transcripts following wounding, instead of the basic PR-protein transcripts that are characteristic of the wounding response (Seo *et al.* 1995). These results suggest that some intermediate or product of the jasmonate biosynthesis pathway normally suppresses SA accumulation following wounding, and that if this suppression is released (such as in TMPIS-1), the resulting phenotype will display the typical SA-mediated stress response. If jasmonates can be shown to accumulate following ozone exposure in tobacco, and if

jasmonates normally suppress SA accumulation, the TMPIS-1 plant would be predicted to accumulate higher levels of SA following ozone exposure, or to accumulate it more quickly, than would the parental genotype. A summary of this hypothetical interplay between ozone exposure, wounding and signaling pathways is presented in Figure 5.2. This summary is based on the results presented in this study as well as previous literature (see Chapter 1 and Discussions in Chapter 2 and 4) and it emphasizes that oxidative stress management in plants is most likely a very complicated process, since ROS can serve both as necessary players in signal transduction, and as a destructive force under adverse environmental situations. First, the figure is composed of pathways that can be viewed as confirmed knowledge in plant signal transduction (pathways with no question marks), such as the JA-biosynthesis pathway, the B-oxidation in the microbodies and the reduction of O_2 in the chloroplasts and the generation of H_2O_2 from $O_2^{\bullet}$. Second, it is composed of pathways that are derived from one or few studies in plants (pathways with one question mark), such as the formation of SA radicals during SA-induced inhibition of CAT and APX, the antagonistic suppression of JA and SA and the formation of H_2O_2 from Criegee radicals. The initial data suggesting such pathways need to be confirmed in further studies. Finally, the figure also includes pathways that are based on analogy to other systems, notably mammalian systems (pathways with two question marks) or are purely speculative. This includes the ROS-activation of MAPK and CDPK and the wound-induced activation of NADPH oxidase.

Figure 5.2 Model illustrating the complex relationship between ozone stress, wound-signaling and antioxidative response.

Abbrevations: Apx; ascorbate peroxidase, Lox; lipoxygenase, Cat; catalase, CDPK; calcium-dependent protein kinase, MAPK; mitogen-activated protein kinase, P-lipase; phospholipase, BA2H; benzoic acid-2 hydroxylase, Ca; carbonic anhydrase, JA; jasmonates, SA; salicylic acid, SAG; glucosylsalicylic acid, AH_2; reduced ascorbate, A; oxidized ascorbate, BA; benzoic acid. Red shading; pathways or reactions contributing to the oxidative stress. Blue shading; pathways or reactions resulting in decreased oxidative stress. One question mark represents pathways based on initial data from one or few studies, whereas two question marks are pathways that have been included simply on the basis of analogy to other systems. Pathways without question mark represent confirmed knowledge of plant systems.


Wounding
O3
"Criegee radicals"
ozonide
H2O
R–CH=O
R'–CH=O
(plasmalemma)
COOH
P-lipase
Lox
JA
O2
NADPH oxidase
O2-
MAPK
OH· ??
Ca+2
CDPK
H2O2
BA
BA2H
SA
SAG
AH2
A
Apx
H2O
(chloroplast)
O2 e- O2- H2O2
B - oxidation H2O2
Cat
H2O + O2
(microbodies)
cell death
gene transcription
(nucleus)
Ca

Eckey-Kaltenbach *et al.* (1994) reported that ozone stress can function as a *cross-inducer*, i.e. it is capable of activating more than one defence pathway. Based on previous literature and the results presented in this study, it is conceivable that ozone can activate at least two different pathways, one involving jasmonate accumulation (wound response), and one involving SA accumulation (pathogen response). Recently, Conconi *et al.* (1996) demonstrated in tomato plants that the jasmonate biosynthesis pathway can be activated by UV-irradiation (UV-B and UV-C). This stress induced higher levels of PinI and II, at both the mRNA and protein levels. The induction could be blocked by SA treatment, but was absent in a tomato line which carries a mutation blocking the jasmonate biosynthesis pathway (Conconi *et al.* 1996). Earlier, Yalpani *et al.* (1994) had reported that UV irradiation also induces SA accumulation in tobacco. These are interesting parallels, since the ozone stress response has frequently been compared to the UV-stress response in tobacco (Willekens *et al.* 1994), in terms of both gene regulation as well as generation of intracellular ROS.

5.2 The importance of cytosolic APX in ozone defence

Transgenic technology is being widely used as a tool to elucidate complex biochemical functions and pathways in plants. The metabolic model used in this study predicted that cytosolic APX could play a critical role in antioxidant defence in tobacco during ozone stress. The experimental results show that transgenic plants possessing reduced cytosolic APX catalytic activity displayed a significantly higher level of ozone injury following ozone exposure than did control plants, indicating that cytosolic APX plays a significant role in ozone defence response. However, overexpressing APX catalytic activity apparently did not alter ozone susceptibility in tobacco. This pattern is not uncommon in transgenic technology; improving the overall capacity of a metabolic function may require introduction of more than one transgene into the genome (Foyer *et*

al. 1995), whereas damaging this function might require only a single mutation (antisense). This concept can be illustrated in a cartoon (Figure 5.3) showing that increasing (APX overexpression) the strength of only one link in a chain (antioxidant scavenging function) will not result in a stronger chain. However, decreasing the strength of only one link in this same chain (APX antisense) will most definitely lead to a weaker chain.

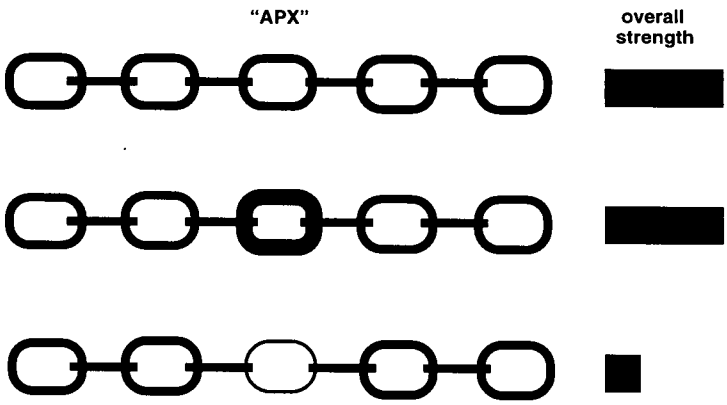


Figure 5.3 Diagram illustrating the analogy between APX activity and chain-strength.

The data presented in this study show that the cytosolic APX antisense gene apparently did not suppress the enzyme activity of APX isoforms that are inactivated in the absence of ascorbate. These isoforms are presumably chloroplastic, although this has not been directly demonstrated. Only cDNA's for cytosolic APX isoforms have recently been cloned from tobacco, but, cDNA's encoding both stromal and thylakoid isoforms have been cloned from pumpkin, spinach and *Arabidopsis.* Since these isoforms show very high homology among species, the sequence information derived from these cDNA's now makes it possible to clone chloroplastic APX cDNA's from tobacco. These could be used as compartment-specific probes in Northern blot analysis of the transgenic plants generated in this study. This analysis would enable us to

determine directly whether, in fact, the chloroplastic APX mRNA levels are also affected by the cytosolic APX antisense genes.

APX participates in an elaborate antioxidant scavenging system which includes an array of different antioxidant enzymes and antioxidants that can collectively minimize the harmful effects of oxidative stress (Foyer *et al.* 1994). Earlier studies have suggested that increasing levels of one antioxidant function can affect the expression of another. For example, Sen Gupta *et al.* (1993) demonstrated that transgenic tobacco plants overexpressing SOD also had higher levels of cytosolic APX mRNA's, suggesting that overexpression of SOD may result in an increase in accumulation of H_2O_2 that can affect, directly or indirectly, expression of genes encoding H_2O_2-scavenging enzymes such as APX. If the tobacco plants expressing the APX-antisense gene have a reduced capacity to scavenge H_2O_2, H_2O_2 might also accumulate more in these plants under conditions such as those following ozone exposure. Furthermore, if H_2O_2 can affect gene expression, the expression of genes that are controlled by H_2O_2 levels, such as those participating in the antioxidant scavenging mechanism, should be altered in the antisense plants. Therefore, investigations of the expression of antioxidant enzymes other than APX in the antisense plants generated in this study might give us more information about how H_2O_2 levels may function as a regulatory factor in oxidative stress management in plants.

Rapid generation of ROS has been observed in plant tissues following different environmental stresses, such as wounding (Olson and Varner 1993), UV-irradiation (Green and Fluhr 1995), chilling (Prasad *et al.* 1994) and pathogen invasion (Legendre *et al.* 1993). Since the transgenic tobacco plants expressing APX antisense gene appear to have a lower capacity to deal with oxidative stress following ozone exposure, it would be interesting to determine whether these plants also show phenotypic differences, in comparison to the parental genotype, when confronted with other stresses that generate oxidative stress (for example: pathogen invasion or UV irradiation). This

could give us information about the importance of APX as a component of the response to different stresses, and about the impact of an potential increase in H_2O_2 levels on stress responses.

Increasing evidence suggests that ROS play an important role in stress signalling in animal cells. A number of targets for such ROS-mediated signalling, including G proteins ($p21^{ras}$), MAP kinase and the NF-κB transcription factor, have been identified (Lander *et al.* 1995). Although the role of ROS in plant signalling is not as well understood, and homologs for NF-κB have yet to be found in plants, MAP kinases have been cloned from several plant species and their importance in stress signalling established (Seo *et al.* 1995). Wu *et al.* (1995) also showed that a transgenic potato plant expressing a fungal glucose oxidase (GO) had an enhanced resistance to both fungal and bacterial diseases, apparently associated with increased levels of endogenous H_2O_2 (GO-catalyzed oxidation of glucose gives H_2O_2). The exact mechanism(s) responsible for the enhanced disease resistance in these plants has not yet been elucidated but the authors speculate it could involve an array of biochemical adapations, including changes in gene regulation (signalling), increased cell wall cross-linking or direct antimicrobial activity of H_2O_2. In contrast, as demonstrated in this study, lowering the H_2O_2 scavenging capacity of plants can have serious consequences for oxidative stress management when those plants are exposed to ozone. Since ROS can serve both as a necessary player in signal transduction, as well as a destructive force under adverse environmental situations, it is clear that oxidative stress management in plants requires subtle and complex metabolic adjustments. Altering oxidative stress defences, or the endogenous ROS levels in plants, through genetic engineering, without compromising other desired qualities, is thus likely to be a challenging task.

Bibliography

Abo-El-Saad, M. and Wu, R. (1995) A rice membrane calcium-dependent protein kinase is induced by gibberellin. Plant Physiol 108:787-793.

Albrecht, T., Kehlen, A., Stahl, K., Knöfel, H.-D., Sembdner, G. and Weiler, E.W. (1993) Quantification of rapid, transient increases in jasmonic acid in wounded plants using a monoclonal antibody. Planta 191:86-94.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J Mol Biol 215:403-410.

Amako, K., Chen, G-X. and Asada, K (1994) Separate assays specific for ascorbate peroxidase and guaiacol peroxidase and for the chloroplastic and cytosolic isozymes of ascorbate peroxidase in plants. Plant Cell Physiol 35:497-504.

Aono, M., Kubo, A., Saji, H., Natori, T., Tanaka, K. and Kondo, N. (1991) Resistance to active oxygen toxicity of transgenic *Nicotiana tabacum* that expresses the gene for glutathione reductase from *Escherichia coli*. Plant Cell Physiol 32:691-697.

Apostol, I., Heinstein, P.F. and Low, P.S. (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol 90:109-116.

Asada, K. (1992) Ascorbate peroxidase - a hydrogen peroxide-scavenging enzyme in plants. Physiol Plant 85:235-241.

Avdiushko, S., Croft, K.P.C., Brown, G.C., Jackson, D.M., Hamilton-Kemp, T.R. and hildebrand, D. (1995) Effect of volatile MeJA on the oxylipin pathway in tobacco, cucumber, and *Arabidopsis*. Plant Physiol 109:1227-1230.

Bahl, A., Loitsch, S.M. and Kahl, G. (1995) Transcriptional activation of plant defense genes by short-term air pollutant stress. Environ Pollut 89:221-227.

Baker, C.J. and Orlandi, E.W. (1995) Active oxygen in plant/pathogen interactions. Ann Rev Phytopathology 33, pp. 304-321.

Baker, C.J., Orlandi, E.W. and Mock, N.M. (1993) Harpin, an elicitor of the hypersensitive response in tobacco caused by *Erwinia amylovora*, elicits active oxygen production in suspension cells. Plant Physiol 102:1341-1344.

Baldwin, I.T., Schmelz, E.A. and Ohnmeiss, T.E. (1994) Wound-induced changes in root and shoot jasmonic acid pools correlate with induced nicotine synthesis in *Nicotiana sylvestris* SPEGAZZINI and COMES. J Chem Ecol 20:2139-2157.

Barford, D. (1991) Molecular mechanisms for the control of enzymatic activity of protein phosphorylation. Biochim Biophys Acta 1133:55-62.

Batini, P., Ederli, L., Pasqualini, S., Antoniellli, M. and Valenti, V. (1995) Effects of ethylenediurea and ozone in detoxificant ascorbic-ascorbate peroxidase system in tobacco. Plant Physiol Biochem 33:717-723.

Becker, K.H., Brockmann, K.J. and Bechara, J. (1990) Production of hydrogen peroxide in forest air by reaction of ozone with terpenes. Nature 346:256-58.

Bell, E., Creelman, R.A. and Mullet, J.E. (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. Proc Natl Acad Sci USA 92:8675-8679.

Bell, E. and Mullet, J.E. (1993) Characterization of an *Arabidopsis* lipoxygenase gene responsive to methyl jasmonate and wounding. Plant Physiol 103:1133-1137.

Blechert, S., Brodschelm, W., Holder, S., Kammerer, L., Kutchan, T.M., Mueller, M.J., Xia, Z-Q. and Zenk, M.H. (1995) The octadecanoic pathway: Signal molecules for the regulation of secondary pathways. Proc Natl Acad Sci USA 92:4099-4105.

Bolwell, G.P. (1996) The origin of the oxidative burst in plants. Biochem Soc Trans 24:438-442

Bors, W., Langebartels, C., Michel, C. and Sandermann Jr., H. (1989) Polyamines as radical scavengers and protectants against ozone damage. Phytochemistry 28:1589-1595.

Bowler, C. and Chua, N-H. (1994) Emerging themes of plant signal transduction. Plant Cell 6:1529-1541.

Bowler, C., Van Montagu, M. and Inze, D. (1992) Superoxide dismutase and stress tolerance. Ann Rev Plant Physiol Plant Mol Biol 43:83-116.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Bradley, D.J., Kjellbom, P. and Lamb, C.J. (1992) Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell wall protein: a novel, rapid defense response. Cell 70:21-30.

Brederode, F.T., Linthorst, H.J. and Bol, J.F. (1991) Differential induction of aquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV-light and wounding. Plant Mol Biol 17:1117-1125.

Brennan, E. and Leone, I.A. (1969) Suppression of ozone toxicity symptoms in virus-infected tobacco. Phytopathology 59:263-264.

Broadbent, P., Creissen, G.P., Kular, B., Wellburn, A.R. and Mullineaux, P.M. (1995) Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. Plant J 8:247-255.

Brown, M., Cox, R., Bull, K.R., Dyke, H., Sanders, G., Fowler, D., Smith, R. and Ashmore, M.R. (1995) Quantifying the fine scale (1KM x 1KM) exposure, dose and effects of ozone: part 2 estimating yield losses for agricultural crops. Water Air Soil Pollut 85:1485-1490.

Bunkelmann, J.R. and Trelease, R.N. (1996) Ascorbate peroxidase: a prominent membrane protein in oilseed glyoxysomes. Plant Physiol 110:589-598.

Bush, D.S. (1993) Regulation of cytosolic calcium in plants. Plant Physiol 103:7-13.

Cadenas, E. (1995) Mechanisms of oxygen activation and reactive oxygen species detoxification. *In* Oxidative Stress and Antioxidant defenses in Biology (Ahmad, S., ed.). New York: Chapman and Hall, pp. 1 - 61.

Castillo, F.J. and Heath, R.L. (1990) Ca2+ transport in membrane vesicles from Pinto bean leaves and its alteration after ozone exposure. Plant Physiol 94:788-795.

Chameides, W.L. , Kasibhatla, P. S. , Yienger, J. and Levy II, H. (1994) Growth of continental-scale metro-agro-plexes, regional ozone pollution, and world food production. Science 264:74-77.

Chameides, W. L. and Lodge, J.P. (1992) Tropospheric ozone: Formation and fate. *In* Surface Level Ozone Exposures and their Effects on Vegetation (Lefohn, A.S., ed.). Chelsea, MI: Lewis Publishers, pp. 5-30.

Chameides, W.L. (1989) The chemistry of ozone deposition to plant leaves: Role of ascorbic acid. Environ Sci Technol 23:595-600.

Chandra, S. and Low, P.S. (1995) Role of phosphorilation in elicitation of the oxidative burst in cultured soybean cells. Proc Natl Acad Sci USA 92:4120-4123.

Chanway, C.P. and Runeckles, V.C. (1984) The role of superoxide dismutase in the susceptibility of bean leaves to ozone injury. Can J Bot 62:236-240.

Chen, G-X and Asada, K. (1989) Ascorbate peroxidase in tea leaves: occurrence of two isozymes and the differences in their enzymatic and molecular properties. Plant Cell Physiol 30:987-998.

Chen, H., Nelson, R.S. and Sherwood, J.L. (1994) Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. BioTech 16:664-668.

Chen, Z., Malamy, J., Henning, J., Conrath, U., Sanches-Casas, P., Silva, H., Ricigliano, J. and Klessig, D.F. (1995) Induction, modification, and transduction of the salicylic acid signal in plant defense responses. Proc Natl Acad Sci USA 92:4134-4137.

Chen, Z., Silva, H. and Klessig, D.F. (1993) Active oxygen species in the induction of plant systemic resistance by salicylic acid. Science 262:1883-1886.

Conklin, P.L., Williams, E.H. and Last, R.L. (1996) Environmental stress sensitivity of an ascorbic acid-deficient *Arabidopsis* mutant. Proc Natl Acad Sci USA 93:9970-9974.

Conklin, P.L. and Last, R.L. (1995) Differential accumulation of antioxidant mRNAs in *Arabidopsis thaliana* exposed to ozone. Plant Physiol 109:203-212.

Conconi, A., Smerdon, M.J., Howe, G.A. and Ryan, C.A. (1996) The octadecanoid signalling pathway in plants mediates a response to ultraviolet radiation. Nature 383:826-829.

Constabel, C.P., Bergey, D.R. and Ryan, C.A. (1995) Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. Proc Natl Acad Sci U S A 92:407-411.

Cote, G.G. and Crain, R.C. (1994) Why do plants have posphoinositides? BioEssays 16:39-46.

Creelman, R.A. and Mullet, J.E. (1995) Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. Proc Natl Acad Sci USA 92:4114-4119.

Creelman, R.A., Tierney, M.L. and Mullet, J.E. (1992) Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression. Proc Natl Acad Sci USA 89:4938-4941.

Dalton, D.A. (1995) Antioxidant defenses of plants and fungi. *In* Oxidative Stress and Antioxidant defenses in Biology (Ahmad, S., ed.). New York: Chapman and Hall, pp. 298 - 355.

Darrall, N.M. (1989) The effect of air pollutants on physiological processes in plants. Plant Cell Environ 12:1-30.

Davis L., Kuehl M. and Battey J. (1994) Preparation and analysis of RNA from eucaryotic cells. *In* Basic methods in molecular biology. Norwalk, CT: Appelton and Lange, pp. 350-355.

Demerjian, K.L. (1986) Atmospheric chemistry of ozone and nitrogen oxides. *In* Air pollutants and their effects on the terrestrial ecosystems (Legge, A.H. and Krupa, S.V., eds.). New York: John Wiley & Sons, pp. 105-127.

Doares, S.H., Narvaez-Vasquez, J., Conconi, A. and Ryan, C.A. (1995) Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol 108:1741-1746.

Doares, S.H., Syrovets, T., Weiler, E.W. and Ryan, C.A. (1995) Oligogalacturonides and chitosan activate plant defensive genes through octadecanoid pathway. Proc Natl Acad Sci USA 92:4095-4098.

Durner, J. and Klessig, D.F. (1995) Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense response. Proc Natl Acad Sci USA 92:11312-11316.

Dwyer, S.C., Legendre, L., Low, P.S. and Leto, T.L. (1996) Plant and human neutrophil oxidative burst complexes contain immunologically related proteins. Biochim et Biophys Acta 1289:231-237.

Eckey-Kaltenbach, H., Ernst, D., Heller, W. and Sandermann Jr., H. (1994) Biochemical plant responses to ozone. IV. Plant Physiol 104:67-74.

Enyedi, A.J., Yalpani, N., Silverman, P. and Raskin, I. (1992) Signal molecules in systemic plant resistance to pathogens and pests. Cell 70:879-886.

Farmer, E.E. and Ryan, C.A. (1992) Octadecanoic precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 4:129-134.

Farmer, E.E. and Ryan, C.A. (1990) Interplant communication: Airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc Natl Acad Sci USA 87:47713-7716.

Fawsett, T.W., Browse, J.A., Volokita, M. and Bartlette, S.G. (1990) Spinach carbonic anhydrase primary structure deduced from the sequence of a complementary DNA clone. J Biol Chem 265:5414-5417.

Feher, J., Csomos, G. and Vereckei, A. (1987) Free Radical Reactions in Medicine (Feher, J., Csomos, G. and Vereckei, A., eds.). Berlin: Springer-Verlag, pp. 19-37.

Foyer, C.H., Souriau, N., Perret, S., Lelandais, M., Kunert, K.J., Pruvost, C. and Jouanin, L. (1995) Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. Plant Physiol 109:1047-1057.

Foyer, C.H., Descourvieres, P. and Kunert, K.J. (1994) Protection against oxygen radicals: an important defence mechanism studied in transgenic plants. Plant Cell Environ 17:507-523.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H. and Ryals, J. (1993) Requirement for salicylic acid for the induction of systemic aquired resistance. Science 261:754-756.

Graham, J.S., Pearce, G., Merryweather, J., Titani, K., Ericsson, L. and Ryan, C.A. (1985) Wound-induced proteinase inhibitors from tomato leaves. The cDNA-deduced primary structure of pre-inhibitor I and its post-translational processing. J Biol Chem 260:6555-6560.

Green, R. and Fluhr, R. (1995) UV-B-induced PR-1 accumulation is mediated by active oxygen species. Plant Cell 7:203-212.

Grimes, H.D., Perkins, K.K. and Boss, W.F. (1983) Ozone degrades into hydroxyl radical under physiological conditions. Plant Physiol 72:1016-1020.

Gross, D. and Parthier, B. (1994) Novel natural substances acting in plant growth regulation. J Plant Growth Regulation 13:93-114.

Gundlach, H., Muller, M.J., Kutchan, T.M. and Zenk, M.H. (1992) Jasmonic acid is signal transducer in elicitor-induced plant cell cultures. Proc Natl Acad Sci USA 89:2389-2393.

Gurr, S.J. and McPherson, M.J. (1991) PCR-directed cDNA libraries. *In* PCR, a practical approach (McPherson, M.J., Quirke, P. and Taylor, G.R., eds.). Oxford: Oxford University Press, pp. 147-165.

Guyton, K.Z., Liu, Y., Gorospe, M., Xu, Q. and Holbrook, N.J. (1996) Activation of mitogen-activated protein kinase by H_2O_2. J Biol Chem 271:4138-4142.

Hahlbrock, K., Scheel, D., Logemann, E., Nurnberger, T., Parniske, M., Reinold, S., Sacks, W.R. and Schmelzer, E. (1995) Oligopeptide elicitor-mediated defense gene activation in cultured parsley cells. Proc Natl Acad Sci USA 92:4150-4157.

Hammond-Kosack, K.E. and Jones, J.D.G. (1996) Resistance gene-dependent plant defense responses. Plant Cell 8:1773-1791.

Heagle, A.S. (1989) Ozone and crop yield. Ann Rev Phytopathology 27:397-423.

Heath, R.I. (1988) Biochemical mechanisms of pollutant stress. *In* Assessment of crop loss from air pollutants (Heck, W.N., Taylor, O,C. and Tingey, D.T., eds.). London: Elsevier, pp. 259-286.

Hewitt, N.C., Kok, G.L. and Fall, R. (1990) Hydroperoxides in plants exposed to ozone mediate air pollution damage to alkene emitters. Nature 344:56-58.

Hoigne J. and Bader H. (1975) Ozonation of water: role of hydroxyl radicals as oxidizing intermediates. Science 190:782-784.

Holmes, D.S. and Quigley, M. (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem 114:193-197.

Horsch, R.B., Fry, J., Hoffmann, N., Neidermeyer, J., Rogers, S.G. and Fraley, R.T. (1988) Leaf disc transformation. *In* Plant Molecular Biology Manual (Gelvin, S.B. and Schilperoort, R.A., eds.). A5: 1 - 9.

Horton, R.F. (1991) Methyl jasmonate and transpiration in barley. Plant Physiol 96:176-1378.

Horvath, D.M. and Chua, N-M. (1994) The role of salicylic acid in systemic acquired resistance. Current Opinion in Biotechnology 5:131-136.

Hossain, M.A., Nakano, Y. and Asada, K. (1984) Monodehydroascorbate reductase in spinach chloroplasts and its participation in regeneration of ascorbate for scavenging hydrogen peroxide. Plant Cell Physiol 25:385-395.

Ishikawa, T., Sakai, K., Yoshimura, K., Takeda, T. and Shigeoka, S. (1996) cDNAs encoding spinach stromal and thylakoid-bound ascorbate peroxidas, differing in the presence or absence of their 3'-coding regions. FEBS Lett 384:289-293.

Ishikawa, A., Tsubouchi, H., Iwasaki, Y. and Asahi, T. (1995) Molecular cloning and characterization of a cDNA for the alpha subunit of a G protein from rice. Plant Cell Physiol 36:353-359.

Joshi, C.P. (1987) Putative polyadenylation signals in nuclear genes of higher plants: a compilation and analysis. Nucleic Acid Res 15:9627-9640.

Kangasjärvi, J., Talvinen, J., Utriainen, M. and Karjalainen, R. (1994) Plant defense systems induced by ozone. Plant Cell Environ 17:783-794.

Kanofsky, J.R. and Sima, P.D. (1995) Singlet oxygen generation from the reaction of ozone with plants. J Biol Chem 270:7850-7852.

Kay, R., Chan, A., Daly, M. and McPherson, J. (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236:1299-1302.

Kirtikara, K. and Talbot, D. (1996) Alteration in protein accumulation, gene expression and ascorbate-glutathione pathway in tomato (Lycopersicon esculentum) under paraquat and ozone stress. J Plant Physiol 148:752-760.

Krupa, S.V., Manning, W.J. and Nosal, M. (1993) Use of tobacco in cultivars as biological indicators of ambient ozone pollution: An analysis of exposure-response relationships. Environ Pollut 90:137-146.

Krupa, S.V. and Manning, W.J. (1988) Atmospheric ozone: Formation and effects on vegetation. Environ Pollut 50:101-137

Kubo, A., Saji, H., Tanaka, K., Tanaka, K. and Kondo, N. (1992) Cloning and sequencing of a cDNA encoding ascorbate peroxidase from *Arabidopsis thaliana*. Plant Mol Biol 18:691-701.

Laisk, A., Kull, O. and Moldau, H. (1989) Ozone concentration in leaf intercellular air spaces is close to zero. Plant Physiol 90:1163-1167.

Lander, H.M., Ogiste, J.S., Teng, K.K. and Novogrodsky, A. (1995) p21ras as a common signaling target of reactive free radicals and cellular redox stress. J Biol Chem 270:21195-21198.

Langebartels, C., Kerner, K., Leonardi, S., Schraudner, M., Trost, M., Heller, W. and Sandermann Jr., H. (1991) Biochemical plant responses to ozone. I Differential induction of polyamine and ethylene biosynthesis in tobacco. Plant Physiol 95:882-889.

Lee, H-L., Leon, J, and Raskin, I. (1995) Biosynthesis and metabolism of salicylic acid. Proc Natl Acad Sci USA 92:4076-4079.

Legendre, L., Yuch, Y.G., Crain, R., Haddock, N., Heinstein, P.F. (1993) Phospholipase C activation during elicitation of the oxidative burst in cultured plant cells. J Biol Chem 268:24559-24563.

Leon, J., Lawton, M.A. and Raskin, I. (1995) Hydrogen peroxide stimulates salicylic acid biosynthesis in tobacco. Plant Physiol 108:1673-1678.

Levine, A., Tenhaken, R., Dixon, R. and Lamb, C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell 79:583-593.

Lois, R. and Hahlbrock, K. (1992) Differential wound activation of members of the phenylalanine ammonia-lyase and 4-coumarate:CoA ligase gene families in various organs of parsley plants. Z Naturforsch 47:90-94.

Lopez, F., Vansuyt, G., Casse-Delbart, F. and Fourcroy, P. (1996) Ascorbate peroxidase activity, not the mRNA level, is enhanced in salt-stressed *Raphanus sativus* plants. Physiol Plant 97:13-20.

Luwe, M.W.F., Takahama, U. and Heber, U. (1993) Role of ascorbate in detoxifying ozone in the apoplast of spinach (*Spinacia oleracea* L.) leaves. Plant Physiol 101:969-976.

Ma, H., Yanofsky, M.F. and Huang, H. (1991) Isolation and sequence analysis of TGA1 cDNAs encoding a tomato G protein alpha subunit. Gene 107:189-195.

Ma, H., Yanofsky, M.F. and Meyerowitz, E.M. (1990) Molecular cloning and characterization of GPA1, a G protein alpha subunit gene from *Arabidopsis thaliana*. Proc Natl Acad Sci USA 87:3821-3825.

Maccarrone , M., Veldink, G.A. and Vliegenthart, J.F.G. (1992) Thermal injury and ozone stress affect soybean lipoxygenases expression. FEBS Lett 309:225-230.

Majeau, N. and Coleman, J.R. (1991) Isolation and characterization of a cDNA coding for Pea chloroplastic carbonic anhydrase. Plant Physiol 95:264-268.

Malamy, J. and Klessig, D.F. (1992) Salicylic acid and plant disease resistance. Plant J 2:643-654.

Manning, W.J. and Krupa, S.V. (1992) Experimental methodology for studying the effects of ozone on crops and trees. In: Surface level ozone exposures and their effects on vegetation. (Lefohn, A.S., ed.). Chelsea, MI: Lewis Publishers, pp. 93-153.

McCurdy, T.R. (1994) Concentration of ozone in the lower troposphere (ambient air). In Tropospheric ozone: human health and agricultural impacts (McKee, D.J., ed.). Boca Raton, FL: Lewis Publishers, pp. 19-37.

McGurl, B., Pearce, G., Orozco-Cardenas, M. and Ryan, C.A. (1992) Structure, expression, and antisense inhibition of the systemin precursor gene. Science 255:1570-1573.

Mehdy, M. C. (1994) Active oxygen species in plant defense against pathogens. Plant Physiol 105:467-472.

Mehlhorn, H., O'Shea, J. and Wellburn, A.R. (1991) Atmospheric ozone interacts with stress ethylene formation by plants to cause visible plant injury. J Exp Bot 42:17-24.

Mehlhorn, H. (1990) Ethylene-promoted ascorbate peroxidase activity protects plants against hydrogen peroxide, ozone and paraquat. Plant Cell Environ 13:971-976.

Mehlhorn, H., Tabner, B.J. and Wellburn, A.R. (1990) Electron spin resonance evidence for the formation of free radicals in plants exposed to ozone. Physiol Plant 79:377-38.

Mehlhorn, H. and Wellburn, A.R. (1987) Stress ethylene formation determines plant sensitivity to ozone. Nature 327:417-418.

Melan, M.A., Dong, X., Endara, M.E., Davis, K.R., Ausubel, F.M. and Peterman, T.K. (1993) An Arabidopsis thaliana lipoxygenase gene is induced by pathogens, abscisic acid, and methyl jasmonate. Plant Physiol 101:441-450.

Menzel, D.B. (1976) The role of free radicals in the toxicity of air pollutants (nitrogen oxides and ozone). *In* Free Radicals in Biology Vol. 2 (Pryor, W.A., ed.). New York: Academic Press, pp. 181-202.

Mittler, R. and Zilinskas, B.A. (1994) Regulation of pea cytosolic ascorbate peroxidase and other antioxidant enzymes during the progression of drought stress and following recovery from drought. Plant J 5:397-405.

Mittler, R. and Zilinskas, B.A. (1992) Molecular cloning and characterization of a gene encoding pea cytosolic ascorbate peroxidase. J Biol Chem 267:21802-21807.

Mittler, R. and Zilinskas, B.A. (1991a) Molecular cloning and nucleotide sequence analysis of a cDNA encoding pea cytosolic ascorbate peroxidase. FEBS Lett 289:257-259.

Mittler, R. and Zilinskas, B.A. (1991b) Purification and characterization of pea cytosolic ascorbate peroxidase. Plant Physiol 97:962-968.

Miyake, C. and Asada, K. (1996) Inactivation mechanism of ascorbate peroxidase at low concentrations of ascorbate; hydrogen peroxide decomposes compound I of ascorbate peroxidase. Plant Cell Physiol 37:423-430.

Miyake C., Cao. W-H and Asada, K. (1993) Purification and molecular properties of the thylakoid-bound ascorbate peroxidase in spinach chloroplasts. Plant Cell Physiol 34:881-889.

Monroy, A.F. and Dhindsa, R.S. (1995) Low-temperature signal transduction: Induction of cold acclimation-specific genes of alfalfa by calcium at 25C. Plant Cell 7:321-331.

Morgan, D.L. and Wenzel, D.G. (1985) Free radical species mediating the toxicity of ozone for cultured rat lung fibroblasts. Toxicology 36:243-251.

Morel, F., Doussiere, J. and Vignais, P.V. (1991) The superoxide-generating oxidase of phagocytic cells. Physiological, molecular and pathological aspects. Eur J Biochem 201:523-546.

Nurnberger, T., Nennstiel, D., Jabs, T., Sacks, W.R., Hahlbrock, K. and Scheel, D. (1994) High affinity binding of a fungal oligopeptide elicitor to parsley membranes triggers multiple defense responses. Cell 78:449-460.

Olson, P.D. and Varner, J.E. (1993) Hydrogen peroxide and lignification. Plant J 4:887-892.

Pallas, J.A., Paiva, N.L., Lamb, C. and Dixon, R.A. (1996) Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. Plant J 10:281-293.

Paradies, I., Konze, J.O. and Elstner, E.F. (1980) Ethylene: indicator but not inducer of phytoalexin synthesis in soybean. Plant Physiol 66:1106-1109.

Pauls, K.P. and Thompson, J.E. (1980) In vitro simulation of senescence-related membrane damage by ozone-induced lipid peroxidation. Nature 283:504-506.

Pearce, G., Strydom, D., Johnson, S. and Ryan, C.A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins. Science 253:895-898.

Pena-Cortes, H., Fisahn, J. and Willmitzer, L. (1995) Signal involved in wound-induced proteinase inhibitor II gene expression in tomato and potato plants. Natl Acad Sci USA 92:4106-4113.

Pena-Cortes, H., Albrecht, T, Prat, S., Weiler, E.W. and Willmitzer, L. (1993) Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. Planta 191:123-128.

Pena-Cortes, H., Sanchez-Serrano, J., Rocha-Sosa, M. and Willmitzer, L. (1988) Systemic induction of proteinase-inhibitor-II gene expression in potato plants by wounding. Planta 174:84-87.

Perl-Treves, R. and Galun, E. (1991) The tomato Cu,Zn superoxide dismutase genes are developmentally regulated and respond to light and stress. Plant Mol Biol 191:745-760.

Pickett, C.B. and Lu, A.Y.H. (1989) Glutathione S-transferase: gene structure, regulation, and biological function. Annu Rev Biochem 58:743-764.

Pitcher, L.H., Brennan, E., Hurley, A., Dunsmuir, P., Tepperman, J.M. and Zilinskas, B.A. (1991) Overproduction of petunia chloroplastic copper/zink superoxide dismutase does not confer ozone tolerance in transgenic tobacco. Plant Physiol 97:452-455.

Prasad, T., Anderson, M.D., Martin, B.A. and Stewart, C.R. (1994) Evidence for chilling-induced stress in maize seedlings and a regulatory role for hydrogen peroxide. Plant Cell 6:65-74.

Price, A., Knight, M., Knight, H., Cuin, T., Tomos, D. and Ashenden, T. (1996) Cytosolic calcium and oxidative plant stress. Biochem Soc Trans 24:479-483.

Rao, M.V., Hale, B.A. and Ormrod, D.P. (1995) Amelioration of ozone-induced oxidative damage in wheat plants grown under high carbon dioxide. Plant Physiol 109:421-432.

Rasmussen, J.B., Hammerschmidt, R. and Zook, M.N. (1991) Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. Plant Physiol 97:1342-1347.

Rawlings, J.O. and Cure, W.W. (1985) The Weibull function as a dose-response model to describe ozone effects on crop yield. Crop Science 25:807-814.

Reinbothe, S., Mollenhauer, B. and Reinbothe, C. (1994) JIPs and RIPs: The regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. Plant Cell 6:1197-1209.

Runeckles, V.C. and Vaartnou, M. (1997) EPR evidence for superoxide anion formation in leaves during exposure to low levels of ozone. Plant Cell Environ 20: in press.

Runeckles, V.C. and Krupa, S.V. (1994) The impact of UV-B radiation and ozone on terrestrial vegetation. Environ Pollut 83:191-213.

Runeckles, V.C. and Chevone, B.I. (1992) Crop responses to ozone. *In* Surface Level Ozone Exposures and their Effects on Vegetation (Lefohn, A.S., ed.). Chelsea, MI: Lewis Publishers, pp. 189-270.

Rushmore, T.H., Morton, M.R. and Pickett, C.B. (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA concensus sequence required for functional activity. J Biol Chem 266:11632-11639.

Ryals, J., Uknes, S. and Ward, E. (1994) Systemic aquired resistance. Plant Physiol 104:1109-1112.

Salisbury, F.B. and Ross, C.W. (1992) Plant Physiology, 3rd edition. Wadsworth Publishing Company, Belmont, California.

Salter, L. and Hewitt, C.N. (1992) Ozone-hydrocarbon interactions in plants. Phytochemistry 31:4045-4050.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Saniewski, M., Nowacki, J. and Czapski, J. (1987) The effect of methyl jasmonate on ethylene production and ethylene-forming enzyme activity in tomatoes. J Plant Physiol 129:175-180.

Sano, H. and Ohashi, Y. (1995) Involvement of small GTP-binding proteins in defense signal-transduction pathways of higher plants. Proc Natl Acad Sci USA 92:4138-4144.

Sano, H., Seo, S., Orudgev, E., Youssefian, S., Ishizuka, K. and Ohashi, Y. (1994) Expression of the gene for a small GTP binding protein in transgenic tobacco elevates endogenous cytokinin levels, abnormally induces salicylic acid in response to wounding, and increases resistance to tobacco mosaic virus infection. Proc Natl Acad Sci USA 91:10556-10560.

Sawadogo, M. and Van Dyke, M.W. (1991) A rapid method for the purification of deprotected oligonucleotides. Nucleic Acid Res 19:674.

Schaller, A. and Ryan, C.R. (1996) Systemin - a polypeptide defense signal in plants. BioEssays 18:27-33.

Schreck, R., Rieber, P. and Baeuerle, P.A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J 10:2247-2258.

Schwacke, R. and Hager, A. (1992) Fungal elicitors induce a transient release of active oxygen species from cultured spruce cells that is dependent on Ca^{2+} and protein-kinase activity. Planta 187:136-141.

Sembdner, G. and Parthier, B. (1993) The biochemistry and the physiological and molecular actions of jasmonates. Annu Rev Plant Physiol Plant Mol Biol 44:569-589.

Sen Gupta, A., Webb, R.P., Holaday, A.S. and Allen, R.D. (1993) Overexpression of superoxide dismutase protects plants from oxidative stress. Plant Physiol 103:1067-1073.

Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y. (1995) Tobacco MAP kinase: A possible mediator in wound signal transduction pathways. Science 270:1988-1992.

Shaaltiel, Y., Glazer, A., Bocion, P.F. and Gressel, J. (1988) Cross-tolerance to herbicidal and experimental oxidants of plant biotypes tolerant to paraquat, sulfur dioxide, and ozone. Pesticide Biochem Physiol 31:13-23.

Shaaltiel, Y. and Gressel, J. (1986) Multienzyme oxygen radical detoxification system correlated with paraquat resistance in *Conyza bonariensis*. Pesticide Biochem Physiol 26:22-.

Sharma, Y.K., Leon, J., Raskin, I. and Davis, K.R. (1996) Ozone-induced responses in *Arabidopsis thaliana*: The role of salicylic acid in the accumulation of defense-related transcripts and induced resistance. Proc Natl Acad Sci U S A 93:5099-5104.

Sharma, Y.K. and Davis, K.R. (1994) Ozone-induced expression of stress-related genes in *Arabidopsis thaliana*. Plant Physiol 105:1089-1096.

Shi, J., Gonzales, R.A. and Battacharyya, M.K. (1995) Characterization of a plasma membrane-associated phosphoinositide-specific phospholipase C from soybean. Plant J 8:381-390.

Shulaev, V., Leon, J. and Raskin, I. (1995) Is salicylic acid a translocated signal of systemic aquired resistance in tobacco? Plant Cell 7:1691-1701.

Sies, H. (1991) Oxidative stress: Introduction. *In* Oxidative Stress: Oxidants and Antioxidants (Sies, H., ed.). London: Academic Press, pp. xv-xxii.

Smirnoff N. and Pallanca, E. (1996) Ascorbate metabolism in relation to oxidative stress. Biochem Soc Trans 24:472-478.

Smith, R.I., Anderson, C.W. and Rivadeneyra, I.C. (1995) Estimation of the uncertainty in crop loss predictions through statistical modelling of the AOT40 levels for ozone. Water Air Soil Pollut 85:1491-1496.

Song, W-C., Funk, C.D. and Brash, A.R. (1993) Molecular cloning of an allene oxide synthase: A cytochrome P450 specialized for the metabolism of fatty acid hydroperoxides. Proc Natl Acad Sci USA 90:8519-8523.

Staskawicz, B.J., Ausubel, F.M., BAker, B.J., Ellis, J.G. and Jones, J.D.G. (1995) Molecular genetics of plant disease resistance. Science 268:661-667.

Staswick, P. (1993) Jasmonic acid signaling in plants. *In* Plant signals in interactions with other organisms (Shultz, J. and Raskin, I., eds.). American Society of Plant Physiologists, pp. 14-23.

Steinberg, J.J., Gleeson, J.L. and Gil, D. (1990) The pathobiology of ozone-induced damage. Arch Environ Health 45:80-87.

Sültenmeyer, D., Schmidt, C. and Fock, H.P. (1993) Carbonic anhydrase in higher plants and aquatic microorganisms. Physiol Plant 88:179-190.

Tanaka, K., Saji, H. and Kondo, N. (1988) Immunological properties of spinach glutathione reductase and inductive biosynthesis of the enzyme with ozone. Plant Cell Physiol 29:637-642.

Tanaka, K., Suda, Y., Kondo, N. and Sugahara, K. (1985) O_3 tolerance and the ascorbate-dependent H_2O_2 decomposing system in chloroplasts. Plant Cell Physiol 26:1425-1431.

Tate, B.F., Schaller, G.E., Sussman, M.R. and Crain, R.C. (1989) Characterization of polyphosphoinositide phospholipase C from plasma membrane of A*vena sativa*. Plant Physiol 91:1275-1279.

Tenhaken, R., Levine, A., Brisson, L.F., Dixon, R.A. and Lamb, C. (1995) Function of the oxidative burst in hypersensitive disease resistance. Proc Natl Acad Sci USA 92:4158-4163.

Tester, M. (1990) Plant ion channels - Whole cell and single channel studies. New Phytol 114:305-340.

Tingey, D.T., Olszyk, D.M., Herstrom, A.A. and Lee, E.H. (1994) Effects of ozone on crops. In Tropospheric ozone: human health and agricultural impacts (McKee, D.J., ed.). Boca Raton, FL: Lewis Publishers, pp. 175-206.

Trewavas, A. and Gilroy, S. (1991) Signal transduction in plant cells. Trends Genet 7:356-361.

Van Camp, W., Willekens, H., Bowler, C., Van Montagu, M., Inze, D., Reupold-Popp, P., Sandermann Jr, H. and Langebartels, C. (1994) Elevated levels of superoxide dismutase protect transgenic plants against ozone damage. Bio/Technology 12:165-168.

Vera-Estrella, R., Higgins, V.J. and Blumwald, E. (1994) Plant defense response to fungal pathogens. Plant Physiol 106:97-102.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, Ward, E., Uknes, S., Kessmann, H. and Ryals, J. (1994) Salicylic acid is not the translocated signal responsible for inducing systemic aquired resistance but is required in signal transduction. Plant Cell 6:959-965.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J-P. and Ryals, J.A. (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085-1094.

Webb, R.P. and Allen, R.D. (1996) Overexpression of pea cytosolic ascorbate peroxidase confers protection against oxidative stress in *Nicotiana tabacum*. Abstract. In supplement to Plant Physiol 111:42.

Wellburn, F.A.M. and Wellburn, A.R. (1996) Variable patters of antioxidant protection but similar ethene emission differences in several ozone-sensitive and ozone-tolerant plant selections. Plant Cell Environ19:754-760.

Whiteside, C. and Hassan, H.M. (1988) Role of oxyradicals in the inactivation of catalase by ozone. Free Radicals Biol Med 5:305-312.

Wildon, D.C., Thain, J.F., Minchin, P.E.H., Gubb, I.R., Reilly, A.J., Skipper, Y.D., Doherty, H.M., O'Donnel, P.J. and Bowles, D.J. (1992) Electrical signalling and systemic proteinase inhibitor induction in the wounded plant. Nature 360:62-65.

Wilkie, T.M. and Yokoyama, S. (1994) Evolution of the G protein alpha subunit multigene family. Soc Gen Physiol Ser 49:249-270.

Willekens, H., Van Camp, W., Van Montagu, M., Inze, D., Langebartels, C. and Sandermann, H. Jr. (1994) Ozone, sulfur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in Nicotiana plumbagnifolia L. Plant Physiol 106:1007-1014.

Wu, G., Shortt, B.J., Lawrence, E.B., Levine, E.B., Fitzsimmons, K.C. and Shah, D.M. (1995) Disease resistance conferred by expression of a gene encoding H_2O_2-generating glucose oxidase in transgenic potato plants. Plant Cell 7:1357-1368.

Yahraus, T., Chandra, S., Legendre, L. and Low, P.S. (1995) Evidence for mechanically induced oxidative burst. Plant Physiol 109:1259-1266.

Yalpani, N., Enyedi, A.J., Leon, J. and Raskin, I. (1994) Ultraviolet light and ozone stimulate accumulation of salicylic acid, pathogenesis-related proteins and virus resistance in tobacco. Planta 193:372-376.

Yamaguchi, K., Hayashi, M. and Nishimura, M. (1996) cDNA cloning of a thylakoid-bound ascorbate peroxidase in pumpkin and its characterization. Plant Cell Physiol 37:405-409.

Yong-Mei, B., Kenton, P., Mur, L., Darby, R. and Draper, J. (1995) Hydrogen peroxide does not function downstream of salicylic acid in the induction of Pr protein expression. Plant J 8:235-245.

Xing, T., Higgins, V.J. and Blumwald, E. (1996) Regulation of plant defense response to fungal pathogens: two types of protein kinases in the reversible phosphorylation of the plasma membrane H^+-ATPase. Plant Cell 8:555-564.

APPENDIX

(i) Table showing comparison of necrosis (% of leaf area) between visual scoring, digital imaging scoring and leaf area meter scoring.

Leaf #	visual scoring[a]	digital image[b]	leaf area meter[c]
1	45	32.3	25.3
2	5	10.6	4.7
3	10	13	8.6
4	5	9.2	4.2
5	40	47.4	38.3
6	50	45.9	33.7

[a] using a subjective scale of 0%, 1 - 5%, 6 - 10%, 11 - 15% etc.

[b] leaves were converted (scanned) to digital images using the IS-500 Digital Imaging System (Alpha Innotech Corporation) and analyzed using NIH Image Version 1.57 software.

[c] every necrotic spot or fleck was printed (manually) on a transparency and the transparency used for the scoring using a Leaf Area Meter (LiCor Model LI-3000).

(ii) Hoagland medium:

Macronutrients

- $Ca(NO_3)_2 \cdot 4H_2O$ — 4 mM
- $MgSO_4 \cdot H_2O$ — 2 mM
- KNO_3 — 6 mM
- $NH_4H_2PO_4$ — 1 mM

Micronutrients

- H_3BO_3 — 45 mM
- $MnSO_4 \cdot H_2O$ — 20 mM
- $CuSO_4 \cdot 5H_2O$ — 0.4 mM
- $ZnSO4 \cdot 7H_2O$ — 0.7 mM
- $(NH_4)6Mo_7O_{24} \cdot 4H_2O$ — 0.2 mM

H_2SO_4 (concentrated) — 5 ml/L

(iii) MS medium and plates

- MS macro salts — 1X
- MS micro salts — 1X
- B5 vitamin — 1X
- sucrose — 30 g/L
- pH to 5.7 with 1 M NaOH
- agar (for plates) — 7 g/L

MS macro stock (20X)

- NH_4NO_3 — 33 g/L
- KNO_3 — 38 g/L
- $MgSO_4 \cdot 7H_2O$ — 8.8 g/L
- KH_2PO_4 — 7.4 g/L
- $CaCl_2 \cdot 2H_2O$ — 4.4 g/L

MS micro stock (500X)

- $MnSO_4 \cdot H_2O$ — 7.8 g/L
- $ZnSO_4 \cdot 7H_2O$ — 6.15 g/L
- H_3BO_3 — 3.1 g/L

- KI	415 mg/L
- $Na_2MoO_4 . 2H_2O$	125 mg/L
- $CuSO_4 . 5H_2O$	12.5 mg/L
- $CoCl_2 . 6H_2O$	12.5 mg/L
B5 stock (100X)	
- Thiamine-HCl	10 mg/L
- Pyridoxin-HCl	50 mg/L
- Nicotinic acid	50 mg/L
- Myo-inositol	10 g/L
- Glycine	200 mg/L

After 25 days, shoots were transferred to Root Induction Medium (including carbenicillin, 200 mg L^{-1}; kanamycin, 50 mg L^{-1})

(iii) MS selection medium and plates (for one liter):

- same as MS medium except:	
- benzyladenine	1 mg
- napthaleneacetic acid	0.1 mg
- agar (for plates)	7 gr
- carbenicillin	200 mg
- kanamycin	100 mg
- vancomycin	500 mg

(iv) Root Induction Medium (for one liter):

- same as MS medium except:	
- carbenicillin	200 mg/L
- kanamycin	50 mg/L

(v) The effects of jasmonates on ozone tolerance in *Arabidopsis thaliana*

In each experiment the 25 to 30 days old plants were first pre-exposed for 2 hrs to MeJA (100ml), 4 hrs before 4 hrs ozone exposure. Each experiment used a different ozone concentration.

Experiment 1: 250 ppb

	no necrosis	necrosis	% leaves with necrosis
Control	43	2	4.4
MeJa	32	4	11.1

Experiment 2: 400 ppb

Control	33	8	19.5
MeJA	11	37	77.1

Experiment 3: 500 ppb

Control	22	35	61
MeJA	8	44	85