Hormonal regulation of radical-induced programmed cell death in ozone-sensitive mutants of *Arabidopsis thaliana*

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Academic dissertation
To be presented for public criticism, with permission of the Faculty of Science,
University of Helsinki, in auditorium 1041 of Biocenter II,
Viikinkaari 5 on November 22, 2002, at 12 o'clock noon

Helsinki 2002

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ISSN 1239-9469
ISBN 952-10-0325-1
ISBN 952-10-0326-X (e-thesis)

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Dedicated to the loving memory of my
Grandfather, Eugene Edwin Overmyer Sr.

Abbreviations used

ABA	abscisic acid
ROS	reactive oxygen species
Apx	ascorbate peroxidase
AsA	ascorbic acid
Col-0	Columbia ecotype of *Arabidopsis*
Cyt-c	cytochrome-c
DAB	3', 3-diaminobenzidine
DPI	diphenylene iodonium
ET	ethylene
GR	glutathione reductase
GST	glutathione-S-transferase
H_2O_2	hydrogen peroxide
JA	jasmonic acid
NahG	salicylate hydroxylase
HR	hypersensitive response
NAD(P)H	nicotinamide adenine dinucleotide (phosphate)
NBT	nitro blue tetrazolium
NO	nitric oxide
NOS	nitric oxide synthase
$O_2^{\cdot -}$	superoxide anion radical
O_3	ozone
$OH^{\cdot}$	hydroxyl radical
PAL	phenylalanine ammonia lyase
PCD	programmed cell death
PR	pathogenesis related (proteins)
SA	salicylic acid
SAR	systemic acquired resistance
SOD	superoxide dismutase
UV	ultra violet (radiation)

Original Publications

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals. Additional unpublished data will also be presented in the text.

I **Overmyer K, Pellinen R, Kuittinen T, Saarma M and Kangasjärvi J.** 2002. Ozone induced programmed cell death in the radical sensitive *rcd1 Arabidopsis* mutant (Submitted to The Plant Journal)

II **Overmyer K, Tuominen H, Kettunen R, Betz C, Langebartels C, Sandermann Jr H and Kangasjärvi J.** 2000. The ozone-sensitive *Arabidopsis rcd1* mutant reveals opposite roles for ethylene and jasmonate signaling pathways in regulating superoxide-dependent cell death. Plant Cell 12: 1849-1862.

III **Wohlgemuth H, Mittelstrass K, Kschieschan S, Bender J, Weigel HJ, Overmyer, K, Kangasjärvi J, Langebartels C and Sandermann Jr H.** 2002. Activation of an oxidative burst is a general feature of sensitive plants exposed to the air pollutant ozone. Plant Cell Environ. 25: 717-726

IV **Tuominen H, Overmyer K, Keinänen M and Kangasjärvi J.** 2002. Mutually antagonistic interactions between ethylene, jasmonic acid and salicylic acid in the ozone-induced oxidative cell death. (Submitted to Plant Cell)

Summary

The gaseous air pollutant ozone (O_3) has become established as a simple and effective way of applying reactive oxygen species (ROS) in order to study ROS signaling in plants. A genetic approach to ROS signaling was taken and mutants, named *rcd* (*radical induced cell-death*), which are hypersensitive to O_3 and superoxide ($O_2^{\bullet-}$) but not hydrogen peroxide (H_2O_2), were isolated and genetically characterized.

The rapid resistance-associated cell death response of plants to pathogens is termed the hypersensitive response (HR). O_3-exposed plants exhibited markers associated with HR including: an oxidative burst of ROS accumulation, cell wall thickenings, PR-protein expression, and both micro and macro scale cell death and their associated local and systemic induced pathogen resistance. It is commonly held that O_3-induced cell death is the result of deleterious firing of the HR program by O_3-derived ROS. This implies that O_3-induced cell death, like HR, is programmed; although this has previously not been shown directly. The morphology of mutant *rcd1* cells undergoing O_3-induced cell death was explored and revealed that O_3-exposed *rcd1* exhibits hallmark characteristics of programmed cell death (PCD) including nuclear shrinkage, chromatin condensation, and nuclear DNA fragmentation. Furthermore, cell death induced by O_3 or exogenous $O_2^{\bullet-}$ application could be reduced or delayed with inhibitors of active metabolism including; Ca^{++} influx, transcription, ATPases, tyrosine kinases, serine-threonine kinases and translation. Inhibition of serine-proteases and caspases resulted in attenuated cell death. These results validate *rcd1* and in general O_3-treated plant systems as models of PCD regulation by ROS.

The mutant *rcd1* allele confers, as a co-dominant trait, a hypersensitivity manifested as increased levels of cell death to O_3, $O_2^{\bullet-}$, avirulent pathogen challenge, but not H_2O_2. Additionally, *rcd1* also has a transient spreading cell death active until circa 12-24 hours post challenge. $O_2^{\bullet-}$ was necessary and sufficient for spreading cell death and $O_2^{\bullet-}$ accumulates in healthy tissue in advance of lesion growth. This $O_2^{\bullet-}$ -driven cell death was ethylene (ET) dependent and could be counteracted by jasmonic acid (JA). Furthermore, ROS accumulation was a general feature in the induction of cell death in O_3-sensitive plants as seen in various tomato and tobacco cultivars, accessions of *Arabidopsis*, and other species.

O_3 induces the biosynthesis of signal molecules such as salicylic acid (SA), JA, and ET. The interaction and balance between these signals are involved in cell death regulation. As SA accumulation is a known requirement for HR-like cell death the effect of the SA degrading *NahG* gene was investigated. Unexpectedly, *NahG* afforded *rcd1* only partial protection thus RCD1 functions downstream or independent of SA. ET-insensitive, SA-insensitive, and SA-deficient plants were tolerant and ET overproducing and JA-insensitive plants were sensitive to ROS. Transcript levels of 130 stress related EST clones were analyzed in a series of mutant backgrounds. This data and exogenous hormone treatments showed that ET, SA, and JA are all mutually antagonistic. Importantly, we show a novel interaction, the suppression of ET signaling by JA. In gene array experiments the transcript levels of JA and abscisic acid (ABA) related genes showed deficiencies in *rcd1*. This together with several auxin-related changes observed in *rcd1* suggests RCD1 is either a commonly recruited signaling link or involved in the interaction and balance of hormone signals, or both. These results suggest a model where SA, ET and their interactions drive lesion initiation and spread while JA counters this in the process of lesion containment.

The isolation of mutants that misregulate O_3-induced ROS signals implies the modulation of ROS signaling leading to the activation of cell death is under genetic control. Possible genetic factors involved could be traditional antioxidant systems but, as shown by the cloning of RCD1, may also include cell death regulatory or signaling genes.

1 Introduction

1.1 Programmed cell death

" And I will show that nothing can happen more beautiful than death."

Walt Whitman

Programmed cell death (PCD), shortly stated, is cellular suicide. As a subject for biological research the process of how cells die has been largely overlooked until only recently. Kerr *et al*. (1971) called attention to a recurrent set of morphological characteristics observed in dying cells and coined the term "apoptosis", which is often used synonymously with PCD in animals. These observations led to the proposal that some deaths in both physiological and pathological situations are suicides. Although now considered a seminal paper, when it was originally published in 1971 the idea of cellular suicide was not well accepted. This was in spite of the fact that the role of cell death in development and tissue homeostasis had been recognized as early as the 1950s (Ellis, R.E. et al., 1991). It took two decades and the isolation of genes dedicated to the control of cell death in humans and the model organism *Caenorhabditis elegans* (worm) before this notion gained broad acceptance (Raff, 1998). Currently PCD has taken its rightful place next to cell division and differentiation, recognized as one of the basic cellular processes determining the function of the cell and the life of the organism. Impressive advances in PCD research have resulted in the definition of pathways dedicated to the regulation of cell death. However, despite the detailed characterization of PCD in animal systems definition of processes involved in the regulation of PCD in plant systems lags behind.

1.1.1 Function of programmed cell death

It has been demonstrated in many biological contexts that PCD performs vital functions and even in some cases can be necessary for life itself. During development PCD helps to sculpt forms. For example the formation the lobed shape of *Monstera deliciosa* leaves is carved out by the developmental death of the tissue between the incipient lobes (Pennell and Lamb, 1997). Similarly in reptiles, birds and mammals the separation of digits in a developing limb buds occur via the death of the interdigital tissue (Wyllie et al., 1980). Also, during flooding induced hypoxia the root cortex is remodeled during the process of aerenchyma formation. Death of select cells within the cortex results in air channels that provide the necessary oxygen for respiration in the root (Pennell and Lamb, 1997; Kawai et al., 1998; Gunawardena et al., 2001).

PCD can also act during development to produce functional structures made of cell corpses. Plants transport water in a conduit consisting of dead cell corpses called tracheary elements (Fukuda, 2000). The conduit is made up of hollow cell remnants with specialized cell wall thickenings, which are laid down by living cells shortly before their death to form the functional vasculature (Pennell and Lamb, 1997; Fukuda, 2000).

PCD is commonly recruited in the removal of cells that have performed a temporary function. As in the degeneration of intersegmental muscles, which are no longer needed in the

adult, in metamorphosing insects (Ellis, R.E. et al., 1991) or the death and resorption of tail of the tadpole (Jacobson et al., 1997). Examples from plants include the death of suspensor cells during embyogenesis as well as aleurone and endosperm cells during seed germination (Beers, 1997; Fath et al., 2000; Young and Gallie, 2000). Additionally, cells shed from the root cap die by PCD (Pennell and Lamb, 1997). A special form of PCD is the senescence of flower petals or leaves (Beers, 1997; Hadfield and Bennett, 1997; Yen and Yang, 1998). This involves the progressive salvaging and export of nutrients from the spent organ prior to its death (Beers, 1997). Interestingly, the term apoptosis coined by Kerr et al. to describe mammalian PCD morphology is derived from the word used in Greek to describe the "dropping off" or "falling off" of petals from flowers or leaves from trees (Kerr et al., 1971).

PCD is involved in the disposal of unwanted or unnecessary cells, for example structures needed only in one sex. Most flowers contain organs of both sexes. However, those flowers that are unisexual are initially formed as perfect flowers (Wu, H.M. and Cheung, 2000), i.e. containing organs of both sexes, and the unneeded organ primordia are subsequently deleted by PCD (Beers, 1997; Wu, H.M. and Cheung, 2000). PCD also functions in a similar manner in the development of the mammalian reproductive system (Jacobson et al., 1997).

Finally, and most importantly for the studies contained in this book, PCD is responsible for the execution of cells that have become dangerous to the well being of the organism as a whole. This can be the result of cell damage, uncontrolled cell-proliferation, misplaced cells, "self"-reactivity in immune cells, or infection (Jacobson et al., 1997; Gilchrist, 1998; Johnstone et al., 2002).

1.1.2 Definitions for programmed cell death

It may be useful to further consider the partially overlapping terminology used to describe PCD and related phenomena. Apoptosis is a descriptive term for a collection of morphological features observed in mammalian cells undergoing one specific type of PCD (Kerr et al., 1971). The key features of apoptosis are nuclear shrinkage, DNA fragmentation, late loss of organelles, cell shrinkage and breakup of the cell and nucleus into membrane bound apoptotic bodies, which are taken up by the surrounding cells. Due to the physical restriction of the cell wall plant cells can obviously never fulfil all of these criteria. Thus the term apoptosis may not be entirely appropriate for the description of cell death in plants even if some apoptotic hallmarks are seen in dying plant cells (Dangl et al., 1996; Jones and Dangl, 1996; Jones, 2000). Necrosis is accidental cell death by overwhelming physical or chemical trauma. Characteristically, necrosis is disorganized and uncontrolled, usually resulting in the death of large groups of cells or whole tissues. Necrotic cells loose membrane and organelle integrity early and DNA fragmentation, if it occurs is random and appears late.

The key characteristics of PCD are the cell autonomous decision to die (triggered from within) and active participation of the cell in its own execution. A major problem with the term PCD lies in the requirement by definition for cell autonomy, i.e. that the decision to die must come entirely from within and is not dependant on signals from other cells or other external factors. Given the element of social control involved in most cell deaths (Raff, 1992) it seems that signals originating from outside the cell are involved in most if not all cell death decisions in multicellular organisms. To strictly limit the term PCD in this way would exclude most deaths.

Physiological cell death is a somewhat broader term for cell deaths that occur via a mechanism that has evolved in an organism specifically to kill its own cells (Vaux, 1993). This term encompasses most if not all of what has been described as PCD and apoptosis and it

has been proposed as a replacement for PCD. The term PCD is often used in a less restricted manner to indicate naturally occurring cell deaths (Ellis, R.E. et al., 1991), with much the same meaning as physiological cell death is defined above, and will be used in this manner throughout this work.

To further complicate the issue not all programmed cell deaths, in both animals and plants, are apoptotic (Schwarts et al., 1993; Levin et al., 1999; Jones, 2000). The range of different phenotypes observed has led to the idea of a spectrum or a continuum of cell death with apoptosis and necrosis at the extremes. In between these extremes there varied mixed morphologies with characteristics of both PCD and necrosis. Some cells die by PCD and only exhibit a subset of the apoptotic features. In other cases death takes place as a part of a developmental program, i.e. it is clearly genetically programmed, but mechanistically shows the features of necrosis.

1.2 Mechanisms and regulation of cell death

1.2.1 Comparative analysis

Great progress has been made in the functional characterization of the regulators of PCD in animals. A driving force in this work has been the isolation of cell death mutants from the *C. elegans* model system (Ellis, R.E. et al., 1991; Hengartner, 1995). From this work a highly conserved pathway for cell death activation has emerged. For the vast majority of cell death regulators characterized in animal systems structural homologs have not been identified in plants. Thus, it is believed that plants have either evolved distinct cell death regulatory machinery or that homologs of animal proteins in plants have sufficiently diverged in structure as to no longer be recognized (Dangl et al., 1996). Some level of conservation has been seen between plants and animals, especially at the level of conserved signaling domains within plant and animal cell death regulators. Thus the animal cell death pathway remains a paradigm for comparative analysis and is described below.

1.2.2 The BCL2-like proteins and mitochondria

The BCL2-like protein (BLP) family represents a large family of cell death regulators, which are located primarily in the outer membrane of the mitochondria (Green and Reed, 1998). They are classified according to whether they are either death inhibiting, such as *Bcl-2, Bcl-X_L* and *CED-9*, or death promoting such *as Bax* (Nunez and Clarke, 1994). Characteristically, BLPs bear conserved BH (Bcl-2 Homology) domains. BLPs form either homo- or heterodimers and the balance of pro-death to pro-survival BLPs acts as a cellular rheostat determining a cell's sensitivity to entering the cell death pathway (Barinaga, 1994). BLPs also interact with a large variety of other proteins and some can form pores in membranes.

There have been no BLPs or BH domain bearing proteins identified in plants (Dangl et al., 1996). However, heterologous expression of mammalian BLPs in plants and yeast has been tested. Both inhibition of cell death by anti-death BLPs and enhanced cell death by pro-death BLPs has been seen in transgenic plants (Lacomme and SantaCruz, 1999; Lam et al., 1999; Mitsuhara et al., 1999; Dickman et al., 2001; Yamada et al., 2001). BAX Inhibitor1 (BI1) is a human protein identified via it ability to rescue yeast against Bax induced death (Lam et al., 1999). BI1 is conserved in plants and exists as a multi-gene family in *Arabidopsis*. In addition to suppressing Bax lethality in yeast and *Arabidopsis* AtBI1 is

rapidly induced in plants by wounding and pathogen challenge (Sanchez et al., 2000; Yamada et al., 2001).

One of the key events regulated by the BLPs is the release of cytochrome c (cyt-c) from the mitochondria into the cytosol (Reed, 1997). This causes the formation of reactive oxygen species (ROS), which acts as a further death-promoting signal. An important protective function for Bcl-2 has been demonstrated to be the prevention of ROS formation (Hockenbery et al., 1993). Also, once released into the cytosol cyt-c acts to promote cell death via its participation in the assembly of the apoptosome.

Caspases are cysteine aspartic proteases and the proteolytic cascade they are involved in is at the core of cell death regulation and execution. They are roughly categorized into upstream regulatory caspases and downstream effector- or executioner-caspases. The apoptosome is a macromolecular complex responsible for caspase activation (Green, 2000). It is composed of the linker molecule Apaf-1, cyt-c and procaspase-9. Cytosolic Cyt-c causes apaf-1 oligomerization, which then functions as a dock for procaspase-9 inducing its autoactivation via cleavage. Caspase-9 then recruits and activates procaspase-3. Active caspase-3 and other executioner (effector) caspases then cleave a wide variety of substrates causing the death of the cell. A further level of regulation comes from the IAPs (inhibitor of apoptosis proteins) through their binding of active caspase-9 and prevention of procaspase-3 activation (Green, 2000). The pro-death molecule Smac/DIABLO, which is coordinately released from the mitochondria with cyt-c, counters this effect by targeting IAPs for proteasomal degradation. A second pathway involving the assembly of a caspase activation complex on the cytoplasmic domain of death receptors can also act upstream in the activation of caspase-3.

Despite the apparent lack of structural conservation of most PCD regulators there are some points that suggest functional conservation of the core PCD regulatory pathways. Cyt-c release from mitochondria has been documented in plant cells undergoing PCD (Sun et al., 1999; Balk and Leaver, 2001; Yu, X.H. et al., 2002). However, the significance of this event has not yet been determined. In plant systems there are currently no known structural or functional homologs of Apaf-1, caspases, or Smac/DIABLO. IAP-like proteins can be found in the *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2000; http://www.tigr.org/tdb/e2k1/ath1/ath1.shtml). There have been no reports addressing whether or not they have a role in the regulation of plant PCD. Heterologous expression of a baculovirus IAP resulted in resistance to death induced by several fungal pathogens (Dickman et al., 2001). A requirement for caspase-like activities has been shown in some plant cell deaths (Lam and del Pozo, 2000).

Among the earliest targets of caspase cleavage during PCD is PARP (poly-ADP ribose polymerase). This event is a defining characteristic of apoptosis and is believed to help maintain the cellular energy balance by preventing depletion of the NAD, a substrate required for PARP activity (Pieper et al., 1999). As in animals plants contain PARP genes that are activated in several stress situations (Babiychuk et al., 1998; Doucet-Chabeaud et al., 2001). PARP cleavage during plant cell deaths has also been documented (D'Silva et al., 1998; Sun et al., 1999; Tian et al., 2000).

1.3 Immunity, reactive oxygen species, and programmed cell death

PCD is an integral feature of the immune response in all eukaryotes (Staskawicz et al., 2001). In animals, the development of the immune system is shaped by PCD (Rathmell and Thompson, 1999). A common strategy in all eukaryotes is the execution of cells that have become infected with a pathogen. Innate immunity is the ability of every cell within an

organism, especially those that are not dedicated immune cells, to fight pathogen infection. In plants there are no dedicated immune cells or a circulatory system for their mobilization. However, all plant cells retain the ability for immune function. This innate immunity is of premier importance for plant health. The controlled sacrifice of infected plant cells and a limited number of surrounding healthy cells is a key event in the limitation of pathogen ingress and coordination of defense response. This form of innate immunity is termed the hypersensitive response (HR) and has become one of the major paradigms for plant cell death control. Despite the ever-increasing importance of agricultural plants for human nutrition and the prominence of *Arabidopsis* as a model organism for comparative biology, little is known about cell death in plants. This is in stark contrast to the detailed knowledge available of the molecular mechanisms controlling cell death in other eukaryotes.

Reactive oxygen species (ROS) are central to the regulation of PCD (Kroemer et al., 1995; Morel and Dangl, 1997). ROS accumulation triggers cell death in plant and animal systems indicating the almost universal nature of their action. Specifically, in plants a high level of ROS production termed the oxidative burst is involved in cell death activation during the HR (Lamb and Dixon, 1997; Morel and Dangl, 1997). In accordance with the role of ROS signaling in cell death and immunity, many plant cell death mutants also have alterations in their regulation of ROS and response to pathogen attack.

Knowledge of the role of cell death and ROS in immunity offers the possibility of developing new plant disease resistance strategies with insights gained from basic research. This could translate into downstream applications with the potential to increase agricultural productivity. While plants in their own right are interesting and important organisms, plant research also benefits other areas. Insights into plant immunity and cell death control have significance for a larger general audience interested in PCD. Several examples testify to the important contributions made by plant research with implications reaching as far as the realm of human health. The foremost example is perhaps the new interest in the human innate immune system, the so-called “undiscovered” branch of the immune system. This has been spurred on by the discovery of human homologs of resistance genes conferring innate immunity in plants (Staskawicz et al., 2001).

The focus of the remainder of this book will be on two plant processes, the hypersensitive response and the ozone (O_3) response, that are unified in their induction of ROS production (the oxidative burst) and cell death. The plant O_3-response represents a vital field of research in its own right. As will become apparent below the linking of PCD to O_3 responses opens new avenues of research with the potential for a deeper understanding of the mechanisms and control of O_3-induced cell death. Conversely, due to its similarity to the hypersensitive response O_3 has the potential to be used as a model system for studying the role of ROS, and other factors, in cell death regulation and immunity.

1.4 The hypersensitive response: a model for the control of programmed cell death

Mutant analysis has contributed greatly to our understanding of the HR, HR-signaling and cell death control. Table 1 summarizes the many mutants that have contributed to this and are discussed in this work. The HR is a general feature of several forms of the plant innate immune response involving the controlled sacrifice of cells at the site of pathogen attack. In addition to the cell death response there is a large battery of induced defenses concomitant with the induction of the HR. The deposition of callous or auto-florescent phenolic compounds is a common histochemical marker of the hypersensitive response. They serve to reinforce cell walls creating a physical barrier that isolates the invading pathogen (Bent,

1996). Additionally, some of these compounds have direct antibiotic properties that inhibit pathogen growth and also prevent secondary infection of the dead tissue.

Table 1: Signaling and cell death mutants discussed in this work

Mutant	Species	Phenotype	Gene	Reference .
axr1 (*auxin resistant 1*)	*Ath*	Auxin insensitive	At1g05180	Leyser et al, 1993
cev1 (*constitutive expresser of VSP1*)	*Ath*	Constitutive jasmonate signal	At5g05170	Ellis et al, 2002
ctr1 (*constitutive triple response1*)	*Ath*	Constitutive ethylene signal	At5g03730	Kieber et al, 1993
coi1 (*coronotine insensitive1*)	*Ath*	Insensitive to coronotine and jasmonate	At2g39940	Xie et al, 1998
dnd1 (*disease-resistance, no death1*)	*Ath*	Resistance without death	At5g15410	Clough et al, 2000
edr1 (*ehanced disease resistance1*)	*Ath*	Virulent pathogen resistance	At1g08720	Frye et al, 2000
eds1 (*ehanced disease susceptibility1*)	*Ath*	loss of disease resistance	At3g48090	Falk et al, 1999
ein2 (*ethylene insensitive 2*)	*Ath*	Ethylene insensitive	At5g03280	Alonso et al, 1999
eto1 (*ethylene overproducer1*)	*Ath*	Ethylene overproduction	unknown	Woeste et al, 1999
etr1 (*ethylene resistant 1*)	*Ath*	Ethylene insensitive	At1g66340	Chang et al, 1993
fad3/7/8 triple mutant (*fatty acid desaturase-3, -7, -8*)	*Ath*	Jasmonate deficient	At2g29980 At3g11170 At5g05580	McConn and Browse, 1996
jar1 (jasmonate resistant1)	*Ath*	Jasmonate insensitive	At2g46370	Staswick et al, 2002
les22 (lesion mimmic 22)	Maize	Lesion mimic	-	Hu et al, 1998
lls1 (lethal leaf spot 1)	Maize	Lesion mimic	-	Gray et al, 1997
lsd1 (lesions simulating disease-resistance1)	*Ath*	Lesion mimic	At4g20380	Dietrich et al, 1997
NahG (salicylate hydroxylase) *	*Ath*	Salicylate deficient	-	Durner et al, 1997
ndr1 (non-race-specific disease resistance1)	*Ath*	Loss of resistance	At3g20600	Century et al, 1997
npr1 (non-expresser PR-proteins1)	*Ath*	Salicylate insensitive	At1g64280	Cao et al, 1997
pad4 (phytoalexin deficient 4)	*Ath*	Phytoalexin deficient	At3g52430	Jirage et al, 1999
rbohD (respiratory burst oxidase homolog D)	*Ath*	Altered ROS production	At5g47910	Torres et al, 2001
vtc1 (vitamin-c 1)	*Ath*	Ascorbate deficient O_3sensitive	At2g39770	Conklin et al, 1999

*NahG plants are not actually a mutant, but rather transgenic plants bearing a bacterial gene, salicylate hydroxylase, which results in an inability to accumulate significant levels of salicylate.

Hypersensitive cell death is regulated genetically and a form of PCD as illustrated by the existence of mutants that spontaneously trigger the response in the absence of pathogens. These mutants have been termed lesion mimic mutants. The requirement for active metabolism in the HR also argues that it is a programmed process (He et al., 1993; He et al., 1994; Morel and Dangl, 1997). Lesion mimic mutants have been isolated from corn, tomato and *Arabidopsis* (Dangl et al., 1996).

Not all lesion mimic mutants represent loci involved directly in PCD control. For example the mutated genes for the maize *les22* (*lesion mimic 22*) and *lls1* (*lethal leaf spot 1*) lesion mimics have been cloned (Gray et al., 1997; Hu et al., 1998). LES22 encodes an enzyme involved in porphyrin metabolism and the LLS1 gene product bears domains suggesting it is an oxidase that acts on a phenolic compound. These mutants do not result in

the activation of defense pathways suggesting that their genes are not directly involved in HR regulation. These mutations lead to metabolic disturbances or the accumulation of toxic products. Apparently plants have a general mechanism that will detect such metabolic disturbances and trigger a default cell death pathway (Richberg et al., 1998). This mechanism could account for the lesion mimic phenotypes seen in a large number of transgenic plants (Mittler and Rizhsky, 2000).

Lesion mimic mutants can be separated into two classes (Dangl et al., 1996). So-called initiation class mutants trigger lesions of determinate size that are similar in appearance to normal HR-lesions triggered by pathogens. Examples of this class are *lsd2* (*lesions simulating disease resistance response2*) and *lsd3* in *Arabidopsis*. The second type of lesion mimic mutants is termed propagation-class mutants. These mutants exhibit spreading cell death. Typical of this class is the *Arabidopsis lsd1* mutant. The existence of these two classes suggests that two genetically distinct processes are involved in lesion formation. First the initiation of cell death and second the spread of cell death in a normal HR to a limited number of surrounding cells. This implies a dialog of signals between dying cells and healthy neighboring cells, the outcome of which determines lesion size (Dangl et al., 1996).

The *lsd1* mutant of *Arabidopsis* is a propagation class mutant and compromised in its ability to halt lesion growth after cell death has been triggered resulting in consumption of the entire leaf (Dietrich et al., 1994). The *LSD1* gene has been identified via a positional cloning strategy (Dietrich et al., 1997). It encodes a novel protein that functions as a negative regulator of cell death and contains three type IV Zn-finger domains. *LSD1* is the defining member of a small gene family. Two LSD-ONE-LIKE (LOL) proteins have been characterized and are also regulators of PCD (Epple et al., 2002).

The HR is associated with race-specific, or gene-for gene, resistance where a plant *Resistance*-gene (*R*-gene) product specifically recognizes an avirulence gene product from the pathogen or the changes it induces (Dangl and Jones, 2001; Nimchuk et al., 2001). This type of plant-pathogen interaction is termed an incompatible interaction. Great advances have been made in our understanding of the pathogen surveillance and recognition machinery. The cloning of a variety of *R*-genes has revealed some common protein structural elements shared between plant R-gene products (Ellis, J. et al., 2000). The proteins encoded by virtually all cloned *R*-genes bear nucleotide-binding domains (NBDs) and leucine rich repeats (LRRs). One subclass of *R*-gene products contains leucine zippers (LZ) and another subclass contains the so-called TIR (toll, interleukin-1-receptor-1, resistance gene) domain, which derives its name from the proteins that share this conserved domain (Wilson et al., 1997). Toll1 from *Drosophila* is a receptor involved in development and defense while the human interleukin-1-receptor-1 has a role in inflammation and other immune responses. In addition to TIR, several other of the above mentioned motifs are also found in animal PCD regulatory proteins and in an ancient pathway involved in development and immunity (reviewed in: Wilson et al., 1997; Dangl and Jones, 2001; Staskawicz et al., 2001).

HR cell death is not an absolute requirement for resistance. Resistance without host cell death has been documented in some plant-pathogen interactions and for example in the *Arabidopsis dnd1* (*defense, no death1*) mutant (Clough et al., 2000; Yu, I.-C. et al., 2000). However, an understanding of when death is required for resistance or what determines resistance in the absence of death is still lacking. In addition to the HR, which occurs in resistant plants (i.e. in incompatible interactions), PCD is involved in other plant pathogen interactions. For example symptom development in susceptible plants (i.e. in compatible interactions) also involves radical production and PCD. The induction of cell death in this case is slow, following pathogen multiplication, making it ineffective a defense.

Some necrotrophic pathogens, which use a strategy of killing plant cells to obtain nutrients, hijack the HR and induce cell death to their benefit. (Morel and Dangl, 1997). Intentional triggering of the HR or the oxidative burst by necrotrophic pathogens has been documented (Govrin and Levine, 2000). Two fungal toxins provide an excellent example of this. Both ALL-toxin produced by *Alternaria alternata* f. sp. *lycopersici* and fumonisin-B produced by *Fusarium monilliforme* are sphinganine analogs and potent inhibitors of cerimide synthase (Gilchrist, 1997). They induce PCD through alterations in the metabolism of these lipid-signaling molecules via poorly understood mechanisms. Interestingly, these toxins have also been used as model systems for the study of plant PCD induction. (Gilchrist, 1997; Asai et al., 2000; Stone et al., 2000).

1.5 Ozone

In the mid-19th century, Schoenbein and Soret discovered and determined the structure of the trimolecular allotrope of oxygen called ozone (O_3; Hogsett et al., 1985). The name O_3 is derived from the Greek word for “smelly” (Hogsett et al., 1985). In contrast to stratospheric O_3, which protects plants from harmful UV-B radiation, tropospheric O_3 is a potent toxin accounting for major loses in both agriculturally important and natural species (Harris and Bailey-Serres, 1994; Kärenlampi and Skärby, 1996; Kley et al., 1999). In the late 1950s O_3 was recognized as the cause of "weather fleck” disease of shade grown wrapper tobacco in the Connecticut Valley (Heggestad and Middleton, 1959) and damage to grape vine leaves in southern California (Richards et al., 1958). These are the first reports of O_3 as a phytotoxic pollutant and the beginning point for the research field of plant O_3 interactions. Great strides have been made in 50 years of research into O_3-plant interactions and still many basic questions remain unanswered.

O_3 is the most wide spread atmospheric pollutant in the industrialized world (Harris and Bailey-Serres, 1994). Estimates in California enumerate O_3-induced yield losses as high as 25% in some O_3-sensitive crops (Harris and Bailey-Serres, 1994). O_3 has been implicated in the forest decline observed in Europe (Harris and Bailey-Serres, 1994; Langebartels et al., 1997; Skärby et al., 1998). O_3-exposure causes a variety of symptoms in plants. The damage-type depends on the concentration and duration of exposure where two classifications are generally recognized. Chronic O_3 is defined as long-term, i.e. for days to weeks, low-level exposure and acute O_3 as short, i.e. for hours, exposures to high-level peaks in O_3 concentration (Kangasjärvi et al., 1994).

Background O_3 concentrations have doubled in the last century (Campbell, 1996). However, O_3-levels generally remain below the threshold that causes visible damage in most locations. Low level, chronically elevated O_3 concentrations cause subliminal metabolic damage; resulting in, for example, reduced crop yields, reduced photosynthetic capacity, alterations in photoassimilate partitioning, and premature senescence (Pell et al., 1997).

The occurrence of short, periodic, high O_3 concentration peaks (episodic O_3), are well documented in industrialized countries and at ever increasing frequency. The damage caused by acute O_3 exposure is readily apparent. When O_3 concentrations even for a short time exceed the tolerance threshold of a given plant it results in the formation of visible damage and even cell death lesions. The physical appearance of this damage is somewhat variable and has been described as necrotic lesions, bifacial flecking, bronzing, and "waterlogging" (Harris and Bailey-Serres, 1994). Generally, O_3-induced cell death foci physically resemble HR lesions. It is this form of O_3 damage that is relevant to the present work.

O_3 requires entry into the apoplast through the stomata to exert its toxicity. The rate of stomatal flux is the first line of defense against O_3. Plants exposed to O_3 under high humidity, when stomata are more open, exhibit enhanced damage (McLaughlin and Taylor, 1981). Conversely, treatment with ABA, which induces stomatal closure, reduces the extent of O_3 damage (Adepipe and Ormrod, 1972). O_3 exposure can also result in stomatal closure (Robinson et al., 1998).

Once inside the leaf O_3 reacts with the cell wall, the plasma membrane, and apoplastic fluids, to produce ROS such as superoxide radicals ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\cdot}$; Hoigné and Bader, 1975; Mehlhorn et al., 1990). O_3 degeneration into these ROS takes place immediately as even when the concentration is high outside of the leaf O_3 is virtually undetectable in the apoplast (Laisk et al., 1989). Via an unknown mechanism, O_3-derived radicals induce the plant itself to produce ROS in an oxidative burst.

1.6 The oxidative burst

The oxidative burst induced either by pathogens or O_3 can occur in two phases where the kinetics of this response depends on the type of plant-pathogen interaction or the O_3-sensitivity of the plant, respectively. The first peak occurs quickly, rising and falling sharply, typically within the first two hours following the beginning of the challenge. Peak one is commonly seen in both sensitive and tolerant plants. During plant-pathogen interactions peak one is common to both incompatible and compatible interactions. The second, more prolonged peak typically begins 3-5 hours after the beginning of exposure but rises much more slowly. Phase two of the oxidative burst peaks at 12-24 hours and slowly recedes. This second phase, where ROS production continues far beyond the end of the O_3-exposure, is typical only of O_3-sensitive plants (Schraudner et al., 1998) or incompatible plant pathogen interactions.

The oxidative burst is common to a large number of other pathogenic stresses in plants. For example, flooding, drought, high light, ionizing radiation, freezing, heat shock, chilling, heavy metals, high salinity, wounding, air pollutants, and pathogen attack can all induce plant ROS production. In some cases ROS production may be incidental to the primary stress while in others ROS are actively produced as a stress signal. ROS-inducing stresses often induce tolerance to other stresses. This phenomenon has been termed "cross tolerance" as illustrates the broad nature of ROS-induced defenses (Bowler and Fluhr, 2000). In particular the O_3-induced oxidative burst closely resembles the pathogen response. The timing and duration of the pathogen-induced burst is the same as that induced by O_3 (Schraudner et al., 1998; Grant and Loake, 2000). The same differential induction of the phase two burst is also seen in this case. Only those plant pathogen interactions that lead to the fast induction of cell death and lesion formation (incompatible interactions) exhibit the second prolonged burst (Grant and Loake, 2000).

1.6.1 Reactive oxygen species produced and their sources

Direct measurement or visualization by staining of O_3-induced ROS production has been reported in birch (*Betula pendula* Roth; Pellinen et al., 1999, 2002), tobacco (Schraudner et al., 1998), and *Arabidopsis* (Rao and Davis, 1999). The ROS detected were the same two major species seen during the pathogen-induced oxidative burst: H_2O_2 and $O_2^{\bullet-}$. In plant pathogen interactions several different mechanisms have been suggested as the source of the oxidative burst. An NADPH oxidase homologous to that of activated mammalian phagocytes

and neutrophils ($gp91^{phox}$), has been considered the most likely source of $O_2^{\bullet-}$ -generation (Dwyer et al., 1996; Keller et al., 1998; Torres et al., 1998). Analysis of *Arabidopsis* mutants of the NADPH oxidase, *rbohD* and *rbohF* (*respiratory burst oxidase homolog*), has confirmed the role of these molecules as a source of $O_2^{\bullet-}$-production in the pathogen-induced oxidative burst (Torres et al., 2001). The neutrophil NADPH oxidase, $gp91^{phox}$, acts as a multi-protein complex. However, novel $gp91^{phox}$ homologs have been discovered in mammals, which like the plant homologs, contain Ca^{++}-binding sites and are able to produce $O_2^{\bullet-}$ without the other subunits of the complex (Lambeth et al., 2000; Lambeth, 2002). There are several potential H_2O_2-generating systems in plants. For example, the following proteins are known to be sources of H_2O_2; the action of diamine oxidase on for example polyamines, oxalate oxidases (germin-like proteins), peroxidases, and the NADPH oxidase in conjunction with either spontaneous or SOD-catalyzed dismutation of $O_2^{\bullet-}$ (Smith, 1985; Baker and Orlandi, 1995; Berna and Bernier, 1999; Bolwell, 1999; Lambeth, 2002; Langabartels et al., 2002).

In plant-pathogen interactions ROS derived from the oxidative burst are thought to directly kill microbes (Bent, 1996). ROS orchestrate a large battery of induced defense responses (Hammond-Kosack and Jones, 1996; Grant and Loake, 2000). These include processes such as cell wall strengthening where ROS drive oxidative cross-linking. Importantly, the HR (cell death), and a plethora of other defense related events are regulated by ROS acting as signal molecules.

1.6.2 The oxidative burst and cell death

It has been proposed that ROS produced during the oxidative burst act as the central regulator of the HR (Levine et al., 1994). A large body of evidence now supports this view. The manipulation of endogenous ROS alters the outcome of cell death signals. In rice cells treated with chitosan elicitor, the induction of cell death was attenuated by the addition of either catalase or a cocktail of antioxidants (Masuta et al., 1991). In soybean catalase inhibitor enhanced cell death triggered by an avirulent pathogen (Levine et al., 1994). Also, catalase-deficient tobacco engineered with anti-sense technology show the induction of cell death under conditions, such as high light, that result in cellular H_2O_2 production (Chamnongpol et al., 1996). Strikingly, pathogen-induced cell death in tobacco was blocked under low oxygen conditions, which suppress the formation of ROS (Mittler et al., 1996). Application of exogenous ROS induces cell death in several systems; for example, bean suspension cell cultures incubated with H_2O_2 (Levine et al., 1994).

Induction of cell death involves the controlled attenuation of ROS detoxification. In situations where ROS are actively involved in the induction of cell death there are constitutive and induced ROS detoxification systems, which could inhibit cell death. To overcome this plants have evolved mechanisms whereby ROS detoxification is specifically suppressed in cells destined to undergo cell death. For example, a key detoxification enzyme, cytosolic ascorbate peroxidase, has been shown to be downregulated postranscriptionally during virus induced cell death of tobacco (Mittler et al., 1998). Other examples of this, as are discussed below, can be found in the action of salicylic acid (SA), which inhibits several detoxification enzymes. Genetic evidence for the role of radicals in regulating cell death has come from the *Arabidopsis lsd1* mutant. Extracellular $O_2^{\bullet-}$ is necessary and sufficient to trigger uncontrolled spreading cell death in *lsd1* (Jabs et al., 1996). This finding indicates that PCD is negatively regulated by LSD1 at a point downstream of extracellular accumulation and also that LSD1 is likely involved in transmitting the signal from the apoplast to the nucleus (Jabs, 1999).

Significantly, LSD1 is required for the induction of Cu/Zn SOD activity by SA (Kliebenstein et al., 1999) confirming its role in the programmatic modulation of ROS signals.

The oxidative burst has been associated with cell death also during the O_3-response. In two examples, the O_3-sensitive Bel-W3 tobacco and the O_3-sensitive *Arabidopsis* accession Cvi-0, respectively, a biphasic O_3-induced oxidative burst and prolonged ROS accumulation result in the activation of cell death (Schraudner et al., 1998; Rao and Davis, 1999).

1.6.3 The oxidative burst and defense activation

Beyond its role in the induction of cell death H_2O_2 from the oxidative burst is believed to be a central coordinator of local and systemic defense signaling. H_2O_2 possesses several properties that enable it to fulfill this role, such as its relative stability, ability to diffuse, and ability to cross membranes. H_2O_2 can move out from the point of production forming a concentration gradient, causing differential concentration dependent effects. The triggering of cell death by H_2O_2 behaves as a threshold phenomenon; however, defense activation is a simple dose dependent response. In this manner cell death and defenses are coordinately induced (Levine et al., 1994). Catalase-deficient tobacco accumulated H_2O_2 under high light leading to the induction of the PR1 gene (Chamnongpol et al., 1996). Alvarez *et al.* (Alvarez et al., 1998) demonstrated a role for H_2O_2 in the establishment of local defense responses and also in the systemic acquired resistance (SAR: discussed at length below). In this study H_2O_2 was shown to be required for the expression of the defense marker genes, PAL, GST, and PR1 in local and systemic tissues of plants challenged with exogenous H_2O_2 or avirulent pathogens (Alvarez et al., 1998).

1.7 Induced defense responses

1.7.1 Pathogenesis Related Proteins

The pathogenesis related (PR)-proteins were first characterized due to their induction during plant-pathogen interactions. They are a very diverse set of proteins and are induced in response to a multitude of stresses. PR-proteins are classified by structural homology into 14 families (Van Loon and Van Strien, 1999). The biochemical activities of some PR-proteins are now known; for example ß-1, 3-glucanases, chitinases, and protease inhibitors, which suggests clear roles in pathogen defense (Kombrink and Somssich, 1997). The activities and roles of the other PR-proteins remain largely unknown (Van Loon and Van Strien, 1999).

Gene regulation studies with PR-proteins have revealed some of the key signal pathways in defense signaling. For example, PR1 is induced primarily by SA (Uknes et al., 1993), while basic glucanase and basic chitinase are regulated by ethylene (Huges and Dickerson, 1991). The plant defensin, PDF1.2, is co-regulated by ET and JA (Penninckx et al., 1998). This allows the use of PR genes as markers for their respective signal transduction pathways and has prompted the discovery of roles for JA and SA in O_3-responses (Langebartels et al., 2000).

1.7.2 Antioxidant response

Detoxification enzymes are responsible for the removal or containment of dangerous ROS. Examples of this are the enzymes of the Halliwell-Asada pathway (Noctor and Foyer, 1998). This pathway is comprised of SOD (superoxide dismutase), ascorbate peroxidase,

dehydroascorbate reductase, and glutathione reductase. This pathway results in the detoxification of $O_2^{\bullet-}$ or H_2O_2 using reducing power ultimately derived from NAD(P)H.

In addition to the ascorbate peroxidase mentioned in connection with the Halliwell-Asada pathway there is also a large collection of other peroxidases, which scavenge ROS and detoxify for example lipid peroxidation products (Baker and Orlandi, 1995). Glutathione-S-transferases (GST) are a very rapidly induced oxidative stress response and are involved in the detoxification and solubilization of toxic lipid oxidation by-products or xenobiotics for their removal from the cell (Edwards et al., 2000). The rapid induction of several classes of GST genes are commonly used as a molecular marker of oxidative stress and cell death. Another important ROS detoxification enzyme is catalase, which directly converts hydrogen peroxide into water (Chamnongpol et al., 1996; Willekens et al., 1997).

There are several normal processes in healthy plants that produce ROS such as at the mitochondrial and chloroplast electron transport chains, free radical catalysis chain reactions during lignification, β-oxidation in peroxisomes, photo-respiration, and signal transduction (Noctor and Foyer, 1998; Vranová et al., 2002). The controlled use and detoxification of ROS in normal plant metabolism is of fundamental importance to plants. Thus detoxification systems have evolved to respond to this need. Furthermore, oxidative stress plays a role in a variety of stress conditions where ROS production or containment has gone awry. These stresses include, for example, drought, photo-oxidative stress, UV-light exposure, heavy metal toxicity, and pathogen challenge (Noctor and Foyer, 1998; Vranová et al., 2002). Detoxification systems, likewise, are thought to play a role in scavenging ROS produced during these stresses. While some stresses seem to have specific responses it is clear that many of these responses are common to many stresses.

O_3 has been shown to induce either the genes or the activities of SOD, glutathione reductase, and ascorbate peroxidase (Kangasjärvi et al., 1994; Rao and Davis, 2001; Langebartels et al., 2002). Furthermore, experiments with transgenic-plants that either over- or under express these various antioxidant genes have demonstrated the roles played by these various antioxidants in O_3 responses (Chamnongpol et al., 1996; Örvar and Ellis, 1997; Willekens et al., 1997). In accordance with the role of ROS-induced cell death and immunity plants with engineered deficits in ROS detoxification exhibited a heightened HR to pathogens (Mittler et al., 1999a).

1.7.3 Non-enzymatic defense systems

1.7.3.1 **Glutathione and ascorbic acid**

In its reduced form glutathione (GSH) is a tripeptide composed of glutamine, cystine, and glycine. GSH is the major currency of reduced sulfur within the cell. During oxidative stress glutathione acts as a protectant on many levels. GSH contributes to the detoxification of H_2O_2 through its role in the Halliwell-Asada pathway. Glutathione peroxidase is another glutathione-linked enzymatic system functioning to clear H_2O_2. Additionally, GSH may act as a primary radical scavenger within the cell. Upon its oxidation GSH is reversibly dimerized via a disulfide bond to form oxidized glutathione GSSG. This disufide is then broken by glutathione reductase, which regenerates reduced glutathione with reducing power coming from NAD(P)H (Noctor and Foyer, 1998).

The GSH pool can act as a redox sensor and result in changes in gene expression. Although the exact mechanism of this is still not understood it is clear that the ratio of oxidized to reduced glutathione is important and the total glutathione pool size is of lesser

importance (Wingsle and Karpinski, 1996). Many of the common antioxidant genes discussed here, such as Cu/Zn SOD and glutathione peroxidase are regulated at some level by alterations in the ratio of reduced to oxidized glutathione (Wingsle and Karpinski, 1996).

Ascorbic acid (AA) is also an important low molecular weight antioxidant and has the ability to act as a primary scavenger of radicals and is a link in the Halliwell-Asada pathway (Noctor and Foyer, 1998). Additionally AA can act as a secondary antioxidant accepting electrons from α-tocopherol, thus contributing to antioxidant maintenance in the lipid environment of membranes. In contrast to some mammals, such as primates and guinea pigs, all plants are able to synthesize their own AA. In light of this fact, and the dietary importance of AA for humans, it is rather surprising that the biosynthetic pathway of AA in plants has been solved only recently (Wheeler et al., 1998; Conklin et al., 1999). The protective role of AA in the plant O_3 response is revealed in the enhanced O_3 sensitivity of some ascorbate-deficient mutants (Conklin et al., 1996, 2000; Smirnoff et al., 2001).

1.7.3.2 **The phenylpropanoids**

Phenolic compounds derived from the phenylpropanoid secondary metabolite pathway have a wide range of important roles within the plant cell; especially in plant stress and pathogen response (Hahlbrock and Scheel, 1989). The stilbenes are phenolic compounds with antimicrobial activity. Some flavonoids are colorless compounds with the ability to absorb light in the UV spectrum; thus they are used as an UV screen by the plant in order to remove dangerous wavelengths of UV light from the spectrum of incoming light. Many flavonoids are also known for their radical scavenging properties. Other phenolics are also radical scavengers such as hydroxycinnamate, especially when it is conjugated to polyamines. SA is also a very important derivative of the phenylpropanoid pathway. Lignin is an important molecule structurally for the plant. Lignin and lignin-like phenolic compounds are used by the plant as a defense response.

1.8 Signaling

1.8.1 Upstream of the oxidative burst

Some details of signaling processes upstream of the oxidative burst have been elucidated. The induction of rapid ion fluxes of H^+, K^+, Cl^-, and Ca^{++} are involved directly in the induction of the oxidative burst. ROS production and cell death can be triggered by the general ionophore amphotericin B, which mimics plasma membrane ion channel activation (Jabs et al., 1997). Ca^{++} is of particular importance in the activation of the NADPH oxidase in both mammalian and plant systems. The multi subunit NADPH oxidase of mammalian neutrophils is a well-characterized model for activation of the oxidative burst. In this system phosphorylation events, small GTPases (guanosine triphosphatases), and phospholipases have also been implicated upstream of ROS production. In plants a rac-family small GTPase has been implicated in ROS production and cell death (Ono et al., 2001). Similarly a GTPase activating protein that regulates the activity of rop (RHO-like small G-protein of plants) is required for ROS signaling in the anoxia response (Baxter-Burrell et al., 2002). A requirement for ion fluxes, in particular Ca^{++}-flux, and kinase activity has been demonstrated for ROS production, phytoalexin accumulation, and defense gene expression in parsley (Renelt et al.,

1993; Zimmermann et al., 1997). Accordingly, treatment with protein phosphatase inhibitors activates the oxidative burst and cell death (Chandra and Low, 1995).

1.8.2 Pathways downstream of resistance genes

The analysis of mutants isolated in screens for loss of disease resistance, so-called enhanced disease susceptibility screens, has identified several loci that encode proteins required for *R*-gene action. EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1), PAD4 (PHYTOALEXIN DEFICIENT 4), and NDR1 (NON-RACE-SPECIFIC DISEASE RESISTANCE 1) all function downstream of disease resistance gene products and are required for resistance. However, the requirement for the downstream signaling pathways defined by these loci differs among *R*-gene products (Aarts et al., 1998). NDR1 is required by several members of the LZ-NBD-LRR class of *R*-gene products, however, is not a requirement of other *R*-gene products. EDS1 and PAD4 are both required for the action of TIR-NBD-LRR class *R*-gene products but not LZ-bearing *R*-gene products. This suggests that there are separate signaling pathways that depend on the domain structure of the *R*-gene products (Aarts et al., 1998). The exception to this is that a number of *R*-gene products, some of which are LZ-containing, do not require NDR1, EDS1, or PAD4. This suggests that there is greater complexity in the system and possibly also additional distinct signaling pathways to be discovered (Glazebrook, 2001).

The gene products of these loci contribute to our understanding of disease resistance signaling. *NDR1* encodes a protein of unknown function that is glycosylated and anchored in the plasma membrane with a glycerol phosphositidyl inositol group (Century et al., 1995). Both *EDS1* and *PAD4* function upstream of SA accumulation and encode proteins bearing lipase-like domains suggesting that they act on lipid substrates. Significantly, *eds1* and *pad4* act as full suppressors and *ndr1* acts as a partial suppressor of *lsd1* spreading cell death. This suggests that they act in signal potentiation in cells peripheral to the infection site (Rustérucci et al., 2001).

1.8.3 Mitogen activated-protein kinases

MAPK (mitogen activated-protein kinase) cascades are central signal transduction links and integration points in plants, yeast and mammals (Zhang, S. and Klessig, 2001). MAPKs (MPKs) are associated in three member kinase modules that act as a signaling cascade. Each trio is composed of a MAPK (MPK), a MAPK kinase (MKK), and a MAPKK kinase (MEKK for MAPK/ERK kinase kinase). MAPK cascades are activated by a wide variety of signals including ABA, auxin, ethylene, ROS, and pathogens (Hirt, 1997; Zhang, S. and Klessig, 2001). CTR1, a member of the ethylene signal transduction pathway identified via mutant analysis, is a raf-like protein kinase (a MEKK) suggesting that ethylene signaling could be transmitted via a MAP kinase cascade (Kieber et al., 1993). Similarly, the *enhanced disease resistance1* (*eds1*) mutant gene also encodes a raf-like kinase (Frye et al., 2000). The first complete MAPK cascade (MEKK1, MKK4/MKK5, MPK3/MPK6) has been identified and functions in *Arabidopsis* innate immunity signaling (Asai et al., 2002). Homologous MAPKs have been shown to be activated by H_2O_2 in tobacco (WIPK, wound-inducible protein kinase and SIPK, salicylic acid-induced protein kinase) and *Arabidopsis* (MPK3 and MPK6) (Kovtun et al., 2000; Samuel et al., 2000). The same two MAPKs, WIPK and SIPK, are activated in O_3-exposed tobacco (Samuel et al., 2000). Furthermore, gain-of-function and loss-of-function experiments with these MAPKs demonstrate that their balanced and tightly regulated activation is indispensable for O_3-tolerance (Samuel and Ellis, 2002).

1.8.4 Nitric oxide

Concomitant with the oxidative burst there is a burst of NO production. NO participates in pathogen defense responses in the regulation of cell death and induction of defenses (Delledonne et al., 1998; Durner et al., 1998). Particularly NO interacts with H_2O_2 to induce cell death in plant cells (Delledonne et al., 1998, 2001). Interestingly, the ratio of NO to ROS is important for death induction. Equal amounts of each results in cell death, however, overproduction of one resulted in scavenging of the other and protection from death induction (Delledonne et al., 2001). Similar to known downstream pathways in mammals NO was shown to signal in plants via cyclic GMP, cyclic ADP ribose, and calcium (Durner and Klessig, 1999).

1.8.5 Hormone Signaling Pathways

1.8.5.1 Salicylic acid

Salicylic acid (SA) is a phenylpropanoid compound that is ubiquitously found in plants (Raskin, 1995). SA has a wide range of effects in plants (Raskin, 1995). Importantly, SA is involved in disease resistance. Beyond its role in the induction of PR proteins and in SAR, which will be discussed below, SA also has a direct role in cell death regulation during the hypersensitive response. As SA is a phenylpropanoid compound it is known that an early step in the biosynthesis is phenylalanine ammonium lyase (PAL). PAL diverts carbon skeletons from the shikimate pathway into secondary metabolism and is the first committed step of the phenylpropanoid pathway. PAL activity is induced upon pathogen challenge and is a commonly used marker of cell death and induced defenses. PAL gene upregulation is required to provide precursors for SA biosynthesis (Mauch-Mani and Slusarenko, 1996).

There is an intimate association between SA-accumulation and cell death. This is supported by a large collection of lesion mimic mutants that, in addition to forming spontaneous lesions, have constitutively elevated levels of SA. These include the *lsd* mutants (*lsd1-lsd7*) and *acd2* (*accelerated cell death2*; Durner et al., 1997). Also, despite the incomplete definition of SA biosynthesis pathway, there is clear evidence of cell death signals such as ROS ($O_2^{\cdot-}$ and /or H_2O_2) and NO being involved in the regulation of key steps in SA biosynthesis during pathogen infection (Draper, 1997; Durner et al., 1997). In parsley suspension cell cultures exogenous SA was shown to enhance both basal and induced ROS production (Kauss and Jeblick, 1995). Thus, SA and ROS have been proposed to be on a positive feedback loop that acts to amplify signals leading to defense and cell-death responses (Draper, 1997; Van Camp et al., 1998).

Identification of SA binding proteins has suggested how SA could lead to enhanced ROS production. Strikingly, two SA-binding proteins are also antioxidant proteins (Durner and Klessig, 1995, 1996). Both catalase and ascorbate peroxidase are inhibited by SA concentrations that can be attained locally in pathogen infected tissue (Durner et al., 1997). Inhibition of these ROS-detoxification proteins could result in enhanced H_2O_2 accumulation.

Arabidopsis genetic screens based on the induction of PR-proteins by SA have contributed to our understanding of SA signal-transduction. Three labs independently isolated alleles of the same gene; *nim1* (*non-inducible-immunity1*; Delaney et al., 1995), *npr1* (*non-inducer of PR proteins1*; Cao et al., 1994), and *sai1* (*salicylic acid insensitive1*; Shah et al., 1997) that confer SA-insensitivity. The gene product encoded by *NPR1/NIM1* has been

cloned and found to share homology with the ankyrin repeat-containing transcriptional regulator IκB (Cao et al., 1997; Ryals et al., 1997). IκB and its structural homologs are involved in an ancient conserved immunity and developmental signaling pathway (Wilson et al., 1997). The interaction of NPR1/NIM1 with the TGA family of bZIP transcription factors is required for the induction of PR1 by SA (Zhang, Y. et al., 1999; Zhou et al., 2000; Fan and Dong, 2002).

1.8.5.2 **Ethylene**

The simple gaseous olefin ethylene (ET) is a classical plant hormone with vital roles in many aspects of plant growth and development (Johnson and Ecker, 1998). Of particular interest is ET's role as a stress hormone. ET evolution is associated with wounding, pathogen attack, anaerobiosis, senescence, heavy metals, and oxidative stresses such as O_3 (Wang et al., 2002). An emerging theme in ET-research is its role as a regulator of PCD. ET is involved in the regulation of PCD in for example the senescence of leaves and flowers (Hadfield and Bennett, 1997), aerenchyma formation (Drew et al., 2000), and pathogen symptom development in susceptible plants (Lund et al., 1998).

Genetic screens in *Arabidopsis* have lead to the definition of a large portion of the ET signal transduction pathway (Johnson and Ecker, 1998). ET insensitive mutants such as *etr1* (*ethylene-resistant1*) and *ein2* (*ethylene-insensitive 2*) and constitutive ET-response mutants such as *eto1* (*ethylene overproducer1*) and *ctr1 (constitutive triple response1)* are powerful tools for dissecting ET-dependent stress responses (Wang et al., 2002).

As long ago as 1976 ET has been recognized as one of the earliest plant responses to O_3-exposure (Tingey et al., 1976). However, it has not been clear weather this ethylene was a part of the O_3-damage mechanism or just an after effect of O_3-wounded tissue. ET is synthesized via a clearly defined and tightly regulated pathway. ET-biosynthesis genes are present in large multi-gene families where different genes or sets of genes respond to various developmental and environmental cues that trigger ET production. The first committed step in ET biosynthesis is the conversion of S-adenosyl methionine (SAM) into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). ACC is then oxidized to ethylene by the action of ACC oxidase (ACO). Within the large *ACS* gene families only one or two genes are activated in response to O_3 (Langebartels et al., 2000). In *Arabidopsis*, *AtACS6* is the only *ACS* gene demonstrated to be upregulated in response to O_3 (Vahala et al., 1998). O_3-inducible ACS genes have also been isolated from other species such as in tomato *LeASC6, LeASC2* and *LeACS1b* (Tuomainen et al., 1997; Moeder et al., 2002), also in potato *StACS4* and *StACS5* (Schlagnhaufer et al., 1997). In potato these two separate *ACS* genes are sequentially induced in response to O_3 (Schlagnhaufer et al., 1997). ACS is widely believed to be the rate-limiting step of ET-biosynthesis as ET-emissions tightly mirror the levels of its immediate precursor, ACC (Kende, 1993). All of the tomato *ACO* genes were at least moderately affected and the *LeACO1* gene was highly induced by O_3-treatment (Tuomainen et al., *1997*). The pattern of O_3-induced *LeACO1* promoter activity correlated with the spatial distribution of subsequent ROS accumulation and cell death (Moeder et al., 2002) Importantly, in this study inhibition of ET biosynthesis or perception blocked ROS accumulation (Moeder et al., 2002).

1.8.5.3 **Jasmonates**

Jasmonic acid (JA) is a recently recognized plant signaling compound with roles in both stress and development (Turner et al., 2002) JA is induced by a wide variety of biotic and abiotic stresses, including O_3, however it is best known for its role in the wound response

(Berger, 2001). In addition to its roles in stress signal transduction JA is an important regulator of plant development. Interestingly, JA plays a particularly important role in flower and pollen development, which are know to require properly regulated PCD for their success. Accordingly, several JA signaling and biosynthesis mutants are male sterile (Feys et al., 1994; Stintzi and Browse, 2000; Berger, 2001; Ishiguro et al., 2001).

This cyclopentone compound is a derivative of the octadecanoid lipid pathway and is derived from linolenic acid. The *fad3/7/8* (*fatty acid deficient-7, -7, -8*) triple mutant lacks linolenic acid and is devoid of JA making it useful in studying JA-responses. Several other JA deficient mutants have been isolated resulting in nearly complete definition of the JA biosynthesis pathway (Berger, 2001; Weber, 2002). Importantly, JA is not the only biologically active molecule on the JA biosynthesis pathway. It is of particular interest that some of these intermediates have been show to be active in defense signaling (Stintzi and Browse, 2000; Kachroo et al., 2001). Also the volatile JA derivative, methyl-JA (MeJA), is an important diffusible molecule involved in both intra- and inter-plant signaling (Seo et al., 2001). A picture is now emerging where not only JA but many other related signal molecules, all derived from fatty acids, act together in signaling. This has led to the concept of the "oxylipin signature", where the full profile of lipid signals taken together determine the signaling outcome. (Berger, 2001; Blée, 2002; Weber, 2002).

The isolation and gene cloning of JA-insensitive mutants, *coi1* and *jar1*, has provided insight into JA-signaling. The *COI1* (*CORONATINE-INSENSITIVE1*) gene encodes an F-box containing E3-ligase implicating ubiquitination and protein degradation in JA-signaling (Xie et al., 1998). The *JAR1* (*JASMONATE-RESISTANT1*) gene product shows adenylation activity toward JA suggesting that conjugation is important for JA-preception (Staswick et al., 2002). Interestingly, *JAR1* defines a large multi gene family, several members of which have adenylation activities toward hormones including auxin and SA. A new allele of the *auxin resistant 1* (*axr1*) locus, which confers auxin-insensitivity, has been isolated as new JA-insensitive mutant (Tiryaki and Staswick, 2002). This further illustrates the common signaling link shared between auxin- and JA-signaling. Furthermore, given the role of regulated proteolysis in auxin-signaling this finding further strengthens the role of the ubiquitin/proteosome pathway in JA-signaling. The processes involved in stress perception upstream of JA accumulation remain largely undefined. However, in tobacco the activation of the MAP kinase WIPK (wound-inducible protein kinase) is required upstream of JA accumulation.

1.9 Resistance phenomena

1.9.1 Systemic Acquired Resistance

Signals emanating from dying cells are also involved in the induction of local and systemic defenses. Prominent among these responses is the so-called systemic acquired resistance (SAR; McDowell and Dangl, 2000). SAR, as implied by its name, results in the establishment of a defense posture in distal tissues that have not yet been pathogen-challenged. This broad-spectrum resistance protects the plant against a wide variety of unrelated pathogens. The mobile transducer of SAR signals to distal tissue remains undefined, however, two key players, salicylic acid and H_2O_2 (Alvarez et al., 1998), are known to be required for establishing SAR. Significantly, H_2O_2, a reactive oxygen species, and salicylic acid, which is a known regulator of ROS, are also both involved in cell death regulation. The death of small groups of cells and its associated micro-oxidative bursts in distal tissue are

necessary for establishing SAR (Alvarez et al., 1998). This exemplifies the tightly linked and intertwined nature of cell death, ROS, and immune response signaling in plants. The establishment of SAR is associated with a reprogramming of transcription where WRKY transcription factors have been assigned a critical role (Maleck et al., 2000). The requirement for SA in SAR was shown using transgenic plants expressing the *NahG* gene. *NahG* is derived from *Pseudomonas putida* and encodes a salicylate hydroxylase, which degrades SA. *NahG* tobacco has drastically reduced SA levels and compromised local and systemic disease resistance (Hammond-Kosack and Jones, 1996; Durner et al., 1997). O_3 results in the induction of SA accumulation as well as the induction of PR proteins. SA-signaling resulted in increased resistance to virulent bacterial pathogens in O_3-challenged *Arabidopsis* (Sharma et al., 1996). Also in tobacco O_3-exposure induced SA accumulation, PR proteins, and resistance to TMV (Yalpani et al., 1994).

1.9.2 Induced systemic resistance

In addition to the SA-dependant SAR there is a second type of systemic resistance termed ISR (induced systemic resistance). ISR is SA-independent however requires the action of ET- and JA-signaling for its activation. This type of resistance can be induced by root-associated bacteria but is more commonly a response to necrotrophic pathogen challenge (Pieterse and van Loon, 1999; Thomma et al., 2001).

1.10 Arabidopsis as a model system for the study of ozone induced cell death

Arabidopsis thaliana has recently become the most widely studied model system in plant biology. A large public effort has been made to promote, coordinate, and standardize the use of *Arabidopsis* as a model system (Meinke and Koornneef, 1997; Meinke et al., 1998). *Arabidopsis* was originally chosen because of its favorable basic characteristics as a model organism (Meinke et al., 1998). *Arabidopsis* is quick to reproduce moving from one generation to the next in 6-10 weeks. Its small size facilitates growing the large population sizes required for mutant screens and other large scale experiments within the confines of facilities typically available at most institutions. The genome consists of only five chromosomes with little repetitive DNA and is believed to be among the smallest size (ca. 0.2 pg; 0,6 x 10^{6} bp) within the angiosperms. Perhaps the greatest advantage of working with *Arabidopsis* is the genome project, which has resulted in the availability of the entire *Arabidopsis* genome sequence in the year 2000 (The Arabidopsis Genome Initiative, 2000; http://www.arabidopsis.org/info/ aboutarabidopsis.html).

The processes responsible for the propagation and execution of the O_3-induced defense and cell death signals are complex, as are the factors contributing to O_3-tolerance or -sensitivity (Wellburn and Wellburn, 1996). Although work with traditional plant model systems has yielded much information about O_3-induced phenomena, well-defined molecular mechanisms, especially at the genetic level, have remained elusive. *Arabidopsis thaliana* has been shown to be a system amenable to the study of O_3-induced defense gene activation (Sharma and Davis, 1994; Conklin and Last, 1995). The simplicity of *Arabidopsis* genetics, easy transformation and the rich resources of existing *Arabidopsis* mutants and cloned genes, invite the possibility of dissecting these complex pathways. The exploitation of these resources and technologies will result in a great increase in our knowledge of the molecular basis of O_3-action and PCD.

2 Aims of the Present Study

The aim of the study was to define ROS and hormone signaling pathways that function in the regulation of hypersensitive-response (HR)-like programmed cell death (PCD). Special emphasis was given to the unraveling of the complicated web of interactions between these pathways, which form a complex and integrated signaling network that regulates PCD. Ozone (O_3) is a convenient tool for delivering ROS to plants and is believed to induce HR-like PCD (Rao et al., 2000). A genetic approach was utilized to address the role of ROS in cell death signaling with the aim to establish and validate the use of O_3-sensitive mutants of *Arabidopsis* as a model system for the study of plant programmed cell death. A parmacological approach was utilized with the aim to establish that an active cell death program is functional during O_3-induced cell death and simultaneously to further define the signaling pathways involved in this process. Utilizing *Arabidopsis* mutants, both our own O_3-sensitive mutants and known hormone signaling mutants, the role of plant hormones and their interactions in the regulation of cell death were targeted for dissection.

Specific goals:

1: The general characterization of a collection of O_3-sensitive mutants of *Arabidopsis*

2: Define the role of PCD and common signaling pathways in O_3- and $O_2^{\bullet-}$-induced cell death of the *rcd1* mutant.

3: Use the *rcd1* mutant system to explore the role of ROS and phytohormones in the regulation of cell death.

4: Validate and widen the role of ROS to other species and field conditions.

5: Genetic dissection of the interactions between the ethylene, salicylic acid and jasmonate signaling pathways and definition of how these hormone interactions function in the regulation of ROS-induced cell death

3 Materials and Methods

3.1 Plant material

Wild type seeds of the Columbia (Col-0) accession were used as the standard background in all *Arabidopsis* studies (I, II, IV). In (III) the following *Arabidopsis* accessions were used; Col-0, Cape Verdi Islands (Cvi-0), Jelinka (JI-1), Lanark (Lan-0), Loch Ness (Lc-0), Lansberg *erecta* (Ler-1), Nossen (No-0), Nieps (Np-0), Slapy (Sap-0), Shokhdarda (Sha), and Wassilewskija (Ws-2). Various signaling mutant and wild type *Arabidopsis* accessions were obtained from the ABRC (*Arabidopsis* biological resource center, Columbus, OH, USA) or the NCSC (Nottingham *Arabidopsis* stock centre, Nottingham, UK) (I-IV). Additional plants used in study (III) were tobacco (*Nicotiana tabaccum* L.) of the BelW3 and Bel-B cultivars; tomato (*Lycopersicon esculentum* Mill.) cultivars, Ailsa Craig, Piedmont, DKR 2003, Trust, Solairo, Money Maker, Roma, and Nikita; *Malva sylvestris* L.; and two *Rumex* species (*R. crispus* and *R. obtusifolius*). Seed sources for species other that *Arabidopsis* are described in (III). O_3-sensitive mutants were isolated as described (II), (Langebartels et al., 2000). The official names of these O_3-sensitive mutants as well as the original line numbers are detailed in table 2. It should be emphasized at this point that early names (*ozone-sensitive -ozs*) are not official mutant names but internal designations only that were assigned prior to publication of the *Arabidopsis* community rules for naming mutants (Meinke and Koornneef, 1997). The internal designations and the official mutant names are: *ozs2* is *rcd1*, *ozs4* is *rcd2*, *ozs3* is *rcd3* and *ozs1* is line 2-40. These names are not to be used and are included here only for reference to early work from the lab. Their identity should not be confused with the other O_3-sensitive mutants, which have been isolated (Conklin et al., 1996, 2000) and given the official name *soz* (*sensitive to ozone*).

3.2 Growth conditions

Arabidopsis growth protocols are all in accordance with standard procedures (Koncz et al., 1992). Plants were grown under 12 hr photoperiod (250 to 350 µmoles $m^{-2}h^{-1}$ irradiance), 22° C / 18° C day/night temperature (70% / 90% relative humidity), in controlled environment growth chambers (Weiss model Bio 1300). For growth room experiments plants were grown in a temperature controlled room with no humidity control and the same temperature and light regime. Plants for O_3-exposures were grown in ambient air. The sowing and potting medium used was a 1:1 mixture of vermiculite and medium peat (Type B2, Kekkilä, Espoo, Finland). Seeds were sown at high density then one-week-old seedlings were transplanted to 9 x 9cm square pots at 5 plants per pot. Plants were either surface watered lightly every day or sub-irrigated every third day with tap water. Growth of other species is described in (III).

3.3 Standard ozone exposure

Unless otherwise indicated, three-week-old *Arabidopsis* plants were exposed to a single pulse of 300 nl/l O_3 of 6 to 8 hours duration beginning at 9:00 am. O_3 was generated

and delivered as described (I-IV). Inhibitor pre-treatments (I) or inhibitor and hormone post-treatments (II) were as described.

3.4 Quantification of cell death

Cell death was quantified as an increase in ion leakage (I, II, IV) or by visual estimation of damaged leaf area (III). Ion leakage was measured as conductivity after incubation of plant material in double distilled water for 1 hour. Total ion content was read after killing the samples by boiling. Ion leakage is expressed as % of total ions.

3.5 Histochemistry

Hydrogen peroxide was stained with 3,3'-diaminobenzidine (DAB) or hexaflourofluorescein and $O_2^{\bullet-}$ with nitroblue tetrazolium (NBT), as described in (II, III). Leaves were cleared for viewing ROS-staining or the accumulation of autofluorescent phenolic compounds by incubating in alcoholic lactophenol (100ºC) or 95% ethanol (40ºC) as in (I-III). TUNEL staining and DAPI counter-staining are described in (I). Transmission electron microscopy was performed as described in (I).

3.6 Reactive oxygen species and pathogen treatments

Excised leaves were treated with $O_2^{\bullet-}$ in vitro by vacuum infiltration of buffer containing xanthine and xanthine oxidase as described in (I, II, IV). H_2O_2 was delivered by direct dilution into a large volume of buffer (50 ml per 10 leaves) prior to infiltration (II). Inhibitors, hormones, antioxidant enzymes, or other reagents were included as indicated (I, II, IV). In (II) *Arabidopsis* rosettes were infiltrated as described with various densities of *Pseudomonas syringae pv. tomato* DC3000 bearing the *avrB* gene on a plasmid. After all the above treatments cell death was quantified as ion leakage.

3.7 Gene expression and hormone determinations

Northern blots and cDNA macroarray with clustering analysis are described in (II, IV). The concentrations of ACC, SA, JA, and the evolution of ET were monitored as described in (II, IV).

3.8 Physiological studies

Inhibition of root growth by JA was determined by growth of seedlings in sterile cultures as described (II). ET action was measured in a triple response assay by determining hypocotyl lengths of three day old etiolated seedlings that were grown in the presence of various concentrations of hormones (IV).

4 Results

4.1 Mutants

4.1.1 Isolation

The gaseous pollutant O_3 has become established as a simple and effective way of applying reactive oxygen species (ROS) in order to study ROS signaling in plants. A genetic approach has been utilized with the aim of dissecting processes involved in the control of ROS induced cell death and defense signaling. As described in tables 2 and 3 *Arabidopsis* mutants that are sensitive to O_3- and exogenous ROS-treatments were used (I-IV). An important part of this approach was the isolation and characterization of our own unique collection of O_3-hypersensitive mutants (I and Table 2).

Table 2: O_3 sensitive mutants of *Arabidopsis thaliana* isolated and utilized in this work.

Mutant	Aliases	Phenotype	Gene	Reference
rcd1 (radical-induced cell death1)	Lines 2-9, 2-20	Ozone sensitive	At1g32230	I, II, IV
rcd2 (*radical-induced cell death2)*	Line 2-3	Ozone sensitive	At2g37860	I, Kollist et al, unpublished
rcd3 (*radical-induced cell death3)*	Line 2-11	Ozone sensitive	unknown	I
line 2-40[*]		Ozone sensitive	unknown	I

[*] Line 2-40 was not given an *rcd* mutant name because of the complicated genetics of its phenotype (see section 4.2.2 below).

Table 3: O_3 sensitive mutants of *Arabidopsis thaliana* from other laboratories that were utilized in this work.

Mutant	Primary Phenotype	Gene(s)	Reference
jar1 (jasmonate resistant1)	Reduced jasmonate sensitivity	At2g46370	I, II, IV, Staswick et al, 2002
coi1 (*coronotine insensitive1*)	Insensitive to jasmonate and the bacterial toxin coronotine	At2g39940	Xie et al, 1998
fad3/7/8 triple mutant (*fatty acid desaturase-3, -7, -8*)	Jasmonate deficient	At2g29980, At3g11170, At5g05580	McConn and Browse, 1996
eto1 (*ethylene overproducer1*)	Ethylene overproduction	unknown	Woeste et al, 1999
ctr1 (*constitutive triple response1*)	Constitutive ethylene signal	At5g03730	Kieber et al, 1993

4.1.2 Timing and pattern of ozone damage formation

The basic timing and sequence of damage formation is similar for *rcd-1, -2, -3* and *line 2-40*. Typically, dark water-soaked lesions appeared at 12 hours, which turn brown and collapse, progressing to a dry lesion with distinct borders by 24 hours. The pattern of affected

tissue differed between mutants (Figure 1). The mutants *rcd2* and 2-40 had randomly distributed lesions found only within the intervascular tissue. Lesions in *rcd1* typically initiate along the leaf margins mid way up the leaf (II). In addition to increased levels of initial cell death, *rcd1* lesions also continue to spread until sometime between 12 and 24 hours after the beginning of the radical treatment (II). In *rcd3* damage was concentrated along the vascular bundles. Frequently, and especially at higher O_3-concentrations, these mutants can exhibit a second mode of damage formation where large areas of confluent lesions collapse entire tissues. In this case visible damage was apparent as a region of turgor loss and tissue collapse as early as 3 hours after the beginning of O_3-exposure.

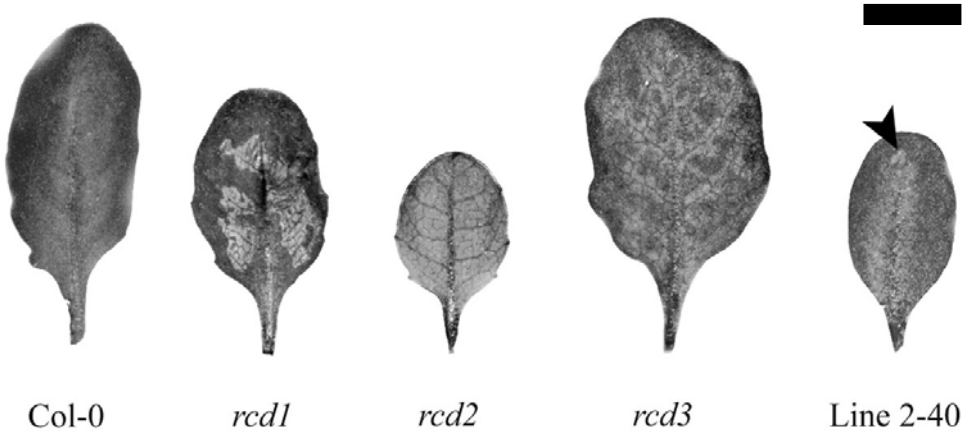

Figure 1: Ozone-exposed *rcd*-class mutants

Ozone-exposed ozone-sensitive Arabidopsis of the *rcd*-mutant class. Plants were 3 weeks old at the time of exposure to 250 nl/l ozone for 6 hours. Photos were taken of representative individual excised leaves at 24 hours after the beginning of the exposure. Collapsed tissue on line 2-40 is indicated with a black arrowhead. Scale bar equals 1 cm. H. Tuominen and K. Overmyer, unpublished results

4.1.3 Additional characteristics of *radical-induced cell death 1*

4.1.3.1 Clean air phenotypes of *radical-induced cell death 1*

Mutant *rcd1* has a more erect rosette architecture and smaller overall rosette size. Its petioles are shorter than seen in the Col-0 wild type. Other leaf characteristics include a change in shape, more oval than oblong in shape, a greater degree of serration on the margin, and a waved relief, compared to the flat to slightly downwardly curled plane of the Col-0 wild-type leaf. Upon flowering further differences are revealed in *rcd1*. The *rcd1* shoot is more highly branched, thinner, and less erect than the Col-0 wild type. More secondary shoots and a generally shorter primary shoot suggest reduced apical dominance. Normal fertility and good seed set is seen in *rcd1* as well as all the other *rcd* mutants presented here. Premature senescence and early flowering are also characteristic of *rcd1*. Interestingly, early flowering, premature senescence, and loss of apical dominance are characteristic of auxin-related mutants.

4.1.3.2 Signaling deficiencies in *radical-induced cell death 1*

Macroarray analysis of gene expression in *rcd1* was performed to identify signaling pathways involved in determining plant O_3-sensitivity. Unexpectedly, the transcript profiles of abscisic acid (ABA) related genes showed deficiencies in *rcd1* (IV). Subsequent analysis has revealed that *rcd1* is roughly as insensitive to ABA as the *abi1* (*ABA insensitive 1*) and *abi2 Arabidopsis* mutants but only when measured as loss of ABA-induced gene expression (P. Pulkkinen, unpublished results). ABA has previously been linked to oxidative stress but never to ozone responses or cell death regulation. This result suggests a new role for ABA signaling in the regulation of ROS induced cell death. Recent results have implicated ROS in ABA signaling processes (Neill et al., 2002). Specifically H_2O_2, produced by a NADPH oxidase, functions downstream from ABA and upstream of Ca^{++}-flux in the regulation of ABA-induced gene expression and stomatal closure. Additionally, *rcd1* showed deficiencies in jasmonate signaling (II, IV), as will be discussed at length below. These findings taken together with the auxin-related changes observed in *rcd1* suggest RCD1 is either a commonly recruited signaling link or involved in the interaction and balance of hormone signals, or both.

4.1.4 Genetics

The mode of inheritance was determined by backcrossing *rcd-1, -2, -3,* and *line 2-40* to the parental Col-0 wild type. Only the codominant *rcd1* mutation exhibited a phenotype, intermediate O_3-sensitivity, in the F_1 generation. Consistent with their recessive modes of inheritance the remaining mutants behaved like wild type plants as heterozygotes in the F_1 generation. Segregation ratios in the F_2 generation and models of heritability are presented in table 4A. To determine that these mutants all represent independent loci genetic complementation tests were performed (II). The vast majority of the F_1 progeny from all of the possible crosses between the *rcd* mutant series were not O_3 sensitive (Table 4B). The only consistent exception was seen in crosses with *rcd1,* which as a codominant trait is expected to yield F_1 progeny that are of intermediate O_3-sensitivity. These results suggest that each of these mutants represent a genetically distinct locus. Further confirmation of this was obtained by the localization of each of these loci to a distinct chromosomal region by genetic mapping (II and table 4C). Mutant line 2-40 was not included in the genetic mapping studies because of its complex genetics. However, the fact that two recessive loci are required for line 2-40's O_3-sensitivity and all the other mutants are monogenically determined would suggest that all of these loci are distinct.

Table 4: The genetics of the *rcd*-class mutants

(A) Inheritance of the ozone-sensitive phenotypes showing the segregation of ozone sensitive phenotypes in the F_2 generation of a backcross between the respective mutants and their wild type Col-0 parental background (B) ozone-sensitive mutants allelism (genetic complementation) tests. Reported is the (number of ozone sensitive individuals)/ (the total number of individuals tested). Plant tested were the F_1 progeny of crosses between the ozone sensitive mutants as indicated. (C) Rough chromosomal mapping positions of mutant loci. Distances given in cM (centi Morgans) were calculated from the % recombination between the marker and the mutant loci using the Kosambi function (Koornneef and Stam, 1992).

A

Mutant	F_2Segregation	ratio / model	X^2	p=	Reference
line 2-40	8/33/69/194 *	1:3:7:21 two recessive	1.19	0.754	K. Overmyer and H. Tuominen, unpublished
rcd1	36/ 66 / 46 [H]	1:2:1 single codominant	3.08	0.214	(II)
rcd3	72 / 218 [I]	1:3 single recessive	0.004	0.946	K. Overmyer and H. Tuominen, unpublished
rcd2	61 / 209 [I]	1:3 single recessive	0.763	0.382	K. Overmyer and H. Tuominen, unpublished

*mutant *line 2-40* showed four different phenotype classes in the F_2 population and is reported as sensitives / intermediate sensitive type 1 / intermediate sensitive type 2 / tolerant

[H] mutant *rcd1* showed three different phenotype classes in the F_2 population and is reported as sensitive / intermediate / tolerant.

[I] Mutants *rcd2* and *rcd3* showed two phenotype classes in the F_2 and are reported as sensitive / tolerant

B

	rcd1	*rcd3*	*rcd2*
2-40	0/15	4/69	1/38
rcd1		7/26[I]	3/18[I]
rcd3			0/8

[I] O_3 damage reported here was typical of the *rcd1* heterozygote and reflects the codominant nature of this mutant rather than a lack of genetic complementation.

C

Mutant	Marker (and position)	Distance to mutation	Reference
rcd1	AT51956 in BAC F23M19 (Ch. I at 55.00 cM)	2.0 cM ± 2.2	(II)
rcd2	M429 (Ch. II at 73.19 cM)	12.5 ± 4.2	K. Overmyer and S. Lång, unpublished
rcd3	AthZFPG (Ch. I at 35.25 cM)	12.8 cM ± 1.0	unpublished

4.2 Ozone induces hypersensitive response-like cell death

Plants exposed to O_3 exhibited characteristics associated with HR. These markers include an oxidative burst (II, III), deposition of autofluorescent phenolic compounds (II), PR-protein expression (II, IV), and both micro (II) and macro scale (I-IV) cell death and their associated local and systemic induced pathogen resistance (Sharma et al., 1996). Thus it is commonly held that O_3-induced cell death is the result of deleterious firing of the HR program by O_3-derived ROS. As has often been noted, this implies that O_3-induced cell death, like the HR, is programmed. This idea has been widely accepted in the review literature (Berger, 2002; Beers and McDowell, 2001; Rao and Davis, 2001). The argument for O_3-induced PCD is quite strong and well documented. However all of the supporting evidence has been circumstantial, thus O_3-induced PCD has not been previously shown directly.

Cell death morphology was explored in *rcd1* (I) and demonstrated that O_3-exposed *rcd1* exhibits hallmark characteristics of PCD including nuclear shrinkage and fragmentation, cytoplasmic vesiculation, chromatin condensation, and nuclear DNA fragmentation.

These morphological studies and others (Koch et al., 2000; Rao and Davis, 2001) further support the existence of O_3-induced PCD. However, there are a number of shortcomings to this comparative morphological approach. Given the wide variety of morphological phenotypes that can be observed during PCD the presence or absence of a given character or set of characters may not be enough to differentiate between PCD and necrosis. Some of the hallmark characteristics of PCD are also observed in necrotic cell death, for example nuclear shrinkage and chromatin chondensation (Wyllie et al., 1980). Also *vice versa*, organellar degeneration, loss of membrane integrity, and swelling, which are typical of necrosis can also been seen in programmed cell deaths especially at late time points (Wyllie et al., 1980). Furthermore, there are also problems with the specificity of the TUNEL method, which was used above to detect nuclear DNA fragmentation. It is well documented that this method should never be used as stand alone evidence of PCD (Collins et al., 1992; Bortner et al., 1995; Dangl et al., 1996). With these concerns in mind a more stringent method of defining the mode of O_3-induced cell death that does not rely on comparisons to apoptotic morphology or the HR was sought after.

PCD is the organized and genetically regulated disassembly of the cell. For its execution this process requires active cellular functions such as energy production, signal transduction, ion fluxes, transcription and translation. This model makes a testable prediction. If O_3 induces PCD then the pharmacological inhibition of active cellular processes should alter the outcome of radical challenge in *rcd1*. A similar approach has been taken to demonstrate active cell death induced by pathogens (He et al., 1993; He et al., 1994), calcium, and H_2O_2 (Levine et al., 1996).

In *rcd1 Arabidopsis* cell death induced by O_3- or exogenous $O_2^{\bullet-}$-application can be reduced or delayed with inhibitors of active metabolism. Utilizing a pharmacological approach a requirement for Ca^{++} influx, transcription , ATPases, Y- kinases, S/T-kinases and translation has been shown (I). Additionally, in tomato both cell death and the oxidative burst induced by O_3 could be reduced by blocking kinase activity (III). The inhibitor studies in (I) were performed on both O_3-treated plants *in planta* and $O_2^{\bullet-}$ (XXO)-treated excised leaves *in vitro*. Use of this *in vitro* system is advantageous in that, unlike O_3-treatment, radical delivery by XXO is not dependent on open stomata. This allows the exclusion of a diminished radical treatment, which could have potentially been caused by unmonitored stomatal closure effects of the inhibitor treatments. This indicates that factors other than stomatal flux have a more significant role in determining O_3 (ROS)-sensitivity in the mutants studied.

Inhibition of serine-proteases and caspases resulted in attenuated cell death (I). Caspases are at the heart of mammalian cell death regulatory cascades. The implication of a caspase-like activity in $O_2^{\bullet-}$ -induced cell death constitutes further evidence of PCD activation in *rcd1* and suggests that RCD1 acts upstream of this proteolytic step in regulating cell death. The enzymatic source of plant caspase-like proteases is currently unknown and the *Arabidopsis* genome reveals no classical caspases. However, a highly conserved family of caspase-like proteins, termed the meta-caspases has been identified (Uren et al., 2000) in *Arabidopsis*. The assignment of caspase activity has not yet been reported for the meta-caspases. However the meta-caspases remain candidates for the caspase-like activity required in this study (I) and elsewhere [reviewed in (Lam and del Pozo, 2000)].

Taken together the above mentioned pharmacological results illustrate directly a role for active cellular participation in O_3-induced cell death and fulfills a major requirement in the demonstration of O_3-induced PCD.

4.2.1 Defining ozone induced cell death

Programmed cell death by definition requires active participation of the cell in its own death and that the decision to die is cell autonomous i.e. triggered from inside the cell. Cell death in radical challenged *rcd1* clearly is cued by an external stimulus, specifically during radical challenge the stimulus is from XXO- or O_3-derived radicals. However, the regulation of O_3-induced spreading cell death in *rcd1* suggests cell autonomous regulation of cell death. Spreading cell death in *rcd1* continues long after the death-initiating stimulus has been removed (II). This phase of damage formation in *rcd1* is transient as lesion growth comes to a halt usually sometime between 12 and 24 hours post radical challenge (II). As is discussed at length below, the spread of cell death is $O_2^{\bullet-}$-dependent and the pattern of $O_2^{\bullet-}$-accumulation suggests that spreading cell death moves as a wave over the leaf actively being passed from one row of cells to the next (II). The fact that the plant can bring this process back under control and halt cell death demonstrates within a single tissue a capability to respond differentially to a cell death cue coming from a neighboring dying cell. Thus, this is a regulated process rather than a runaway process, which implies cell autonomy.

Taken together all of the above mentioned morphological and pharmacological results illustrate directly a role for PCD in O_3-induced cell death. Given that O_3-induced cell death requires active cellular participation and the decision to die is cell autonomous, it is a case of PCD. These findings validate the use of *rcd1* and in general O_3-exposed plants as experimental model systems for the study of PCD, ROS, as well as their interaction and regulation.

Although O_3 can induce PCD it should not be implied that all O_3-induced cell death is PCD. It has been suggested that both death by rampant oxidation and PCD may occur depending on the magnitude of O_3-induced oxidative stress (Pell et al., 1997). Ultrastructural characterization of plants exposed to high concentrations of O_3 has revealed morphology consistent with necrotic cell death (Pell and Weissberger, 1976; Holopainen et al., 1992). Evidence has been presented showing both O_3-induced necrotic- and HR-like cell death where the mechanism of cell death was dependent on genotype (Rao and Davis, 1999; Koch et al., 2000). Furthermore, the possibility that mosaics of apoptotic and necrotic cells occur in the same O_3-exposed tissue cannot be excluded either. Mixtures of cells bearing signs of different modes of death within the same tissue have been described in the study of cell death in mammals (Levin et al., 1999). Another intriguing variation on this is that signals emanating from a few cells that have undergone necrotic cell death by rampant oxidation may trigger surrounding cells to die by PCD resulting in large areas of affected tissue and lesion

formation. This is analogous to the penumbral or secondary cell death seen at the periphery of acute hypoxic or traumatic lesions, which has been described extensively in mammals (Jacobson et al., 1997).

4.2.2 Implications of ozone-sensitive mutants

The isolation of O_3-sensitive mutants of *Arabidopsis* (II) provides direct proof that plant O_3-sensitivity is under genetic control. The existence of genetic programs that control the activation of the HR is revealed in the form of lesion mimic mutants. Some lesion mimic mutants in *Arabidopsis* are compromised in their ability to control the HR and form lesions in the absence of pathogen attack (Dangl et al., 1996). This class of mutants has also been described extensively in maize (Johal et al., 1995). The isolation of mutants that trigger cell death upon challenge with normally sub-lethal doses of ROS, such as the *rcd* mutants (II, Table 2), especially when supported by evidence of PCD involvement, represent a new class of lesion mimic mutants that misregulate ROS signals leading to cell death. An understanding of the function of their mutant protein products will aid in the unraveling of plant PCD control and the determinants of plant O_3-sensitivity. Finally, as these mutants are O_3-sensitive their very existence points to a genetic program controlling O_3-induced processes and may be taken as genetic evidence of O_3-induced PCD.

Not all O_3- or ROS-sensitive mutants will necessarily be effected in cell death regulatory machinery as deficits in radical detoxification or other defense pathways will also lead to ROS- or O_3-sensitivity. This is illustrated by the ascorbate deficient *vitamin c1* (*vtc1*) mutant, which was originally named *sensitive to ozone1* (*soz1*) and isolated based in its O_3-sensitivity (Conklin et al., 1996; Conklin et al., 1999). Interestingly, in a null allele of *VTC1,* isolated as an embryo lethal mutant under the name *cytokinesis1* (*cyt1*), embryo abortion involves a massive deposition of callous material (Smirnoff et al., 2001). As the mode of O_3-induced cell death in *vtc1* has not been explored it cannot be excluded that this mutant may also yield insight into the interaction of ROS and PCD. Thus, radical sensitive mutants, like *rcd1* and perhaps *vtc1*, provide further genetic evidence for the role of ROS in PCD regulation.

Characterization of the antioxidant response of *rcd1* at the gene expression (II, IV) and enzyme activity level (II) revealed no gross deficiencies. This suggests that *rcd1* is deficient in cell death control rather than radical containment, *per se*. Several lines of research support the notion that regulated alterations in radical containment systems are intimately associated with cell death control. The control of radical detoxification and the control of PCD, it seems, are not necessarily mutually exclusive. $O_2^{\bullet-}$ is involved in the misregulated cell death of the *lsd1* lesion mimic mutant (Jabs et al., 1996) and the down regulation of ascorbate peroxidase during viral induced PCD in tobacco (Mittler et al., 1999b) provide a few examples of programmatic upregulation of radical accumulation. The high level of $O_2^{\bullet-}$-accumulation in O_3-exposed *rcd1* (II) implies that ROS detoxification systems are either programmatically down regulated or low to begin with. Although this remains unclear as neither ROS-production nor ROS-detoxification were directly measured. Inhibition patterns suggest that $O_2^{\bullet-}$-accumulation is extracellular making the apoplast the important compartment for this response (II). Accordingly, treatment of *rcd1* with other agents that cause oxidative stress in the cytoplasm and other intracellular compartments, such as photo-inhibitory conditions, UV-B radiation, or paraquat did not cause a differential response as compared to wild type plants (II). In fact, *rcd1* was even more tolerant than the Col-0 wild type plants to the herbicide paraquat, which causes toxicity via the production of $O_2^{\bullet-}$ in the chloroplasts. This indicates that the ROS sensitivity of *rcd1* is specific to the apoplast and underscores the importance of

compartmentalization in ROS phenomena. Furthermore, this widens the scope of previous findings which have shown alterations in specific subcellular compartments, such as peroxisomes, chloroplasts, cytosol, apoplast, and mitochondria, being involved in oxidative stress (Jabs et al., 1996; Willekens et al., 1997; Noctor and Foyer, 1998; Vanacker et al., 1998; Karpinski et al., 1999; Jones, 2000; Noctor et al., 2002).

A recurrent theme in this work has been the similarities between O_3-response and the pathogen-response. However, there are also clear differences between O_3- and pathogen-responses. O_3-exposure results in the activation of pathogen independent defense pathways. For example, in parsley UV-B exposure results in the biosynthesis of flavone glycosides but not phytoalexins, while fungal elicitor treatment results in the accumulation of furanocoumarin phyoalexins but not flavones. Although these two pathways are not normally cross-activated O_3-treatment results in the activation of both (Sandermann, 1996). This indicates that O_3 is able to simultaneously activate multiple stress pathways. While O_3-induced processes clearly represents a model for the activation of pathogen cell death and defense responses careful dissection of O_3 induced pathways will be necessary in order to delineate pathogen-like responses from other mitigating pathways activated in parallel.

4.2.3 Ozone induced cell death: How many pathways lead to cell death?

One interesting question raised by this work is that of the number of distinct mechanisms or pathways that lead to O_3-induced cell death. Both within and between species there are a wide variety of symptoms associated with O_3-damage. To what extent do the terms used to describe O_3-damage represent subjective terminology describing the same process? Conversely, how many cases are these manifestations of distinct processes?

O_3-exposed tomato and tobacco typically form damage in the perivascular tissue at the mid-vein and in the vicinity of secondary or lower order vasculature, respectively (III). Similar damage patterns have also been seen in these species in previous studies (Tuomainen et al., 1997; Schraudner et al., 1998). O_3-sensitive birch typically has collapsed intervascular tissue where the lesion boundaries seem to be limited by the vasculature (Tuomainen et al., 1996). O_3-sensitive hybrid poplar exhibits a damage pattern where the whole leaf appears to be “burned” and dead (Koch et al., 1998). Delineation of these processes would be of great interest.

The O_3-sensitive mutants isolated here (II; Table 1) all exhibited different characteristic patterns of damage formation. This implies that damage formation is mechanistically distinct in these mutants. This assertion may be tested using genetic analysis of double mutants. The creation of double mutant lines with these O_3-sensitive mutants will result in the ordering of these genes or processes along a defined genetic pathway. In this manner the epistatic relationships of various processes contributing to O_3-sensitivity can be determined. Alternatively this analysis may reveal multiple independent pathways leading to cell death.

Interestingly, there are parallels between the damage patterns seen in these mutants and those observed in other species. For example the perivascular damage in mutant *rcd3* is reminiscent of damage on O_3-sensitive tomato cultivars. Cloning of the mutant gene that confers O_3-sensitivity in mutant *rcd3* will allow targeted studies in tomato. Ultimately this line of investigation has the potential to determine if the similarity between mutant *rcd3 Arabidopsis* and O_3-sensitive tomato cultivars is just superficial or truly reflects a common underlying mechanism. Similarly, different lesion mimics resemble different types of cell

death. Some are more like the HR while others are more similar to disease symptoms caused by a particular pathogen (Dangl et al., 1996).

4.3 The role of reactive oxygen species and the oxidative burst

The oxidative burst is a common response, if not principally then at least as a secondary effect, to virtually every biotic and abiotic stress (Vranová et al., 2002). Accordingly, O_3-exposure induces the oxidative burst (II, III) (Schraudner et al., 1998; Pellinen et al., 1999; Rao and Davis, 1999). In mutant *rcd1* plants treatment with exogenous $O_2^{\bullet-}$ and inhibition of cell death by diphenylene iodonium (DPI) indicated that $O_2^{\bullet-}$-production is necessary and sufficient for O_3-induced cell death (II). Challenge by avirulent pathogens, which is known to induce an oxidative burst in *Arabidopsis,* also triggered *rcd1* lesions (II). Interestingly, treatment with H_2O_2 did not differentially induce cell death in *rcd1* to levels higher than seen in Col-0 wild type plants (II). In addition to the elevated levels of initial cell death, *rcd1* exhibits spreading cell death. Patterns of $O_2^{\bullet-}$-accumulation and inhibition studies indicate that this spreading cell death is $O_2^{\bullet-}$-dependent (II). This is reminiscent of the propagation class lesion mimic mutant, *lsd1* (Jabs et al., 1996), suggesting that RCD1 may act on the same pathway as LSD1 in the regulation of radical accumulation and cognate cell death.

As the studies cited above represent a rather limited number of species tested for the presence of the O_3-induced oxidative burst a wider collection of different species, cultivars, and accessions were assayed for ROS production (III). These studies indicate that the accumulation of either $O_2^{\bullet-}$ or H_2O_2 is a general feature of cell death induction in O_3-sensitive plants as seen in 8 tomato cultivars, 11 accessions of *Arabidopsis*, tobacco, *Rumex*, and *Malva* (III). As cell death was attenuated by application of inhibitors of radical accumulation it can be concluded that the oxidative burst is required for cell death induction in tomato and *Malva* as well as *Arabidopsis* (III). The further significance of this work is that plants were also examined under field conditions (III), indicating that the O_3-induced oxidative burst is not limited to experimental chambers but also occurs under natural field conditions.

The inhibition of ROS accumulation by DPI suggests that an NADPH oxidase is the source of these radicals (II, III). This mechanism of production is valid for $O_2^{\bullet-}$ as well as H_2O_2, which can be produced from $O_2^{\bullet-}$ either spontaneously or by the activity of SOD. Conclusions based on the use of DPI may be challenged based on concerns about the specificity of this inhibitor. DPI is a general inhibitor of flavin containing oxidases thus potential activity against other targets may account for its cell death inhibitory action. For example, a role for nitric oxide (NO) in regulating cell death and defense responses has been shown in plants (Durner and Klessig, 1999). In these studies DPI was used as an inhibitor of NOS (nitric oxide synthase), which is also a flavin containing oxidase. It is conceivable that DPI blocks O_3-induced cell death via inhibition of NO synthesis (NOS). This finding in itself would be of general interest. A role for NO in O_3-induced damage has been suggested previously (Mehlhorn and Wellburn, 1987; Neighbour et al., 1990) but this area of research has remained unexplored. However, several lines of evidence argue against NO synthase as the target of DPI action in O_3-exposed plants. First, the pattern of $O_2^{\bullet-}$ accumulation in *rcd1* (II) strongly implicates $O_2^{\bullet-}$ in the process of spreading cell death. Second the dose-response curve of cell death inhibition by DPI (II) matches the kinetics of NADPH oxidase inhibition in both plant and animal systems (Cross and Jones, 1986; Jabs et al., 1996). Furthermore, when DPI was used as an inhibitor of NOS, high concentrations (250 µM) were required (Durner et al., 1998) while only 1-20µM for damage inhibition in O_3-exposed *rcd1* (II).

Finally, mutants of the *rboh*s (*respiratory burst oxidase homologs*, i.e. the NADPH oxidases) have been used to demonstrate a definitive role for these molecules in radical production, cell death, and pathogen response (Torres et al., 2001). No typical NOS has been identified in plant systems thus far. Notwithstanding these arguments, epistasis studies with *rboh* mutants and O_3-sensitive accessions such as *rcd1* will be required for the resolution of this issue.

Several plant species have been shown to predominantly accumulate one particular ROS species; such as tomato and tobacco (III), which accumulate H_2O_2 or mutant *rcd1 Arabidopsis*, *Rumex* and *Malva*, which accumulate predominantly $O_2^{\bullet-}$ (II, III). *Arabidopsis* behaved differently with regard to the ROS species detected. The accumulation of either H_2O_2 or $O_2^{\bullet-}$, or both, was detected depending on which accession was assayed (III). As lesions developed on at least one representative accession for each of the ROS accumulation patterns (III) it is clear that H_2O_2 as well as $O_2^{\bullet-}$ can actively induce cell death in *Arabidopsis*. In *Arabidopsis* accessions that accumulated both $O_2^{\bullet-}$ and H_2O_2 the pattern of ROS accumulation did not coincide suggesting these ROS are produced by distinct mechanisms (III). This is in accordance with the findings of Pellinen *et al.* (1999) where temporally and spatially distinct ROS accumulation patterns were detected in O_3 exposed birch. The inhibitor sensitivities of these ROS indicated they are derived from distinct sources.

It is important to note that although a clear requirement for $O_2^{\bullet-}$ has been concluded in some cases (II, III) this does not rule out the possibility that $O_2^{\bullet-}$ acts either via or in conjunction with another ROS species downstream of $O_2^{\bullet-}$. This is illustrated by the functional and rapid conversion of $O_2^{\bullet-}$ into H_2O_2 as suggested by a DPI sensitive source of H_2O_2 in the presence of very light or non-detectable $O_2^{\bullet-}$-production in tomato leaves (III) or spinach hypocotyls (Ogawa et al., 1997). $O_2^{\bullet-}$ and H_2O_2 may only be the lead species of a multitude of ROS in a given plant species. NBT and DAB staining are by no means specific.

The above results taken together demonstrate the complexity of the O_3-induced oxidative burst and the oxidative burst in general. Many claims have been made concerning the importance of one reactive oxygen species or another (Levine et al., 1994; Jabs et al., 1996; Dangl et al., 1996; Glazener et al., 1996). However, although particular radical species may predominate in one given situation clearly there is variety in the ROS that can regulate the plant defense and cell death responses even within one plant species.

4.4 Hormonal regulation of cell death

4.4.1 Salicylic acid promotes cell death

SA accumulation is a known requirement for HR-like cell death. SA also accumulated in O_3-exposed plants (IV) where the high levels of SA accumulation correlated with lesion formation in *rcd1* and *jar1*and *jar1ein2*. SA accumulation was also shown in *Arabidopsis* where it was either correlated with induction or was required for the induction of molecular markers of the HR (PR1, and GST) by O_3 (IV; Sharma et al., 1996). The *Arabidopsis* ecotype Cvi-0 is also highly O_3-sensitive and hyperaccumulates SA upon O_3-exposure (Rao et al., 2000). When transformed or crossed with *NahG* Cvi-0 and *jar1* became markedly more O_3-tolerant (IV; (Rao et al., 2000)). Similarly, expression of *NahG* in the tobacco cultivar Xanthi also resulted in reduced lesion formation upon O_3-exposure (Örvar et al., 1997). Furthermore, when exogenous SA was added to $O_2^{\bullet-}$ treated Col-0 it significantly enhanced the induction of

cell death (IV; Mazel and Levine, 2001). Clearly, in several situations SA has a role in promoting cell death in O_3-exposed plants.

Genetic evidence of SA's role in the regulation of cell death comes form lesion mimic mutants that accumulate high levels of SA and require SA for lesion formation (Weymann et al., 1995). Other lesion mimic mutants form lesions independent of SA accumulation (Hunt et al., 1997). The genetic ordering of cell death in these mutants both upstream and downstream of SA accumulation further supports the above mentioned signal amplification loop (Hunt et al., 1997).

Expression of *NahG* in the *rcd1* background or blocking SA signaling in the *rcd1 npr1* double mutant diminished but did not abolish O_3-symptom development (I). This shows that O_3 induced cell death in *rcd1* is made up of both SA-dependent and SA-independent components. This result is consistent with the loss of a SA-dependent feed-forward amplification loop that promotes cell death. However, the primary defect leading to cell death is SA independent in the *rcd1* mutant. This is seen as a high level of O_3-induced cell death that is retained in the *rcd1 NahG* and *rcd1 npr1* double mutants (I) and suggests that RCD1 functions downstream or independent of SA accumulation and NPR1 in the regulation of cell death. In contrast, O_3-induced cell death in *jar1* is completely abolished in *jar1 NahG* (IV). The placement of cell death in *rcd1* downstream and in *jar1* upstream of SA accumulation provides direct genetic evidence of an SA mediated cell death amplification loop active in O_3-exposed plants (Figure 2).

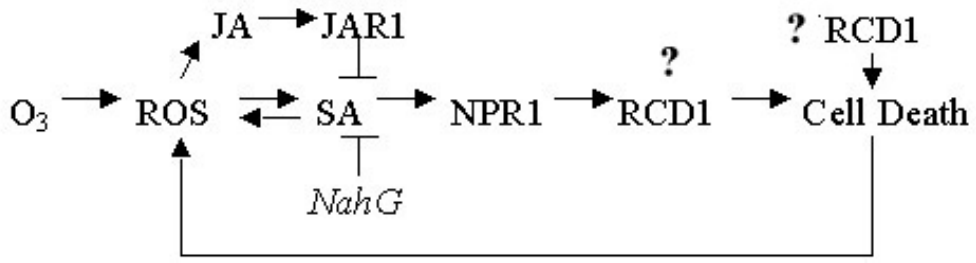

Figure 2: Salicylic acid and ozone induced cell death

The genetic relationship of SA accumulation and SA signaling to the *RCD1* and *JAR1* gene products as inferred from double mutant studies. Abbreviations used: O_3, ozone; ROS, reactive oxygen species; JA, jasmonic acid, SA, salicylic acid; JAR1, JASMONATE RESISTANT1; NahG, bacterial salicylate dehydroxylase; NPR1, NON-EXPRESSER OF PR GENES1; RCD1, RADICAL-INDUCED CELL DEATH1. RCD1 is placed with question marks to indicate it is either downstream or independent of SA accumulation and NPR1 signaling.

4.4.2 Ethylene promotes cell death

ET is one of the earliest known plant responses to O_3-treatment. Accordingly high levels of ET evolution have been detected in O_3-exposed *Arabidopsis* (II, IV). ET evolution levels are correlated with O_3-sensitivity (Wellburn and Wellburn, 1996). This correlation has been noted as early as 1976 by (Tingey et al., 1976). Also in the O_3-sensitive mutants of *Arabidopsis* described here there is good agreement between ET evolution at 4.5 hours and leaf damage at 24 hours (Figure 3).

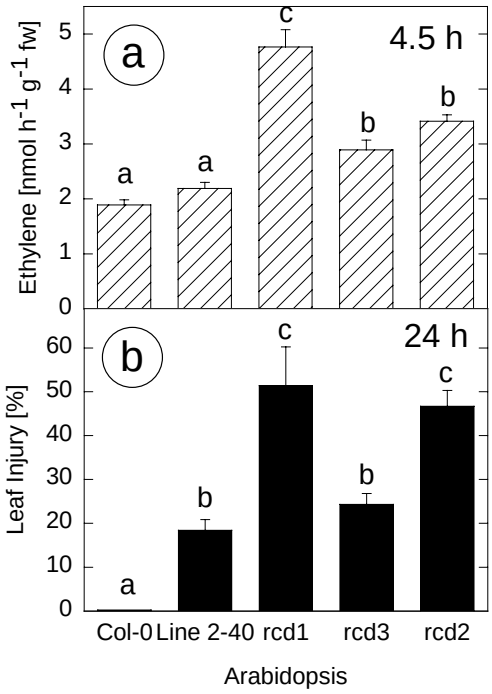

Figure 3: Ethylene evolution vs. cell death

Early ethylene evolution is a good marker of later damage formation. Ozone-induced ethylene evolution at 4.5 hours after exposure begin (A) and damaged leaf area (%) at 24 hours after an 8 hour 300 ul/l ozone-exposure. Bars labeled with the same letter do not differ significantly ($p>0.05$). C. Betz, C. Langebartels, and H. Sandermann, unpublished results.

The limiting step in ET synthesis is the production of the direct ET precursor ACC by ACC synthase (ACS; Johnson and Ecker, 1998). ACS is generally encoded by large multigene-families whose members are differentially regulated (Johnson and Ecker, 1998). In *Arabidopsis* a single O_3-induced *ACS* gene, *AtACS6*, has been identified (Vahala et al., 1998) although the *AtACS2* gene may also be involved (IV, supplemental table). In O_3-exposed *rcd1* the specific transcriptional upregulation of *AtACS6* is followed by and tightly mirrors both ACC accumulation and ET evolution (II). As has been noted (Wang and Ecker, 2002) *rcd1* has lost the ability to attenuate O_3-induced ET production as compared to small tightly regulated ET burst seen in Col-0 (II, IV). This makes *rcd1* an interesting and important mutant for exploring the regulation of stress ET biosynthesis.

Although ET evolution patterns have suggested a role in symptom development it has remained unclear whether this was a cause of damage or just an effect of the "wounded" tissue (Tingey et al., 1976; Heath and Taylor, 1997). A number of studies now suggest a causal role for ET in damage formation.

When the ET precursor, ACC, is added exogenously to $O_2^{\bullet-}$-treated leaves *in vitro* it enhanced cell death in both *rcd1* and Col-0 (II, IV) in an EIN2 dependent manner (II). Furthermore, O_3-treated plants that were post incubated in ET developed more damage than clean air controls, where again the effect was EIN2 dependent (II). Consistent with a death promoting effect for ET, the ET-insensitive *ein2* mutant was much more tolerant to high concentrations of O_3 than Col-0 (II, IV) and exhibited diminished O_3-induced $O_2^{\bullet-}$-accumulation (II). Conversely, the ET-overproducing mutant, *eto1* (*ethylene over-producer 1*) is O_3-hypersensitive (Table 3; Figure 4).

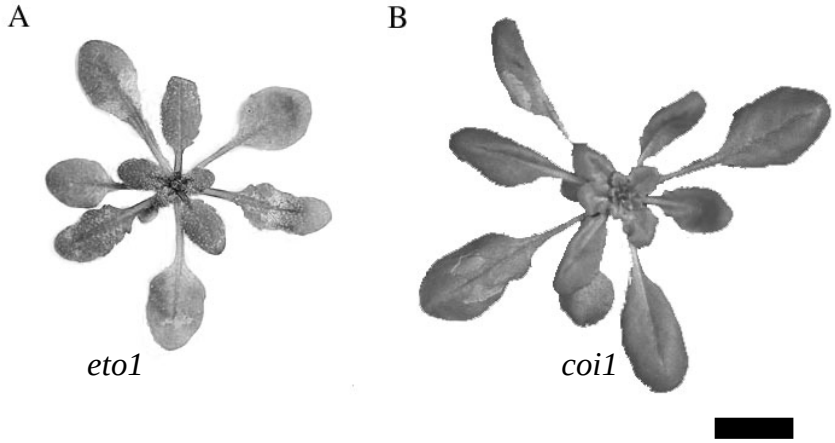

Figure 4: Ozone sensitivity of *eto1* and *coi1*

Ozone-induced damage on the ethylene over producing mutant *eto1* (*ethylene overproducer1*) (A) and the jasmonate insensitive *coi1* (*coronitine insensitive1*) mutant (B). Photos were taken at 24 hours after the beginning of a 6 hour 250 nl/l ozone-exposure. Scale bar is the same for both (A) and (B) and equals 1 cm. H. Tuominen and K. Overmyer, unpublished results.

Studies addressing the epistasis of ET-signaling and cell death in *rcd1* or *jar1* were conducted utilizing an antagonist of ET action and the *rcd1ein2* and *jar1ein2* double mutants (II, IV, Figure 5). These studies indicate that ET is required for full O_3-symptom development in both of these mutants (II, IV, Figure 5). However, some initial cell death was apparent in O_3-exposed both *rcd1ein2* and *jar1ein2* double mutants indicating that lesion initiation is ET-independent (II, IV). Specifically in *rcd1* ET was required for $O_2^{\bullet-}$-accumulation and spreading cell death (II). Treatment of O_3-exposed *jar1* with the ET-antagonist, NBD (Figure 5) demonstrates a requirement for ET-signaling in spreading cell death in this mutant as well. Likewise, it has been reported that NBD halts lesion development and H_2O_2 accumulation in O_3-exposed tomato (Bae et al., 1996; Moeder et al., 2002).

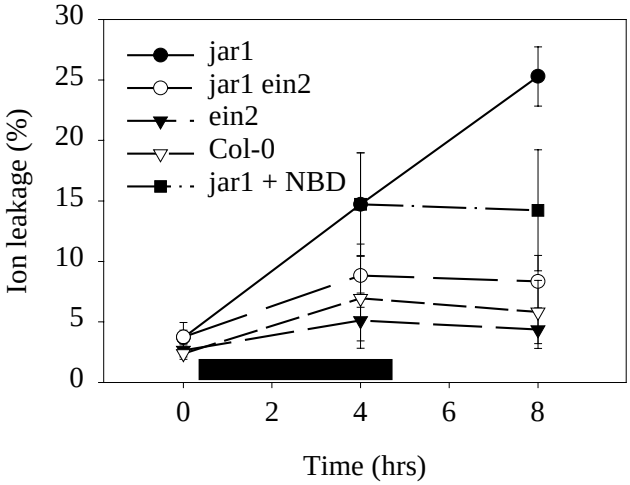

Figure 5: Role of ethylene in jar1 cell death

Ion leakage (% of total ions +/- SD) induced by a 4 hour 250 nl/l ozone-exposure (indicated with the black bar). NBD (norbornadiene: an antagonist of ethylene perception) treatment was from 4 to 8 hours. Remaining samples were incubated in clean air between 4 and 8 hours. Control values at 4 and 8 hours not shown and do not deviate significantly ($p<0.05$) from the values given in the 0 hour control.

These results are consistent with the role assigned to ET in the regulation of the pathogen triggered cell death. In plants challenged with a compatible bacterial pathogen ET signaling was required for symptom development (Bent et al., 1992) This type of cell death, which develops more slowly that the HR, appears to be mechanistically related to O_3-induced spreading cell death. The HR triggered in an incompatible interaction, however, developed fully in ET-insensitive plants (Bent et al., 1992). Thus, there seems to be a core cell death pathway that is ET-independent. This does not exclude the possibility that ET-dependent cell death can contribute to lesion size in incompatible interactions. In fact, manipulation of ET-signaling has been shown to alter lesion size in at least two different incompatible interactions (Ohtsubo et al., 1999; Ciardi et al., 2001).

In the 1980s it was suggested that ethylene and O_3 could react to produce water-soluble, free-radical, aldehyde derivatives (Elstner et al., 1985; Mehlhorn and Wellburn, 1987). Such products are highly reactive and efficient inducers of self-propagating lipid peroxidation chain reactions. This model predicts that ethylene can promote oxidative damage without the action of ethylene signaling. The demonstrated requirement for ET-signaling in O_3-symptom development in *Arabidopsis* (II, IV) refutes this model. However, this does not exclude the possibility that differing ET-mediated mechanisms of damage induction can be active under various conditions of exposure or in different plant species. This point is illustrated by non-ET-signaling dependent death promotion by ET that seems to function in O_3 exposed hybrid aspen (Vahala et al., 2002).

Both the loss-of-function and gain-of-function experiments elaborated above reveal a requirement for ethylene synthesis and signaling in cell death. Thus it can be concluded that the O_3-induced ethylene burst in *Arabidopsis* is not just a wound response or an indicator of damage (Tingey et al., 1976; Heath and Taylor, 1997). Rather this ET is an active programmed response involved in the control of the damage process. This is consistent with the role of ET as a regulator of PCD that has recently gained appreciation (Wang et al., 2002).

Based on work with plants undergoing HR the oxidative cell death cycle has been proposed (Van Camp et al., 1998). In this model, which is further supported by the results presented here (II, IV), ROS, SA and cell death function in a self-amplifying feed-forward loop in the regulation of cell death. ET-signaling is ordered upstream of $O_2^{\bullet-}$-accumulation and cell death (II). This attributes a previously unknown role to ET together with ROS as positive co-regulators of cell death. Furthermore, this finding expands the role of ET as a cell death regulator, which had been observed in development, to the realm of plant pathology and stress biology. In light of these findings it has been proposed that ET be included as a death-promoting factor in the oxidative cell death cycle model (II).

4.4.3 Jasmonate protects against reactive oxygen species induced cell death

Transient spreading cell death in *rcd1* makes this mutant a model system relevant for the study of both lesion spread and containment (II). Gene expression analysis with JA-inducible marker genes in *rcd1* suggested that JA could be a factor involved in lesion containment (II). The O_3-sensitivity of JA mutants further supports this idea. The JA-insensitive mutants *jar1* (II, IV) and *coi1* (*coronatine-insensitive 1*; Table 3; Figure 4) as well as JA biosynthesis mutant *fad 3/7/8* triple mutant (*fatty-acid deficient-3, -7, and -8*; Table 3) are all highly O_3-sensitive. Furthermore, *jar1* exhibits a transient spreading cell death phenotype and a pattern of $O_2^{\bullet-}$-accumulation similar to that observed in *rcd1* (II). Post-treatment of O_3-exposed *rcd1* with JA halts spreading cell death providing direct evidence of

JA's role in lesion containment (II). Similarly, pre-treatment of tobacco or the O_3-sensitive Cvi-0 *Arabidopsis* accession diminished O_3-damage (Örvar et al., 1997; Rao et al., 2000). Finally JA treatment of $O_2^{\bullet-}$-challenged Col-0 *in vitro* offered a modest level of protection as compared to leaves treated with $O_2^{\bullet-}$ alone (IV).

As will be discussed below JA has several effects mediated through its interactions with other hormones that contribute to its role as a negative regulator of cell death. These studies however do not exclude the possibility of direct regulation of cell death by JA. In particular there are a few studies suggesting such a role of JA. One of the few plant cell death regulators identified via a connection to mammalian PCD regulatory systems AtBI-1 is wound responsive and has reduced expression levels in *coi1* (Sanchez et al., 2000) suggesting it is JA regulated. Proteases have a key role in the regulation of PCD in plants, including radical challenged *rcd1* (I). Many protease inhibitors are known to be induced by JA, including cystatin, which was shown to have a role in regulating H_2O_2 induced cell death in parsley (Solomon et al., 1999).

It has been suggested that anti cell death function of JA makes it an important plant defense against necrotrophic pathogens, whose pathogenicity strategy relies of killing plant cells before exploiting them as a source of nutrition (Thomma et al., 2001). This view is strengthened by reports of increased cell death facilitating the growth of the necrotroph, *Botrytis* (Govrin and Levine, 2000) Strikingly, *Botrytis* grows better on mutants with deficiencies in JA-signaling (Berger, 2001) and plant sensitivity to *Botrytis* infection correlates well with O_3-sensitivity (Tiedemann, 1997). This makes O_3-responses a particularly good model for pathogenesis by necrotrophs (Berger, 2001).

4.4.4 Hormone interactions in cell death regulation

In order to facilitate their initial characterization, hormone signal transduction pathways have necessarily been conceptualized as linear and independent. However, as more details of these signaling pathways become available increasingly a new picture is emerging. The ET, SA, and JA pathways do not operate independently but rather are linked together in a complex web of interactions. It has been suggested that the overall sensitivity of the plant cell to a given hormone is at least partially established in the interplay of multiple hormones (Ghassemian et al., 2000). This point is richly illustrated by studies of plant defense signaling (Glazebrook, 2001; Thomma et al., 2001; Feys and Parker, 2000).

Consistent with its role as a model for oxidative burst dependent pathogen responses the O_3-response contributes to our understanding of how stress hormone interactions modulate defense and cell death signaling. Gene expression studies and the O_3-sensitivity phenotypes of signaling mutants (and double mutants) indicate that mutually antagonistic interactions are prevalent between ET, SA and JA (IV). This mutual antagonism was observed under both oxidative stress and normal growth conditions indicating that it is general in its nature (IV). A discussion of these interactions follows below.

4.4.4.1 Jasmonate as an antagonist of ethylene signaling

The inhibition of ET-action by JA was inferred, but not show directly, from the phenotype of the *cev1* (*constitutive expresser of vegetative storage protein 1*) mutant (Ellis, C.

and Turner, 2001). Significantly, the studies presented here (IV) provide direct evidence of this novel hormone interaction.

Cell death containment by JA involves an antagonistic interaction with ET. $O_2^{\bullet-}$-accumulation and spreading cell death in *rcd1* are ET-dependent (II). The fact that JA is able to halt this process (II) implies that JA can act as an antagonist that overcomes the cell death promoting effect of ET. At the level of gene expression JA was found to antagonize ET-action especially in the regulation of ET-responsive element binding proteins (IV). Furthermore, in an *in vitro* cell death assay JA diminished the cell death promoting effect of the ET precursor ACC and $O_2^{\bullet-}$ (IV).

JA could potentially mediate this antagonistic effect via the attenuation of ET-biosynthesis or the modulation of ET-sensitivity. ET evolution studies show that JA does not attenuate ET-biosynthesis while blocking spreading cell death. As shown in figure 6, between the 4hr and 8 hr time points, while JA overcomes ET-dependent spreading cell death, ET-evolution is equal or slightly greater in JA treated plants as compared to clean air controls where lesions are actively spreading. This suggests that JA mediates its antagonistic effect via changes in ET-sensitivity rather than ET-biosynthesis. One testable prediction stemming from this model is that JA-insensitive mutants, which lack antagonism of ET-sensitivity by JA, would be hypersensitive to ET.

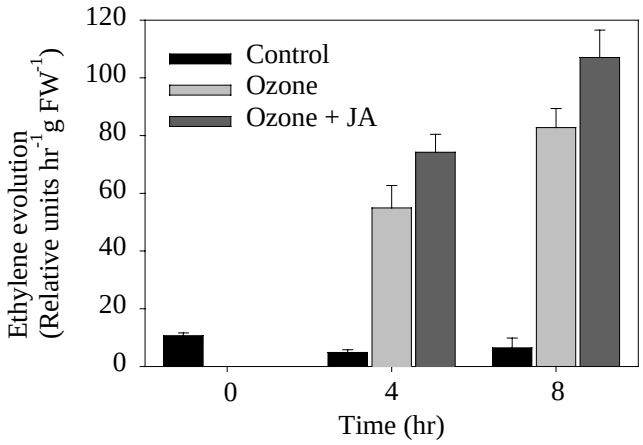

Figure 6: Effect of jasmonate on ozone-induced ethylene

Ethylene (ET) evolution induced by a 4 hour 250 nl/l ozone-exposure. Ozone-exposure was from 0-4 hours followed by clean air. Ozone + JA (jasmonate) was ozone from 0-4 hours followed by 1,4 uM jasmonate from 4-8 hours. Values are expressed as relative units of ET hr-1 g-1FW +/- SD. The 0 hour timepoint represents ET from 0-1,5 hours. The 4 hour timepoint represents ET from 4-5,5 hours. The 8 hour timepoint represents the ET from 4-8 hours (while lesions were spreading).

The JA-insensitive *jar1* mutant is ET-hypersensitive (IV). Consistent with a large body of previous data it has been confirmed that high ET-evolution is a general characteristic of O_3-sensitive plants (Figure 3; II, IV). Furthermore, ET-signaling is required for full O_3-symptom development (II, IV). The high level of O_3-sensitivity and low O_3-induced ET-evolution in *jar1* (II, IV) appear to contradict the above assertions. Yet in spite of low levels of O_3-induced ET in *jar1*, double mutant analysis and ET-antagonist studies (IV, Figure 5) demonstrate a requirement for ET in *jar1* O_3-pathogenesis. These issues could all be explained by the ET-hypersensitivty of *jar1*, thus prompting us to test this model. Gene-expression analysis with ET-marker genes and physiological studies using the triple response assay a gauge of ET-action both indicate that *jar1* is hypersensitive to ET (IV). Interestingly, increased hormone accumulation is associated with a loss of biosynthesis feedback control and can been seen as a common symptom of hormone-insensitive mutants (IV). Conversely, hormone-hypersensitivity could be expected to heighten feedback control of biosynthesis. This offers explication for the decreased ET-evolution seen in *jar1* (II, IV).

Further direct evidence of ET-antagonism by JA was obtained by the application of JA to plants that have the ET-signaling pathway activated. Exogenous JA counteracted the

inhibition of hypocotyl elongation, measured in the triple response assay, in Col-0 that was exogenously fed the ET-precursor ACC and in the constitutive ET-mutant *eto1* (*ethylene-overproducer 1*) but not in another similar mutant *ctr1* (*constitutive triple response 1*; IV). The *ctr1* mutant activates ET-signaling downstream of the ET-receptor whereas ACC treated Col-0 and *eto1* activate the ET-pathway at the level of ET-production, i.e. upstream of ET-perception. This genetic dissection demonstrates that JA acts to antagonize ET-action downstream from ET-evolution but upstream from CTR1.

As only the ET receptor lies between ETO1 and CTR1, placement of JA action between these proteins suggests a mechanism for JA-antagonism of ET-sensitivity. There is a possibility that JA could somehow modify the ET-receptor or interfere with its assembly. The *ran1* mutant illustrates that there are side branches of the ET-pathway capable of altering ET-sensitivity that are genetically placed upstream of the ET-receptor but not involved with ET-biosynthesis (Hirayama et al., 1999). However a more parsimonious explanation would be the modulation of ET-receptor levels by JA. Decreasing or increasing the levels of the ET-receptor have been shown to result in increased and decreased ET-sensitivity, respectively (Hua and Meyerowitz, 1998; Hackett et al., 2000; Ciardi et al., 2001). Published micro array data shows that at least one of the ET-receptors, ERS2, is JA-inducible (Schenk et al., 2000). Based on these results I propose that JA decreases ET sensitivity via the transcriptional activation of ET-receptor gene(s) and subsequent increases in the protein levels of ET-receptors, which are known to act as negative regulators of ET-signaling.

4.4.4.2 Synergistic regulation of defense genes by ethylene and jasmonate

A fundamental difference was noted in how JA interacts to regulate some defense genes as compared to cell death. On one hand cooperative action was seen. JA and ACC synergistically induced *CHIB* expression (Figure 7). In tobacco, ET and JA have been shown to synergistically induce *PR1b* and *PR5* (Xu et al., 1994). In contrast, exogenous MeJA countered ethylene action in the triple response assay and overcame the ACC enhancement of $O_2^{\bullet-}$-induced cell death (IV). It is not know what role such defense genes play in plant O_3-responses. However, O_3-induced cell death closely imitates and may serve as a model for pathogenesis by necrotrophic pathogens (Tiedemann, 1997; Berger, 2001). In this biological context a reason for such interactions may be found. Most pathogens induce an ET burst in plants. In the case of necrotrophs the death promoting effects of ET works to the pathogen's advantage (Thomma et al, 2001). JA can reduce ET-sensitivity, preventing the deleterious effects of cell death and thus is a particularly important defense against necrotrophs.

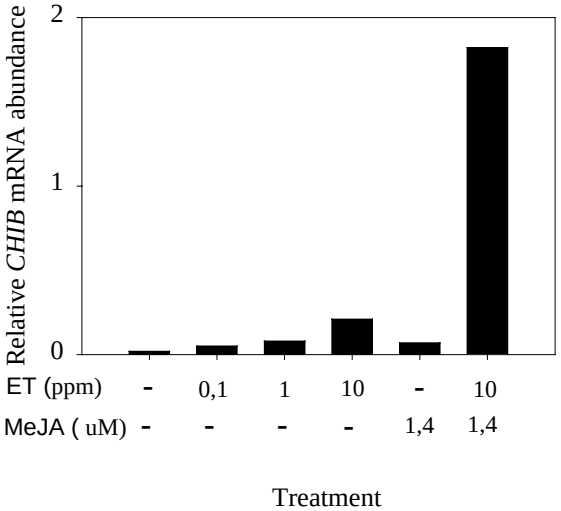

Figure 7: *ChiB* induction by ethylene and jasmonate

ChiB (basic chitinase) gene expression induced in 3 week old soil grown Col-0 Arabidopsis by hormone treatments as indicated. The (-) symbol indicates no treatment. Plant were exposed to hormones in the vapor phase in gas tight glass jars and sampled at 48 hours for gene expression analysis. Experiment repeated twice with similar results. Representative experiment shown.

Interestingly, synergistic induction of defense genes by JA and ET allows the plant to maintain a high defense profile even while the ET component of defense gene induction is diminished due to the attenuation of ET sensitivity by JA. A similar situation is seen also between JA and SA, which are antagonists in cell death regulation but synergistically induce *PR1*b (Xu et al., 1994; Niki et al., 1998).

4.4.4.3 Antagonism of jasmonate by ethylene and salicylic acid

The antagonism of ET and SA against JA biosynthesis and signaling is well documented (Norman-Setterblad et al., 2000; Rao et al., 2000; Ellis, C. and Turner, 2001; Kloek et al., 2001). Macroarray gene expression analysis of O_3 exposed signaling mutants indicated that JA-signaling was antagonized by both ET and SA (IV). These interactions were confirmed in gene expression studies utilizing exogenous hormone treatments and pathway specific marker genes (IV). These interactions apparently play a role in lesion formation as addition of exogenous ET or SA to JA treated leaves enhanced cell death in a $O_2^{\bullet-}$-triggered in vitro cell death assay (IV). Additionally, in genetic epistasis studies *ein2* and *NahG*, respectively, either partially or fully suppressed the O_3-sensitive phenotypes of *jar1* (IV). This indicates that the pro-death effects of SA and ET, respectively, are completely and partially epistatic to the pro-survival effects of the JA pathway. Furthermore, during O_3-exposure JA levels rise however JA-dependent signaling remains unchanged suggesting that JA pathway signaling is suppressed (IV). Thus, suppression of the JA-pathway has a role in O_3-symptom development. This conclusion was also reached by Rao et al. (2000) based on studies with the O_3 sensitive Cvi-0 accession of *Arabidopsis*.

As ET is a JA antagonist (IV) it is conceivable that the JA-insensitivity of *jar1*could be the result of its ET-hypersensitivity, rather than *vice versa*. This model predicts that JA insensitivity in *jar1* is ET-signaling dependent. Table 5 shows that *jar1ein2* roots are fully insensitive to inhibition of elongation by JA. This indicates that the *jar1* MeJA insensitive phenotype is fully expressed in the ethylene insensitive *ein2* mutant background and therefore cannot be dependent on ET-signaling. Thus it can be concluded that ET-hypersensitivity in *jar1* is an effect rather than a cause of JA-insensitivity.

Table 5: MeJA sensitivity of the *jar1 ein2* double mutant

The MeJA insensitivity of *jar1* is not dependent on ethylene signaling. Root length in mm ± sd (n =10) of 7 day old seedlings grown vertically on 0.5 x ms plates containing 10 µM MeJA. Experiment was performed two times with similar results. Representative results shown. Results followed by a different letter are significantly different ($p < 0.05$) according to one-way ANOVA with Tukey's HSD test.

Genotype	Root length (mm ± sd)
Col-0	6.50 ± 1,35 [a]
ein2	6.95 ± 1,83 [a]
jar1	18.85 ± 3,85 [b]
jar1 ein2	20.05 ± 5,51 [b]

4.4.4.4 Jasmonate suppresses salicylic acid biosynthesis and signaling

JA antagonism of SA-dependent gene expression was apparent in macro array analyses and confirmed by exogenous hormone experiments with PR1 as a marker gene (IV). JA also modestly reversed the death-enhancing effect of SA in the $O_2^{\bullet-}$-triggered *in vitro* cell death assay (IV). SA hyperaccumulates in JA-signaling deficient mutants (IV) indicating that JA antagonizes SA at the level of biosynthesis in addition to signaling.

The O_3-induced biosynthesis of signal molecules such as SA, JA, and ET (IV) provides further evidence of the similarities between biotic- and O_3-pathologies. The magnitude and timing of hormone accumulation supports the above conclusions concerning antagonistic interactions. The interaction and balance between these signals are involved in cell death regulation (II, IV).

Mutual antagonism in hormone signaling may be advantageous to the plant in several ways. In some cases these hormones induce each other's biosynthesis. The induction of an antagonist may serve to shutdown or reset a signaling pathway allowing renewed responsiveness to changing signaling demands. Interplay between combinations of different hormones would allow for a greater diversity of responses. This concept has been proposed by Xu (Xu et al., 1994) where varied hormone combinations are proposed to generate specific "signature sets" leading to a downstream responses appropriate to a given stress situation.

These hormone interactions may offer explication of a long-standing enigma concerning how O_3 alters plant-pathogen interactions. O_3 has been shown in some cases to induce pathogen resistance and SAR (Sharma et al., 1996). In other cases O_3 is known to be detrimental for plants weakening them and making them more susceptible to pathogen attack (Sandermann et al., 1998). Plant sensitivities to O_3 vary greatly and the O_3-induced hormone interactions are very complex. In light of this complexity and natural variation, variety in O_3's effect on plant-pathogen interactions should not be surprising. In the face of varied pathogenicity strategies utilized by pathogens plants counter with different defense systems, such as the ET-JA mediated resistance against necrotrophs and the SA-dependant SAR pathway against biotrophs. Interestingly, diseases caused by bacterial and obligate fungal pathogens are almost always reduced by O_3 (Hughes and Laurence, 1984). Conversely, diseases caused by necrotrophic fungi are almost always promoted by plant O_3-exposure (Hughes and Laurence, 1984). Examples where increased resistance to one type of pathogen results in increased susceptibility to another are prevalent (Felton et al., 1999; Bostock, 1999; Paul et al., 2000). The O_3-induced changes in hormone concentrations and their interactions (IV) suggest many possibilities for tipping the balance in favor or against any given pathogen.

4.5 Summary: the current model of ozone induced cell death

The above results are summarized together with current findings from the literature, which have been previously discussed above, to form a model of how O_3-induced cell death is regulated in *Arabidopsis*.

Once inside the leaf, O_3-derived ROS mimic the oxidative burst and are perceived by the plant by unknown $O_2^{\bullet-}$, peroxide, or redox sensitive receptors. These ROS then trigger an active oxidative burst of $O_2^{\bullet-}$, which is produced from an NADPH oxidase (II). This occurs in cells that are preconditioned for sensitivity to ROS (Figure 8 A; I, II).

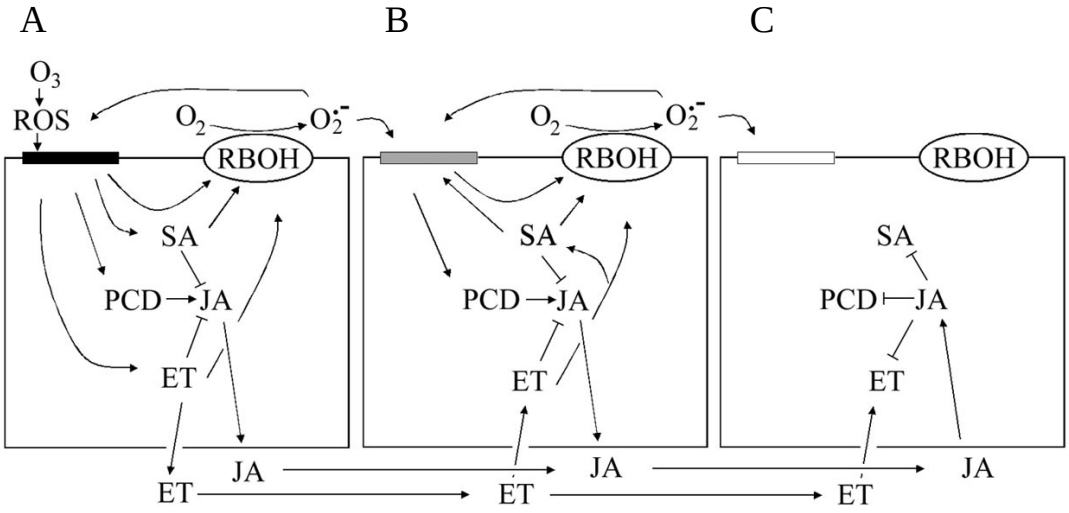

Figure 8: The regulation of ozone-induced cell death

Three cells are depicted each representing a different phase of cell death regulation are (A) lesion initiation, (B) lesion spread, and (C) lesion containment. The salient features of this model are elaborated below: (A) In ROS-sensitive cells O_3-derived ROS trigger an oxidative burst of $O_2^{\bullet-}$ from the RBOH (NADPH oxidase). SA and ET potentiate ROS action and suppress JA-action resulting in the activation of cell death. Preconditioned sensitivity to ROS signals is depicted by the black box. (B) ET from cells destined to die moves to other cells and primes them, via the action of ET-dependent SA, for competency to ROS signals, represented by the grey box, resulting in lesion spread. During this phase ET and SA continue to promote cell death through the suppression of JA and petentiation of ROS-action. (C) JA derived from dying cells moves outside of the lesion ahead of lesion spread. This JA suppresses the pro-death actions of SA and ET resulting in a lack of competency to perceive death promoting ET and ROS signals, which is depicted as an open box. These events result in lesion containment. Abreviations used: O_3, ozone; RBOH, respitory burst oxidase homolog, which is an NADPH oxidase; SA, salicylic acid; JA, jasmonates; ET, ethylene; PCD, programmed cell death; $O_2^{\bullet-}$, superoxide; O_2, oxygen.

Activation of the NADPH oxidase by pathogens in several systems requires ion fluxes including H^+ and Ca^{++}, as well as protein kinases (Hahlbrock et al., 1995). The requirement for kinases, Ca^{++}-, and H^+-fluxes in the induction of O_3-induced cell death most likely occurs at this level (I). In wild type plants ROS-action is amplified and death of a few individual cells is thus induced through the accumulation of O_3-induced SA (I, IV). This action of SA is countered by JA signaling, which is apparently activated in surrounding cells, as it prevents cell death from spreading (Figure 8 C; II, IV). In *rcd1* plants SA accumulation is bypassed and cell death is induced to a higher level than in wild type (I). During this initial cell death process JA signaling is suppressed by SA and ET (Figure 8A; IV). From these initial sites of cell death a burst of ET production occurs (II, IV). This ET spreads to surrounding cells and induces competency for cell death spread (Figure 8 B; II). In tomato ET signaling was required for SA accumulation and cell death spread during symptom formation after virulent

pathogen attack (O' Donnell et al., 2001). I suggest that this model, where ET-dependent SA is required for spreading cell death, also functions in O_3-exposed plants (Figure 8 B). This model is consistent with the cooperative action of SA and ET seen during O_3 symptom development (IV). But to which signal do ET (and SA) primed cells become competent? $O_2^{\bullet-}$ is actively produced in lesions and then during spreading cell death in a row of cells in advance of cell death spread (II). This pattern suggests that $O_2^{\bullet-}$ is the death-inducing signal to which ET and ET-dependent SA prime cells. Thus ET and $O_2^{\bullet-}$ conspire in a feed forward chain reaction that is responsible for spreading cell death. The $O_2^{\bullet-}$ signal is passed forward a few rows of cells at a time from dying cells to cells primed by ET. Cell death results in the production of wound JA, which triggers the JA-dependent process of lesion containment. JA can antagonize lesion spread in several ways (Figure 8 C), for example, through the suppression of SA biosynthesis and signaling (IV). Also, attenuation of ET-sensitivity by JA contributes to halting cell death spread (II, IV). Alternatively, JA may also have direct pro-survival effects through the induction of Bax inhibitor proteins and protease inhibitors.

During the signaling and execution of O_3-induced cell death downstream of the oxidative burst a number of processes are also now know to be active. Activation of a ROS-induced transcriptional program and new protein synthesis are required for O_3-induced cell death (I). Execution of O_3-induced cell death most likely is achieved via multiple mechanisms but can follow a program leading to dead cells with several of the morphological characteristics of classical PCD (I).

4.6 Future prospects

The next logical step in this work will be the cloning of mutant genes that confer O_3-sensitivity and the definition of functions for the proteins they encode. To this end the cloning of the *rcd1* gene is underway. The map based cloning of *rcd1* led to the identification of a C to T transition within the conserved splice site of the predicted intron III exon IV junction of the gene At1g32230. This mutational model predicts truncation of the protein at a stop codon following the translation of a 22 amino acid missense peptide within the unexcised intron. The mapping and identification of this mutation strongly suggests it is responsible for the *rcd1* phenotypes. This is however subject to confirmation pending genetic complementation. At1g32230 encodes a novel plant protein containing several conserved domains, which suggest possible functions. The first domain is a poly ADP-ribose polymerase (PARP)-like domain. RCD1 is clearly not a classical PARP as the homology is limited to the PARP catalytic domain and lacks the DNA-binding zinc-finger domain. Yet the possibility of RCD1 being a novel PARP cannot be excluded as the catalytic activity of this protein has not been shown yet. Furthermore, the possibility remains that RCD1 may perform one of the related enzymatic activities such as mono (ADP-ribos)yl transferase (mADPRT) activity or even (ADP-ribos)yl cyclase (ADPrC). All these activities share NAD binding, NAD glycohydrolase activity, and similar catalytic domains (Ziegler, 2000). PARPs covalently modify proteins and other targets by the addition of long branched poly ADP-ribose polymers (D'Amours et al., 1999). If RCD1 has PARP activity then *rcd1* will be the first plant PCD mutant whose gene cloning lead to a direct connection with established mammalian apoptosis models. mADPRTs are largely unexplored in plants. In mammals mono (ADP-ribos)ylation of proteins acts as a reversible post-translational covalent modification, much like phosphorylation, which can modify protein activity (Ziegler, 2000). Mono (ADP-ribos)ylation has also been documented for non-protein low molecular weight substrates. Remarkably, mADPRT activity can be strongly induced by and is involved in NO signaling (Brüne and Lapetina, 1989). Cyclic ADP ribose (cADPR) has been demonstrated to function as a signaling intermediate acting downstream of NO and ABA in plants, however the identity of the plant ADPrC remains unknown (Wu, Y. et al., 1997; Klessig et al., 2000). Dysfunction in

NO signaling, which is known to be a potent inducer of plant PCD, could provide an interesting field for building models to explain *rcd1* radical sensitivity. Similarly, cADPR involvement would offer a tempting explanation for *rcd1* ABA insensitivity.

Interestingly, RCD1 contains a protein interaction domain where in classical PARPs the DNA binding zinc-finger would be found. This protein interaction domain, the so called WWE domain (Aravind, 2001) for 3 of its conserved amino acids, is found both in a subclass of PARPs and in some proteins bearing E3 ubiquitin ligase domains, which are involved in ubiquitination of proteins targeting them for degradation in the proteosome. It is predicted that the WWE domain mediates specific protein-protein interactions between two WWE -domain containing proteins. However, this model still requires experimental confirmation. The potential link to the ubiquitin/proteosome system invites the possibility that RCD1 and other WWE containing proteins are regulated via controlled degradation. Alternatively, RCD1 may be involved in the regulation of ubiquitination and subsequent regulated protein turnover via the modification, such as the (ADP-ribos)ylation, of ubiquitin ligases. Another interesting question to be raised is if the PARP homology in RCD1 extends over the site where PARPs are cleaved by caspases. If RCD1 were also a target for caspase cleavage this would provide a link to known cell death regulatory pathways. Furthermore, the significance of PARP cleavage in PCD regulation would be widened by this finding. These functions suggested by the domain structure of RCD1 are consistent with our conclusion that RCD1 is either a commonly recruited signaling link and/or involved in the interaction and balance of hormone signals.

Although entirely speculative at this stage, the above models illustrate the diverse and rich fields of inquiry opened up by the application of a genetic approach to the problem of plant ROS signaling. The cloning of mutant genes conferring O_3-sensitivity and elucidating functions of their products will further contribute to our understanding of plant ROS signaling, plant-O_3-interactions, and PCD control.

5 Acknowledgements

My doctoral studies were begun at the University of Kuopio in the Department of Ecology and Environmental Sciences in 1995 in the group of Dr. Jaakko Kangasjärvi. My thanks to the Department Head, Professor Lauri Kärenlampi for facilities and a good working environment in the Department in Kuopio during the years 1995-1997. I owe a great debt to Lauri Kärenlampi and Jaakko Kangasjärvi for all they did to make it possible for me to come to Finland.

Following the relocation of the Kangasjärvi group from Kuopio to Helsinki my work continued in the Institute of Biotechnology and the Department of Bioscience's Division of Genetics. Thanks to Professor Mart Saarma and Professor Tapio Palva for providing facilities in Helsinki during the years 1997 to 2002.

Special thanks to Professor Mart Saarma both as a collaborator in my work and for the wonderful job he has done in making the Institute of Biotechnology such a vibrant and stimulating workplace of international character.

I would like to express my thanks to the official pre-reviewers of this thesis, Professor Alte Bones and Docent Hans Thordal-Christensen for their review.

I wish to thank my primary advisor Professor Jaakko Kangasjärvi for all that he has contributed to my education as a scientist; for all the discussions, encouragement, and especially for the freedom he has allowed me to develop and pursue my own ideas. Thanks also to my second advisor Dr. Christian Langebartels. Especially during the writing of the thesis his comments and criticism were vital to improving the written work.

This work benefited from the excellent technical help of Mika Korva, Arja Ikävalko, Anne Aronta, Mirva Lehtinen, and Amy Warren. A warm thanks to Timo Oksanen for his patience and expertise in teaching me about the superb ozone exposure systems he has built in Kuopio and Helsinki. I also wish to thank Arja Valomäki in the Genetic Division's office in Helsinki for the many times she has provided me help.

To all of my coworkers and colleagues I would like to express my heartfelt thanks. What a boring dry pursuit science would be without all of you! Thanks for all the discussions, lunches, coffee breaks, social events, evenings out, and parties. Also thanks for all the help; each of you has contributed in so many ways to make this thesis work possible.

To the early crew from Kuopio; Dr. Riikka Pellinen, Dr. Merja Utriainen, Jaana Tuomainen, Markus Kiiskinen, Maarit Sillanpää, and Minna Korhonen, thank you especially for all of your warmth and humor that eased my transition to life in Finland. Thanks also to some of the other scientists I had the pleasure to meet at the University of Kuopio; Dr. Elina Oksanen, Dr. Seija Antonen, Dr. Anu Wulff, Professor Sirpa Kärenlampi, and Professor Seppo Lapinjoki.

To the Helsinki members of the group: Dr. Markku Keinänen, Dr. Mikael Brosché, Raili Ruonala, Dr. Reetta Kettunen, Dr. Hannes Kollist, Dr. Hannele Tuominen, Pinja Pulkkinen, Airi Tauriainen, Dr. Saara Lång, Jorma Vahala, and Reetta Ahlfors, thanks for all the good times! Jorma, thanks for all the insanity that helped keep me sane, it was great to share the "thesis writing" with you. Keep mixin' & scratchin' homeboy (are you threatening me?). Hannele thanks for all the discussions and lunches, Markku -god of chemistry and statistics, Saara-great friend and officemate, Raili-famous horizontal desk organisational system, Reetta- speak up you don't talk enough.

Thanks to all those around in the Department and the BI: Professor Alan Schulman, Professor Teemu Teeri, Dr. Ykkä Helariutta, Dr. Kurt Fagerstedt, Dr. Martin Romantschuk, Dr. Sari Tähtiharju, Ilkka Tamminen, Dr. Marcos Montesano, Dr. Pekka Heino, Annikki Welling (super genius), Dr. Karen Sims-Huopaniemi, Tarja Saloranta, Tuula Puhakainen, Roosa Laitinen, Heidi Hyytiäinen, Elina Helenius, Dr. Günter Brader, Kristian Aspegren, Martin Bonke, Ari Pekka Mähönen, Dr. Pia Runeberg-Roos, Tero Kuittinen, Dr Mika Kotilainen. Thanks to you all for making this such a great place to work!

Thanks to my family at home in the USA: Erik, Sandy, Brandon, Jacob, Kristen, Kylie, Kelsey, Cedric, Jamila, Mom and Dad. I have been missing you all so much all these years while I have been living in Europe. Thank you for all of your love, support and unwavering belief in the things I can accomplish.

Finally to my wife Margit and our children, Rebecca and Andreas. Without you none of this would have been possible. Thanks for all the love, support, and balance you bring to my life. Also, thank you for your tolerance and sacrifice during the long hours I required at work to finish this thesis.

Helsinki October 2002

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