Ozone-Induced Signaling in *Arabidopsis thaliana*

Reetta Ahlfors

Department of Biological and Environmental Sciences
Faculty of Biosciences and
Finnish Graduate School in Plant Biology
University of Helsinki
Finland

Academic dissertation
To be presented for public criticism, with permission of the Faculty of Biosciences,
University of Helsinki, in the auditorium 1 of the Infocenter Korona,
Viikki, on April 25, 2008, at 12 o´clock noon.

Helsinki 2008

Supervisors: Professor Jaakko Kangasjärvi
Department of Biological and Environmental Sciences
University of Helsinki, Finland

Doctor Mikael Brosché
Department of Biological and Environmental Sciences
University of Helsinki, Finland

Reviewers: Professor Eva-Mari Aro
Department of Biology
University of Turku, Finland

Professor Elina Oksanen
Department of Biology,
University of Joensuu, Finland

Opponent: Professor Stanislaw Karpinski
Department of Plant Genetics, Breeding and Biotechnology
Warsaw Agricultural University, Poland

ISSN 1795-7079
ISBN 978-952-10-4606-3
ISBN 978-952-10-4607-0 (PDF)

Yliopistopaino
Helsinki 2008

[Fæ:ntæ:stik]!

Table of Contents

Abbreviations

$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Superoxide radical
ABA	Abscisic acid
ACC	1- Aminocyclo-propane-1-carboxylic acid
ACS	ACC synthase
AOX	Alternative oxidase
Ca^{2+}	Calcium
Col-0	Columbia-0 ecotype
CTR/*CTR*/*ctr*	Constitutive triple response protein/wild type gene/mutant gene
DND/*DND*/*dnd*	Defense no death protein/wild type gene/mutant gene
DREBP	Drought response element binding protein
EIN/*EIN*/*ein*	Ethylene insensitive protein/wild type gene/mutant gene
EMS	Ethylmethylsulfonate
EREBP	Ethylene responsive element binding protein
ERF	Ethylene response factor
ERK	Extracellular response kinase
ERS	Ethylene response sensor
ETR	Ethylene receptor
H_2O_2	Hydrogen peroxide
HR	Hypersensitive response
JA	Jasmonic acid
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NahG	Salicylate hydroxylate
NO	Nitric oxide
NOA/*NOA*/*noa*	Nitric oxide associated protein/wild type gene/mutant gene

NOS	Nitric oxide synthase
NPR/*NPR*/*npr*	Non expressor of PR proteins protein/wild type gene/mutant gene
O_3	Ozone
$OH^{\cdot -}$	Hydroxyl radical
$ONOO^-$	Peroxynitrite
PCD	Programmed cell death
RCD/*RCD*/*rcd*	Radical-induced cell death protein/wild type gene/mutant gene
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
S/T	Serine/threonine
SA	Salicylic acid
SAM	S-adenosyl methionine
SAR	Systemic acquired resistance
SIPK	Salicylic acid-induced protein kinase
SNP	Sodium nitroprusside
SOD	Superoxide dismutase
TMV	Tobacco mosaic virus
UV	Ultraviolet
WIPK	Wound-induced protein kinase
VSP	Vegetative storage protein
XO	Xantine oxidase
XXO	Xantine xantine oxidase

Original publications

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

I Ahlfors R, Lång S, Overmyer K, Jaspers P, Brosché M, Tauriainen A, Kollist H, Tuominen H, Belles-Boix E, Piippo M, Inzé D, Palva E.T and Kangasjärvi J (2004). Arabidopsis RADICAL-INDUCED CELL DEATH1 belongs to the WWE protein-protein interaction domain protein family and modulated abscisic acid, ethylene and methyl jasmonate responses. Plant Cell 16: 1925-1937.

II Ahlfors R, Macioszek V, Rudd J, Brosché M, Schlichting R, Scheel D and Kangasjärvi J (2004). Stress hormone-independent activation and nuclear translocation of mitogen-activated protein kinases in *Arabidopsis thaliana* during ozone exposure. Plant J 40:512-522.

III Overmyer K, Brosché M, Pellinen R, Kuittinen T, Tuominen H, Ahlfors R, Keinänen M, Saarma M, Scheel D and Kangasjärvi J (2005). Ozone-nduced programmed cell death in the Arabidopsis *radical-induced cell death1* mutant. Plant Phys. 137, 1092-1104.

IV Ahlfors R, Brosché M, Kollist H and Kangasjärvi J (2008). The role of nitric oxide in the regulation of ozone-induced cell death (Manuscript).

Summary

Tropospheric ozone (O_3) is one of the most common air pollutants in industrialized countries, and an increasing problem in rapidly industrialising and developing countries in Asia, Africa and South America. Elevated concentrations of tropospheric O_3 can lead to decrease in photosynthesis rate and therefore affect the normal metabolism, growth and seed production. Acute and high O_3 episodes can lead to extensive damage leading to dead tissue in plants. Thus, O_3 derived growth defects can lead to reduction in crop yield thereby leading to economical losses. Despite the extensive research on this area, many questions remain open on how these processes are controlled. In this study, the stress-induced signaling routes and the components involved were elucidated in more detail starting from visual damage to changes in gene expression, signaling routes and plant hormone interactions that are involved in O_3-induced cell death.

In order to elucidate O_3-induced responses in *Arabidopsis*, mitogen-activated protein kinase (MAPK) signaling was studied using different hormonal signaling mutants. MAPKs were activated at the beginning of the O_3 exposure. The activity of MAPKs, which were identified as AtMPK3 and AtMPK6, reached the maximum at 1 and 2 hours after the start of the exposure, respectively. The activity decreased back to clean air levels at 8 hours after the start of the exposure. Both AtMPK3 and AtMPK6 were translocated to nucleus at the beginning of the O_3 exposure where they most likely affect gene expression. Differences were seen between different hormonal signaling mutants. Functional SA signaling was shown to be needed for the full protein levels and activation of AtMPK3. In addition, AtMPK3 and AtMPK6 activation was not dependent on ethylene signaling. Finally, jasmonic acid was also shown to have an impact on AtMPK3 protein levels and AtMPK3 activity.

To further study O_3-induced cell death, an earlier isolated O_3 sensitive *Arabidopsis* mutant *rcd1* was mapped, cloned and further characterized. *RCD1* was shown to encode a gene with WWE and ADP-ribosylation domains known to be involved in protein-protein interactions and cell signaling. *rcd1* was shown to be involved in many processes including hormonal signaling and regulation of stress-responsive genes. *rcd1* is sensitive against O_3 and apoplastic superoxide, but tolerant against paraquat that produces superoxide in chloroplast. *rcd1* is also partially insensitive to glucose and has alterations in hormone responses. These alterations are

seen as ABA insensitivity, reduced jasmonic acid sensitivity and reduced ethylene sensitivity. All these features suggest that RCD1 acts as an integrative node in hormonal signaling and it is involved in the hormonal regulation of several specific stress-responsive genes.

Further studies with the *rcd1* mutant showed that it exhibits the classical features of programmed cell death, PCD, in response to O_3. These include nuclear shrinkage, chromatin condensation, nuclear DNA degradation, cytosol vesiculation and accumulation of phenolic compounds and eventually patches of HR-like lesions. *rcd1* was found to produce extensive amount of salicylic acid and jasmonic acid in response to O_3. Double mutant studies showed that SA independent and dependent processes were involved in the O_3-induced PCD in *rcd1* and that increased sensitivity against JA led to increased sensitivity against O_3. Furthermore, *rcd1* had alterations in MAPK signature that resembled changes that were previously seen in mutants defective in SA and JA signaling.

Nitric oxide accumulation and its impact on O_3-induced cell death were also studied. Transient accumulation of NO was seen at the beginning of the O_3 exposure, and during late time points, NO accumulation coincided with the HR-like lesions. NO was shown to modify defense gene expression, such as, SA and ethylene biosynthetic genes. Furthermore, *rcd1* was shown to produce more NO in control conditions. In conclusion, NO was shown to be involved in O_3-induced signaling leading to attenuation of SA biosynthesis and other defense related genes.

1. Introduction

Due to the sessile nature of plants, they are forced to cope with changing environmental conditions, disease and pathogen attacks, not forgetting the constantly changing threat of various air pollutants. In order to survive these stress situations, plants have developed many strategies to adapt their metabolism and defense. The mechanisms behind the survival require complex signaling. It is important to further our knowledge on why and how plant responses against stresses, since plants are the primary energy producers converting light energy to chemical energy. This energy is used to oxidize water in order to reduce carbon dioxide for synthesizing carbohydrates. At the same time oxygen is released. Because we are entirely dependent on plants, we need research and deeper understanding on plant stress responses. How a plant senses the cues from the surrounding environment, then transduces the signals and responds to the stress, is an important area of research that a wide field of plant researchers is trying to address with the aim of securing our food and living resources in the future.

When plant experiences stress, a typical phenomenon is the formation of reactive oxygen species that activate different signaling routes. These signaling pathways lead, for instance, to induction of senescence, reduction of dry matter production and yield losses. Stress condition can, depending on the situation, also lead to cell death and may eventually kill the whole plant. The chapters in the introductory part of this thesis aim to give a short overview on the formation of reactive oxygen species, formation of cell death and the hormonal signaling involved therein. During this thesis work, ozone, a common tropospheric air pollutant responsible for vast crop losses in industrialized countries, and in industrialising and developing countries, was used to create reactive oxygen species and cell death in a model plant *Arabidopsis thaliana*. The ozone-induced signaling pathways involved in cell death were studied in more detail. In the Results and discussion –part, the research made during this thesis is discussed and hypotheses on the composition and interactions between different signaling pathways are made.

1.1 Reactive oxygen species, ROS

Reactive oxygen species (ROS), such as super oxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot -}$), are produced in plants continuously as by-products in electron transport chain in chloroplast (Asada, 1999), mitochondria and in the plasma membrane cytochrome *b*-mediated electron transfer. ROS can also form when molecular oxygen reacts with transition metal ions (Fe^{2+}, Cu^{2+}) and semiquinones acting in the reaction as e^- donors. These reactions include Fenton reaction and Haber-Weiss reaction. $O_2^{\cdot -}$ and its protonated form $^{\cdot}O_2H$ exist in equilibrium state. $^{\cdot}O_2H$ is hydrophilic molecule and it is capable of penetrating membranes. During normal growth conditions, $O_2^{\cdot -}$ and $^{\cdot}O_2H$ are disproportionated to H_2O_2 and O_2 in aqueous solutions either spontaneously or by superoxide dismutase (SOD) (Scandalios, 1993; Streller and Wingsle, 1994; Wojtaszek, 1997). From the ROS formed in plants hydroxyl radical ($OH^{\cdot -}$) is the strongest oxidant initiating reactions with organic molecules. Fenton reaction, together with Haber-Weiss reaction, are significant sources of $OH^{\cdot -}$ (Wojtaszek, 1997). Haber-Weiss reaction produces relatively low amounts of $OH^{\cdot -}$, but during Fenton reaction high amount of $OH^{\cdot -}$ is formed. In summary, the following chemical reactions lead to production of ROS in plants during normal metabolism:

Superoxide disproportionation:

$$2\ ^{\cdot}O_2H \leftrightarrow H_2O_2 + O_2$$

$$2\ ^{\cdot}O_2H + O_2^{\cdot -} + H^+ \leftrightarrow H_2O_2 + O_2$$

$$2 \cdot O_2^{\cdot -} + 2\ H^+ \leftrightarrow H_2O_2 + O_2$$

Fenton reaction:

$$H_2O_2 + Fe^{2+}\ (Cu^+) \rightarrow Fe^{3+}\ (Cu^{2+}) + \cdot OH + OH^-$$

$$O_2^{\cdot -} + Fe^{3+}\ (Cu^{2+}) \rightarrow Fe^{2+}\ (Cu^+) + O_2$$

Haber-Weiss reaction:

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

During stress situations, the formation ROS increases and plants exhibit several mechanisms to limit this ROS formation, but when the production of ROS exceeds the degrading capacity it leads to oxidative stress. Thus, excess ROS can lead to injury, but formation of ROS also has another important function; ROS are important signaling molecules that control several processes including pathogen defense, programmed cell death during abiotic and biotic stresses and stomatal responses (Foyer and Noctor, 2005; Karpinski et al., 1999). The production and scavenging of ROS will be discussed below in addition to the role of ROS in plant signaling.

1.2 Sources of ROS in plants

Plants accumulate ROS continuously and this accumulation is strictly controlled. The major sources of ROS are chloroplasts (Asada, 2006), mitochondria, plasmalemma-bound NAD(P)H oxidases, cell wall-associated peroxidases, peroxisomes and glyoxysomes (del Rio et al., 2006).

In chloroplast, there are two main processes where ROS are formed during photosynthesis. Direct photoreduction occurs when $O_2^{\cdot -}$, is produced as a side product from photosynthetic electron transport from photosystem I and $^1O_2$ is produced from photosystem II. Under normal conditions, the produced $O_2^{\cdot -}$ is quickly metabolized to H_2O_2 by superodixe dismutase, SOD. Singlet oxygen, $^1O_2$, is quenched by carotenoids involved in photosystem antenna complexes and also by tocopherols (Asada, 2006). In addition, during photorespiration ROS, more precisely H_2O_2, is formed when Rubisco catalyzes a competitive reaction where oxygen is favored over CO_2 leading to formation of glycolate. Glycolate is then transported to peroxisomes where in subsequent oxidation H_2O_2 is formed (Nyathi and Baker, 2006).

In mitochondria, ROS, mainly $O_2^{\cdot -}$, and also H_2O_2, are produced due to leakage in electron transport chain by NADH dependent dehydrogenases, ubiquinone radical and by complex III (Jezek and Hlavata, 2005). In addition, cytochrome C

oxidase and alternative oxidase in mitochondria produce nitric oxide, also considered as a form of ROS, as a side product from electron transport chain (Planchet et al., 2005, Gupta et al., 2005). In mammalian cells mitochondria have been reported to be the major source of ROS (Halliwell, 1989), but in plants the relative mitochondrial ROS production is quite low (Purvis, 1997).

During photorespiration, peroxisomes produce $O_2^{\cdot -}$ most likely by xantine oxidase/xantine dehydrogenase (Corpas et al., 2001). In addition, NADH dependent proteins are found to produce $O_2^{\cdot -}$ in peroxisomes. Furthermore, H_2O_2 is produced in peroxisomes via B-oxidation (Nyathi and Baker, 2006), peroxisomal sulfite oxidases (Nakamura et al., 2002) and Sarcosine oxidases (Goyer et al., 2004). Also glyoxysomes produce H_2O_2 during fatty acid oxidation (del Rio et al., 2002).

1.3 Scavenging of ROS

The accumulation of ROS is tightly controlled by nonenzymatic and enzymatic scavenging mechanisms. From the most common forms of ROS, superoxide and hydrogen peroxide are far less reactive than hydroxyl radical. Since $OH^{\cdot -}$ is produced as an intermediate by reactions of $O_2^{\cdot -}$ and H_2O_2, the reactions leading to its generation are controlled.

Nonenzymatic antioxidants scavenge ROS by acting as “redox buffers” and include ascorbate, glutathione (GSH), tocopherol, flavonoids, alkaloids and carotenoids. During stress situations, the amount of GSH has been shown to increase (Noctor et al., 2002). In addition, elevated levels of xanthophyll resulted in enhanced stress tolerance (Davison et al., 2002). Elevated citrulline levels have been suggested to lead to increased oxidative stress tolerance by scavenging $OH^{\cdot -}$ (Akashi et al., 2001). It is essential for a plant to contain high ratio of reduced ascorbate and GSH over oxidized. This is accomplished in reactions catalyzed by glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) where NADPH is used as reducing power.

Enzymatic ROS scavenging mechanisms include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and catalase (CAT). SOD dismutates $O_2^{\cdot -}$ to H_2O_2 and catalase converts H_2O_2 to water and oxygen. APX

uses two molecules of ascorbate to reduce H_2O_2 to water and and simultaneously two molecules of monodehydroascorbate is formed that are consequently reduced by MDHA reductases. GPX catalyzes the reaction between H_2O_2 and two molecules of reduced monomeric glutathione. The glutatione disulfide formed in this reaction is reduced back to glutathione by glutahione reductase. Since the extent of oxidative stress is determinated by the balance of antioxidant capacity and the quantity of $O_2^{\cdot -}$, H_2O_2 and $OH^{\cdot -}$, the equilibrium between the production and scavenging of ROS is important. Consequently, modification of the enzymatic balance can lead to enhanced ROS tolerance (Willekens et al., 1997), but it can also lead to unexpected sensitivity. For example, Rizhsky et al. (2002) showed that plants lacking both APX and CAT are less sensitive against oxidative stress, whereas the single antisense plant with either suppressed APX or CAT are more sensitive than the double antisense plants.

1.4 ROS production during pathogen attack

Quick production of ROS is a common reaction in defense against pathogens. This production is also called an oxidative burst and it involves the production of ROS, mainly $O_2^{\cdot -}$ and H_2O_2 at the site of infection (Apostol et al., 1989). Superoxide generation is involved in a broad range of plant-pathogen interactions and several enzymes have been implicated to be involved in it.

Oxidative burst in plants resembles the oxidative burst found in animals. In animals, NADPH-dependent oxidase system, found in phagocytes and B lymphocytes, has been shown to catalyze the production of superoxide in order to kill invading micro-organisms. The core of this Phagocyte oxidase is composed of $p40^{PHOX}$, $p47^{PHOX}$, $p67^{PHOX}$, $p22^{PHOX}$ and $gp91^{PHOX}$. From these, the two latter ones have been located to membrane secretory vesicles, and the rest are located in cytosol. Antibodies against the human $p47^{PHOX}$ and $p67^{PHOX}$ cross react also with plant proteins of similar size (Desikan et al., 1996), but later on after the publication of *Arabidopsis* genome, no homologs of the mammalian $p47^{PHOX}$ and $p67^{PHOX}$ were found in the *Arabidopsis* genome (Dangl and Jones, 2001). On the other hand, many respiratory burst oxidase homologs (*rboh*) of $gp91^{PHOX}$ have been found in plants (Keller et al., 1998, Torres et al., 1998). For example, genetic evidence from knock-out studies using *Arabidopsis*

AtrbohD and *AtrbohF* indicate that at least these two genes eliminate the ROS production during disease resistance against avirulent pathogens (Torres et al., 2002). The difference between $gp91^{PHOX}$ and the plant *rboh* action is that plant Rboh proteins are stimulated directly by Ca^{2+} and they can produce $O_2^{\cdot -}$ in the absence of additional cytosolic components (Sagi and Fluhr, 2001). This is due to the main structural difference between $gp91^{PHOX}$ and plant *rboh* homologs, since there are two Ca^{2+}-binding EF-hands located in the N-terminal end of the plant *rboh* homologs. Furthermore, ROS production by AtrbohD has been shown to require both Ca^{2+} binding and phosphorylation (Ogasawar et al., 2008). Nevertheless, these EF-hands are present in other mammalian NADPH-oxidase homologs, in so called NOX family (Torres and Dangl, 2005), and therefore, plant Rboh proteins actually resemble more this NOX family of NADPH oxidases. For instance, this NOX family includes the NOX5-like isoforms (up to 10 isoforms per genome) found in plants and NOXes (Ancestral-type NOX1-4 and NOX5-like isoform NOX5) and DUOXes (DUOX1-2) found in animals. The function of these NOX enzymes in animals include host defense, post-translational modification of proteins in addition to regulation of growth and differentiation (Bedard and Krause, 2007). In comparison, the plant NOX homologs have been connected to defense (Torres et al., 2002) and wounding (Sagi and Fluhr, 2001), stomatal closure (Kwak et al., 2003) and root and root hair growth responses (Joo et al., 2001, Foreman et al., 2003).

Another possible mechanism of ROS production during biotic stresses has been proposed to be the peroxidases found in the apoplastic space. They are normally involved in the synthesis of lignin from phenolic substrates, but also NADPH and related products can be used as substrates. This enables peroxidases to have NADPH-oxidase activity producing H_2O_2 and superoxide, and potentially hydroxyl radicals (Chen and Schopfer, 1999).

1.5 Hypersensitive response

The oxidative burst is one of the most characterized phenomenon in plant-pathogen interactions, and this controlled burst of oxidative molecules is a part of defense responses, called hypersensitive response (HR). In HR, recognition of a

pathogen triggers a localized resistance reaction that is seen as a limited degree of death on neighboring cells at the pathogen infection site (Lamb and Dixon, 1997). During HR, a biphasic burst of ROS is evident around the pathogen infection site and this ROS production is NADPH oxidase-dependent. HR directly affects the pathogen growth by acidification of cytosol and cross-linking the proteins and phenolic compounds of the cell wall and also triggers micro-HR in distant tissues, thereby inducing systemic acquired resistance, SAR (Alvarez et al., 1998). During SAR plant can develop immunity to different pathogens with a requirement of SA signaling (Gaffney et al., 1993). SAR can be triggered by many pathogens that cause necrosis, either as a part of HR or as a symptom of disease. Recently, Park et al. (2007) illustrated using tobacco that the mobile signal for SAR is methyl salicylate, MeSA, and therefore demonstrated for the first time how a signal can be transferred from one part of a plant to distant tissues in order to develop immunity.

1.6 O_3-induced ROS production resembles hypersensitive response

Ozone, O_3, is a relatively reactive molecule with many roles. In the stratosphere, O_3 is known to protect earth form excess UV radiation. In contrast, tropospheric O_3 is known to be a highly reactive molecule causing severe damage in both plants and animals (Pell et al., 1997). Essentially in southern, heavily populated regions the problem is severe. This is because tropospheric O_3 is formed when nitrous oxides, NOx, produced by factories and cars, react with oxygen in the presence of heat and sun light thereby forming O_3. Naturally, the direct effects of O_3 are seen in industrialized areas, but the problem spreads, when wind transfers the formed O_3. Albeit the levels of NOx in industrialized countries show reduction in emission, the NOx effects are an increasing problem in developing countries (IPCC fourth assesment report, http://www.ipcc.ch/ipccreports/ar4-wg1.htm; Sitch et al., 2007). During the last 100 years, the tropospheric O_3 levels have increased 2-3 fold (Laurila and Lättilä, 1994, Skärby et al., 1994), indicating that even more severe and wide spread problems caused by O_3 can be expected in the future (Madden and Hogsett, 2001). For example, Sitch et al. (2007) estimated that the O_3 effect will raise even three fold in South America, Africa and Asia during next 100 years. Long term O_3 exposure leads to reduction of

photosynthesis and growth of sensitive species, eventually leading to crop losses and decreased growth of forest trees (Ashmore, 2005; Wittig et al., 2007). On the other hand, high and acute pulse of O_3 causes visible damage in plants. These damage patterns resemble lesions caused by pathogens not only physically, but also molecular features are alike. O_3 induces HR-like response, similar to that induced by pathogens (Rao et al., 2000a; Sandermann et al., 1998; Sharma et al., 1996). Comparable to plant-pathogen interactions, O_3 activates an oxidative burst and concomitant accumulation of ROS (Overmyer et al., 2000; Pellinen et al., 1999; Rao and Davis, 1999; Rao et al., 2000a; Schraudner et al., 1998). Because of the gaseous nature of O_3, it is also an effective tool to study plant stress responses in laboratory conditions without actually touching the plant.

O_3 induces oxidative stress in plants by producing ROS. This O_3-induced oxidative burst is biphasic: Once O_3 enters the leaf through open stomata (Rich et al., 1970) it reacts instantaneously with the surrounding membranes leading to production on ROS, such as $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$ (Lamb and Dixon, 1997). The production of ROS leads to alterations in the permeability of plasma membranes and lipid compositions (Heath, 1987). It has to be taken into account that ROS can directly affect the conformation of proteins and lipids. The primary burst of ROS initiates a signaling cascade leading to a secondary wave of ROS production. These include also nitric oxide, NO. NO can react with $O_2^{\cdot-}$, forming peroxynitrite radical, $ONOO^-$. This is a powerful radical that can damage all biomolecules and can also lead to further formation of $OH^{\cdot}$ (Halliwell, 2006; Lamattina et al., 2003). Naturally, these different forms of ROS are also formed in plant at low levels during normal growth conditions in mitochondria and chloroplasts. The problem arises under stress situations, when the production of ROS exceeds the degradation capacity leading to oxidative stress. This O_3-induced ROS formation activates several pathways, including Ca^{2+} and MAPK signaling pathways, in addition to ethylene, salicylic acid and jasmonic acid dependent pathways eventually leading to induction of defense reactions leading to programmed cell death (Rao et al., 2000b; Samuel et al., 2000; Sandermann, Jr. et al., 1998; Sharma et al., 1996; Overmyer et al., 2000). These signaling pathways will be introduced in the following chapters.

1.7 Programmed cell death, PCD

Programmed cell death (PCD) is a genetically controlled system of self-destruction, where unwanted cells are eliminated. PCD is widely studied in animals and plants and the induction of PCD is a part of HR. In contrast to necrosis that occurs due to an acute tissue injury, PCD is a regulated process. In animals, PCD can be divided to apoptosis and autophagy. The molecular and biochemical markers in plant PCD meet the criteria of animal apoptosis. In animals, during PCD a burst of ROS leads to changes in calcium fluxes and caspase activation. Also mitochondrial membrane depolarization occurs, leading to changes in mitochondrial pore size and release of cytochorome C oxidase and activation of caspases. In addition, cell shrinkage, DNA fragmentation, formation of micronuclei and plasma membranes blebbing is seen (Falcieri et al., 1994). The main difference between mammalian apoptosis and necrosis is the use of ATP, energy. Necrosis is said to be occurring spontaneously in response so sudden injury/stress leading to immediate damage, without consuming the cell's energy resources. In contrast, apoptosis uses ATP and therefore is considered to be an active reaction (Gilchrist, 1998).

PCD is a known phenomenon during plant-pathogen interaction as well as a response to O_3 exposure in sensitive species (Dangl et al., 1996; Rao et al., 2000a). PCD is also a common feature in plant growth and development: PCD can be detected during floral organ abortion when male/female flowers are formed and during tapetal layer generation. PCD also occurs during terminal tracheary element differentiation, senescence, leaf sculpture and aerenchyma formation. For example, aerenchyma formation by PCD under hypoxia is a genetic and tissue -specific program (Muhlenbock et al., 2007). Other important PCD actions are evident in death of root cap cells, pathogen attack and trichome development. In addition, PCD is known to be involved in aleurone degeneration, degeneration of suspensor, degeneration of endosperm, and also during megaspore abortion.

All types of ROS can initiate PCD and this involves an orchestra of signaling components that lead to changes in gene expression and growth/stress responses. The web of signaling induced by ROS includes changes in ion fluxes, changes in the activity of mitogen-activated protein kinases, in addition to accumulation

of other signaling components, such as plant hormones. These responses are introduced in following chapters.

1.8 Signaling involved in PCD

1.8.1 Mitogen-activated protein kinases, MAPKs

Mitogen-activated protein kinases (MAPK) are one of the most studied components in both mammalian and plant signal transduction pathways. Overall, the phosphorylation reactions are an important factor in signal transduction pathways forwarding the signals, but also combining different signaling routes.

MAPKs are a subfamily of protein kinases and they act in signaling cascades composed of mitogen-activated protein kinase kinase kinase (MAPKKK) that phosphorylates mitogen-activated protein kinase kinase (MAPKK, also called as MEK), which then finally phosphorylates MAPK (Tena et al., 2001). MAPKs are activated by phosphorylation of tyrosine and serine residues of the TxY-motif found in the activation loop, whereas MAPKKs are activated by the phosphorylation of the S/T-X_{3-5}-S/T motif by MAPKKKs (Jonak et al., 2002).

In mammals, many MAPK pathways, such as stress activated protein kinase/c-Jun N-terminal kinase cascades, p38 MAPK cascades and the ERK-MAPK kinase cascades have been shown to function in response to stress (Beck et al., 1999). In plants, only a few complete signaling cascades with direct substrate have been illustrated this far. These include NtMEK-SIPK/WIPK cascade in stress ethylene biosynthesis in tobacco (Kim et al., 2003), MKK3-MPK6 cascade in jasmonic acid signaling in *Arabidopsis* (Takahashi et al., 2007), MKK4/5-MPK6 cascade in tobacco/*Arabidopsis* (Liu and Zhang, 2004) and NPK1-NQK1/NtMEK1-NRK1 cascade during cytogenesis in tobacco (Soyano et al., 2003). In addition to the stress-inducible nature of MAPKs, the same MAPKs can also have a developmental function. Wang et al. (2007) reported a whole MAPK cascade functioning in stomatal development composed of YODA (MAPKKK), MKK4/MKK5 and finally MPK3/MPK6. This

suggests that MAPK cascade can integrate signaling branches between environmental and developmental responses.

Sequencing of the *Arabidopsis* genome revealed 20 genes encoding for MAPKs, 10 encoding for MAPKK and 60 putative genes encoding for MAPKKK (MAPK Group, 2002). The amount of different kinases also indicates that there is putative complexity and cross-talk between MAPK cascades. Naturally, another important point of signal convergence and modulation is the counteracting MAPK phosphatases (MKP). *Arabidopsis* genomes has five MKPs, from which AtMKP1 has been shown to interact with AtMPK6 during salt stress (Ulm et al., 2001; Ulm et al., 2002) and AtMKP2 has been shown to interact with both AtMPK3 and AtMPK6 (Lee and Ellis, 2007).

1.8.2 Nitric oxide, NO

NO is a molecule with diverse signaling functions in different species (Beligni and Lamattina, 2001). It has a capability to diffuse across membranes and through cytoplasm due to its lipophilic features. The molecule's half life is less than 10 seconds and it is able to diffuse with a speed up to 50 µm/s. NO is capable of either lose or gain an electron and thereby form different structures, mainly nitrosonium cation (NO^+) and nitroxyl radical (NO^-). In addition, it readily reacts with other molecules forming reactive nitrogen species (RNS), such as peroxynitrite $ONOO^-$, nitrotyrosine, dinitrogen trioxide N_2O_3 and nitrogen dioxide NO_2. The formed RNS can react with DNA, lipids, proteins and carbohydrates leading to impaired cellular functions. RNS can induce post-translational modifications through the thiol group nitrosylation of cysteines. For example, NO reacts with tyrosine and cysteine residues of protein kinases and also through reactions with heme groups, thiols and metal clusters (Stamler et al., 2001).

In plants, NO regulates many developmental and stress-inducible processes during plant life. For instance, NO has been shown to be involved in germination, root growth, gravitropic bending, stomatal closure, flowering, orientation of pollen tubes, hypoxia, iron availability, adaptation to stresses and finally, in cell death (Delledonne, 2005).

In animals, NO is synthesized by enzyme nitric oxide synthase (NOS). NO synthase catalyzes the formation of L-citrulline and NO from L-arginine using $NADPH^+$ and oxygen as co-substrates and enzyme bound heme, tetrahydrobiopterin (H_4B), calmoduling, FAD and FMN as co-factors. The first NO synthase found in mammals was endothelium-derived nitric oxide synthase eNOS, which was found to be involved in smooth muscle relaxation. Later on, neuronal NOS, nNOS, was found to be involved in neuronal communication. NO synthases were thought to act only in a continuous manner, until researchers found the inducible NOS, iNOS, from macrophages that were capable of producing high amounts of NO to act as a cytotoxic compound with antimicrobial effects (Nathan and Xie, 1994; Wendehenne et al., 2001). A few years ago, a mitochondrial NOS, mtNOS, was isolated from rat liver (Schild et al., 2003).

In plants, NO is synthesized via enzymatic and non-enzymatic steps. Slow formation of NO occurs from nitrite at neutral pH (Yamasaki, 2000). NO can be synthesized non-enzymaticly by reduction of nitrite to NO in the apoplastic space (Bethke et al., 2004) or by enzymatic nitrite reduction reactions, catalyzed by xanthine oxidase (XO, yet no clear evidence in plants) (Li et al., 2004), plasma-membrane bound nitrite:NO reductase (Ni-NOR, in roots) (Stohr et al., 2001) and nitrate reductase (NR, in chloroplasts) (Klepper, 1990; Yamasaki and Sakihama, 2000). During recent years, the existence of NOS-like activity in plants has been under extensive studies, but no clear evidence has yet been provided. Increased production of L-citrulline form L-arginine has been noticed during incompatible interactions between *Arabidopsis* and *Pseudomonas syringae* (Zeidler et al., 2004), tobacco and TMV (Durner et al., 1998), and between soybean and *Diaporthe phaseololum* (Modolo et al., 2002) indicating the possible existence of NOS activity in plants. The protein AtNOA1 (Nitric oxide associated 1, former AtNOS1, Nitric oxide synthase 1) was the first protein connected to have a role in NO synthesis in mitochondria (Guo and Crawford, 2005, Zemojtel et al., 2006), but the actual role in NO synthesis is still unclear (Crawford et al., 2006).

1.8.3 Ethylene

The gaseous plant hormone ethylene has a variety of different functions and its synthesis is an elicitor of morphological changes in all stages during plant life cycle (Johnson and Ecker, 1998). Ethylene is synthesized via the Yang cycle (also called Methione cycle). In this cycle methionine is converted by methionine adenosyltransferase (MAT/SAM synthetase) to S-adenosyl-L-methionine (AdoMet/SAM) via 5´-methylthioadenosine (MTA), 5´-methylthioribose (MTR), MTR-1-phosphate (MTR-1-P) and 2-keto-methylthiobutyrate (KBM). After this, AdoMet is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme ACC synthase, and finally ACC is converted to ethylene by ACC oxidase.

Ethylene is perceived by two-component type receptors, composed of a sensor histidine kinase and a response regulator domain. The sensor histidine kinase phosphorylates an internal histidine residue according to environmental signals and thereafter the response regulator receives that phosphate group on a conserved aspartate residue. *Arabidopsis* has five ethylene receptors (ETR1, EIN4, ETR2, ERS1 and ERS2) that perceive ethylene. From these, ETR1 and ERS1 share the highest homology belonging to subfamily 1. The main difference between these two is that ERS1 lacks the C-terminal receiver domain. Subfamily 2 consists of ETR2, ERS2 and EIN4 that all lack the conserved motifs within the histidine kinase domains and they have an additional putative signal sequence at the C-terminal end of the protein.

Under normal conditions, ethylene receptors function as negative regulators of the downstream signal transduction pathway. In the absence of ethylene receptors remain active (Hall et al., 2000). ETR1 has been shown to directly interact with CTR1 (Huang et al., 2003), a putative MAPKKK that negatively regulates downstream responses (Kieber, 1997) after which there is a putative MAPK cascade consisting of MAPKK and MAPK that represses the downstream elements, positive ethylene signal regulators: EIN2 (Alonso et al., 1999) and EIN3/EIL1/EIL2 (Chao et al., 1997). Ouaked et al. (2003) suggested using heterologous system that *Medigago* MAPKK and *Arabidopsis* MPK6 would act downstream of ETR1 but upstream of EIN2, placing this kinase cascade after CTR1. Later on, this finding has been criticized and therefore the MAPK cascade following CTR1 remains unknown (Ecker, 2004). Taken together, when ethylene binds to a receptor, it inactivates the receptor. The inactivated receptor cannot interact with CTR1 therefore activating the EIN2 N-ramp

metal transport-type protein that forwards the signal to nucleus and via EIN3/EIL1/EIL2 and other ethylene related transcription factors, ERFs, and ethylene response element binding proteins, EREBPs, that interacts with the promoters of the targets genes finally leading to ethylene responses (Solano et al., 1998; Wang et al., 2002). Recently, a MKK9-MPK3/MPK6 cascade was found to promote EIN3-medited transcription (Yoo et al., 2008). Yoo et al. (2008) illustrated an unconventional role for CTR1 acting as blocker of a MKK9-MPK3/MPK6 cascade and simultaneously enhancing EIN3 degradation. This implies a divergence on ethylene signaling pathway downstream of CTR1 and upstream from EIN3.

The formation of ethylene is tightly controlled by the ACC synthase activity (Yang and Hoffman, 1984). When an antisense ACS from tomato was introduced to tobacco, it improved the plant's O_3 tolerance. This was due to the suppression of endogenous ACS thereby decreasing O_3-induced ethylene formation (Nakajima et al., 2002). In addition to ACS, also ACC oxidase activity is an important factor in controlling ethylene biosynthesis under O_3 (Tuomainen et al., 1997). Both of these enzymes are also activated at the gene expression level, but most importantly, they are also regulated post-translationally, by protein kinases. Liu and Zhang (2004) showed that AtACS2 and AtACS6 are directly phosphorylated and thereby stabilized by AtMPK6 that, in turn, is regulated by the upstream kinases MKK4 and MKK5 in *Arabidopsis*. In addition, NtMEK2-SIPK/WIPK cascade has been shown to be involved in ethylene biosynthesis in tobacco in response to wounding and viral infection (Kim et al., 2003). Recently, the counteracting phosphatase in ethylene synthesis pathway was described by Schweighofer et al. (2007) who showed that the PP2C-type phosphatase AP2C1 interacts with AtMPK6 thereby negatively regulating ethylene levels. The same phosphatase was also shown to interact with AtMPK4 and affect JA levels thereby suggesting a novel link between the regulation of these two pathways (Schweighofer et al., 2007). Another regulation point of ethylene formation is the convergence of two metabolic pathways, polyamine synthesis and ethylene synthesis. Both of these pathways are competing for the same substrate, SAM (S-adenosyl-L-methionine) derived from the Yang cycle. In addition, an interesting regulation point in ethylene biosynthesis is the capability of NO to inhibit MAT1 (methionine adenosyltransferase, also known as SAM synthase) that produces the methionine needed in ethylene biosynthesis by S-nitrosylation (Lindermayr et al., 2005; Lindermayr et al., 2006).

Ethylene production is known to correlate with the O_3-induced leaf damage (Tingey et al., 1976). Later on, ethylene and SA were found to have a common role in O_3-induced lesion propagation (Overmyer et al., 2000; Pell et al., 1997; Rao et al., 2000a; Rao and Davis, 2001) and in contrast, ethylene and JA have been shown to function antagonistically (Tuominen et al., 2004**)**. Naturally, ethylene is also an important plant growth hormone, but its role in stress signaling is evident. The complexity of the ethylene signaling and biosynthesis regulation suggests that additional regulation steps and new potential signal convergence points will be found in the future. For example, there have been suggestions whether there would be an alternative pathway for ethylene signal to be transduced. This assumption has been made on the basis that even the strong alleles of *ctr1* still respond to ethylene (Kieber et al., 1993).

1.8.4 Salicylic acid

Salicylic acid (SA) and its volatile derivate, Methyl salicylate (MeSA) have been known for a long time to be important signaling molecules (Shulaev et al., 1997). SA also has a role in maintaining the redox state of glutathione. Therefore, SA is an important factor in activation of antioxidant responses. SA can also directly affect ROS levels through inhibition of catalase activity therefore leading to an increase in H_2O_2 concentration (Chen et al., 1993). SA can be synthesized either using phenylalanine as a substrate or through the isochorismate pathway. SA synthesis is induced in response to various stresses, such as UV (Nawrath et al., 2002), cold (Scott et al., 2004), heat (Clarke et al., 2004; Larkindale et al., 2005), pathogens (Wildermuth et al., 2001) and O_3 (Rao and Davis, 1999). SA has been shown to be involved in O_3-induced lesion propagation since the transgenic *NahG Arabidopsis* line, where SA accumulation is compromised, does not show O_3-induced lesion formation (Overmyer et al., 2000; Rao et al., 2000a). Naturally, SA has also a role in plant development. For example, SA has been shown to regulate flowering time in non-stressed *Arabidopsis* and also SA regulates the transition to flowering during UV-C stress (Martinez et al., 2004). In addition, SA has been shown to control gene expression during senescence (Morris et al., 2000).

SA is required for induction of defense –related genes, such as PR1, and it is an essential part of SAR (Dempsey et al., 1999; Durrant and Dong, 2004). An important component in the SA signaling is the NPR1-dependent signaling pathway. NPR1 has been suggested to have a role in detoxification of SA and in feedback regulation of SA biosynthesis, in addition to transcriptional regulation of SA responsive genes (Cao et al., 1997; Kinkema et al., 2000). MAP kinases are an important factor in regulation of SA synthesis. For example, SA has been shown to activate SIPK in tobacco (Kumar and Klessig, 2000; Zhang et al., 1998**)**. Seo et al. (2007) showed that SIPK and WIPK are required for down-regulation of SA accumulation after wounding. In contrast, Samuel and Ellis (2002) suggested that SA signaling is not needed for the activation of p46 (presumably SIPK) during O_3 exposure.

Studies have shown that during cell death, SA acts antagonistically and/or synergistically between ethylene and JA pathways (Asai et al., 2000; Overmyer et al., 2000; Rao et al., 2000b). In addition, SA has been shown to mediate the crosstalk between JA and ethylene pathways (Spoel et al., 2003). Furthermore, SA has been shown to be required for stress-induced ethylene formation in *Arabidopsis* (Rao et al., 2002). In contrast, Ogawa et al. (2007) showed that the level of SA is not regulated by ethylene in O_3-exposed *Arabidopsis* if O_3 exposure does not generate lesions. Thus, considerable complexity is seen on the regulation of SA signaling, and the signal regulation differs between plant species. For example, in tomato SA has a role during later stages in plant pathogen interactions whereas in *Arabidopsis*, SA has a role during the early time points of pathogen attack (Zhou et al., 1998).

1.8.5 Jasmonic acid

Jasmonic acid, JA, is a fatty acid derivate synthesized from 18-carbon linoleic acid. It is converted from released membrane lipids by a series of enzymatic steps. JA and its derivate methyl jasmonate (MeJA) are important factors in responses to stresses such as wounding, O_3 and UV-B exposure (Conconi et al., 1996; Overmyer et al., 2000; Örvar et al., 1997) and defense against pathogens (Kloek et al., 2001; Thomma et al., 1999). The precursor of JA, OPDA, has also been shown to act as a physiological signal for defense, inducing resistance in the absence of JA (Stintzi et al.,

2001). Complex interactions are evident between the different members of the jasmonate family leading to a possibility to orchestrate specific responses from array of signals (Reymond et al., 2000).

Studies using JA insensitive mutants such as *jar1*, *coi1* and *jin1* have illustrated the complexity of JA signaling. *jar1* has been shown to be involved in various stress responses. JAR1 has been shown to positively regulate jasmonate signaling by initiating the JA-Ile conjugation leading to JA responses (Staswick and Tiryaki, 2004). Since JAR1 has been shown to be related to adenylate-forming enzymes, it could possibly provide also an alternative pathway for JA methylation (Staswick et al., 2002). Studies using the *jin1* mutant (also known as *ATMYC2*) have shown that JA negatively regulate *jin1/ATMYC2* via MKK3-MPK6 cascade (Takahashi et al., 2007). In addition, AtMYC2 has been shown to function as transcriptional activation in ABA signaling (Abe et al., 2003). Furthermore, JA is also capable of regulating ethylene biosynthesis by ACC conjugation (Staswick and Tiryaki, 2004) further demonstrating the interactions between different hormone signaling routes. Experiments with *coi1* have shown that COI1 is required for all JA responses. *coi1* is more susceptible against necrotropich pathogens and to aphid infestation (Ellis et al., 2002; Thomma et al., 1998). Recently, COI1 was reported to be the receptor for JA, binding the JA-Ile conjugate. COI1 was shown to be an F-box protein functioning as a core in jasmonate signaling depending on SCF^{COI1}-type E3 ligases similar to TIR1, an auxin receptor (Chini et al., 2007; Tan et al., 2007; Thines et al., 2007). When JA-Ile binds to the SCF^{COI1} ubiquitin ligase, it leads to subsequent degradation of the JAZ1 repressor protein by ubiquitin-mediated protein degradation leading to transcriptional activation of jasmonate responses (Chini et al., 2007; Thines et al., 2007). Taken together, E3 ligases mediate the transfer of ubiquitin molecules to target proteins that will then undergo proteolytic degradation in the 26S proteasome (Hochstrasser, 1996; Deshaies, 1999). The regulation of protein turnover is a common control element in many processes. Interactions between different hormone signaling routes have been suggested to converge at this point. Nevertheless, further studies will be needed to establish linkage between different signaling routes in the regulation of protein turnover.

JA production in plants leads to induction of many genes, such as *VSP1* (Benedetti et al., 1995) and *PDF1.2* (Penninckx et al., 1998), *CHI-B* and *Thi2.1* (Ellis and Turner, 2001). Overmyer et al. (2000) showed that jasmonate signaling is needed

for the containment of O_3-induced lesion formation. In addition, Rao et al. (2000) showed that pretreatment with MeJA decreased the amount of H_2O_2 formed after O_3 exposure and completely abolished the O_3-induced cell death. Furthermore, Tuominen et al. (2004) showed that JA and ethylene function antagonistically during O_3-induced lesion formation where ethylene can suppress the protective role of JA. A similar effect is seen during wounding; the JA activated genes are down-regulated by ethylene (Lorenzo et al., 2004). In addition, JA and SA signaling are also known to interact. JA and SA signaling may activate different plant defense genes (Thomma et al., 1998) or they can act antagonistically on the same genes (Doares et al., 1995; Felton et al., 1999). In *Arabidopsis*, JA negatively regulates SA-responsive genes (Kloek et al., 2001; Petersen et al., 2000), and the antagonistic effect requires NPR1, the regulatory protein required in SA signal transduction (Cao et al., 1997; Genoud et al., 2002; Kinkema et al., 2000). In conclusion, additional studies will be needed to understand the complexity of JA signaling and to elucidate the signal convergence points on this complicated signaling pathway.

1.8.6 Abscisic acid

Abscisic acid, ABA, is a sesquiterpenoid hormone that has roles in different aspects of plant life, including seed development, adaptation to abiotic stresses like drought, cold and salinity (Finkelstein et al., 2002), sugar sensing together with ethylene (Gazzarrini and McCourt, 2001) and stomatal closure (Kwak et al., 2003). ABA is also reported to be involved in hydrogen peroxide and NO signaling in guard cells (Bright et al., 2006). ABA induces a plethora of signaling events in plants that includes activation of MAP kinases (Lu et al., 2002; Yoshida et al., 2002), protein phosphatases (Leung et al., 1997), RNA binding proteins (Hugouvieux et al., 2001; Lu and Fedoroff, 2000; Xiong et al., 2001) and transcription factors (Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Giraudat et al., 1992; Lopez-Molina and Chua, 2000). Mutant approaches have accumulated interesting data on ABA signaling. For example, *abi1* and *abi2* that both encode for a protein phophatase 2C are strongly ABA insensitive leading to impaired stomatal closure (Finkelstein et al., 2002; Leung et al., 1997). It is known that ROS intermediates one branch of the ABA signaling during

stomatal closure by inducing an increase in cytosolic Ca^{2+} (Lee et al., 1999; McAinsh et al., 1996; Zhang et al., 2001). The involvement of ROS is seen in the NADPH oxidase catalytic subunit single mutant *AtrbohF* and in the double mutant *AtrbohD AtrbohF* that have impaired stomatal closure (Kwak et al., 2003). ABA has an important role in regulating other hormones. For example, increased ethylene production increases the biosynthesis rate of ABA (Grossmann, 2003), and ethylene also modifies glucose signaling through ABA (Gazzarrini and McCourt, 2001; Leon and Sheen, 2003). Furthermore, ABA and ethylene have been shown to act antagonistically (Fedoroff, 2002).

During recent years, exceeding progress has been made in the field of ABA signaling. For example, cyclic ADP ribose has been shown to mediate ABA signaling (Wu et al., 1997), and interestingly, cyclic ADP ribose has been shown act as a second messenger together with ABA in humans (Bruzzone et al., 2007). Several proteins have been indicated to be responsive for the ABA perception in plants: The Mg-chelatase H subunit (also called ABAR) from *Vicia faba* was found to be a putative ABA receptor (Shen et al., 2006; Zhang et al., 2002). It has also been shown to have a role in plastid –to-nuclear signaling and chlorophyll biosynthesis (Mochizuki et al., 2001). In addition, Mg-chelatase H subunit has been shown to have a role in ABA responses in stomatal movement, gene expression and in germination and post-germination growth. However, the molecular mechanism behind this ABA receptor with dualistic functions needs to be further clarified. Another candidate for ABA perception is GCR2 (G PROTEIN-COUPLED RECEPTOR 2). GCR2 is a G protein coupled, membrane-localized ABA receptor and it has been shown to bind ABA in *E. coli* (Liu et al., 2007b). Nevertheless, it is still unclear whether the two GCR2 related genes in *Arabidopsis* have a function in ABA responses. Gao et al. (2007) showed that GCR2 has no role in ABA controlled seed germination. A third protein, FCA (FLOWERING TIME CONTROL PROTEIN A), an RNA-binding protein involved in controlling flowering time was suggested to act as an ABA receptor. Other studies with *fca1* mutant showed that this suggested ABA receptor is not involved in ABA responses in guard cells and seeds (Razem et al., 2006). In addition to these receptors, a membrane bound Leu-rich repeat receptor-like kinase 1 (RPK1) has been suggested to perceive the ABA signal on the plasma membrane (Osakabe et al., 2005).

1.9 O_3-induced cell death cycle, an emerging picture of complex interactions

O_3 induces a complicated web of signaling, including ROS and plant hormones in addition to other signaling components. As concluded earlier, O_3-induced signaling resembles signaling involved in plant-pathogen interactions. Van Camp et al. (1998) proposed a model for cell death during plant-pathogen interaction. In this model, after infection H_2O_2 is produced and this induces the production of ethylene and SA eventually leading to cell death. In addition, pathogen infection leads to production of NO that affects the antioxidant and *PR* gene expression, and therefore could potentiate the cell death cycle. A few years later, Overmyer et al., (2000) isolated a novel O_3 sensitive mutant, *rcd1*. This mutant was described to be sensitive against $O_2^{\cdot -}$, but not to H_2O_2. Using *rcd1* together with mutants involved in ethylene and JA signaling, Overmyer et al. (2000) proposed an O_3-induced cell death cycle -model, based on the model by Van Camp et al. (1998). In this model (Figure 1, adapted from Overmyer et al., 2000 and Van Camp et al., 1998), O_3 or X/XO induces production of $O_2^{\cdot -}$ that leads to cell death. Cell death leads to increased accumulation of $O_2^{\cdot -}$ therefore creating a loop/cycle of amplified cell death. Ethylene together with SA and NO amplifies this cycle (marked as + in Figure 1), whereas JA inhibits $O_2^{\cdot -}$ accumulation and contains lesions (marked as ⊤ in Figure 1). This model is still valid, but many questions remain open on signaling interactions during O_3 exposure, and most likely other hormones and signaling molecules are involved in this cycle.

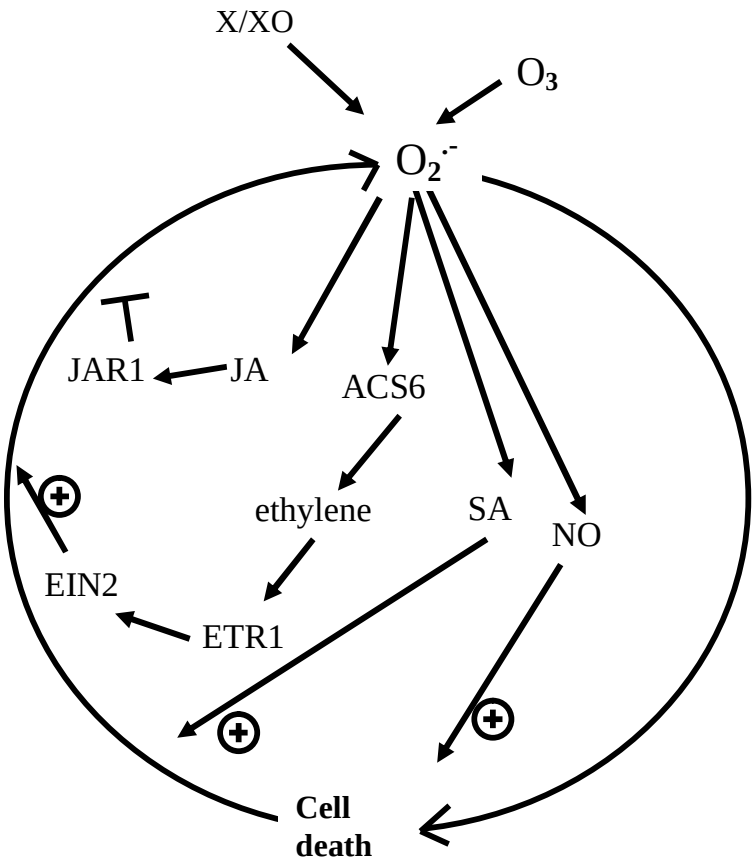

Figure 1. O_3-induced cell death cycle –model, modified from Overmyer et al. (2000) and Van Camp et al. (1998). See text for details.

2. Aims of the present study

The aim of this study was to investigate the interactions behind the O_3-induced cell death signaling in *Arabidopsis thaliana*, the model plant of molecular biology. A mutant approach was used to dissect the signaling routes leading to cell death. The studies I-IV were carried out to elucidate *in planta* how O_3 affects the MAPK signature and how different hormonal mutants differ in this response. Another aim was to map and characterize the O_3 sensitive mutant *rcd1*. Furthermore, it was essential to dissect and compare the nature of cell death found in *rcd1* when compared to Col-0 wild type in order to dissect the nature of cell death in response to O_3. Finally, the function of the signaling molecule NO during O_3-induced signaling and cell death was addressed.

Specific aims were as follows:

1. To clone and characterize the *rcd1* mutant
2. To study how the MAPK signature is affected in hormonal signaling mutants during O_3 exposure
3. To dissect the O_3-induced cell death in *rcd1* mutant
4. To study the role of nitric oxide in O_3-induced cell death

3. Materials and methods

3.1 Plant material

Arabidopsis thaliana Columbia-0 (Col-0) wild type seeds were used in all studies. In addition *ethylene resistant 1* (*etr1*), *ethylene insensitive 2* (*ein2*), *nonexpressor of PR genes 1* (*npr1*) mutants and NahG (over expression of *Pseudomonas putida* gene encoding salicylate hydroxylase that degrades SA into catechol) were used in study (II). In study III, Landesberg *erecta* (L*er*) and *abi2* were used. Knockout mutant of *Atnoa1* (*Arabidopsis nitric oxide associated 1*) were used in study IV. Seeds of ecotypes and mutants were obtained from ABRC (http://www.Arabidopsis.org/abrc) and Nottingham Arabidopsis Stock Centre (http://nasc.nott.ac.uk).

3.2 Growth conditions

Plants were grown under 12 hr photoperiod (light intensity 250 µmol m^{-2} s^{-1}), 22 °C / 18 °C day/night temperature (70% / 90% relative humidity) in controlled environment growth chambers (Weiss model Bio 1300). Plants were sown in potting mixture 1:1 of peat (type B2, Kekkilä, Espoo, Finland) and vermiculite. Seeds were sown at high density and after one week they were transplanted to 7 x 7 cm pots, 5 plants / pot. Plants were sub-irrigated twice a week with tap water.

3.3 O_3 treatments

21-23 day old plants were exposed to single pulse of 350 ppb of O_3 for six to eight hours beginning at 9:00 am, if not otherwise indicated. O_3 was generated as

described in I-IV. Inhibitor studies and other treatments combined with O_3 treatments were done as described in (I-IV).

3.4 Kinase assays

Proteins were extracted in extraction buffer (25 mM Tris pH 7.8, 75 mM NaCl, 15 mM EGTA, 15 mM glycerophosphate, 15 mM 4-nitrophenylpyrophosphate, 10 mM $MgCl_2$, 1 mM DTT, 1 mM NaF, 0.5 mM NA_3VO_4, 0.5 mM PMSF, 10 µg ml^{-1} leupeptin, 10 µg ml^{-1} aprotinin, 0.1% Tween-20). Protein quantifications were performed with Bradford assay (Bio-Rad Laboratories GmbH, Munich, Germany). SDS-PAGE gels were semi-dry blotted onto nitrocellulose membrane (Porablot-NCL; Machery-Nagel, Duren, Germany). MAPK Western blot analyses were performed using phosphor-p44/42 MAPK antibody (Cell signaling technology, Beverly, MA, USA) and blotting grade affinity purified GOAT anti-Rabbit IgG (H þ L)-HRP conjugate (Bio-Rad Laboratories GmbH). Detection was done using ECLþPlus (Amersham-Pharmacia Biotech, Freiburg. Br., Germany) and Kodak BioMax MR-1 film (Amersham Biosciences, Piscataway, NJ, USA). After detection, membranes were stained for total proteins with 1% amido black in 7% v/v acetic acid.

Leaf extracts containing 100 µg of total protein were immunoprecipitated for 1 h at 4 °C together with MAPK-specific antiserum pre-coupled to protein-A Sepharose (Amersham-Pharmacia Biotech). Subsequent washing and in vitro myelin basic protein (MBP) phosphorylation reactions were as described previously (Kroj et al., 2003). Reactions were stopped by the addition of SDS sample buffer and boiling. The proteins were then separated by SDS-PAGE and MBP phosphorylation was determined by phosphorimaging. Two independent experiments with two to five repeats within each experiment were used for quantitative analysis of kinase activity by densitometry of ^{32}P-labeled PAGE-separated MBP using the ImageQuant software (Molecular Dynamics, Krefeld, Germany).

3.5 Gene expression profiling

RNA was extracted with Qiagen-RNeasy Plant kit (Qiagen, Hilden, Germany). Total RNA was separated on formaldehyde-agarose gels and transferred to nylon membrane (Roche, Indianapolis, IN, USA). The membranes were hybridized in Church buffer (Church and Gilbert, 1984). Gene-specific DNA probes (I-IV) were amplified with PCR and labeled with Ready-To-Go DNA Labeling Beads (Amersham Biosciences, Buckinghamshire, UK). The microarray studies (I) containing 6500 genes was hybridized with probes prepared from 23 day old Col-0 and *rcd1-1* RNA. Six biological repeats (each 5 to 10 plants) were pooled into pairs of two, each of the three repeats were labeled with cy3 and cy5 and with the dyes swapped for a total of six hybridizations. The image analysis was with GenePixPro 5.0 (Axon Instruments, Union City, CA). Visually bad spots or areas and low intensity spots were excluded. Low intensity spots were determined as spots where <55% of the pixels had intensity above the background þ1 SD in either channel. The GenePixPro 5.0 data was imported into GeneSpring 6.0 (Silicon Genetics, Redwood City, CA) and normalized with the Lowess method. The background subtracted median intensities were used for calculations. Expression of 92 stress- and defense-related genes was characterized in samples collected from 3-week-old plants with macroarrays described in Overmyer et al. (2000) and Tuominen et al. (2004). Gene expression was quantified by hybridization of a ^{33}P-labeled cDNA probe prepared from each mRNA sample and normalized by division with the mean expression level of two constitutively expressed genes, ACT2 (At3g18780) and ACT8 (At1g49240).

3.6 Cloning and complementation of *rcd1*

Visual identification of the recessive *rcd1* habitus was used to select 2000 homozygous *rcd1/rcd1* individuals from more than 10,000 F2 progeny of *rcd1* x Ler cross. Plants were genotyped with simple sequence length polymorphic and cleaved-amplified polymorphic sequence markers. Candidate genes were amplified from *rcd1-1* using Pfu polymerase (Promega, Madison, WI) and sequenced with internal primers. BLAST and PSI-BLAST searches (Altschul et al., 1990) of the nonredundant protein

database (National Center for Biotechnology Information) were performed to find homologs of RCD1.

For complementation, *RCD1* and *rcd1* cDNAs were prepared from leaf total RNA by RT-PCR according to the manufacturer's instructions (One-Step RT-PCR; Qiagen, Hilden, Germany) using gene-specific primers (59-TTACAATCCACCTGCACCTTC- 39 and 59-ATGGAAGCCAAGATCGTCA-39) and Hot Start Taq DNA polymerase (Promega). PCR products were cloned into pGEMTEasy vector (Promega), confirmed by sequencing, cloned (NotI) into pART27 binary vector (Gleave, 1992), and introduced into *Agrobacterium tumefaciens* strain C58C1pGV2260 by electroporation. Plants were transformed using the floral dip method (Clough and Bent, 1998). Kanamycin-resistant T1 plants were confirmed by PCR and DNA gel blot analyses. As a complementation test, surface-sterilized T2 seeds were germinated on 1% agar MS plates containing 1.0 mM paraquat. To determine O_3 sensitivity, 21- to 28 day old T2 plants were exposed to O_3 for 4 h with 300 ppb. Cell death was measured by ion leakage from rosette leaves as described in Overmyer et al. (2000).

3.7 NO staining

NO staining was performed according to (Guo et al., 2003). Leaves were stained with 15 μM DAF-FM-DA (4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate, Molecular Probes) in loading buffer (5 mM MES/KOH, pH 5.7, 0.25 mM KCl, 1 mM $CaCl_2$). Leaves were collected from plants exposed to 350 ppb of O_3 for 0.5, 1.5, 3.0, 8.0 hours, additional samples were taken 24 hours after the start of an 8 hour O_3 exposure. Leaves were collected into 2 ml eppendorf tubes covered with foil and incubated in dark for 30 min. Thereafter leaves were placed into loading buffer only. Fluorescent signals were detected using a confocal microscope (Leica TCS SP2 AOBS). The dye was excited at 488 nm, and images were collected at emission 515-560 nm. To visualize background cells chlorophyll fluorescence was collected with other channel at 600-650. For whole plant fluorescence measurements 4 plants/time point together with 4 control plants/time point were weighted and frozen in liquid nitrogen. Samples were ground in liquid nitrogen and diluted to DAF-FM-DA buffer (5mM MES-KOH pH 5.7,

0.25 mM KCl, 1 mM $CaCl_2$). Samples were centrifuged 2 x 15 000 rpm for 5 min, +1 °C. Samples were pipetted to 96 well fluorescence-free plate with 15 µM DAF-FM-DA final concentrations using DMSO as background controls. Fluorescence was measured with Versa fluorometer.

3.8 Hormone treatments

For ABA treatment, sterilized seeds were sown on medium containing half-strength MS salts (Sigma-Aldrich, St. Louis, MO), 0.1% MES, 2% sucrose, and 0.8% Bacto-agar, pH 5.7. After vernalization for 3 days at 48 °C, the plates were incubated in controlled growth chambers (Sanyo, Sakata, Japan) at 22 °C temperature and 70 % relative humidity under a 12 h photoperiod. At 4 d, seedlings were transferred to 12- well plates containing 1.5 ml of the same medium. At 14 d, 200 ml of ABA, ACC, and/or MeJA solutions were added to the plates. Plants were harvested after 48 h. For triple response assay, surface-sterilized seeds were sown on MS with 0, 1, or 3 mM ACC, vernalized for 5 d at 4 °C, and incubated in a growth chamber at 22 °C temperature and 70% relative humidity in darkness for 3 d. To assess glucose sensitivity, seeds were sown on MS plates supplemented with 0, 2, 4, or 6% glucose and incubated in the same conditions except for constant light for 4 d.

3.9 Hormone measurements

JA and SA were extracted and quantified with [1,2-^{13}C]JA and [^{13}C1]SA as internal standards as described by (Baldwin et al., 1997), with the modifications described by (Vahala et al., 2003). For ethylene measurements 21-22 d old plants were collected and sealed in 14 ml glass vials with 500 µl water. Plants were incubated for 2 hours and 1 ml sample was analyzed by a flame ionization gas chromatograps (Varian 3700, Varian Inc. Walnut Creek, CA, USA) equipped with a porapak Q column (80-100 mesh, 1 m x 3.2 mm). Column, injector and detector temperatures were 40 °C, 150 °C, and 200 °C, respectively.

4. Results and discussion

4.1 RCD1 belongs to a novel protein family with a potential role in protein-protein interactions

Initially, the *rcd1* mutant was isolated as an O_3 sensitive mutant from EMS-mutagenized seed. It develops lesions in response to extracellular superoxide and O_3. The lesions in *rcd1* are not induced by hydrogen peroxide and once the lesion formation is initiated, lesions rapidly expand from leaf margins through intervascular tissue (Overmyer et al., 2000). In addition to the sensitivity against extracellular ROS, *rcd1* is tolerant against paraquat (I), which produces $O_2^{\cdot -}$ in the chloroplasts. Besides altered $O_2^{\cdot -}$ sensitivity, *rcd1* has other phenotypes, including slightly smaller, more erect rosette, altered, curvy leaf shape and earlier flowering (I, Overmyer et al., 2000).

The mutation locus for O_3 sensitive *rcd1* was positioned to chromosome 1, to the gene At1g32230 (I). The mutation in *rcd1* was due to a single C-to-T transition on the antisense strand resulting in a G-to-A transition at the splice site on exon-intron junction leading to a premature stop codon (I). Complementation tests over-expressing the mutant form of *rcd1* in Col-0 confirmed the phenotypes (I). Another allele of *rcd1*, *rcd1-2*, has a similar kind of missense mutation and a premature stop codon (Fujibe et al., 2004). The predicted RCD1 protein is presumed to be targeted to nucleus (I). Supporting the nuclear localization, in yeast-two-hybrid experiments RCD1 interacts with several transcription factors, such as DREB2A (Belles-Boix et al., 2000). Indeed, later on Katiyar-Agarwal et al. (2006) showed that RCD1 is located in nucleus.

RCD1 belongs to a gene family of six members (I, Fig. 2). The other members of the family are called as SIMILAR-TO-RCD-ONE 1-5 (SRO1-5). According to database searches, SRO1 shares the highest similarity, 76 % with RCD1. SRO2-5 share only 43-49 % similarity with RCD1 and they lack the predicted WWE-protein-protein interaction site (I, Fig. 2). The mutations in the two alleles of *rcd1*, *rcd1-1* and *rcd1-2*, lead to premature stop-codon (Fig. 2) and therefore leading to truncated protein (II, Fujibe et al., 2004).

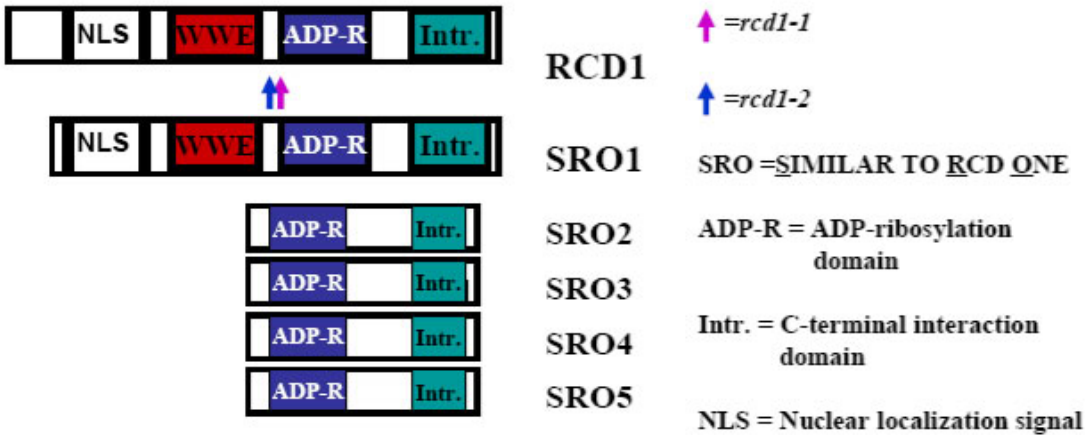

Figure 2. RCD1 is a part of protein family of six members that all harbor the predicted ADP-ribosylation domain and the C-terminal protein-protein interaction domain. Arrows indicate the position of *rcd1-1* and *rcd1-2* mutations.

Previously, Aravind (2001) described a WWE-module, found from proteins associated with ubiquitination and poly-ADP-ribosylation. WWE was named for the two conserved tryptophan residues and one conserved glutamate residue found in the sequence. While searching for evolutionary conservation, Aravind (2001) found WWE homologs from animals and one WWE homolog from *Arabidopsis*. This *Arabidopsis* homolog was RCD1. The appearance of a WWE domain in RCD1 (I, Fig. 2) suggests its involvement in protein-protein interactions and possibly also in cell-to-cell signaling. Studies using *Drosophila* deltex-proteins involved in Notch signaling pathway showed that WWE domain architecture resembles the architecture of ubiquitin ligases. Tandem pair of WWE domains has been shown to form clefts for ligand binding in Deltex-proteins involved in cell fate determination (Zweifel et al., 2005). Deltex-proteins with WWE domains are known to interact with Notch receptors that are involved in cell fate determination during development and throughout life (Kanwar and Fortini, 2004). Notch signaling is an evolutionary conserved mechanism that transmits signals between cells that are directly contacted. The signal initiates when a ligand binds to a Notch-receptor leading to its proteolysis, releasing an intracellular signaling fragment leading to transcriptional regulation of downstream nuclear genes (Fostier et al., 1998; Selkoe, 2003). The mutations in Notch pathway have been connected to a wide variety of diseases in humans, such as T cell lymphomas, neurological disorders and developmental disorders (Artavanis-Tsakonas et al., 1999; Bulman et al., 2000;

Weng et al., 2004). Previously, Belles-Boix et al. (2000) showed that RCD1 is capable to interact with its C-terminal domain with proteins involved in salt and dehydration stress, putative transcription factors and with other proteins with regulatory function (I). RCD1 has been shown to bind to itself (Lin and Heaton, 2001), but future studies will show, whether RCD1 is also able to interact with other proteins/ WWE domains with its N-terminal WWE-domain.

In addition to the WWE domain, RCD1 and the other members of the gene family are predicted to have an ADP-ribosylation domain (I, Fig. 2.). Mono-ADP-ribosylation is a reversible, covalent posttranslational modification of proteins in which the ADP-ribose moiety of NAD^+ is transferred to an acceptor protein with the simultaneous release of nicotinamide. In mammals, mono-ADP ribosylation has been demonstrated for heterotrimeric GTP-binding proteins, small GTPases, ER-resident glucose regulatory protein 78, tubulin, actin, elongation factor 2, mitochondrial glutamate dehydrogenase (GDH), and for histones. In addition, mono-ADP-ribosyltransferases have been shown to ribosylate free amino acids, DNA and RNA. Both, mono-ADP ribosylation and poly-ADP ribosylation have been connected with cell death processes in animals (Hassa et al., 2006). It is known that the over-activation of poly-ADP ribose polymerase by massive DNA damage causes cell death via NAD^+ and ATP depletion in mammals (Berger and Petzold, 1985). In humans, poly-ADP-ribosylation has been shown to mediate glutamate-nitric oxide neurotransmissions (Pieper et al., 2000).

Unfortunately, poly- and mono-ADP ribosylation in plants is not yet as well understood as in mammalian field. Thus far, ADP-ribosylation activity for RCD1 has not been shown, but future studies will hopefully give further information. Nevertheless, the potential role of RCD1 in ADP-ribosylation suggests that the protein could be involved in direct modification of target proteins.

4.2 *rcd1* has functional early O_3-induced signaling but has altered NO-SA levels

Sensing ROS and early ROS-induced signaling are important factors in determination between defense and cell death. O_3 enters the plant leaf through the open stomata and it immediately reacts with cell walls and finally with plasma membranes

creating ROS in the apoplastic space of the leaf. *rcd1* has more open stomata, but it is completely capable of closing stomata as wild type Col-0 (I). In principle, the more open stomata could, at the early time points, allow more O_3 to enter the leaf therefore increasing the toxic effect of O_3 in *rcd1*. However, most likely the signaling events leading to O_3 sensitivity in *rcd1* are more complex.

A rapid production of NO in the stomata was found to be a characteristic incident during the first steps of sensing the ROS in O_3 exposed plants (IV). Later on this NO production was seen to spread to the adjacent epidermal cell and finally to mesophyll cells in studied plants (IV). These results suggest that NO production is an important feature of early O_3-induced signaling on the stomata level. *rcd1* produced more NO in clean air conditions compared to Col-0 and this appeared to be derived from guard cells (IV). In addition, *rcd1* has more open stomata (I). Katiyar-Agarwal et al. (2006) showed using GUS fusions that the *RCD1* promoter is active in stomata. These results raise a question of the possible interaction between NO and RCD1. For example, since NO is known to induce stomatal closure (Desikan et al., 2002; García-Mata and Lamattina, 2002), and *rcd1* has more open stomata (I), there is a possibility that *rcd1* produces more NO in order to close its stomata. However, this is an unlikely situation, as *rcd1* has normal stomatal responses (I). Nevertheless, it is still possible that the increased NO level in clean air affects the O_3-induced signaling in *rcd1*.

NO is known to affect the gating of Ca^{2+} -dependent K^+ channels, K^+ channels, Ca^{2+} and Na^+ channels (Bolotina et al., 1994; Tang et al., 2001; Renganathan et al., 2002; Sokolovski and Blatt, 2004) and induce stomatal closure (Desikan et al., 2002; García-Mata and Lamattina, 2002) demonstrating that NO is an important signalling molecule in the early signalling leading to stomatal closure. *rcd1* produces more NO in clean air condition (IV) as well as SA under O_3 (III) when compared to wild type. Previously, Yu et al. (2000) identified a mutant named as *defense no death1*, *dnd1*. DND1 is known to encode the same protein as AtCNGC2, which was previously identified as a cyclic nucleotide gated channel that can allow passage of Ca^{2+}, K^+ and other cations (Leng et al., 1999; Yu et al., 2000). The *dnd1* mutation has been shown to cause constitutive systemic acquired resistance and exhibit elevated levels of SA (Clough et al., 2000). Recently, Ali et al. (2007) showed that treatment of *dnd1* with SNP (an NO producing chemical) partially overrides the lack of HR in *dnd1* in response to an avirulent pathogen. This led to a suggestion that NO is required but not sufficient for the signaling pathway leading to HR. The results seen in (III) support this finding,

given that O_3 induces ROS production, including H_2O_2 (III), SNP treatment did not induce cell death, but combined O_3 and SNP treatment had an additive impact on cell death (IV). Previously, NO and H_2O_2 have been shown to co-operate in cell death (Zago et al., 2006; Zaninotto et al., 2006). In addition, a tight link has been shown between H_2O_2 and SA balance during stress (Mateo et al., 2006). Recently, Ogawa et al. (2007) demonstrated that SA is synthesized in *Arabidopsis* mainly through isochorismate (ICS) pathway under O_3. They also showed that this O_3-induced SA signaling down-regulates SA biosynthesis. The results in shown in (IV) using SNP and O_3 treatment both separately and together show that the genes involved in SA biosynthesis or accumulation (ICS1 and ALD1) are up regulated in both treatments, but the O_3 activation is attenuated when the two treatments are combined (IV). Also, results shown in (III) indicate that SA is involved in lesion propagation (III). These results suggest that NO could also have a role in lesion containment by attenuation of SA biosynthesis and accumulation genes, in addition to a role in lesion initiation. Moreover, results in (IV) support the tight interaction between ROS-SA (Mateo et al., 2006) emphasizing the ROS-SA balance in the induction of cell death and the feedback regulation of SA. In conclusion, according to these studies it is conceivable that NO has a dualistic function during cell death by involvement in the early steps of ROS signaling besides being involved in lesion containment by modification of NO-SA balance. Nevertheless, further studies are needed to elucidate how NO modifies SA biosynthesis during cell death.

4.3 O_3 activates MAPKs and functional hormone signaling is needed for an appropriate MAPK response in *Arabidopsis* mutants during O_3 exposure

Plant MAPK cascades are known to be involved in signaling cascades during biotic and abiotic stresses, as well as, during development. Two *Arabidopsis* MAPKs, AtMPK3 and AtMPK6 and their orthologs in other plant species have been shown to be activated in response to stress (Desikan et al., 2001; Ichimura et al., 2000; Kovtun et al., 2000; Nuhse et al., 2000; Yuasa et al., 2001; Zhang and Klessig, 1998). In more detail, O_3 has been shown to quickly activate ERK-type MAPKs, more

specifically SIPK and WIPK in tobacco (Samuel et al., 2000; Samuel and Ellis, 2002). The intrest in paper (II) was to study O_3-induced MAPK activity *in planta*, using *Arabidopsis* (II). In addition, the intrest was in the translocation of the two MAPKs, AtMPK3 and AtMPK6, *in planta* during O_3 exposure (II). Indeed, O_3 induced MAPK activation and translocation of AtMPK3 and AtMPK6 from cytosol to nucleus (II) where they phosphorylate transcription factors (Asai et al., 2002) thereby most likely affect gene expression.

Thus, O_3 activated both AtMPK3 and AtMPK6 (II). Logically, this kind of protein kinase activation also requires a phosphatase for counteraction in order to reset the system. In accordance with this, Lee and Ellis (2007) described that AtMKP2, a MAPK phosphatase, inactivates both AtMPK3 and AtMPK6 under O_3. In addition, AtMKP2 is translocated to nucleus as a response to O_3 (Lee and Ellis, 2007). This confirms the results shown in (II) that the signaling pathway needed for the O_3 response requires the transportation of AtMKP3/6 to nucleus in order to affect gene expression (II). Furthermore, this illustrates the signaling cascade components in the early O_3-induced signaling; In order to balance and down-regulate O_3-induced signal leading to changes in gene expression, AtMPK2 is also transported to nucleus for the counterbalancing phosphatase action thereby resetting/modifying the signal pathway by inactivating AtMPK3/6 (Lee and Ellis, 2007).

Besides the activation and translocation of AtMPK3 and AtMPK6 during O_3 exposure, emphasis was made in (II) in finding out how the MAPK signature is affected in different *Arabidopsis* mutants defective in hormone signaling pathways known to be involved in O_3 responses. The ethylene pathway was addressed using *etr1* and *ein2*, both involved in ethylene perception and downstream signaling. The JA pathway was addressed using *jar1*, a mutant resistant to JA. SA signaling pathway was addressed using NahG (incapable in accumulating SA) and *npr1* (impaired in SA signal transduction) (II). In addition, MPK signature was also studied in the O_3 sensitive mutant *rcd1* (III):

AtMPK3 activation was found to be partially dependent on functional SA signaling (II). The impairment of either SA production (NahG) or insensitivity (*npr1*) lead to lowered AtMPK3 protein levels in NahG and *npr1*, therefore functional SA signaling is needed for the accumulation of AtMPK3 in clean air and in response to O_3-induced stress (II). Another aspect on the SA-MPK signaling pathway gave the result shown in (III) that O_3 sensitive *rcd1* has a lowered AtMPK3 activity level in response to

O_3 (III). Since *rcd1* produces more SA in response to O_3 (III), it suggests that *rcd1* is somehow defective in functional SA-MPK signaling in a similar manner as the mutants NahG and *npr1* in study (II). Overall, a typical feature for many lesion mimic mutants is the high accumulation of SA (Lorrain et al., 2003). Furthermore, the double mutant studies showed that blocking SA production in *rcd1* gives only partial protection against O_3 (III). This suggests that the O_3-induced HR-like cell death in *rcd1* is not due to the changes in SA hormone levels *per se*, but is more likely due to misfunction in SA signaling or SA feedback regulation. In addition, these data further support the argument that functional SA signaling is needed for the O_3-induced MAPK activity (II). Nevertheless, more studies will be needed to dissect this signaling route in more detail.

MAPK actions in ethylene pathway were studied using *etr1* and *ein2* mutants (II). *etr1* exhibits slightly prolonged AtMPK3 and AtMPK6 activity whereas no major differences in AtMPK3 and AtMPK6 activity were seen between Col-0 and *ein2*. As concluded in (II), this suggests that ethylene might have a secondary negative regulatory role on AtMPK3/6 activity in O_3-exposed plants. Then again, *rcd1* has an earlier peak activity of AtMPK6 (III) and earlier and higher rate of ethylene biosynthesis than Col-0 as a response to O_3 (Overmyer et al., 2000). This could be due to the more open stomata found in *rcd1* (I). This might facilitate more O_3 to enter the plant leaf at the early time points of the O_3 exposure (II), but there are alternative reasons for the early AtMPK6 activation.

Spanu et al. (1994) used kinase inhibitors to prove that kinases are needed for keeping ACS active and thereby keeping the ethylene biosynthesis active. Kim et al. (2003) showed that expression of the activated form of NtMEK2 activated SIPK (corresponding to the *Arabidopsis* MPK6) and resulted in an increase in ethylene biosynthesis via increasing the ACS activity post-transcriptionally. In addition, studies with loss of function *mpk6* mutants showed that AtMPK6 phosphorylates serine residues of ACS6 and ACS2 leading to accumulation of the ACS6 and ACS2 proteins and cellular ACS activity and thereby to a higher ethylene production. Phosphorylation of ACS6 and ACS2 prevents their degradation by the ubiquitin-26S-proteasome pathway (Liu and Zhang, 2004). These data suggests that the alterations in ethylene biosynthesis in *rcd1* could be due to the post-translational misregulation of AtMPK6 during the O_3 response. This is explained by an earlier AtMPK6 activity in *rcd1* (III), which in turn would activate ACS6 earlier compared to wild type. Thus, this would prevent the degradation of ACS6 and thereby ACS6 activity would remain high in *rcd1*.

This suggests a role for RCD1 in ethylene responses and/or that RCD1 is needed for the fine-tuning of AtMPK6 activity and thereby ACS6 activity.

In conclusion, the results in (II) support the fact that there are most likely other different/parallel pathways where AtMPK6 action lies. Ouaked et al. (2003) suggested that AtMPK6 would act downstream of *ctr1*, a putative MPKKK found in ethylene pathway. This would mean that if AtMPK6 activity lies downstream of CTR1 the AtMPK6 activity should be blocked in *etr1* mutant. As mentioned previously, criticism against this observation has been depicted (Ecker, 2004). In accordance with Ecker (2004), the data in (II) indicated that *mpk6* would not act on this pathway, but rather has a role in a parallel pathway. This assumption is supported by the very recent publication by Joo et al. (2008), where they described a parallel pathway in ethylene signaling downstream form CTR1 that included a MKK9-MPK3/MPK6 cascade. Nevertheless, it appears that MAPK-hormone signaling includes complex feed-back regulation between hormone biosynthesis and perception. Therefore more studies are needed to further clarify the possible site of MAPK action in hormonal signaling.

4.4 Higher NO production in *rcd1* is possibly a secondary effect from changes in multiple pathways

rcd1 has elevated NO levels in clean air when compared to wild type (IV). Where this excess NO is derived from, remains a question to be studied further in the future, but there are still many aspects to consider. Experiments by Planchet et al. (2005) and Gupta et al. (2005) using tobacco suspension cell cultures of wild type and NR deficient *nia*-double mutants showed that there was still low rates of NO produced. Interestingly, this low production was removed by an AOX inhibitor, SHAM (salicylhydroxamic acid). This made the authors to conclude that the mitochondrial electron transport chain, more precisely cytochrome C oxidase and alternative oxidase, produces continuously small amounts of NO. In addition, Huang et al. (2002) showed that NO treatment induces AOX transcript accumulation and activity levels, and this induction was independent from SA. In contrast, SA has been shown to specifically induce AOX (Norman et al., 2004; Rhoads and McIntosh, 1992). On the other hand, Millar and Day (1996) suggested that AOX plays a role in NO tolerance of higher

plants. This is due to the ability of NO to inhibit cytochrome C oxidase thereby to induce AOX transcription (Millar and Day, 1996; Vanlerberghe et al., 2002). Furthermore, Amirsadeghi et al. (2006) showed that tobacco plants lacking AOX are less susceptible than the wild type against SA- and NO-induced cell death. These are very interesting observations, since *rcd1* is known to have higher SA levels (III) and approximately 10 times higher *AOX1a* transcript levels than wild type (I). Whether higher AOX transcript levels lead to actual higher AOX protein levels in *rcd1* remains to be shown, but it is tempting to speculate about the reasons for higher transcript levels. The higher NO levels in *rcd1* could be due to the possible higher amount of AOX1a protein in *rcd1* and therefore this would lead to higher "background" levels of NO produced by mitochondrial electron transport chain during clean air conditions.

Another source for the higher NO levels in *rcd1* could be a secondary effect through ethylene biosynthesis. *rcd1* has been shown to produce more ethylene in clean air conditions (Overmyer et al., 2000) and it has higher ACC synthase (Overmyer et al., 2000) and ACC oxidase (I) transcript levels than wild type. Ethylene has been shown to upregulate AOX transcript levels (Ederli et al., 2006). An important side product from ethylene biosynthesis is cyanide that is produced from the ACC, the precursor of ethylene (Goudey et al., 1989). Therefore elevated ethylene levels will inevitably lead to higher cyanide accumulation that is detoxified either by sulfurtransferases or ß–cyano-L-alaninen synthase (Maryama et al., 2001; Meyer et al., 2003; Westley, 1973; 1981). Furthermore, cyanide has been shown to induce AOX transcript in tobacco and maize by inhibiting cytochrome C oxidase (Polidoros et al., 2005; Sabar et al., 2000). Taken together, with increased NO and cyanide levels that both can inhibit cytochrome C oxidase (Huang et al., 2002) and thereby increase the AOX transcript levels, these two possible routes could also lead to increased AOX transcript levels seen in *rcd1* (I).

A possible route for elevated NO levels and AOX transcript levels is also the polyamine biosynthesis pathway. Polyamines are synthesized in *Arabidopsis* from arginine by arginine decarboxylase, using S-adenosyl-methione (SAM/AdoMet, the intermediate product from ethylene biosynthesis) that is synthesized by MAT/SAM synthase, which activity is inhibited by NO (Lindermayr et al., 2005; Lindermayr et al., 2006). Interestingly, *rcd1* has been shown to have elevated levels of arginine (Sipari, Blomster, Keinänen and Kangasjärvi, unpublished results), which could provide the precursor for elevated polyamine levels through the polyamine biosynthesis route.

Furthermore, polyamines have been suggested to activate NO production in plants (Tun et al., 2006; Yamasaki and Cohen, 2006). Arginine is also known to be the starting material for NOS-type NO synthesis. However, NOS produces NO and citrulline in 1:1 proportion to arginine, and *rcd1* has less citrulline than wild type (Sipari, Blomster, Keinänen and Kangasjärvi, unpublished results), thus it is unlikely that the NO would be derived from NOS-type reaction.

Finally, *rcd1* has higher paraquat tolerance than Col-0 (I), which has also been shown to be a consequence of elevated NO levels in rice (Hung et al., 2002). In Figure 3, all these NO interaction-aspects are shown with respect to the higher induction/expression in *rcd1* (arrows).

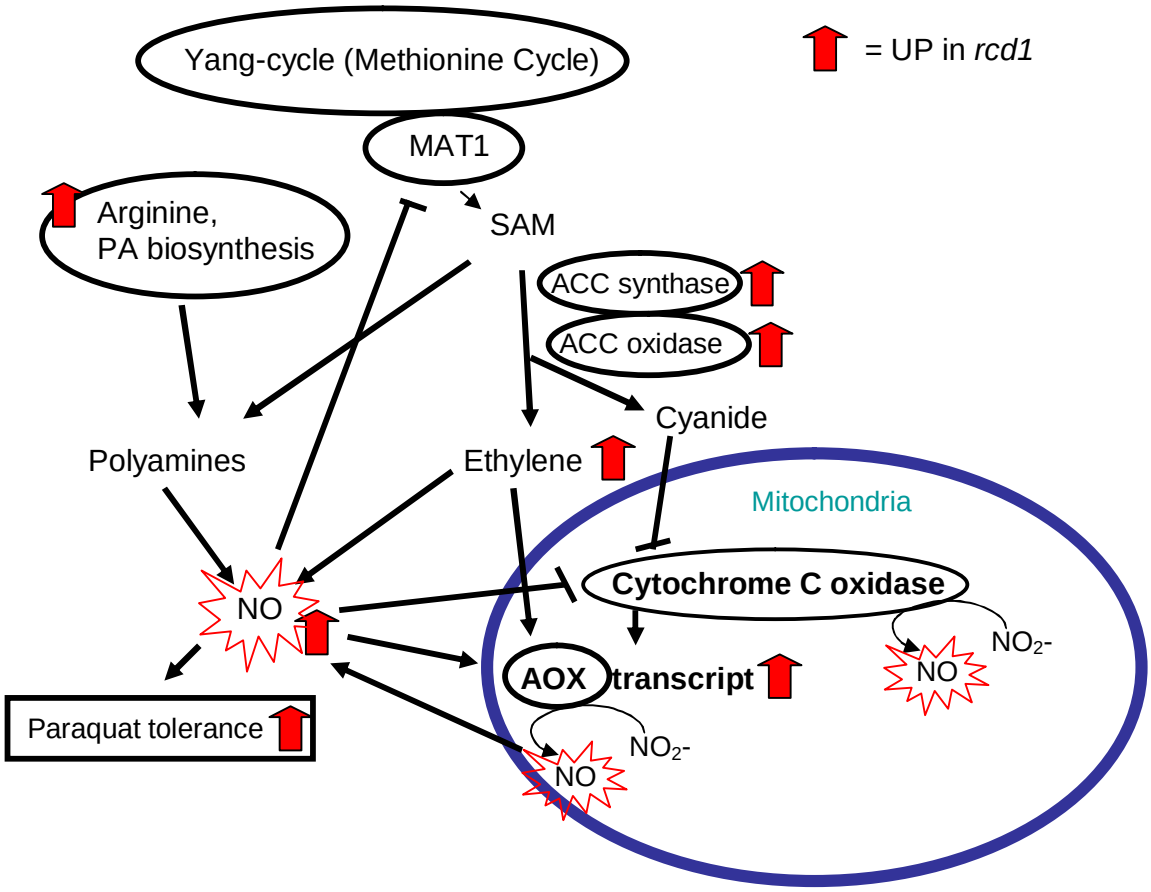

Figure 3. Possible NO interactions found in plants with respect to changes found in *rcd1* (arrows), see text for further information.

4.5 *rcd1* has altered ethylene and ABA signaling responses

The *rcd1* mutant has many phenotypes; including earlier flowering, curlier leaves, O_3 sensitivity, alterations in hormone perception and regulation of gene expression (I). Besides these, *rcd1* has a partial glucose insensitive phenotype (I). *rcd1* is more tolerant against glucose than Col-0 and *ein2* is glucose hypersensitive (I). Interestingly, the *rcd1 ein2* double mutant is only partially insensitive to glucose (I) suggesting a role for RCD1 in sugar signaling. One of the first indications of the cross-talk of ethylene and sugar signaling pathways were presented by Zhou et al. (1998) who published the mutant *gin1* (*glucose insensitive 1*) and revealed the connection between the sugar and ethylene signaling pathways downstream of ETR1. Later on, this *gin1* mutant was actually found to be the *aba2* mutant that has altered ABA biosynthesis. *ABA2* has been suggested to act as a late stress-responsive gene in fine tuning ABA biosynthesis under stress (Lin et al., 2007). A similar kind of ethylene and ABA interaction can be seen in *rcd1* (I). *rcd1* is ABA insensitive and has alterations in ABA responsive genes (I). Also, *rcd1* displays normal triple response phenotype, but has higher ethylene production and altered ethylene biosynthesis related gene expression (II, III). This led Wang et al. (2002) to suggest a role for RCD1 as a regulator of ethylene production. It is intriguing that *aba2* shows a similar, although stronger, sugar insensitive phenotype with connection to ethylene signaling as *rcd1*. In addition, microarray- and macroarray together with northern analyzes show differences in expression of ABA related gene expression levels in *rcd1* (II, III). Furthermore, *rcd1* has impaired induction of some of the ABA and cold responsive genes together with lower basal levels of RAB18 mRNA (I). This suggests that *rcd1* is not deficient in ABA biosynthesis, but it is rather deficient in ABA signaling.

4.6 RCD1 has a role in stress signaling

An interesting feature of the many roles of *rcd1* is its role in salt stress induced signaling. Studies with *sos* (*salt overly sensitive*) mutants have shown that salt stress involves Ca^{2+}-signaling, which is sensed by SOS3 (Ishitani et al., 2000; Zhu et al., 1998). SOS3 then activates SOS2, a serine/threonine kinase (Halfter et al., 2000; Liu et

al., 2000). Together, SOS2 and SOS3 regulate SOS1, a plasma membrane Na^+/H^+ exchanger (Qiu et al., 2002). Interestingly, Katiyar-Agarwal et al. (2006) showed that this plasma membrane-localized Na^+/H^+ exchanger SOS1 interacts through its predicted cytoplasmic tail with RCD1 *in vitro* and *in vivo* and therefore RCD1 has a role in salt tolerance. In addition, they demonstrated that RCD1 is localized predominantly in nucleus under control conditions, as predicted in (I). Also Fujibe et al. (2006) showed that RCD1 is localized in nucleus. But additionally, Katiyar-Agarwal et al. (2006) showed that RCD1 is localized also in cytosol close to the cell periphery as well as in nucleus during oxidative and salt stress. Since RCD1 interacts with transcription factors (II, Belles-Boix et al., 2000) it is quite natural that it is found in the nucleus, but the cytosolic location under stress (Katiyar-Agarwal et al., 2006) is an interesting observation. This could possibly lead to another branch studied in the salt stress signaling pathway, the so called unfolded protein response, implicated in endoplasmic reticulum stress (ER-stress) signaling. An important phenomenon in ER-stress is the movement of transcription factors by proteins from cytosol to nucleus and the degradation of unwanted proteins. It is tempting to speculate that RCD1 could act as a scaffold protein in the degradation machinery during this response:

During stress, unfolded proteins accumulate to the lumen of the ER since the normal protein folding or secretory responses are inhibited (Urade, 2007). This leads to transit of non-active transcription factors to Golgi-complex. In the Golgi-complex the protein is cleaved yielding a free cytoplasmic domain that is an active transcription factor. This then moves to nucleus to interact with chaperones and eventually leading to transcriptional activation of target genes (Rutkowski and Kaufman, 2004). In *Arabidopsis*, ER-stress has been reported to evoke changes in gene expression involved in up-regulation of chaperones and vesicle trafficking. Also up-regulation of the degradation of unwanted proteins and attenuation of secretory genes, mostly cell wall proteins, was seen (Martinez and Chrispeels, 2003). Recently, Liu et al. (2007a) characterized a salt stress response pathway that resembles an ER-stress response. This pathway involves a substilisin-like serine protease that targets a membrane associated bZIP factor. This AtbZIP17 functions as a stress sensor/transducer. During salt stress AtbZIP17 is cleaved and its N-terminal part is translocated to nucleus where it activates gene expression. Interestingly, *rcd1* shows similar changes in gene expression/protein interaction as seen during ER-stress (I, III, Blomster and Jaspers et al., unpublished, Martinez and Chrispeels, 2003), for example: differential regulation of cold related gene

cor6.6 (I) and peroxidase *ATP24a* (III). Of course, this could be just a common stress response seen in *rcd1*, but it is tempting to speculate that RCD1 could act in the cytosol in the proteosomic pathway involved in ER-stress response and/or in nucleus interacting with transcription factors in response to ER-stress. Nevertheless, further studies will be needed in order to understand the function of dualistic location of RCD1 shown in Katiyar-Agarwal et al. (2006).

Another aspect in salt stress responses and RCD1 was revealed when Borsani et al. (2005) showed that a member of a RCD1 gene family is involved in salt stress responses through a novel signaling mechanism. SRO5 expression is induced by salt and this salt induction is needed for siRNA induction. In addition, they showed that SRO5 forms a natural *cis*-antisense gene pair with *P5CDH* that encodes for a enzyme catalyzing an intermediate found in proline synthesis and catabolism. Disruption of P5CDH in yeast leads to decreased growth and ROS accumulation (Nomura and Takagi, 2004; Deuschle et al., 2001). This interesting genepairing-phenomenon further indicates that RCD1 gene family is an important node in stress signaling. Future studies are needed to show how this gene family modulates cell signaling under stress and what the roles of each individual member of this protein family are.

4.7 *rcd1* is a ROS sensitive mutant that combines different hormonal signaling routes

rcd1 is an O_3 sensitive mutant where O_3 damage is measurable already at two hours after the start of the exposure (Overmyer et al., 2000). Under O_3, *rcd1* exhibits typical characteristics of PCD, such as nuclear shrinkage, chromatin condensation, degradation of nuclear DNA, cytosol vesiculation and accumulation of phenolics and eventually patches of HR-like lesions. These characteristics did not exist in the Col-0 wild type (III).

O_3 exposure is known to increase SA and JA levels in Col-0 wild type (Rao et al., 2000b). In contrast to other lesion mimic mutants, the levels of these hormones are normal in *rcd1* mutant in clean air conditions, but higher under O_3, when compared to Col-0 (III). Nevertheless, double mutant studies showed that PCD in *rcd1* is not entirely dependent on higher levels of SA since deficiency/insensitivity against

SA reduced the cell death phenotypes of *rcd1* only slightly and insensitivity against JA actually increased the cell death in *rcd1* (III). This indicates that both of these hormones modify progress of cell death in *rcd1*.

The *rcd1* mutation causes multiple changes in gene expression as response to hormone treatments, such as impaired induction of CHIB mRNA as a response to ACC treatment and VSP1 induction as a response to MeJA treatment (I). Taken together, with altered sugar sensitivity (I) and differential expression of ABA related genes (I) these results points the way for a role for RCD1 at the cross point of signal transduction pathways and the malfunction of the protein would lead to the many changes seen in the mutant. Further on, the potential interactions with transcription factors and the possible dualistic interaction features of RCD1, having a WWE domain in addition to C-terminal interaction domain, suggest a role for RCD1 on a downstream interaction point. Nevertheless, using these results we cannot separate between the primary and secondary responses in *rcd1* because of the complexity in hormone signaling routes. This could be solved by using an inducible system, such as RNAi-lines, which would facilitate a targeted timing for the disruption of the RCD1 dependent signaling route.

Considering what RCD1 actually does, an appealing role for RCD1 would be “a gatekeeper” –protein, a protein that is part of the final steps when the decisions are made between altered gene expression leading to cell death and/or protein degradation. For comparison, Larsen and Cancel (2004) published an interesting mutant called *rce1* that has changes in basic chitanase and defensin responses in addition to altered ethylene biosynthesis gene expression. *RCE1* was found to encode for a RUB1, which has been demonstrated to function in the covalent attachment of RUB1 to SCF-ubiquitin ligase complex. The authors concluded that RCE1 is a new component regulating ethylene biosynthesis pathway and defense gene expression. It is tempting to speculate a similar kind of role for *rcd1* at the cross point of protein degradation and gene expression regulation. This kind of role is partly supported by the observation of the low expression levels of RCD1 (I) indicating a tight regulation of the *RCD1* gene expression levels. In addition, the probable capability of both C- and N-terminal ends to interact with other proteins (I) and the putative ADP-ribosylation activity of RCD1 further suggest a role in post-translational regulation.

5. Concluding remarks

This study demonstrates the complexity of O_3/apoplastic ROS –induced signaling with emphasis on MAPK-, hormone- and NO- signaling during cell death. O_3 treatment induced transient activation of AtMPK3 and AtMPK6 and this activation was not dependent on ethylene signaling, but ethylene has probable secondary effects on the MAPK function. Conversely, SA signaling was shown to be needed for the full activation of AtMPK3 by O_3. AtMPK3 was shown to respond both transcriptionally and translationally during O_3 exposure. In addition, both AtMPK3 and AtMPK6 were shown to be translocated to nucleus during O_3 exposure where they are likely to phosphorylate target transcription regulators.

Furthermore, this study illustrates the significance of RCD1 on the cross-road of different signaling pathways acting as an integrative node in stress signaling. RCD1 belongs to an (ADP-ribosyl)transferase domain containing family of six proteins. RCD1 harbors a WWE -domain that has been associated with protein-protein interactions. The sensitivity of RCD1 against apoplastic ROS in contrast to the tolerance against chloroplastic $O_2^{\cdot-}$ further defines the multiple levels of plant stress signaling. The altered ethylene and ABA regulated gene expression together with altered ethylene, MeJA, glucose and stomatal responses suggest a role for RCD1 in the convergence point of signaling. In addition, studies defining the cell death phenomena in *rcd1* show that RCD1 operates on the signaling pathway leading to cell death and that functional hormone responses are needed for the O_3 tolerance.

This study shows that NO production is an important hallmark in O_3-induced signaling. NO modifies gene expression under O_3. *rcd1* has altered NO accumulation possibly providing a loop for altered stomatal responses seen in *rcd1* mutant. In summary, this work demonstrates that NO is required but not sufficient for cell death and furthermore, this study emphasizes the importance of ROS-NO balance in O_3-induced cell death.

Studies within this dissertation give us a glimpse on the complexity of the signaling present in plants. As described in previous chapters, the interactions between the components involved in the cell death phenomena have a very complicated nature. This study illustrates these interactions a step deeper, but in addition many new questions have arisen from the results. It is evident that more studies are needed in order

to understand the interactions and counteractions during cell death. The web of O_3-induced signaling resembles a puzzle of many pieces. The pieces are starting to find their place and we are able to recognize the big picture, but more research is needed to finish the puzzle.

6. Future prospects

An important aim in the future is to define the function of RCD1. Finding the interaction partners for the WWE- and C-terminal end of RCD1 will reveal the pathways that RCD1 function on. In addition, the potential ADP-ribosylation activity of RCD1 should be addressed in the future. Further on, the impact of ADP-ribosylation on modifying different signaling routes should be studied. There are also various aspects yet to be solved on how hormonal signaling is deficient / changed in *rcd1*. For example, *rcd1* mutant phenotype indicates changes in auxin signalling. Nevertheless, the role of auxin in *rcd1* mutant in development as well as in response to stress has not thus far been evaluated. In addition, cytokinin signaling in *rcd1* remains to be elucidated. Furthermore, the influence of decreased levels of citrulline found in *rcd1* on stress tolerance should be studied. Illustrating the role of the *RCD1*-gene family in stress-induced signaling will enlighten new aspects on the field of plant stress signaling. In addition, many questions remain open on the interaction between NO and hormone signaling. Therefore, the details in the communication between NO and hormone signaling during stress situations require further evaluation.

Acknowledgements

This work was carried out in Professor Jaakko Kangasjärvi's Plant Stress Group at Plant Biology in the Department of Biological and Environmental Sciences at the University of Helsinki and in Professor Dirk Scheel's group at the Institute of Plant Biochemistry, Halle, Germany. I would like to thank Prof. Jaakko Kangasjärvi, Prof. Kurt Fagerstedt, Prof. Yrjö Helariutta and Prof. Tapio Palva for providing good working environment and facilities in Plant Biology as well as in Genetics and in the Institute of Biotechnology. I would also like to thank Prof. Dierk Scheel for the excellent working environment and facilities in IPB, Halle, Germany.

Academy of Finland and University of Helsinki have financially supported this thesis work, I wish to thank these organizations for making it possible to do this thesis.

I am grateful to Prof. Elina Oksanen and Prof. Eva-Mari Aro for reviewing and commenting this thesis. I would like to thank my supervisor Prof. Jaakko Kangasjärvi for all these years I´ve been in the group. I would also like to thank Dr. Mikael Brosché for excellent scientific correspondence during past years. In addition, I wish to thank all the co-authors.

I started in the group as a master student year 2000 and then continued to doctoral studies 2002. During these years I've come to know so many fantastic persons, that I consider myself extremely lucky to work and study in such an environment. I would like to thank the Kangasjärvi group members, present, former and visitors: Airi Lamminmäki, Hannele Tuominen, Amy Warren, Hannes Kollist, Kirk Overmyer, Maarit Sillanpää, Markku Keinänen, Riikka Pellinen, Pinja Jaspers, Saara Lång, Raili Ruonala, Tiina Blomster, Jorma Vahala, Jarkko Salojärvi, Michael Wrzackzek, Triin Vahisalu, Mikael Brosché, Niina Idänheimo, Tuomas Puukko, Tuula Puhakainen and Annikki Welling, thank you all for creating such an explicit working environment! Tiina –my official symbiotic part at work, my very good and understanding friend, thank you! Annikki- vertaistuen ja reflection kautta voittoon! Thank you for the indispensable friendship! Raili- writing the thesis at the same time and sharing the mutual feelings concerning the process was invaluable, thank you for that and for the enormous support! Pinja- thank you for the friendship, watching your dreadlocks grow has been an efficient reminder how time flies while doing this thesis, thank you! Jorma- thanks for being such a… grand/splendid/good friend and scientist during these years!

Michi Wrrzäkzeks –one more tequila, and one might learn how to write your last name, thanks! Kirk- thanks for insulting me even overseas, but mainly thanks for being such a good friend and scientist (you a_ _e)! Mikael –(Also) The Most Fantastic Person one could ever work with! Thank you! Triin -thank you for being a good friend/neighbor/colleague! Hannes -Boarding completed-Kollist, thank you! Saara -thank you for proving that it is possible to write understandable lab notes! Airi-tehopakkaus-Lamminmäki -thank you! Niina -thank you for the family values. Maarit –always so calm, thank you. Hannele, the Respectable Science Woman, thank you! Tuula -thank you for the friendship! Riikka –I´m so excited and I can't hide it, thank you! Jarkko –the statistic wizard, thank you! Markku –thank you for explaining how to collect saliva from certain bugs, one never knows when that information comes handy.

I would like to thank fellows from the Tapio Palva´s group, Ykä Helariutta´s group and Kurt Fagerstedt´s group. In addition, Assistant Ahlfors expresses gratitude to Assistant Tarja Kariola for being such an excellent Assistant. Gunter Brader –Thank You for always helping me! Elina Helenius –thank you for our motivational corridor chats. Anne Honkanen –thank you for the never ending positive attitude.

I would like to thank Marjukka Uuskallio, Marja Tomell, Arja Ikävalko, Leena Laakso and Pekka Lönqvist for technical help. I wish to thank Arja Välimäki, Pirjo Cantell and Leena Häyrinen for the secretarial help. In addition, I would like to thank the whole Plant Biology personnel.

I wish to thank all the people in IPB, Halle, Germany. Especially I would like to thank Jason Rudd and Violetta Macioszek for the friendship and scientific discussions during the year I spent there.

I would like to thank all my friends outside the university. Especially I would like to thank Jonna, Sannu, Eliisa and Eeva. You have been an important source of energy, friendship and parties; you are especially good in reminding me of the possibility that there might be life outside the university.

I wish to thank my family and relatives for all the support during these years. Especially I would like to thank my mother Seija, father Timppa and Hanne, and my sister Riikka for the encouragement and tolerance during these years. Finally, I would like to thank Jarkko for all the love, support and compassion that made the last steps during this thesis work a lot more enjoyable.

Helsinki, April 2008

References

Abe,H., Urao,T., Ito,T., Seki,M., Shinozaki,K., and Yamaguchi-Shinozaki,K. (2003). Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell *15*, 63-78.

Akashi,K., Miyake,C., and Yokota,A. (2001). Citrulline, a novel compatible solute in drought-tolerant wild watermelon leaves, is an efficient hydroxyl radical scavenger. Febs Letters *508*, 438-442.

Ali,R., Ma,W., Lemtiri-Chlieh,F., Tsaltas,D., Leng,Q., von Bodman,S., and Berkowitz,G.A. (2007). Death don't have no mercy and neither does calcium: Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2 and innate immunity. Plant Cell *19*, 1081-1095.

Alonso,J.M., Hirayama,T., Roman,G., Nourizadeh,S., and Ecker,J.R. (1999). EIN2, a bifunctional transducer of etyhylene and stress responses in *Arabidopsis*. Science *284*, 2148-2152.

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

Alvarez,M.E., Pennell,R.I., Meijer,P.J., Ishikawa,A., Dixon,R.A., and Lamb,C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. Cell *92*, 773-784.

Amirsadeghi,S., Robson,C.A., McDonald,A.E., and Vanlerberghe,G.C. (2006). Changes in plant mitochondrial electron transport alter cellular levels of reactive oxygen species and susceptibility to cell death signaling molecules. Plant and Cell Physiology *47*, 1509-1519.

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

Aravind,L. (2001). The WWE domain: a common interaction module in protein ubiquitination and ADP ribosylation. Trends Biochem. *26*, 273-275.

Artavanis-Tsakonas,S., Rand,M.D., and Lake,R.J. (1999). Notch Signaling: Cell Fate Control and Signal Integration in Development. Science *284*, 770-776.

Asada,K. (1999). The water-water cycle in chloroplasts: Scavenging of active oxygens and dissipation of excess photons. Annu. Rev. Plant Physiol. Plant Mol. Biol. *50*, 601-639.

Asada,K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol. *141*, 391-396.

Ashmore,M.R. (2005) Assessing the future global impacts of ozone on vegetation. Plant, Cell and Environ. *28*, 949-964.

Asai,T., Stone,J.M., Heard,J.E., Kovtun,Y., Yorgey,P., Sheen,J., and Ausubel,F.M. (2000). Fumonisin BI-induced cell death in Arabidopsis protoplasts requires jasmonate-, ethylene-, and salicylate-dependent signaling pathways. Plant Cell *12*, 1823-1835.

Asai,T., Tena,G., Plotnikova,J., Willmann,M.R., Chiu,W.L., Gomez-Gomez,L., Boller,T., Ausubel,F.M., and Sheen,J. (2002). MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature *415*, 977-983.

Baldwin,I.T., Zhang,Z.P., Diab,N., Ohnmeiss,T.E., McCloud,E.S., Lynds,G.Y., and Schmelz,E.A. (1997). Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotine in *Nicotiana sylvestris*. Planta *201*, 397-404.

Beck,K.F., Eberhardt,W., Frank,S., Huwiler,A., Messmer,U.K., Muhl,H., and Pfeilschifter,J. (1999). Inducible NO synthase: Role in cellular signalling. Journal of Experimental Biology *202*, 645-653.

Bedard,K. and Krause,K.H. (2007). The NOX family of ROS-generating NADPH oxidases: Physiology and pathophysiology. Physiological Reviews *87*, 245-313.

Beligni,M.V. and Lamattina,L. (2001). Nitric oxide: a non-traditional regulator of plant growth. Trends Plant Sci. *6*, 508-509.

Belles-Boix,E., Babiychuk,E., van Montagu,M., Inzé,D., and Kushnir,S. (2000). CEO1, a new protein from *Arabidopsis thaliana*, protects yeast against oxidative damage. FEBS Lett. *482*, 19-24.

Benedetti,C.E., Xie,D., and Turner,J.G. (1995). COI1-Dependent Expression of an Arabidopsis Vegetative Storage Protein in Flowers and Siliques and in Response to Coronatine or Methyl Jasmonate. Plant Physiol. *109*, 567-572.

Berger,N.A. and Petzold,S.J. (1985). Identification of Minimal Size Requirements of Dna for Activation of Poly(Adp-Ribose) Polymerase. Biochemistry *24*, 4352-4355.

Bethke,P.C., Badger,M.R., and Jones,R.L. (2004). Apoplastic synthesis of nitric oxide by plant tissues. Plant Cell *16*, 332-341.

Bolotina,V.M., Najibi,S., Palacino,J.J., Pagano,P.J., and Cohen,R.A. (1994). Nitric-Oxide Directly Activates Calcium-Dependent Potassium Channels in Vascular Smooth-Muscle. Nature *368*, 850-853.

Borsani,O., Zhu,J., Verslues,P.E., Sunkar,R., and Zhu,J.K. (2005). Endogenous siRNAs Derived from a Pair of Natural cis-Antisense Transcripts Regulate Salt Tolerance in Arabidopsis. Cell *123*, 1279-1291.

Bright,J., Desikan,R., Hancock,J.T., Weir,I.S., and Neill,S.J. (2006). ABA-induced NO generation and stomatal closure in Arabidopsis are dependent on H_2O_2 synthesis. Plant J. *45*, 113-122.

Bruzzone,S., Moreschi,I., Usai,C., Guida,L., Damonte,G., Salis,A., Scarfi,S., Millo,E., De Flora,A., and Zocchi,E. (2007). Abscisic acid is an endogenous cytokine in human granulocytes with cyclic ADP-ribose as second messenger. Proc. Natl. Acad. Sci. USA *104*, 5759-5764.

Bulman,M.P., Kusumi,K., Frayling,T.M., McKeown,C., Garrett,C., Lander,E.S., Krumlauf,R., Hattersley,A.T., Ellard,S., and Turnpenny,P.D. (2000). Mutations in the human *Delta* homologue, *DLL3*, cause axial skeletal defects in spondylocostal dysostosis. Nature Genetics *24*, 438-441.

Cao,H., Glazebrook,J., Clarke,J.D., Volko,S., and Dong,X. (1997). The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell *88*, 57-63.

Chao,Q., Rothenberg,M., Solano,R., Roman,G., Terzaghi,W., and Ecker,J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell *89*, 1133-1144.

Chen,S.X. and Schopfer,P. (1999). Hydroxyl-radical production in physiological reactions - A novel function of peroxidase. Eur. J. Biochem. *260*, 726-735.

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

Chini,A., Fonseca,S., Fernandez,G., Adie,B., Chico,J.M., Lorenzo,O., Garcia-
Casado,G., Lopez-Vidriero,I., Lozano,F.M., Ponce,M.R., Micol,J.L., and Solano,R. (2007). The JAZ family of repressors is the missing link in jasmonate signalling. Nature *448*, 666-671.

Church,G.M. and Gilbert,W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA *81*, 1991-1995.

Clarke,S.M., Mur,L.A.J., Wood,J.E., and Scott,I.M. (2004). Salicylic acid dependent signaling promotes basal thermotolerance but is not essential for acquired thermotolerance in Arabidopsis thaliana. Plant J. *38*, 432-447.

Clough,S.J., Fengler,K.A., Yu,I.C., Lippok,B., Smith,R.K., Jr., and Bent,A.F. (2000). The *Arabidopsis dnd1 "defense, no death"* gene encodes a mutated cyclic nucleotide-gated ion channel. Proc. Natl. Acad. Sci. USA *97*, 9323-9328.

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

Corpas,F.J., Barroso,J.B., and del Río,L.A. (2001). Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells. Trends Plant Sci. *6*, 145-150.

Crawford,N.M., Galli,M., Tischner,R., Heimer,Y.M., Okamoto,M., and Mack,A. (2006). Plant nitric oxide synthase: back to square one - Response. Trends Plant Sci. *11*, 526-527.

Dangl,J.L., Dietrich,R.A., and Richberg,M.H. (1996). Death don't have no mercy: Cell death programs in plant-microbe interactions. Plant Cell *8*, 1793-1807.

Dangl,J.L. and Jones,J.D.G. (2001). Plant pathogens and integrated defence responses to infection. Nature *411*, 826-833.

Davison,P.A., Hunter,C.N., and Horton,P. (2002). Overexpression of beta-carotene hydroxylase enhances stress tolerance in *Arabidopsis*. Nature *418*, 203-206.

del Rio,L.A., Corpas,F.J., Sandalio,L.M., Palma,J.M., Gomez,M., and Barroso,J.B. (2002). Reactive oxygen species, antioxidant systems and nitric oxide in peroxisomes. J. Exp. Bot. *53*, 1255-1272.

del Rio,L.A., Sandalio,L.M., Corpas,F.J., Palma,J.M., and Barroso,J.B. (2006). Reactive oxygen species and reactive nitrogen species in peroxisomes. Production, scavenging, and role in cell signaling. Plant Physiol. *141*, 330-335.

Delledonne,M. (2005). NO news is good news for plants. Curr. Opin. Plant Biol. *8*, 390-396.

Dempsey,D.A., Shah,J., and Klessig,D.F. (1999). Salicylic acid and disease resistance in plants. Critical Reviews in Plant Sciences *18*, 547-575.

Deshaies,R. (1999). Mechanism and regulation of the SCF ubiquitination pathway. Kidney International *56*, 1186-1187.

Desikan,R., Griffiths,R., Hancock,J., and Neill,S. (2002). A new role for an old enzyme: Nitrate reductase-mediated nitric oxide generation is required for abscisic acid-induced stomatal closure in *Arabidopsis thaliana*. Proc. Natl. Acad. Sci. USA *99*, 16314-16318.

Desikan,R., Hancock,J.T., Coffey,M.J., and Neill,S.J. (1996). Generation of active oxygen in elicited cells of *Arabidopsis thaliana* is mediated by a NADPH oxidase-like enzyme. FEBS Lett. *382*, 213-217.

Desikan,R., Hancock,J.T., Ichimura,K., Shinozaki,K., and Neill,S.J. (2001). Harpin induces activation of the Arabidopsis mitogen-activated protein kinases AtMPK4 and AtMPK6. Plant Physiol. *126*, 1579-1587.

Deuschle,K., Funck,D., Hellmann,H., Daschner,K., Binder,S., and Frommer,W.B. (2001). A nuclear gene encoding mitochondrial triangle(1)-pyrroline-5-carboxylate dehydrogenase and its potential role in protection from proline toxicity. Plant J. *27*, 345-355.

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

Durner,J., Wendehenne,D., and Klessig,D.F. (1998). Defense gene induction in tobacco by nitric oxide, cyclic GMP, and cyclic ADP-ribose. Proc. Natl. Acad. Sci. USA *95*, 10328-10333.

Durrant,W.E. and Dong,X. (2004). Systemic acquired resistance. Annu. Rev. Phytopathol. *42*, 185-209.

Ecker,J.R. (2004). Reentry of the ethylene MPK6 module. Plant Cell *16*, 3169-3173.

Ederli,L., Morettini,R., Borgogni,A., Wasternack,C., Miersch,O., Reale,L., Ferranti,F., Tosti,N., and Pasqualini,S. (2006). Interaction between nitric oxide and ethylene in the induction of alternative oxidase in ozone-treated tobacco plants. Plant Physiol. *142*, 595-608.

Ellis,C., Karafyllidis,L., and Turner,J.G. (2002). Constitutive activation of jasmonate signaling in an Arabidopsis mutant correlates with enhanced resistance to Erysiphe cichoracearum, Pseudomonas syringae, and Myzus persicae. Mol. Plant-Microbe Interact. *15*, 1025-1030.

Ellis,C. and Turner,J.G. (2001). The Arabidopsis mutant *cev1* has constitutively active jasmonate and ethylene signal pathways and enhanced resistance to pathogens. Plant Cell *13*, 1025-1033.

Falcieri,E., Gobbi,P., Zamai,L., and Vitale,M. (1994). Ultrastructural features of apoptosis. Scanning Microscopy *8*, 653-666.

Fedoroff,N.V. (2002). "Crosstalk" in abscisic acid signaling. Science *140*, re10.

Felton,G.W., Korth,K.L., Bi,J.L., Wesley,S.V., Huhman,D.V., Mathews,M.C., Murphy,L.B., Lamb,C., and Dixon,R.A. (1999). Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. Curr. Biol. *9*, 317-320.

Finkelstein,R.R., Gampala,S.S.R., and Rock,C.D. (2002). Abscisic acid signaling in seeds and seedlings. Plant Cell *14*, S15-S45.

Finkelstein,R.R. and Lynch,T.J. (2000). The Arabidopsis abscisic acid response gene ABI5 encodes a basic leucine zipper transcription factor. Plant Cell *12*, 599-609.

Finkelstein,R.R., Wang,M.L., Lynch,T.J., Rao,S., and Goodman,H.M. (1998). The Arabidopsis Abscisic Acid Response Locus ABI4 Encodes an APETALA 2 Domain Protein. Plant Cell *10*, 1043-1054.

Foreman,J., Demidchik,V., Bothwell,J.H.F., Mylona,P., Miedema,H., Torres,M.A., Linstead,P., Costa,S., Brownlee,C., Jones,J.D.G., Davies,J.M., and Dolan,L. (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature *422*, 442-446.

Fostier,M., Evans,D.A.P., rtavanis-Tsakonas,S., and Baron,M. (1998). Genetic characterization of the Drosophila melanogaster Suppressor of deltex gene: A regulator of notch signaling. Genetics *150*, 1477-1485.

Foyer,C.H. and Noctor,G. (2005). Redox homeostasis and antioxidant signaling: A metabolic interface between stress perception and physiological responses. Plant Cell *17*, 1866-1875.

Fujibe,T., Saji,H., Arakawa,K., Yabe,N., Takeuchi,Y., and Yamamoto,K.T. (2004). A methyl viologen-resistant mutant of *Arabidopsis*, which is allelic to ozone-sensitive *rcd1*, is tolerant to supplemental UV-B irradiation. Plant Physiol. *134*, 275-285.

Fujibe,T., Saji,H., Watahiki,M.K., and Yamamoto,K.T. (2006). Overexpression of the *RADICAL-INDUCED CELL DEATH1* (*RCD1*) Gene of Arabidopsis Causes Weak *rcd1* Phenotype with Compromised Oxidative-Stress Responses. Bioscience Biotechnology and Biochemistry *70*, 1827-1831.

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

Gao,Y., Zeng,Q., Guo,J., Cheng,J., Ellis,B.E., and Chen,J-G. (2007). Genetic characterization reveals no role for the reported ABA receptor, GCR2, in ABA control of seed germination. Plant J. *52*, 1001-1013.

García-Mata,C. and Lamattina,L. (2002). Nitric oxide and abscisic acid cross talk in guard cells. Plant Physiol. *128*, 790-792.

Gazzarrini,S. and McCourt,P. (2001). Genetic interactions between ABA, ethylene and sugar signaling pathways. Curr. Opin. Plant Biol. *4*, 387-391.

Genoud,T., Buchala,A.J., Chua,N.H., and Metraux,J.P. (2002). Phytochrome signalling modulates the SA-perceptive pathway in *Arabidopsis*. Plant J. *31*, 87-95.

Gilchrist,D.G. (1998). Programmed cell death in plant disease: The purpose and promise of cellular suicide. Annu. Rev. Phytopathol. *36*, 393-414.

Giraudat,J., Hauge,B.M., Valon,C., Smalle,J., Parcy,F., and Goodman,H.M. (1992). Isolation of the Arabidopsis *ABI3* Gene by Positional Cloning. Plant Cell *4*, 1251-1261.

Goudey J.S., Tittle F.L., Spencer M.S. (1989) A role of ethylene in the metabolism of cyanide by higher plants. Plant Physiology *89*, 1306–1310.

Goyer,A., Johnson,T.L., Olsen,L.J., Collakova,E., Shachar-Hill,Y., Rhodes,D., and Hanson,A.D. (2004). Characterization and metabolic function of a peroxisomal sarcosine and pipecolate oxidase from *Arabidopsis*. Journal of Biological Chemistry *279*, 16947-16953.

Grossmann, Klaus. (2003) Mediation of Herbicide Effects by Hormone Interactions. Journal of Plant Growth Regulation *22*, 109-122.

Guo,F.Q. and Crawford,N.M. (2005). *Arabidopsis* Nitric Oxide Synthase1 is targeted to mitochondria and protects against oxidative damage and dark-induced senescence. Plant Cell *17*, 3436-3450.

Guo,F.Q., Okamoto,M., and Crawford,N.M. (2003). Identification of a plant nitric oxide synthase gene involved in hormonal signaling. Science *302*, 100-103.

Gupta,K.J., Stoimenova,M., and Kaiser,W.M. (2005). In higher plants, only root mitochondria, but not leaf mitochondria reduce nitrite to NO, in vitro and in situ. J. Exp. Bot. *56*, 2601-2609.

Halfter,U., Ishitani,M., and Zhu,J.K. (2000). The *Arabidopsis* SOS2 protein kinase physically interacts with and is activated by the calcium-binding protein SOS3. Proc. Natl. Acad. Sci. USA *97*, 3735-3740.

Hall,A.E., Findell,J.L., Schaller,G.E., Sisler,E.C., and Bleecker,A.B. (2000). Ethylene perception by the ERS1 protein in Arabidopsis. Plant Physiol. *123*, 1449-1457.

Halliwell,B. (1989). Tell Me About Free-Radicals, Doctor - A Review. Journal of the Royal Society of Medicine *82*, 747-752.

Halliwell,B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. *141*, 312-322.

Hassa,P.O., Haenni,S.S., Elser,M., and Hottiger,M.O. (2006). Nuclear ADP-ribosylation reactions in mammalian cells: Where are we today and where are we going? Microbiol. Mol. Biol. Rev. *70*, 789-829.

Heath,R.L. (1987). The biochemistry of ozone attack on the plasma membrane of the plant cells. In Recent Advances in Phytochemistry. Phytochemical Effects of Environmental Compounds, J.A.Saunders, L.Kosak-Channing, and E.E.Conn, eds. (New York: Plenum Press), pp. 29-54.

Hochstrasser,M. (1996). Protein degradation or regulation: Ub the judge. Cell *84*, 813-815.

Huang,X., von Rad,U., and Durner,J. (2002). Nitric oxide induces transcriptional activation of the nitric oxide-tolerant alternative oxidase in *Arabidopsis* suspension cells. Planta *215*, 914-923.

Huang,Y.F., Li,H., Hutchison,C.E., Laskey,J., and Kieber,J.J. (2003). Biochemical and functional analysis of CTR1, a protein kinase that negatively regulates ethylene signaling in *Arabidopsis*. Plant J. *33*, 221-233.

Hugouvieux,V., Kwak,J.M., and Schroeder,J.I. (2001). An mRNA cap binding protein, ABH1, modulates early abscisic acid signal transduction in *Arabidopsis*. Cell *106*, 477-487.

Hung,K.T., Chang,C.J., and Kao,C.H. (2002). Paraquat toxicity is reduced by nitric oxide in rice leaves. J. Plant Physiol. *159*, 159-166.

Ichimura,K., Mizoguchi,T., Yoshida,R., Yuasa,T., and Shinozaki,K. (2000). Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. Plant J. *24*, 655-665.

Ishitani,M., Liu,J., Halfter,U., Kim,C.H., Shi,W and Zhu,J.K. (2000). SOS3 function in plant salt tolerance requires N-myristolytion and calcium binding. Plant Cell *12*, 1667-1677.

Jezek,P. and Hlavata,L. (2005). Mitochondria in homeostasis of reactive oxygen species in cell, tissues, and organism. International Journal of Biochemistry & Cell Biology *37*, 2478-2503.

Johnson,P.R. and Ecker,J.R. (1998). The ethylene gas signal transduction pathway: A molecular perspective. Annu. Rev. Genet. *32*, 227-254.

Jonak,C., Okresz,L., Bogre,L., and Hirt,H. (2002). Complexity, cross talk and integration of plant MAP kinase signalling. Curr. Opin. Plant Biol. *5*, 415-424.

Joo,J.H., Bae,Y.S., and Lee,J.S. (2001). Role of auxin-induced reactive oxygen species in root gravitropism. Plant Physiol. *126*, 1055-1060.

Kanwar,R. and Fortini,M.E. (2004). Notch Signaling: A Different Sort Makes the Cut. Curr. Biol. *14*, 1043-1045.

Karpinski,S., Reynolds,H., Karpinska,B., Wingsle,G., Creissen,G., and Mullineaux,P. (1999). Systemic signaling and acclimation in response to excess excitation energy in *Arabidopsis*. Science *284*, 654-657.

Katiyar-Agarwal,S., Zhu,J., Kim,K., Agarwal,M., Fu,X., Huang,A., and Zhu,J.K. (2006). Colloquium Paper: The plasma membrane Na+/H+ antiporter SOS1 interacts with RCD1 and functions in oxidative stress tolerance in *Arabidopsis*. Proc. Natl. Acad. Sci. USA *103*, 18816-18821.

Keller,T., Damude,H.G., Werner,D., Doerner,P., Dixon,R.A., and Lamb,C. (1998). A plant homolog of the neutrophil NADPH oxidase gp91^{phox} subunit gene encodes a plasma membrane protein with Ca^{2+} binding motifs. Plant Cell *10*, 255-266.

Kieber,J.J. (1997). The ethylene response pathway in Arabidopsis. Annu. Rev. Plant Physiol. Plant Mol. Biol. *48*, 277-296.

Kieber,J.J., Rothenberg,M., Roman,G., Feldmann,K.A., and Ecker,J.R. (1993). *CTR1*, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the Raf family of protein kinases. Cell *72*, 427-441.

Kiiskinen,M., Korhonen,M. and Kangasjärvi,J. (1997) Isolation and characterization of cDNA for a plant mitochondrial phosphate translocator (*Mpt1*): ozone stress induces *Mpt1* mRNA accumulationin birch (*Betula pendula* Roth). Plant Mol. Biol. *35*, 271–279.

Kim,C.Y., Liu,Y., Thorne,E.T., Yang,H., Fukushige,H., Gassmann,W., Hildebrand,D., Sharp,R.E., and Zhang,S. (2003). Activation of a stress-responsive mitogen-activated protein kinase cascade induces the biosynthesis of ethylene in plants. Plant Cell *15*, 2707-2718.

Kinkema,M., Fan,W., and Dong,X. (2000). Nuclear Localization of NPR1 Is Required for Activation of PR Gene Expression. Plant Cell *12*, 2339-2350.

Klepper,L. (1990). Comparison between NOx Evolution Mechanisms of Wild-Type and *nr1* Mutant Soybean Leaves. Plant Physiol. *93*, 26-32.

Kloek,A.P., Verbsky,M.L., Sharma,S.B., Schoelz,J.E., Vogel,J., Klessig,D.F., and Kunkel,B.N. (2001). Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (*coi1*) mutation occurs through two distinct mechanisms. Plant J. *26*, 509-522.

Kovtun,Y., Chiu,W.L., Tena,G., and Sheen,J. (2000). Functional analysis of oxidative stress activated mitogen-activated protein kinase cascades in plants. Proc. Natl. Acad. Sci. USA *97*, 2940-2945.

Kroj,T., Rudd,J., Nürnberger,T., Gäbler,Y., Lee,J., and Scheel,D. (2003). Mitogen-activated protein kinases play an essential role in oxidative burst-independent expression of pathogenesis-related genes in parsley. J. Biol. Chem. *278*, 2256-2264.

Kumar,D. and Klessig,D.F. (2000). Differential induction of tobacco MAP kinases by the defense signals nitric oxide, salicylic acid, ethylene, and jasmonic acid. Mol. Plant-Microbe Interact. *13*, 347-351.

Kwak,J.M., Mori,I.C., Pei,Z.M., Leonhardt,N., Torres,M.A., Dangl,J.L., Bloom,R.E., Bodde,S., Jones,J.D.G., and Schroeder,J.I. (2003). NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J. *22*, 2623-2633.

Lamattina,L., Garcia-Mata,C., Graziano,M., and Pagnussat,G. (2003). Nitric oxide: The versatility of an extensive signal molecule. Annu. Rev. Plant Biol. *54*, 109-136.

Lamb,C. and Dixon,R.A. (1997). The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. *48*, 251-275.

Larkindale,J., Hall,J.D., Knight,M.R., and Vierling,E. (2005). Heat stress phenotypes of Arabidopsis mutants implicate multiple signaling pathways in the acquisition of thermotolerance. Plant Physiol. *138*, 882-897.

Larsen,P.B. and Cancel,J.D. (2004). A recessive mutation in the RUB1-conjugating enzyme, RCE1, reveals a requirement for RUB modification for control of ethylene biosynthesis and proper induction of basic chitinase and *PDF1.2* in *Arabidopsis*. Plant J. *38*, 626-638.

Laurila,T. and Lättilä,H. (1994). Surface ozone exposures measured in Finland. Atmospheric Environment *28*, 103-114.

Lee,J.S. and Ellis,B.E. (2007). Arabidopsis MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. Journal of Biological Chemistry *282*, 25020-25029.

Lee,S., Choi,H., Suh,S., Doo,I.S., Oh,K.Y., Jeong Choi,E., Schroeder Taylor,A.T., Low,P.S., and Lee,Y. (1999). Oligogalacturonic Acid and Chitosan Reduce Stomatal Aperture by Inducing the Evolution of Reactive Oxygen Species from Guard Cells of Tomato and Commelina communis. Plant Physiol. *121*, 147-152.

Leng,Q., Mercier,R.W., Yao,W.Z., and Berkowitz,G.A. (1999). Cloning and first functional characterization of a plant cyclic nucleotide-gated cation channel. Plant Physiol. *121*, 753-761.

Leon,P. and Sheen,J. (2003). Sugar and hormone connections. Trends Plant Sci. *8*, 110-116.

Leung,J., Merlot,S., and Giraudat,J. (1997). The Arabidopsis *ABSCISIC ACID-INSENSITIVE2* (*ABI2*) and *ABI1* genes encode homologous protein phosphatases 2C involved in abscisic acid signal transduction. Plant Cell *9*, 759-771.

Li,H.T., Samouilov,A., Liu,X.P., and Zweier,J.L. (2004). Characterization of the effects of oxygen on xanthine oxidase-mediated nitric oxide formation. Journal of Biological Chemistry *279*, 16939-16946.

Lin,B. and Heaton,L.A. (2001). An *Arabidopsis thaliana* protein interacts with a movement protein of *Turnip crinkle virus* in yeast cells and *in vitro*. J. Gen. Virol. *82*, 1245-1251.

Lin,P.C., Hwang,S.G., Endo,A., Okamoto,M., Koshiba,T., and Cheng,W.H. (2007). Ectopic Expression of *ABSISIC ACID 2/GLUCOSE INSENSITIVE 1* in Arabidopsis Promotes Seed Dormancy and Stress Tolerance. Plant Physiol. *143*, 745-758.

Lindermayr,C., Saalbach,G., Bahnweg,G., and Durner,J. (2006). Differential inhibition of *Arabidopsis* methionine adenosyltransferases by protein S-nitrosylation. Journal of Biological Chemistry *281*, 4285-4291.

Lindermayr,C., Saalbach,G., and Durner,J. (2005). Proteomic identification of S-nitrosylated proteins in Arabidopsis. Plant Physiol. *137*, 921-930.

Liu,J.P., Ishitani,M., Halfter,U., Kim,C.S., and Zhu,J.K. (2000). The *Arabidopsis thaliana* SOS2 gene encodes a protein kinase that is required for salt tolerance. Proc. Natl. Acad. Sci. USA *97*, 3730-3734.

Liu,J.X., Srivastava,R., Che,P., and Howell,S.H. (2007a). Salt stress responses in Arabidopsis utilize a signal transduction pathway related to endoplasmic reticulum stress signaling. Plant J. *51*, 897-909.

Liu,J.X., Srivastava,R., Che,P., and Howell,S.H. (2007b). An endoplasmic reticulum stress response in *Arabidopsis* is mediated by proteolytic prosessing and nuclear relocation of a membrane-associated transcription factor, bZIP28. Plant Cell *19*, 4111-4119.

Liu,X.G., Yue,Y.L., Li,B., Nie,Y.L., Li,W., Wu,W.H., and Ma,L.G. (2007). A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. Science *315*, 1712-1716.

Liu,Y. and Zhang,S. (2004). Phosphorylation of 1-Aminocyclopropane-1-Carboxylic Acid Synthase by MPK6, a Stress-Responsive Mitogen-Activated Protein Kinase, Induces Ethylene Biosynthesis in Arabidopsis. Plant Cell *16*, 3386-3399.

Lopez-Molina,L. and Chua,N.H. (2000). A null mutation in a bZIP factor confers ABA-insensitivity in *Arabidopsis thaliana*. Plant and Cell Physiology *41*, 541-547.

Lorenzo,O., Chico,J.M., Sánchez-Serrano,J.J., and Solano,R. (2004). *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated responses in Arabidopsis. Plant Cell *16*, 1938-1950.

Lorrain,S., Vailleau,F., Balagué,C., and Roby,D. (2003). Lesion mimic mutants: keys for deciphering cell death and defence pathways in plants? Trends Plant Sci. *8*, 263-270.

Lu,C. and Fedoroff,N. (2000). A Mutation in the Arabidopsis *HYL1* Gene Encoding a dsRNA Binding Protein Affects Responses to Abscisic Acid, Auxin, and Cytokinin. Plant Cell *12*, 2351-2366.

Lu, C., Han M.H., Guevara-Garcia A., Feforoff N. (2002) Mitogen –activated protein kinase signaling in postgermination arrest of development by abscisic acid. Proc. Natl. Acad. Sci. USA *14*, 15812-15817.

Madden,M.C. and Hogsett,W.E. (2001). A historical overview of the ozone exposure problem. Human and Ecological Risk Assessment *7*, 1121-1131.

MAPK Group (2002). Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci. *7*, 301-308.

Martinez,C., Pons,E., Prats,G., and Leon,J. (2004). Salicylic acid regulates flowering time and links defence responses and reproductive development. Plant J. *37*, 209-217.

Martinez,I.M. and Chrispeels,M.J. (2003). Genomic analysis of the unfolded protein response in Arabidopsis shows its connection to important cellular processes. Plant Cell *15*, 561-576.

Maruyama A., Saito K., Ishizawa K. (2001). Beta-cyanoalanine synthase and cysteine synthase from potato: molecular cloning, biochemical characterization, and spatial and hormonal regulation. Plant Mol Biol *46*, 749–760.

Mateo,A., Funck,D., Muhlenbock,P., Kular,B., Mullineaux,P.M., and Karpinski,S. (2006). Controlled levels of salicylic acid are required for optimal photosynthesis and redox homeostasis. J. Exp. Bot. *57*, 1795-1807.

Meyer,T., Burow,M., Bauer,M., and Papenbrock,J. (2003). *Arabidopsis* sulfurtransferases: investigation of their role during senescence and in cyanide detoxification. Planta *217*, 1-10.

McAinsh,M.R., Clayton,H., Mansfield,T.A., and Hetherington,A.M. (1996). Changes in stomatal behaviour and guard cell cytosolic free calcium in response to oxidative stress. Plant Physiol. *111*, 1031-1042.

Millar,A.H. and Day,D.A. (1996). Nitric oxide inhibits the cytochrome oxidase but not the alternative oxidase of plant mitochondria. Febs Letters *398*, 155-158.

Mochizuki,N., Brusslan,J.A., Larkin,R., Nagatani,A., and Chory,J. (2001). *Arabidopsis genomes uncoupled 5* (*GUN5*) mutant reveals the involvement of Mg-chelatase H subunit in plastid-to-nucleus signal transduction. Proc. Natl. Acad. Sci. USA *98*, 2053-2058.

Modolo,L.V., Cunha,F.Q., Braga,M.R., and Salgado,I. (2002). Nitric oxide synthase-mediated phytoalexin accumulation in soybean cotyledons in response to the *Diaporthe phaseolorum* f. sp. *meridionalis* elicitor. Plant Physiol. *130*, 1288-1297.

Morris,K., Mackerness,S., Page,T., John,C.F., Murphy,A.M., Carr,J.P., and Buchanan-Wollaston,V. (2000). Salicylic acid has role in regulating gene expression during leaf senescence. Plant J. *23*, 677-685.

Muhlenbock,P., Plaszczyca,M., Plaszczyca,M., Mellerowicz,E., and Karpinski,S. (2007). Lysigenous aerenchyma formation in *Arabidopsis* is controlled by *LESION SIMULATING DISEASE1*. Plant Cell *19*, 3819-3830.

Nakajima,N., Itoh,T., Takikawa,S., Asai,N., Tamaoki,M., Aono,M., Kubo,A., Azumi,Y., Kamada,H., and Saji,H. (2002). Improvement in ozone tolerance of tobacco plants with an antisense DNA for 1-aminocyclopropane-1-carboxylate synthase. Plant Cell Environ. *25*, 727-736.

Nakamura,T., Meyer,C., and Sano,H. (2002). Molecular cloning and characterization of plant genes encoding novel peroxisomal molybdoenzymes of the sulphite oxidase family. J. Exp. Bot. *53*, 1833-1836.

Nathan,C. and Xie,Q.W. (1994). Regulation of Biosynthesis of Nitric-Oxide. Journal of Biological Chemistry *269*, 13725-13728.

Nawrath,C., Heck,S., Parinthawong,N., and Metraux,J.P. (2002). EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell *14*, 275-286.

Noctor,G., Gomez,L., Vanacker,H., and Foyer,C.H. (2002). Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. J. Exp. Bot. *53*, 1283-1304.

Nomura,M. and Takagi,H. (2004). Role of the yeast acetyltransferase *Mpr1* in oxidative stress: Regulation of oxygen reactive species caused by a toxic proline catabolism intermediate. Proc. Natl. Acad. Sci. USA *101*, 12616-12621.

Norman,C., Howell,K.A., Millar,A.H., Whelan,J.M., and Day,D.A. (2004). Salicylic Acid Is an Uncoupler and Inhibitor of Mitochondrial Electron Transport. Plant Physiol. *134*, 492-501.

Nuhse,T.S., Peck,S.C., Hirt,H., and Boller,T. (2000). Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK6. Journal of Biological Chemistry *275*, 7521-7526.

Nyathi,Y. and Baker,A. (2006). Plant peroxisomes as a source of signalling molecules. Biochimica et Biophysica Acta-Molecular Cell Research *1763*, 1478-1495.

Ogasawara,Y., Kaya,H., Hiraoka,G., Yumoto,F., Kimura,S., Kadota,Y., Hishinuma,H., Senzaki,E., Yamagow,S., Nagata,K., Nara,M., Suzuki,K., Tanokura,M., and Kuchitsu, K. (2008) Synergistic Activation of *Arabidopsis* NADPH Oxidase AtrbohD by Ca^{2+} and Phosphorylation, http://www.jbc.org/cgi/doi/10.1074/jbc.M708106200.

Ogawa,D., Nakajima,N., Tamaoki,M., Aono,M., Kubo,A., Kamada,H., and Saji,H. (2007). The isochorismate pathway is negatively regulated by salicylic acid signaling in O_3-exposed *Arabidopsis*. Planta *226*, 1277-1285.

Ouaked,F., Rozhon,W., Lecourieux,D., and Hirt,H. (2003). A MAPK pathway mediates ethylene signaling in plants. EMBO J. *22*, 1282-1288.

Overmyer,K., Tuominen,H., Kettunen,R., Betz,C., Langebartels,C., Sandermann,H., Jr., 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.

Park,S.W., Kaimoyo,E., Kumar,D., Mosher,S., and Klessig,D.F. (2007). Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. Science *318*, 113-116.

Pell,E.J., Schlagnhaufer,C.D., and Arteca,R.N. (1997). Ozone-induced oxidative stress: Mechanisms of action and reaction. Physiol. Plant. *100*, 264-273.

Pellinen,R., Palva,T., and Kangasjärvi,J. (1999). Subcellular localization of ozone-induced hydrogen peroxide production in birch (*Betula pendula*) leaf cells. Plant J. *20*, 349-356.

Penninckx,I.A.M.A., Thomma,B.P.H.J., Buchala,A., Métraux,J.P., and Broekaert,W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell *10*, 2103-2113.

Petersen,M., Brodersen,P., Naested,H., Andreasson,E., Lindhart,U., Johansen,B., Nielsen,H.B., Lacy,M., Austin,M.J., Parker,J.E., Sharma,S.B., Klessig,D.F., Martienssen,R., Mattsson,O., Jensen,A.B., and Mundy,J. (2000). *Arabidopsis* MAP kinase 4 negatively regulates systemic acquired resistance. Cell *103*, 1111-1120.

Pieper,A.A., Blackshaw,S., Clements,E.E., Brat,D.J., Krug,D.K., White,A.J., Pinto-Garcia,P., Favit,A., Conover,J.R., Snyder,S.H., and Verma,A. (2000). Poly(ADP-ribosyl)ation basally activated by DNA strand breaks reflects glutamate-nitric oxide neurotransmission. Proc. Natl. Acad. Sci. USA *97*, 1845-1850.

Planchet,E., Gupta,K.J., Sonoda,M., and Kaiser,W.M. (2005). Nitric oxide emission from tobacco leaves and cell suspensions: rate limiting factors and evidence for the involvement of mitochondrial electron transport. Plant J. *41*, 732-743.

Polidoros,A.N., Mylona,P.V., Pasentsis,K., Scandalios,J.G., and Tsaftaris,A.S. (2005). The maize *Alternative oxidase 1a* (*Aox1a*) gene is regulated by signals related to oxidative stress. Redox Report *10*, 71-78.

Purvis,A.C. (1997). Role of alternative oxidase in limiting superoxide production by plant mitochondria. Physiol. Plant. *100*, 165-170.

Qiu,Q.S., Guo,Y., Dietrich,M.A., Schumaker,K.S., and Zhu,J.K. (2002). Regulation of SOS1, a plasma membrane Na^+/H^+ exchanger in *Arabidopsis thaliana*, by SOS2 and SOS3. Proc. Natl. Acad. Sci. USA *99*, 8436-8441.

Rao,M.V. and Davis,K.R. (1999). Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis*: the role of salicylic acid. Plant J. *17*, 603-614.

Rao,M.V. and Davis,K.R. (2001). The physiology of ozone-induced cell death. Planta *213*, 682-690.

Rao,M.V., Koch,J.R., and Davis,K.R. (2000a). Ozone: a tool for probing programmed cell death in plants. Plant Mol. Biol. *44*, 345-358.

Rao,M.V., Lee,H.I., Creelman,R.A., Mullet,J.A., and Davis,K.R. (2000b). Jasmonic acid signaling modulates ozone-induced hypersensitive cell death. Plant Cell *12*, 1633-1646.

Rao,M.V., Lee,H.I., and Davis,K.R. (2002). Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. Plant J. *32*, 447-456.

Razem,F.A., El-Kereamy,A., Abrams,S.R., and Hill,R.D. (2006). The RNA-binding protein FCA is an abscisic acid receptor. Nature *439*, 290-294.

Renganathan,M., Cummins,T.R., and Waxman,S.G. (2002). Nitric oxide blocks fast, slow, and persistent Na+ channels in C-type DRG neurons by S-nitrosylation. Journal of Neurophysiology *87*, 761-775.

Reymond,P., Weber,H., Damond,M., and Farmer,E.E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell *12*, 707-719.

Rhoads,D.M. and McIntosh,L. (1992). Salicylic-Acid Regulation of Respiration in Higher-Plants - Alternative Oxidase Expression. Plant Cell *4*, 1131-1139.

Rich,W., Wagoner,P.E., and Tomlinson,H. (1970). Ozone uptake by bean leaves. Science *169*, 79-80.

Rizhsky,L., Hallak-Herr,E., Van Breusegem,F., Rachmilevitch,S., Barr,J.E., Rodermel,S., Inze,D., and Mittler,R. (2002). Double antisense plants lacking ascorbate peroxidase and catalase are less sensitive to oxidative stress than single antisense plants lacking ascorbate peroxidase or catalase. Plant J. *32*, 329-342.

Rutkowski,D.T. and Kaufman,R.J. (2004). A trip to the ER: coping with stress. Trends Cell Biol. *14*, 20-28.

Sabar,M., De Paepe,R., and de Kouchkovsky,Y. (2000). Complex I impairment, respiratory compensations, and photosynthetic decrease in nuclear and mitochondrial male sterile mutants of *Nicotiana sylvestris*. Plant Physiol. *124*, 1239-1249.

Sagi,M. and Fluhr,R. (2001). Superoxide production by plant homologues of the gp91(phox) NADPH oxidase. Modulation of activity by calcium and by tobacco mosaic virus infection. Plant Physiol. *126*, 1281-1290.

Samuel,M.A. and Ellis,B.E. (2002). Double jeopardy: both overexpression and suppression of a redox-activated plant mitogen-activated protein kinase render tobacco plants ozone sensitive. Plant Cell *14*, 2059-2069.

Samuel,M.A., Miles,G.P., and Ellis,B.E. (2000). Ozone treatment rapidly activates MAP kinase signalling in plants. Plant J. *22*, 367-376.

Sandermann,H., Jr., Ernst,D., Heller,W., and Langebartels,C. (1998). Ozone: an abiotic elicitor of plant defence reactions. Trends Plant Sci. *3*, 47-50.

Scandalios,J.G. (1993). Oxygen stress and superoxide dismutases. Plant Physiol. *101*, 7-12.

Schild,L., Reinheckel,T., Reiser,M., Horn,T., Wolf,G., and Augustin,W. (2003). Nitric oxide produced in rat liver mitochondria causes oxidative stress and impairment of respiration after transient hypoxia. FASEB J. *17*, 2194-2201.

Schraudner,M., Moeder,W., Wiese,C., Van Camp,W., Inzé,D., Langebartels,C., and Sandermann,H., Jr. (1998). Ozone-induced oxidative burst in the ozone biomonitor plant, tobacco Bel W3. Plant J. *16*, 235-245.

Schweighofer,A., Kazanaviciute,V., Scheikl,E., Teige,M., Doczi,R., Hirt,H., Schwanninger,M., Kant,M., Schuurink,R., Mauch,F., Buchala,A., Cardinale,F., and Meskiene,I. (2007). The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in *Arabidopsis*. Plant Cell *19*, 2213-2224.

Scott,I.M., Clarke,S.M., Wood,J.E., and Mur,L.A.J. (2004). Salicylate accumulation inhibits growth at chilling temperature in Arabidopsis. Plant Physiol. *135*, 1040-1049.

Selkoe,D.J. (2003). Folding proteins in fatal ways. Nature *426*, 900-904.

Seo,S., Katou,S., Seto,H., Gomi,K., and Ohashi,Y. (2007). The mitogen-activated protein kinases WIPK and SIPK regulate the levels of jasmonic and salicylic acids in wounded tobacco plants. Plant J. *49*, 899-909.

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

Shen,Y.Y., Wang,X.F., Wu,F.Q., Du,S.Y., Cao,Z., Shang,Y., Wang,X.L., Peng,C.C., Yu,X.C., Zhu,S.Y., Fan,R.C., Xu,Y.H., and Zhang,D.P. (2006). The Mg-chelatase H subunit is an abscisic acid receptor. Nature *443*, 823-826.

Shulaev,V., Silverman,P., and Raskin,I. (1997). Airborne signalling by methyl salicylate in plant pathogen resistance. Nature *385*, 718-721.

Sitch,S., Cox,P.M., Collins,W.J. and Huntingford,C. (2007) Indirect radiative forcing of climate change through ozone effects on the land-carbon sink. Nature *448*, 791-795.

Skärby,L., Grennfelt,P., Karlsson,P.E., Karlsson,G.P., Pleijel,H., Selldén,G., Wallin,G., Mikkelsen,T., Mortensen,L., Ro-Poulsen,H., Huttunen,S., Kärenlampi,L., Ojanperä,K., Sutinen,S., Tuovinen,J.P., Aarnes,H., Eriksen,A.B., and Mortensen,L. (1994). Critical levels for tropospheric ozone -Concepts and criteria tested for Nordic conditions. TemaNord *592*, 1-25.

Sokolovski,S. and Blatt,M.R. (2004). Nitric Oxide Block of Outward-Rectifying K+ Channels Indicates Direct Control by Protein Nitrosylation in Guard Cells. Plant Physiol. *136*, 4275-4284.

Solano,R., Stepanova,A., Chao,Q., and Ecker,J.R. (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes & Dev. *12*, 3703-3714.

Soyano,T., Nishihama,R., Morikiyo,K., Ishikawa,M., and Machida,Y. (2003). NQK1/NTMEK1 is a MAPKK that acts in the NPK1 MAPKKK-mediated MAPK cascade and is required for plant cytokinesis. Genes & Dev. *17*, 1055-1067.

Spanu,P., Grosskopf,D.G., Felix,G., and Boller,T. (1994). The apparent turnover of 1-aminocyclopropane-1-carboxylate synthase in tomato cells is regulated by protein phosphorylation and dephosphorylation. Plant Physiol. *106*, 529-535.

Spoel,S.H., Koornneef,A., Claessens,S.M.C., Korzelius,J.P., van Pelt,J.A., Mueller,M.J., Buchala,A.J., Metraux,J.P., Brown,R., Kazan,K., van Loon,L.C., Dong,X.N., and Pieterse,C.M.J. (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell *15*, 760-770.

Stamler,J.S., Lamas,S., and Fang,F.C. (2001). Nitrosylation: The prototypic redox-based signaling mechanism. Cell *106*, 675-683.

Staswick,P.E. and Tiryaki,I. (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell *16*, 2117-2127.

Staswick,P.E., Tiryaki,I., and Rowe,M.L. (2002). Jasmonate response locus *JAR1* and several related Arabidopsis genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell *14*, 1405-1415.

Stintzi,A., Weber,H., Reymond,P., Browse,J., and Farmer,E.E. (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentones. Proc. Natl. Acad. Sci. USA *98*, 12837-12842.

Stohr,C., Strube,F., Marx,G., Ullrich,W.R., and Rockel,P. (2001). A plasma membrane-bound enzyme of tobacco roots catalyses the formation of nitric oxide from nitrite. Planta *212*, 835-841.

Streller,S. and Wingsle,G. (1994). *Pinus sylvesteris* L. needles contain extracellular CuZn superoxide dismutase. Planta *192*, 195-201.

Takahashi,F., Yoshida,R., Ichimura,K., Mizoguchi,T., Seo,S., Yonezawa,M., Maruyama,K., Yamaguchi-Shinozaki,K., and Shinozaki,K. (2007). The mitogen-activated protein kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. Plant Cell *19*, 805-818.

Tan,X., Calderon-Villalobos,L.I.A., Sharon,M., Zheng,C.X., Robinson,C.V., Estelle,M., and Zheng,N. (2007). Mechanism of auxin perception by the TIR1 ubiquitin ligase. Nature *446*, 640-645.

Tang,X.D., Daggett,H., Hanner,M., Garcia,M.L., McManus,O.B., Brot,N., Weissbach,H., Heinemann,S.H., and Hoshi,T. (2001). Oxidative Regulation of Large Conductance Calcium-activated Potassium Channels. J. Gen. Physiol. *117*, 253-274.

Tena,G., Asai,T., Chiu,W.L., and Sheen,J. (2001). Plant mitogen-activated protein kinase signaling cascades. Curr. Opin. Plant Biol. *4*, 392-400.

Thines,B., Katsir,L., Melotto,M., Niu,Y., Mandaokar,A., Liu,G.H., Nomura,K., He,S.Y., Howe,G.A., and Browse,J. (2007). JAZ repressor proteins are targets of the SCFCO11 complex during jasmonate signalling. Nature *448*, 661-666.

Thomma,B.P.H.J., Eggermont,K., Penninckx,I.A.M.A., Mauch-Mani,B., Vogelsang,R., Cammue,B.P.A., and Broekaert,W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. PNAS *95*, 15107-15111.

Tingey,D.T., Standley,C., and Field,R.W. (1976). Stress ethylene evolution: a measure of ozone effects on plants. Atmospheric Environment *10*, 969-974.

Torres,M.A. and Dangl,J.L. (2005). Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr. Opin. Plant Biol *8*, 397-403.

Torres,M.A., Dangl,J.L., and Jones,J.D.G. (2002). *Arabidopsis* $gp91^{phox}$ homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. USA *99*, 517-522.

Torres,M.A., Onouchi,H., Hamada,S., Machida,C., Hammond-Kosack,K.E., and Jones,J.D.G. (1998). Six *Arabidopsis thaliana* homologues of the human respiratory burst oxidase (*$gp91^{phox}$*). Plant J. *14*, 365-370.

Tun,N.N., Santa-Catarina,C., Begum,T., Silveira,V., Handro,W., Floh,E.I.S., and Scherer,G.F.E. (2006). Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant and Cell Physiology *47*, 346-354.

Tuomainen,J., Betz,C., Kangasjärvi,J., Ernst,D., Yin,Z.H., Langebartels,C., and Sandermann,H., Jr. (1997). Ozone induction of ethylene emission in tomato plants: regulation by differential transcript accumulation for the biosynthetic enzymes. Plant J. *12*, 1151-1162.

Tuominen,H., Overmyer,K., Keinänen,M., Kollist,H., and Kangasjärvi,J. (2004). Mutual antagonism of ethylene and jasmonic acid regulates ozone-induced spreading cell death in *Arabidopsis*. Plant J. *39*, 59-69.

Ulm,R., Ichimura,K., Mizoguchi,T., Peck,S.C., Zhu,T., Wang,X., Shinozaki,K., and Paszkowski,J. (2002). Distinct regulation of salinity and genotoxic stress responses by Arabidopsis MAP kinase phosphatase 1. Embo Journal *21*, 6483-6493.

Ulm,R., Revenkova,E., di Sansebastiano,G.P., Bechtold,N., and Paszkowski,J. (2001). Mitogen-activated protein kinase phosphatase is required for genotoxic stress relief in Arabidopsis. Genes & Dev. *15*, 699-709.

Urade,R. (2007). Cellular response to unfolded proteins in the endoplasmic reticulum of plants. Febs Journal *274*, 1152-1171.

Vahala,J., Keinänen,M., Schützendübel,A., Polle,A., and Kangasjärvi,J. (2003). Differential effects of elevated ozone on two hybrid aspen genotypes predisposed to chronic ozone fumigation: role of ethylene and salicylic acid. Plant Physiol. *132*, 196-205.

Van Camp,W., van Montagu,M., and Inzé,D. (1998). H_2O_2 and NO: redox signals in disease resistance. Trends Plant Sci. *3*, 330-334.

Vanlerberghe,G.C., Robson,C.A., and Yip,J.Y.H. (2002). Induction of mitochondrial alternative oxidase in response to a cell signal pathway down-regulating the cytochrome pathway prevents programmed cell death. Plant Physiol. *129*, 1829-1842.

Wang,H.C., Ngwenyama,N., Liu,Y.D., Walker,J.C., and Zhang,S.Q. (2007). Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. Plant Cell *19*, 63-73.

Wang,K.L.C., Li,H., and Ecker,J.R. (2002). Ethylene biosynthesis and signaling networks. Plant Cell *14*, S131-S151.

Wendehenne,D., Pugin,A., Klessig,D.F., and Durner,J. (2001). Nitric oxide: comparative synthesis and signaling in animal and plant cells. Trends Plant Sci. *6*, 177-183.

Weng,A.P., Ferrando,A.A., Lee,W., Morris,J.P., Silverman,L.B., Sanchez-Irizarry,C., Blacklow,S.C., Look,A.T., and Aster,J.C. (2004). Activating mutations of *NOTCH1* in human T cell acute lymphoblastic leukemia. Science *306*, 269-271.

Westley J. (1973). Rhodanese. Adv Enzymol *39*, 327–368.

Westley J. (1981). Thiosulfate:cyanide sulfurtransferase (rhodanese). Methods Enzymol *77*, 285–291.

Wildermuth,M.C., Dewdney,J., Wu,G., and Ausubel,F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defense. Nature *414*, 562-565.

Willekens,H., Chamnongpol,S., Davey,M., Schraudner,M., Langebartels,C., van Montagu,M., Inzé,D., and Van Camp,W. (1997). Catalase is a sink for H_2O_2 and is indispensable for stress defence in C3 plants. EMBO J. *16*, 4806-4816.

Wittig,V.E., Ainsworth,E.A. and Long,S.P. (2007). To what extent do current and projected inicreases in surface ozone affect photosynthesis and stomatal conductance of trees? A meta-analytic review fo the last 3 dacades of experiments. Plant, Cell and Environ. *30*, 1150-1162.

Wojtaszek,P. (1997). Oxidative burst: an early plant response to pathogen infection. Biochem. J. *322*, 681-692.

Wu,Y., Kuzma,J., Maréchal,E., Graeff,R., Lee,H.C., Foster,R., and Chua,N.H. (1997). Abscisic acid signaling through cyclic ADP-ribose in plants. Science *278*, 2126-2130.

Xiong,L., Gong,Z., Rock,C.D., Subramanian,S., Guo,Y., Xu,W., Galbraith,D., and Zhu,J.K. (2001). Modulation of abscisic acid signal transduction and biosynthesis by an Sm-like protein in *Arabidopsis*. Dev. Cell *1*, 771-781.

Yamasaki,H. (2000). Nitrite-dependent nitric oxide production pathway: implications for involvement of active nitrogen species in photoinhibition *in vivo*. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences *355*, 1477-1488.

Yamasaki,H. and Cohen,M.F. (2006). NO signal at the crossroads: polyamine-induced nitric oxide synthesis in plants? Trends Plant Sci. *11*, 522-524.

Yamasaki,H. and Sakihama,Y. (2000). Simultaneous production of nitric oxide and peroxynitrite by plant nitrate reductase: in vitro evidence for the NR-dependent formation of active nitrogen species. Febs Letters *468*, 89-92.

Yang,S.F. and Hoffman,N.E. (1984). Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. *35*, 155-189.

Yoo,S.D., Cho,Y.H., Tena,G., Xiong,Y., and Sheen,J. (2008). Dual control of nuclear EIN3 by bifurcate MAPK cascades in C2H4 signalling. Nature *451*, 789-795.

Yoshida,K.T., Fujiwara,T., and Naito,S. (2002). The synergistic effects of sugar and abscisic acid on *myo*-inositol-1-phosphate synthase expression. Physiol. Plant. *114*, 581-587.

Yu,I.C., Fengler,K.A., Clough,S.J., and Bent,A.F. (2000). Identification of Arabidopsis mutants exhibiting an altered hypersensitive response in gene-for-gene disease resistance. Mol. Plant-Microbe Interact. *13*, 277-286.

Yuasa,Y., Ichimura,K., Mizoguchi,T., and Shinozaki,K. (2001). Oxidative stress activates ATMPK6, an Arabidopsis homologue of MAP kinase. Plant and Cell Physiology *42*, 1012-1016.

Zago,E., Morsa,S., Dat,J.F., Alard,P., Ferrarini,A., Inze,D., Delledonne,M., and Van Breusegem,F. (2006). Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. Plant Physiol. *141*, 404-411.

Zaninotto,F., La Camera,S., Polverari,A., and Delledonne,M. (2006). Cross talk between reactive nitrogen and oxygen species during the hypersensitive disease resistance response. Plant Physiol. *141*, 379-383.

Zeidler,D., Zahringer,U., Gerber,I., Dubery,I., Hartung,T., Bors,W., Hutzler,P., and Durner,J. (2004). Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defense genes. Proc. Natl. Acad. Sci. USA *101*, 15811-15816.

Zemojtel,T., Frohlich,A., Palmieri,M.C., Kolanczyk,M., Mikula,I., Wyrwicz,L.S., Wanker,E.E., Mundlos,S., Vingron,M., Martasek,P., and Durner,J. (2006). Plant nitric oxide synthase: a never-ending story? Trends Plant Sci. *11*, 524-525.

Zhang,D.P., Wu,Z.Y., Li,X.Y., and Zhao,Z.X. (2002). Purification and identification of a 42-kilodalton abscisic acid-specific-binding protein from epidermis of broad bean leaves. Plant Physiol. *128*, 714-725.

Zhang,S., Du,H., and Klessig,D.F. (1998). Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora* spp. Plant Cell *10*, 435-449.

Zhang,S. and Klessig,D.F. (1998). The tobacco wounding-activated mitogen-activated protein kinase is encoded by *SIPK*. Proc. Natl. Acad. Sci. USA *95*, 7225-7230.

Zhang,X., Zhang,L., Dong,F., Gao,J., Galbraith,D.W., and Song,C.P. (2001). Hydrogen peroxide is involved in abscisic acid-induced stomatal closure in *Vicia faba*. Plant Physiol. *126*, 1438-1448.

Zhou,L., Jang,J.C., Jones,T.L., and Sheen,J. (1998a). Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. Proc. Natl. Acad. Sci. USA *95*, 10294-10299.

Zhou,N., Tootle,T.L., Tsui,F., Klessig,D.F., and Glazebrook,J. (1998b). *PAD4* functions upstream from salicylic acid to control defense responses in Arabidopsis. Plant Cell *10*, 1021-1030.

Zhu,J.K., Liu,J.P., and Xiong,L.M. (1998). Genetic analysis of salt tolerance in Arabidopsis: Evidence for a critical role of potassium nutrition. Plant Cell *10*, 1181-1191.

Zweifel,M.E., Leahy,D.J., and Barrick,D. (2005). Structure and Notch receptor binding of the tandem WWE domain of Deltex. Structure *13*, 1599-1611.

Örvar,B.L., McPherson,J., and Ellis,B.E. (1997). Pre-activating wounding response in tobacco prior to high-level ozone exposure prevents necrotic injury. Plant J. *11*, 203-212.