Reactive oxygen species and SRO proteins as regulators of gene expression in

Arabidopsis thaliana

Tiina Blomster

Faculty of Biological and Environmental Sciences

Viikki Doctoral Programme in Molecular Biosciences

University of Helsinki

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Supervisors

Professor Jaakko Kangasjärvi
Department of Biosciences
University of Helsinki, Finland

Docent Kirk Overmyer
Department of Biosciences
University of Helsinki, Finland

Reviewers

Professor Paula Elomaa
Department of Agricultural Sciences
University of Helsinki, Finland

Professor Hely Häggman
Department of Biology
University of Oulu, Finland

Opponent

Professor Frank Van Breusegem
Department of Plant Systems Biology
Ghent University, Belgium

Custos

Professor Ykä Helariutta
Department of Biosciences
University of Helsinki, Finland

"To infinity … and beyond!"

Buzz Lightyear (from Toy Story)

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Original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals (I-IV). The publications are reprinted with the kind permission from Blackwell Publishing (I), BioMed Central (II) and the American Society of Plant Biologists (III). Additional unpublished data is presented in the thesis.

I) Jaspers P, **Blomster T**, Brosché M, Salojärvi J, Ahlfors R, Vainonen JP, Reddy RA, Immink R, Angenent G, Turck F, Overmyer K, Kangasjärvi J (2009) Unequally redundant RCD1 and SRO1 mediate stress and developmental responses and interact with transcription factors. Plant J 60: 268-279

II) Jaspers P*, Overmyer K*, Wrzaczek M*, Vainonen JP, **Blomster T**, Salojärvi J, Reddy RA, Kangasjärvi J (2010) The RST and PARP-like domain containing SRO protein family: analysis of protein structure, function and conservation in land plants. BMC Genomics 11: 170

III) **Blomster T**, Salojärvi J, Sipari N, Brosché M, Ahlfors R, Keinänen M, Overmyer K, Kangasjärvi J (2011) Apoplastic reactive oxygen species transiently decrease auxin signaling and cause stress-induced morphogenic response in Arabidopsis. Plant Physiol 157: 1866-1883

IV) **Blomster T***, Brosché M*, Salojärvi J, Sipari N, Lamminmäki A, Cui F, Narayanasamy S, Reddy RA, Keinänen M, Overmyer K, Kangasjärvi J. Transcriptomics and functional genomics of ROS-induced cell death regulation by *RADICAL-INDUCED CELL DEATH1*. Manuscript.

* Equal contribution

Author´s contribution

I) TB isolated *rcd1-3*, *rcd1-4*, and *sro1-1* mutants and identified the *rcd1 sro1* plants. TB cloned the promoter reporter lines and initialized the complementation lines. TB performed the microarray hybridizations. This publication was part of PJ´s doctoral thesis.

II) TB isolated the *sro5-2* mutant, carried out microarray hybridizations and designed qPCR primers. This publication was part of PJ´s doctoral thesis.

III) TB performed plant experiments, RNA isolation, and microarray hybridizations. NS and TB collected samples for the microarrays, NS ground the samples, scanned the microarrays and analyzed hormone concentrations. KO and TB performed SIMR experiments. JS analyzed the microarray data and publicly available data with input from TB, MB and KO, and performed statistical analysis. MB, KO and TB designed and performed qPCR experiments. TB, MB, KO and JK wrote the paper.

IV) Microarray practical work was done as in III by TB and NS. TB, MB, AL, FC and RAR performed ion leakage experiments. JS and SN made statistical analysis and data clustering with input from MB and TB. TB, MB, KO and JK wrote the manuscript.

Abbreviations

$^1O_2$	singlet oxygen		PAMP	pathogen-associated molecular pattern
ABA	abscisic acid			
AFB	auxin signaling F-box protein		PARP	poly-ADP-ribose polymerase
AOX	alternative oxidase		PCD	programmed cell death
APX	ascorbate peroxidase		PQ	paraquat (methyl viologen)
ARF	auxin response factor		PR	pathogenesis-related
Aux/IAA	auxin/IAA		PS	photosystem
BR	brassinosteroid		PTI	pathogen-triggered immunity
BTH	SA-analog benzothiadiazole S-methylester			
			qPCR	quantitative real-time PCR
CAT	catalase		RBOH	respiratory burst oxidase homolog
CK	cytokinin			
COI	coronatine insensitive		RCD	radical-induced cell death
Col-0	Columbia-0 ecotype		ROS	reactive oxygen species
ERF	ethylene-responsive element binding factor		RST	RCD1-SRO-TAF4 domain
			SA	salicylic acid
ETI	effector-triggered immunity		SAUR	small auxin up-regulated
flg22	flagellin peptide		SCF	SKP1-CULLIN-F-box
GA	gibberellin		SIMR	stress-induced morphogenic response
GO	gene ontology			
H_2O_2	hydrogen peroxide		SKP	s-phase kinase associated protein
HR	hypersensitive response			
IAA	indole-3-acetic acid		SOD	superoxide dismutase
ICS	isochorismate synthase		SRO	similar to rcd-one
JA	jasmonic acid		TAF	TBP-associated factor
MPK	mitogen-activated protein kinase		TBP	TATA-box binding protein
			TF	transcription factor
NO	nitric oxide		TIR	transport inhibitor response
NPR	non-expressor of PR proteins		UPOX	upregulated by oxidative stress
$O_2^{\bullet-}$	superoxide			
O_3	ozone		UV	ultraviolet
$OH^{\bullet}$	hydroxyl radical			

Abstract

Life on earth is largely dependent on plants, which provide us energy and oxygen. Plants are sessile organisms adapted to their respective growth environments. However, these environmental conditions are rarely constant and changes in growth conditions require modifications in plant metabolism. Abiotic environmental factors may suddenly become limited or excessive, or a pathogen attack may cause biotic stress. Reactive oxygen species (ROS) are by-products of normal aerobic metabolism and their production is enhanced by biotic and abiotic stresses. ROS serve as signaling molecules, which regulate expression of stress-responsive genes together with other signaling pathways in order to achieve appropriate responses to the suboptimal environment. Human activities also modify the environment for instance by increasing levels of air pollutant ozone (O_3) which is a ROS causing foliar damage in sensitive species and cultivars. Therefore, understanding the mechanisms governing plant stress tolerance is of increasing importance. Importantly for this work, O_3 is known to cause production of apoplastic ROS in plants similar to other stresses and was therefore used here as a tool to study stress signaling.

The *Arabidopsis thaliana* mutant *radical-induced cell death1* (*rcd1*) harbors several stress phenotypes related to ROS signaling. In order to identify novel genes and signaling pathways regulating plant stress responses, *RCD1* and its homologs were studied further. RCD1 belongs to a plant-specific SIMILAR TO RCD-ONE (SRO) gene family present in all land plants analyzed. The molecular function of SROs remains unknown as they have no poly(ADP-ribose) polymerase (PARP) activity and lack catalytic amino acids in the conserved PARP catalytic domain. However, SROs are able to interact with transcription factors via their C-terminal RST domain which suggests that they may regulate gene expression. In addition to altered rosette morphology, *rcd1* has changes in gene expression in normal growth conditions which may partially be attributed to RCD1 protein-protein interactions. However, *sro1* and *sro5* plants are similar to wild-type Col-0 regarding their growth habitus and gene expression. *A. thaliana* paralogs RCD1 and SRO1 share unequal genetic redundancy: the *rcd1 sro1* double mutant is stunted and SRO1 can partially complement *rcd1*. Transcriptomic analysis of apoplastic ROS-induced signaling triggered by O_3 treatment revealed altered expression of thousands of genes in a time-dependent manner. In *rcd1*, this response was exaggerated, which may explain the triggering of programmed cell death in O_3-treated *rcd1*. Transcription factor WRKY70 was identified as a positive regulator of cell death, putatively acting through altered balance of salicylic acid and jasmonic acid signaling. A transient decrease in auxin signaling together with altered expression of auxin-responsive transcripts by apoplastic ROS was observed. Decreased auxin signaling did not affect the extent of cell death caused by acute O_3, but caused more severe morphological changes in chronically O_3-treated plants. Altogether, these results suggest that auxin-ROS interaction modulates plant development under stress.

1 Introduction

1.1 Reactive oxygen species (ROS): matters of life and death

1.1.1 Aerobic metabolism produces ROS

Aerobic metabolism by definition uses oxygen (O_2) in several vital reactions. O_2 is the final electron acceptor in the mitochondrial electron transport chain of respiration releasing energy from carbohydrates to cells. In plants, O_2 is formed after hydrolysis of water (H_2O) in the chloroplasts, and the electrons are utilized in photosynthesis to capture the energy of light in biomolecules. However, these electron transport reactions (redox reactions) and energy transfers also give rise to reactive oxygen species (ROS). ROS are oxygen forms, which are strong oxidants in biological systems and can therefore cause oxidative damage to proteins, lipids and nucleic acids with harmful effects on cellular functions. ROS include for instance hydrogen peroxide H_2O_2, superoxide $O_2^{\bullet-}$, singlet oxygen $^1O_2$ and hydroxyl radical $OH^{\bullet}$ (Fig. 1). These ROS differ in their chemical properties: for example H_2O_2 can diffuse across membranes, unlike $O_2^{\bullet-}$ which is a charged molecule. Specific water channels, aquaporins, also assist the diffusion of H_2O_2 (Bienert et al., 2007). Different ROS may also convert into other ROS non-enzymatically or enzymatically, for instance superoxide dismutases (SODs) catalyze the formation of H_2O_2 from $O_2^{\bullet-}$ (Fig. 1). This type of conversion is also a detoxification process, because $O_2^{\bullet-}$ is more reactive and thus more harmful than H_2O_2. For the most reactive ROS, $OH^{\bullet}$, there is no known enzymatic detoxification system so plants must simply avoid its formation from Haber-Weiss reaction (from ROS precursors H_2O_2 and $O_2^{\bullet-}$) and from Fenton reaction requiring transition metal ions (Fe^{2+} and Cu^{2+}). Therefore iron-binding ferritin proteins limit $OH^{\bullet}$ formation and are considered as part of ROS defense. Other systems for ROS detoxification include low molecular weight antioxidants, such as ascorbate and glutathione.
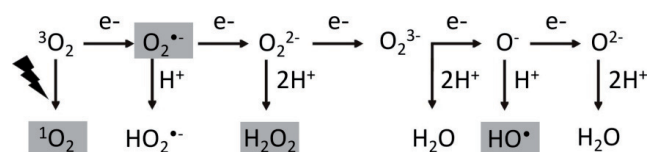
Figure 1. Formation of ROS (in gray boxes) by energy transfer (marked with lightning) or redox reactions. Adapted after Apel and Hirt (2004).

Plant cells are divided into several compartments each carrying out their specific functions. Similarly, also the ROS forming reactions, the particular ROS produced and the detoxification pathways are characteristic for each subcellular compartment. Chloroplasts produce mostly $^1O_2$ and $O_2^{\bullet-}$ as metabolic side-products and mitochondria $O_2^{\bullet-}$. $^1O_2$ is quenched by α-tocopherol and carotenoids in the chloroplast, whereas both chloroplasts and mitochondria contain specific SODs for conversion of $O_2^{\bullet-}$ into H_2O_2. Mitochondrial, chloroplastic and also cytosolic H_2O_2 can be reduced into H_2O by ascorbate peroxidases (APXs) utilizing ascorbate as the reductant. The recycling of oxidized ascorbate back to the reduced form, the Halliwell-Asada pathway, includes glutathione and several enzymes (monodehydroascorbate reductase, dehydroascorbate reductase, gluthatione reductase and reduced ferredoxin). Large quantities of H_2O_2 formed in peroxisomes during photorespiration and in fatty acid catabolism in glyoxysomes are reduced into H_2O by catalases (CAT) at the respective subcellular locations. Taken together, normal plant metabolism produces several interconvertible ROS hazardous for life, which differ in their chemical properties, sites of production and their detoxification pathways.

1.1.2 ROS production in response to stress

The oxidative state of a cell is determined by the balance between ROS formation and detoxification. Disturbance in this balance can be caused by environmental factors increasing ROS formation thereby creating so called oxidative stress. The increased ROS production by environmental perturbations can be divided into two main mechanisms: metabolic accumulation and regulated burst accumulation (Apel and Hirt 2004; Foyer and Noctor 2009). Firstly, metabolic ROS accumulation may increase at the sites of electron transport in chloroplasts and mitochondria when these electron transport chains become overreduced and can no longer adequately accept electrons. For instance, high light stress inhibits function of photosystem II (PSII) proteins (a process called photoinhibition), causing energy from exited chlorophyll to be transferred to O_2 resulting in the increase of $^1O_2$. To reduce photoinhibition, plants revert to alternative electron sinks such as photorespiration or the water-water cycle (Asada 1999). Photorespiration increases ROS in peroxisomes (Apel and Hirt 2004). The role for plant mitochondria in ROS production is estimated to be miniscule compared to chloroplasts and peroxisomes under normal and high light conditions (Foyer and Noctor 2003). In addition to the major role of photosynthetic light reactions as ROS sources, the presence of mitochondrial alternative oxidase enzyme complex (AOX) provides an optional, plant-specific electron sink in mitochondria without producing energy (Maxwell et al., 1999). The increase of ROS in chloroplast and mitochondria has been mainly attributed to abiotic stresses such as high light, cold, heat and mechanical stress with a concomitant increase in ROS detoxification processes (Apel and Hirt 2004).

Due to the intimate linkage of ROS and metabolism, ROS formation is an important cue for plants to adjust their metabolism according to the environmental change. The role of ROS as

signaling molecules and not only toxic by-products of aerobic metabolism has nowadays been widely accepted. The dual nature of ROS is also indicated by the capability of plant cells to actively produce ROS, which is the second mechanism by which ROS concentrations are regulated by environmental factors. The rapid production of large amounts of ROS, the so called oxidative burst, by enzymes localized to plasma membrane and cell walls was first discovered in response to biotic stress. Plasma membrane bound $O_2^{\bullet-}$-generating NADPH-oxidases are called RESPIRATORY BURST OXIDASE HOMOLOGS (RBOHs) according to the similarity with enzyme complexes in mammalian neutrophils (Torres et al., 2002; Suzuki et al., 2011). This demonstrates that the ROS signaling network has similarities between plants and animals. There is genetic evidence that RBOHD and RBOHF are the main $O_2^{\bullet-}$ producers in plant defense using the model species *Arabidopsis thaliana* (Torres et al., 2002) but also other sources of apoplastic ROS, such as peroxidases, may play an important role in some cases of plant-pathogen interactions (Bindschedler et al., 2006). In plants, cell wall bound peroxidases participating in lignin formation may act also as NADPH oxidases and produce $O_2^{\bullet-}$ and consequently H_2O_2. Horse-radish peroxidase has been demonstrated also to catalyze formation of highly reactive $OH^{\bullet}$ in the presence of $O_2^{\bullet-}$ and H_2O_2 (Chen and Schopfer 1999). Also xanthine and amine oxidases may cause ROS production during pathogen attack. The increase of extracellular ROS has been interpreted as an attempt to kill the invading pathogen similarly to the action of phagocytes in animal cells. Recently it was shown that NADPH-oxidase activity causes changes in the cellular pH, which drives the antipathogenic effect (Segal 2008). Similarly, alkalinization of apoplastic environment is co-occurring with RBOH activity in plants due to Ca^{2+} influx (Monshausen et al., 2009), which may lead to elevated peroxidase activity and H_2O_2 production in the apoplast (Bolwell et al., 2002).

Importance of endogenously produced ROS as signals promoting plant stress survival and fitness is reinforced by the simultaneous decrease in antioxidant defenses, which is not usually the case in abiotic stress responses. However, ROS production and accumulation in response to environmental pertubations is complex with spatial and also temporal variation. Recently RBOHD was shown to generate $O_2^{\bullet-}$ in response to high light, wounding, heat, cold and salt stress as well (Miller et al., 2009). Therefore, abiotic stresses induce ROS production also in the apoplast and not merely in the electron transport chains of the mitochondria or chloroplast. Plants defective in RBOH function exhibit reduced salt tolerance (Leshem et al., 2007; Kaye et al., 2011). In addition to apoplastic ROS, pathogens may cause also mitochondrial ROS production (Amirsadeghi et al., 2007). Plants impaired in mitochondrial complex II and mitochondrial ROS production have increased pathogen susceptibility and partially lack plant hormone salicylic acid (SA) responses (Gleason et al., 2011b). Nuclear ROS accumulation has been observed in tobacco treated with fungal elicitor protein cryptogein (Ashtamker et al., 2007) and in salt-treated Arabidopsis roots (Kaye et al., 2011), but no specific role for nuclear ROS in stress signaling has yet been demonstrated.

In Arabidopsis at least 289 genes are directly involved in the production or detoxification of ROS (Gechev et al., 2006) thereby establishing a basal network for ROS signaling. It is noteworthy that although chloroplast and mitochondria possess their own genomes, still the majority of their proteins are encoded in the nucleus. Retrograde signaling, the process by which organellar signals affect nuclear gene expression is yet largely unresolved. Because ROS-derived signals interact with other signaling molecules, such as lipid-derived messengers, plant hormones and nitric oxide (NO), the number of genes involved in the fine-tuning of ROS signaling is even larger.

1.1.3 ROS in plant growth, development and stomatal function

ROS are important signaling molecules mediating stress responses, but ROS production is also necessary for plant development. Interestingly, analysis regarding the evolutionary history of $O_2^{\bullet-}$-generating NADPH oxidases within the plant kingdom revealed that the basal plants do have a sophisticated antioxidative capacity, but they lack the NADPH oxidases, which have arisen with increased plant size presumably for advanced signaling purposes (Mittler et al., 2011). ROS produced by RBOHD triggers systemic signaling across large distances (Miller et al., 2009), which is in line with this interpretation. The *rbohd* and *rbohf* plants are smaller than the respective wild type (Torres et al., 2002), which supports the importance of ROS production in also plant development and growth. RBOH-produced ROS in stomatal guard cells regulate plant gas exchange by promoting stomatal closure (Kwak et al., 2003; Joo et al., 2005a). ROS are produced by NADPH oxidases also in the elongating root hairs (Foreman et al., 2003), pollen tubes (Potocký et al., 2007) and maize (*Zea mays*) leaves (Rodríguez et al., 2007). Concomitantly, the halt of *Z. mays* leaf elongation caused by salt stress could be reversed by ROS application (Rodríguez et al., 2004). However, it has also been frequently reported that plants defective in the antioxidant defenses or under constitutive stress have reduced growth, which highlights the importance of well-controlled ROS concentrations. Stress-induced morphogenetic response (SIMR) is a term used to describe similar alterations in plant growth and architecture caused by different stresses. These features include increased number of lateral organs (roots and shoots), decreased cell divisions in the apical meristems and decreased cell elongation. At molecular level SIMR is not well understood, but it is likely to encompass interaction between ROS and plant hormone auxin (Potters et al., 2007). Several plant hormones regulate plant growth and development also via ROS-dependent signals. This provides a regulatory node between development and stress responses, which will be addressed in chapter 1.2.

1.1.4 ROS have unique and overlapping functions in stress signaling

Increased ROS levels are among the most rapidly responding markers of several stresses. How are these signals transmitted further and converted into cellular responses? Many aspects of ROS signaling are still unknown, starting from the ROS perception. Several mechanisms for sensing extracellular ROS have been proposed, including yet unidentified ROS-receptors on the plasma membrane. Lipid peroxidation products resulting from oxidative damage could also account for secondary messengers sensed by the plant. Furthermore, the general redox-status of the cell may regulate the activity of transcription factors (Wormuth et al., 2007). An example of plant-specific redox-sensitive transcriptional regulator is NON-EXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) (discussed in detail in chapter 1.2.2). ROS perception and signaling in plants may be a combination of all these mechanisms. Intracellular ROS signals may be transmitted from one part of the cell to another, for instance ROS accumulation in chloroplasts and mitochondria is regulating the expression of nucleus-encoded genes (Woodson and Chory 2008). Enhancement of vesicle trafficking has been established as a ROS response, which may allow ROS themselves to be transported within the cell as intracellular signaling molecules (Leshem et al., 2007; Kaye et al., 2011). In the algae *Chlamydomonas reinhardtii*, chloroplast-derived $^1O_2$ can diffuse to nucleus and therefore act as putative intracellular signaling molecule (Fischer et al., 2007).

The signaling role of ROS has been studied by addressing the ROS-regulated transcriptome. Changes in transcript abundance can be measured reliably and in a high-throughput manner with the microarray hybridization-method. Different ROS in different subcellular compartments have unique effects on gene expression, but a subset of genes is also similarly regulated by different ROS (Gadjev et al., 2006; Suzuki et al., 2011). A fruitful approach in modulating ROS concentrations in specific subcellular compartments has been with the engineering of the antioxidative enzymes. High light induces abundant changes in gene expression, and the role of photorespiratory H_2O_2 was addressed with peroxisomal catalase-deficient Arabidopsis (Vandenabeele et al, 2004). Lack of cytosolic ASCORBATE PEROXIDASE1 (APX1) increases H_2O_2 in the cytosol, but also has a detrimental effect on the chloroplast redox status (Davletova et al., 2005a). Paraquat (PQ, also known as methyl viologen) is a herbicide, which causes chloroplastic $O_2^{\bullet-}$ (and subsequently H_2O_2) production by accepting electrons from PSI. Plants overexpressing thylakoid-specific APX (tAPX) are tolerant to oxidative stress caused by PQ (Murgia et al., 2004) and have reduced pathogen growth (Yao and Greenberg 2006). In contrast, overexpression of tAPX enhanced the chloroplastic $^1O_2$ responses including electrolyte leakage and gene expression (Laloi et al., 2007), indicating that specific ROS even within the same subcellular compartment may antagonize one another.

The role of chloroplastic $^1O_2$ as signaling molecule has been studied with the conditional *fluorescent* (*flu*) mutant. In darkness, the *flu* mutant accumulates protochlorophyllide, which is degraded after dark-light switch thus producing $^1O_2$ within chloroplast. This leads to rapid

lipid oxidation, changes in gene expression and growth cessation (op den Camp et al., 2003). Studies with *flu* mutant and high light treatment of wild type plants share largely overlapping gene expression, which is expected due to the high amount of $^1O_2$ (instead of H_2O_2 or $O_2^{•-}$) produced under both conditions (González-Pérez et al., 2011). Furthermore, gene expression in the *flu* mutant after $^1O_2$ induction differs from the changes in gene expression caused by PQ. This reinforces the unique signaling properties of different ROS even within the same subcellular compartment (op den Camp et al., 2003). However, because PQ needs to be externally applied it is postulated that a longer response time is needed compared tofor instance for the dark-light shift response of the *flu* mutant (Gadjev et al., 2006).

Interestingly, high light-induced transcriptional changes are similar to *abscisic acid deficient1* (*aba1*) and *more axillary branching4* (*max4*) mutants lacking carotenoids that are required for dissipation of thermal energy and ROS detoxification (González-Pérez et al., 2011). Because *aba1* and *max4* are defective in the biosynthesis of plant hormones abscisic acid (ABA) and strigolactone, respectively, this result provides a link between $^1O_2$ and hormone signaling. Recently another research group showed that the lack of carotenoids lutein and zeaxanthin causes selective increase in $^1O_2$ under high light stress, but no significant overlap with *flu* gene expression was detected (Alboresi et al., 2011). Shao et al. (2007) presented a hypothesis that the differing location of $^1O_2$ production (within the chloroplast) in *flu* and carotenoid-deficient plants (peripherally at thylakoid membranes or within PSII reaction centers, respectively) could contribute to the non-overlapping transcriptional responses.

As discussed previously, the stress-induced increases in ROS are complex. Gadjev et al. (2006) studied the overlap between ROS-triggered and stress-induced gene expression. Quite unexpectedly transcripts specific to $^1O_2$ were the most abundant group of ROS-responsive genes induced by abiotic stresses. Clustering of transcripts responsive to biotic, abiotic and chemical stresses identified more than hundred universal stress genes, such as *UPREGULATED BY OXIDATIVE STRESS1* (*UPOX1*) and *SIMILAR TO RCD-ONE5* (*SRO5*) (Ma and Bohnert 2007). Ultimately, different ROS might regulate the same transcription factor, but by modification of different redox-sensitive cysteine groups of protein. H_2O_2 and $^1O_2$ induce the *HSP70A::luciferase* reporter construct via distinct promoter elements in the algae *C. reinhardtii* (Shao et al., 2007). The same approach in Arabidopsis could be extremely informative when used to dissect the temporal role of individual ROS in stress responses. It has been reported that the *ascorbate peroxidase* (*apx*) and *catalase* (*cat*) double mutant has increased stress tolerance compared to the respective single mutants (Rizhsky et al., 2002). Later a constitutively active DNA damage repair mechanism protecting the DNA from oxidative damage in *apx cat* plants was discovered (Vanderauwera et al., 2011). Therefore, perturbations of the ROS signaling network may result in enhanced sensitivity to another type of ROS, and either to increased or decreased stress tolerance.

1.1.5 Programmed cell death and ROS

1.1.5.1 Programmed cell death in plants

For eukaryotes, the controlled disposal of certain cells during growth and development is important for the benefit of the whole organism. Programmed cell death (PCD) is defined by a series of genetically encoded events leading to the controlled disposal of the protoplast content. PCD comes in many forms during plant development: in the formation of xylem vessels, flower and pollen development, aerenchyma formation in response to flooding, senescing leaves turning yellow, shaping of leaf margins or the break-down of grain endosperm to nourish the germinating seedling (Greenberg 1996; van Doorn and Woltering 2005; Gunawardena 2008). PCD can be viewed as a series of time-dependent events: first, signaling leads to the cellular commitment to PCD followed by the loading of vacuole with signal-specific enzymes (proteases, cellulases, chitinases, phytoalexins and/or nucleases). Thereafter, PCD takes place due to Ca^{2+} flux and vacuole collapse, and the cell remnants are further processed according to the developmental fate of the cell (Jones 2001). In addition to genetically encoded developmental programs, also abiotic and biotic stresses induce PCD (Love et al., 2008). To dissect PCD from external, irreversible injury causing passive cell death, hallmarks of PCD such as chromatin condensation, fragmentation of nuclear DNA into ladders, nuclear shrinkage, ATP depletion and vesiculation of cytosol have been extensively studied (Jabs 1999; Jones 2001; Overmyer et al., 2005; van Doorn et al., 2011). According to recent view, plant PCD may be divided into necrotic and vacuolar cell death both with distinct features, which may be partially overlapping in several known cases of PCD (van Doorn et al., 2011). It has been concluded that plant PCD does not fulfill the criteria for the apoptotic cellular suicide pathway conserved in animal cells, because formation of apoptotic bodies engulfed by surrounding cells is hindered by the plant cell walls. However, several commonalities between PCD in plants and apoptosis exist, such as ROS signaling (Jabs 1999). Therefore, ROS are instrumental for both plant life and death. Due to the genetic programming of plant PCD, so called lesion mimic mutants with either spontaneous run-away cell death or conditional cell death induced for instance by light (long day) or SA have been isolated to elucidate signaling pathways resulting in PCD (Lorrain et al., 2003).

1.1.5.2 Hypersensitive response

Hypersensitive response (HR) includes induction of local and systemic defense responses to pathogens and a form of rapid plant PCD. HR cell death encompasses features of both vacuolar and necrotic PCD (van Doorn et al., 2011). For the HR cell death to occur, plants have to first recognize the pathogen. Pathogen presence results in conserved pathogen-associated molecular patterns (PAMPs), which may be for instance flagellin, harpin, glucan, chitosan, lipopolysaccharides or peptidoglycanes depending on the pathogen in question

(Torres 2010). PAMPs are recognized by the plant with pattern recognition receptors on the cell surface or in the cytoplasm leading to PAMP-triggered immunity (PTI). However, pathogenic effector molecules, so called virulence factors, may disturb PTI, which allows the disease to progress. In this compatible interaction, disease but no HR cell death occurs. In an incompatible plant-pathogen interaction the attacker´s effector molecules are avirulent, they are identified by the plant and counteracted by *R* (*Resistance*)-genes. This line of defense is called effector-triggered immunity (ETI), which typically includes HR cell death. This interaction halts the spread of the disease; therefore, the plant and pathogen are incompatible. Clearly an elegant molecular warfare takes place between the pathogen and the plant during attempt to infect and defend, respectively (Jones and Dangl 2006). DAMPs (damage-associated molecular patterns), such as oligogalacturonides derived from damaged plant cell walls, may induce similar responses as PAMPs (Hématy et al., 2009).

ROS have a prominent role in the establishment of HR resulting in both local and systemic defense. Local production of $O_2^{\bullet-}$ in response to avirulent pathogen was first reported in *Phytophtora infestans* infected potato tubers, whereas a virulent race of the same pathogen did not elicit ROS production (Doke 1985). Since then, it has been shown that ROS production in incompatible plant-pathogen interactions is biphasic and consists of an early and late apoplastic oxidative bursts, whereas the early apoplastic ROS burst is detected in both compatible and incompatible pathogen responses (Baker and Orlandi 1995; Lamb and Dixon 1997). Apoplastic ROS are produced by NADPH oxidases and peroxidases, which is followed by defense gene activation, cell wall fortification and, in incompatible plant-pathogen encounters, HR cell death (Torres et al., 2006; Torres 2010).

Both PTI (with PAMPS) and ETI (with effectors) induce ROS production (Torres 2010). The ETI reaction is generally considered stronger than PTI, and HR cell death is also more characteristic to ETI (Jones and Dangl 2006). However, flagellin (a PAMP) is able to trigger HR cell death in Arabidopsis (Naito et al., 2008), and also in other species such as rice, tomato and tobacco (Taguchi et al., 2003). Lipid signals from the chloroplast have been shown to activate NADPH oxidases and increase ROS production and HR cell death in response to avirulent bacteria *Pseudomonas* challenge (Yaeno et al., 2004). PCD may be triggered by H_2O_2 , $O_2^{\bullet-}$ and $^1O_2$ (Gechev et al., 2006). Levine et al. (1994) established that H_2O_2 is sufficient and required for PCD. Decreased antioxidative capacity has been shown to increase high-light induced active cell death similar to hypersensitive cell death in catalase-deficient tobacco (Dat et al., 2003) and Arabidopsis (Vandenabeele et al., 2004). However, cell death in the lesion mimic mutant *lesions simulating disease resistance1* (*lsd1*) can be initiated with $O_2^{\bullet-}$ but not H_2O_2 (Jabs et al., 1996). RBOHD is not necessary for pathogen-induced cell death, but it limits the spread of the HR cell death (Torres et al., 2005). Altogether, the roles of ROS in hypersensitive cell death and the importance of HR in the establishment of successful defense may depend on the plant species and pathogen in question.

1.1.5.3 Ozone is an abiotic inducer of defense responses

Ozone (O_3) is an air pollutant with increasing concentrations in the troposphere due to industrialization (Sitch et al., 2007). O_3 is also a ROS which is harmful to plant and animal life. O_3 enters the plants via stomata, which are central in regulation of O_3 entry and quantity of oxidative stress caused (Vahisalu et al., 2008; Brosché et al., 2010). Once inside the foliar tissue, O_3 reacts with plant cell walls, apoplastic fluid and plasma membranes degrading rapidly into H_2O_2, $O_2^{\bullet-}$ and $OH^{\bullet}$ (Heath 1994; Rao et al., 2000a). Consequently, concentration of O_3 inside the leaf even during O_3 treatment is close to zero (Laisk et al., 1989). Apoplastic ascorbate has been regarded as the first defense against O_3 (Conklin and Barth 2004; Baier et al., 2005). Reduced ascorbate levels resulted in O_3-sensitivity in *vitamin c defective1* (*vtc1*) Arabidopsis mutant (Conklin et al., 1996; Conklin et al., 2000) and the cytosolic dehydroascorbate reductase mutant with significantly decreased apoplastic ascorbate content is highly O_3-sensitive (Yoshida et al., 2006). However, reaction between ascorbate and O_3 may also yield $^1O_2$, and therefore add yet another ROS to the oxidative load generated by O_3 (Kanofsky and Sima 1995; Sandermann 2008).

Apoplastic ROS formed as degradation products of O_3 may cause some damage within the tissues, but more importantly, they activate signaling events such as Ca^{2+} influx into the cytosol (Clayton et al., 1999; Evans et al., 2005) and endogenous production of ROS by plant cells (Rao and Davis 1999; Pellinen et al., 1999; Overmyer et al., 2000; Wohlgemuth et al., 2002; Mahalingam et al., 2006). Controlled O_3 treatments have indicated that endogenous ROS production is biphasic in O_3-sensitive species and Arabidopsis mutants, similar to incompatible plant-pathogen interactions, whereas only the early ROS production peak is present in O_3-tolerant plants (Schraudner et al., 1998; Joo et al., 2005a). The first ROS peak originates from stomatal guard cell chloroplasts and NADPH oxidases, which together trigger ROS production in adjacent epidermal cells (Joo et al., 2005a). Within minutes after the start of the O_3 treatment ROS production in guard cell chloroplasts coincides with rapid transient stomatal closure, which is dependent on the activation of guard-cell specific SLOW ANION CHANNEL-ASSOCIATED1 (SLAC1) anion channel (Vahisalu et al., 2008; Vahisalu et al., 2010). Peroxidases and oxidases localized at the cell wall may also be involved in the early endogenous ROS production (Pellinen et al., 1999). The later endogenous ROS burst is NADPH-oxidase dependent (Joo et al., 2005a). Intracellular ROS accumulation in cytoplasm, mitochondria and peroxisomes has also been observed after O_3-treatment in birch (*Betula pendula*)(Pellinen et al., 1999).

In addition to ROS, O_3 induces production of NO (Mahalingam et al., 2006; Ederli et al., 2006; Ahlfors et al., 2009), which is known to regulate both plant development and stress responses (reviewed in Moreau et al., 2010). Plant hormones ethylene, jasmonic acid (JA), SA and ABA are produced in O_3-treated plants in a time-dependent manner (discussed in detail later in chapter 1.2.). Altogether, although the mechanisms by which O_3-derived apoplastic ROS are initially perceived are not completely characterized, they are known to activate a complex signaling network composed of early and late events (summarized in Fig. 2).
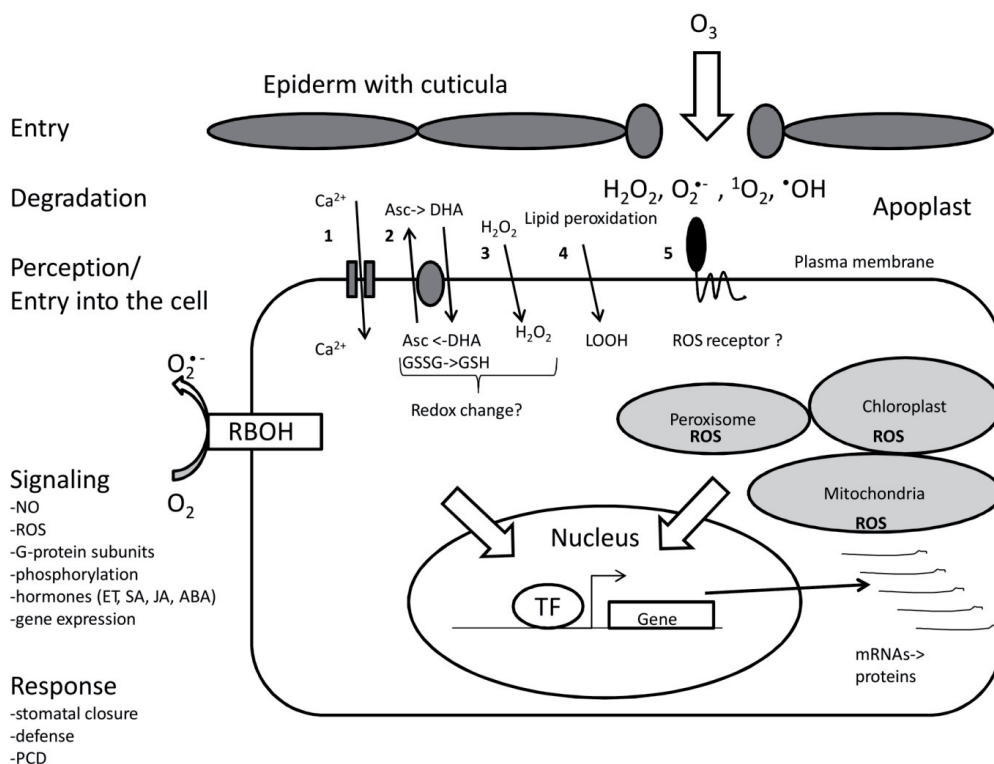
Figure 2. Summary of the O_3 entry, degradation, putative perception mechanisms and signaling events regulating gene expression and downstream responses. 1) calcium channels, 2) ascorbate oxidation and transport 3) direct diffusion of H_2O_2 across the plasma membrane 4) lipid peroxidation and 5) putative transmembrane ROS receptor. Asc, ascorbate; DHA, dehydroascorbate; LOOH, lipid hydroperoxide; TF, transcription factor; ET, ethylene, SA; salicylic acid, JA, jasmonic acid, ABA, abscisic acid, GSSG and GSH, oxidized and reduced glutathione, respectively.

The overlap between molecular responses caused by O_3, pathogens and other stresses has been noticed almost 20 years ago (Kangasjärvi et al., 1994). In Arabidopsis, O_3 was shown to increase transcripts encoding antioxidative and defense-related proteins (*SOD*, *PHENYLALANINE AMMONIA LYASE (PAL)*, *GLUTATHIONE S-TRANSFERASE (GST),* peroxidase and PR- proteins) (Sharma and Davis 1994; Sharma et al., 1996). Recently it was shown that O_3-derived changes in the expression of cysteine-rich receptor like kinases are the more similar to pathogen treatments than to for instance high light stress (Wrzaczek et al., 2010). Furthermore, O_3-induced PCD is largely similar to cell death occurring in HR, and this has allowed the use of O_3 as a noninvasive method for studying the signaling leading to programmed cell death (Rao et al., 2000a; Kangasjärvi et al., 2005). In comparison to other ROS sources and treatments causing ROS production, O_3 causes large-scale changes in gene expression in Arabidopsis (Gadjev et al., 2006). Number of O_3-responsive genes is dependent on the O_3 concentration, duration of O_3 exposure and experimental methods including the hybridization platform used to analyze the transcript abundance (summarized in Table I). In general, acute O_3-exposure induces genes mostly related to pathogen defense and cell death, whereas chronic O_3 mostly represses gene expression and may cause premature senescence.

Table I. Studies analyzing O₃-responsive gene expression using either suppression subtractive hybridization (SSH) or array hybridization techniques. NA, not available in the publication; * experiment information in the databases varies (also 1h 200 ppb O_3 treatment has been reported).

Reference	Hybridization platform	Plant material	Plant age	O_3 treatment	Time points	O_3-responsive genes	Database accession number
Mahalingam et al., 2003	cDNA macroarray 1K	Col-0	4 weeks	350 ppb 1h	1h	238	NA
Mahalingam et al., 2003	SSH stress library	Col-0	4 weeks	350 ppb 6h	1, 3, 6, 9, 12, 24h	257	NA
Mahalingam et al., 2003	SSH stress library	Col-0	3-4 weeks	150 ppb 6h for 6 days	2, 4, 6 days	69	NA
Tamaoki et al., 2003	cDNA macroarray 12K	Col-0	2 weeks	200 ppb 12h	12 h	205	NA
Ludwików et al., 2004	Affymetrix 22K (ATH1)	Col-0	4 weeks	350 ppb 6h	3h, 6h	2385	NA
Miyazaki et al., 2004	Oligoarray 26K (Qiagen)	Col-0 ?	?	1.2 x ambient (60-90 ppb)	8-12 days	635	NA
Tuominen et al., 2004	cDNA array (126 oligos)	Col-0	3 weeks	250 ppb 6h	2h, 8h	NA (84 according to Table S1)	NA
Li et al, 2005	Oligoarray 26 K	Col-0, WS and Cvi-0	?	1.2 x ambient (60-90 ppb)	8-12 days	630 (Col-0)	NA
Mahalingam et al., 2005	cDNA macroarray (1391 oligos)	Col-0	4 weeks	350 ppb 6h	3, 6, 9, 12h	200 (max. 533)	NA
D'Haese et al., 2006	Oligoarray 21,5 K (Agilent 2)	Col-0	3 weeks	150 ppb 8h for 2 days	2 days	582	NA
Gadjev et al., 2006	Affymetrix 22K (ATH1)	Col-0	2 weeks	500 ppb 6h	6h	approximately 2250	NASCARRAYS-26 (also GSE5722, E-MEXP-342*)
Mahalingam et al., 2006	Oligoarray 26K	Ws-0	4 weeks	300 ppb, 6h	1, 4, 8, 12 and 24h	330 (max. 371)	NA
Tosti et al., 2006	Affymetrix 22K (ATH1)	Col-0	4 weeks	300 ppb 6h	3h, 12h	569 (max. 2601)	NA
Ludwików et al., 2009	Affymetrix 22K (ATH1)	Col-0 and abi1td	4 weeks	350 ppb for 9 h	3h, 6h	NA (for Col-0)	E-MEXP-1863

HR-like cell death caused by O_3 treatment has been shown to be positively regulated by plant hormones ethylene and SA, whereas JA reduces O_3-triggered cell death lesions (Kangasjärvi et al., 2005). Reports of increased O_3 sensitivity due to a lack of mitochondrial ALTERNATIVE OXIDASE1a (AOX1a) in tobacco suggest that mitochondrial ROS are important for O_3-induced cell death (Pasqualini et al., 2007). Cyclic nucleotide-gated ion (Ca^{2+}) channel lacking from *defense no death1* (*dnd1*) is required for O_3-induced cell death and signaling (Overmyer et al., 2005; Wrzaczek et al., 2010). Inhibitors of proteases, kinases, transcription and translation blocked O_3-induded cell death, which highlights the role of active signaling and gene expression in PCD regulation (Overmyer et al., 2005). Anion channels (anion efflux), Ca^{2+} uptake and ROS were positive regulators of O_3-induced PCD in cultured Arabidopsis suspension cells and increased levels of VACUOLAR PROCESSING ENZYMEγ (VPEγ, At4g32940) transcript and protein were observed (Kadono et al., 2010). VPEs have caspase-like protease activity and may be directly involved in PCD execution (Hara-Nishimura and Hatsugai 2011). Altogether, plant hormones, Ca^{2+}, ROS and active signaling events including transcription have been shown to be required for O_3-induced PCD. However, *in planta* O_3 may induce additionally also direct necrotic damage or chlorosis, which may coexist with HR-like damage (Pell et al., 1997; Overmyer et al., 2008). Therefore careful assessment of morphological markers is required to determine the type of cell death in O_3-treated plants (Overmyer et al., 2005). HR-related cell death is perhaps the best studied form of PCD in plants, and therefore many genes involved in the regulation of PCD have been identified from plants altered in pathogen-triggered PCD. Using O_3 in the study of for novel PCD -associated genes offers the advantage of screening large mutant populations with easily adjustable amount of stress applied without dependence of particular pathogen.

1.2 Plant hormone signaling

By definition, plant hormones are naturally occurring, organic substances which influence physiological processes at low concentrations. These processes entail all aspects of plant growth, development and stress responses. Plant hormones may directly affect the tissues in which they are synthesized, but also hormone gradients regulating plant development in different parts are formed by hormone transport, for instance from root to shoot (cytokinin, CK) or shoot to root (auxin). The advance of molecular tools in plant biology, especially with the model plant Arabidopsis, has significantly increased our understanding of plant hormone signaling within the last decade. Importantly, receptors for all major plant hormones apart from SA have been identified. The main mode of action for plant hormones is signaling via receptor binding, which results in changes in gene expression and eventually in adjustments in plant metabolism. Molecular studies on hormone signaling have been possible prior to detailed knowledge on the receptors by analysis of hormone-regulated transcripts. Individual hormones work in a complex web of crosstalk of antagonistic and synergistic effects between other hormones and signaling molecules, such as secondary

messengers ROS or NO. Hormonal interactions may be classified into three groups: i) direct crosstalk i.e. targeting the same gene or protein, ii) indirect crosstalk at the level of perception, biosynthesis, transport or degradation and iii) co-regulation of the same process via several input pathways determining the output response (Chandler 2009). Comparative analysis of Arabidopsis seedling transcriptome after treatments with seven individual hormones regulating altogether 4666 genes revealed that a low amount of transcripts is regulated by more than one hormone. Instead, the transcripts responsive to different hormones were suggested to overlap at the level of biological processes (Nemhauser et al., 2006). Furthermore, the number of transcripts regulated by each phytohormone varied between 125 (gibberellic acid) and 2936 (ABA) (Nemhauser et al., 2006).

In the next chapters, the hormonal signaling pathways regulating gene expression are introduced together with their role in plant stress responses and development as well as selected interactions with other signaling pathways.

1.2.1 Ethylene

The gaseous plant hormone ethylene, a simple hydrocarbon, regulates many aspects of the plant life such as fruit ripening, leaf abscission, seed dormancy and senescence (Davies 2004). A well-studied ethylene-mediated growth response is the so called triple response with characteristic stunted dark-grown seedlings with exaggerated apical hooks and hypocotyl swelling (Neljubow 1901). Increased ethylene production is a rapid response to numerous biotic and abiotic stresses therefore creating the concept of "stress ethylene" (Yang and Hoffman 1984). Ethylene is produced from the methionine (Yang) cycle intermediate S-adenosyl methionine (SAM) in two consecutive reactions catalyzed by 1-aminocyclopropane-1-carboxylic acid (ACC) synthases (ACSs) and ACC oxidases (ACOs), respectively (Yang and Hoffman 1984). The ACS activity has been estimated to be the rate-limiting step of ethylene biosynthesis (Liu and Zhang 2004). Phosphorylation by MITOGEN-ACTIVATED PROTEIN KINASE6 (MPK6) stabilizes the ACS2 and ACS6 proteins and leads to increased ethylene biosynthesis (Liu and Zhang 2004; Joo et al., 2008). MPK6 and MPK3 are themselves rapidly activated by phosphorylation after apoplastic ROS treatment onset independently from ethylene, JA or SA (Ahlfors et al., 2004b), which suggests that ethylene biosynthesis represents a node between hormone and ROS signaling. Increased *ACS6* expression is a marker for O_3-induced gene expression (Vahala et al., 1998; Ahlfors et al., 2009) and also *ACS2* transcript levels increase in O_3-treated Arabidopsis (Overmyer et al., 2005). ACC oxidases are induced by O_3 in tomato (*Lycopersicon esculentum*) (Moeder et al., 2002).

Ethylene is perceived by a family of five two-component histidine kinase receptors residing in the endoplasmic reticulum, which act as negative regulators of ethylene signaling (Hua and Meyerowitz 1998). In the absence of ethylene they are activating a negative regulator

of ethylene signaling, Raf-like kinase CONSTITUTIVE TRIPLE RESPONSE1 (CTR1) (Huang et al., 2003). CTR1 further inactivates downstream positive ethylene signaling component ETHYLENE INSENSITIVE2 (EIN2), which is a protein of unknown biochemical function and upstream of EIN3 and EIN3-like (EIL) transcription factors. Binding of ethylene inactivates the ethylene receptors hence inactivating CTR1, activating EIN2, EIN3 and EILs and allowing ethylene-mediated gene expression. The primary targets of EIN3 and EILs are transcription factors *ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR1* (*ERF1*) and *ETHYLENE-RESPONSIVE DNA BINDING FACTOR1* (*EDF1*) to *EDF4*, which further broaden ethylene signaling to a second wave of ethylene-responsive genes (reviewed in Stepanova and Alonso 2009). These include for instance other ERFs, which bind to the ethylene responsive promoter element GGC box and may act as either positive or negative regulators of ethylene signaling (Fujimoto et al., 2000; Nakano et al., 2006). Treatment with ethylene precursor ACC altered expression of 500 genes in Arabidopsis seedlings (Nemhauser et al., 2006). Ethylene decreases expression of F-box ubiquitin ligases EBF1 and EBF2, which target EIN3 and its homologue EIL1 for ubiquitin-mediated degradation (An et al., 2010). Also EIN2 can be targeted for degradation (Qiao et al., 2009).

Ethylene production is an early in response to O_3, occurring after MPK activation and simultaneously with the first endogenous ROS burst in the mesophyll (Kangasjärvi et al., 2005). Increased ethylene production compared to tolerant plants is a marker for O_3-sensitivity across several species and cultivars (Overmyer et al., 2003). Ethylene production in O_3-treated plants is SA-dependent (Rao et al., 2002). Interestingly, ethylene signaling is also required for a PAMP -triggered oxidative burst (Mersmann et al., 2010), which places ethylene upstream of ROS production in chloroplasts. Ethylene signaling is known to interact with most, if not all, plant hormones (Yoo et al., 2009). In fact, this crosstalk is likely the reason why ethylene has such a wide range of effects on plant development and stress responses (Stepanova and Alonso 2009).

1.2.2 Salicylic acid

Salicylic acid (SA) is a phenolic plant hormone, which is involved in plant developmental processes such as seed germination, senescence and flowering (Rivas-San Vicente and Plasencia 2011). However, SA has preliminarily been regarded as a stress hormone, because it is produced in response to abiotic and especially biotic stresses (Vlot et al. 2009 and references therein). Arabidopsis mutants with constitutively higher SA levels have in general reduced growth, whereas in SA-depleted plants (for instance *NahG* plants transformed with bacterial SA-degrading salicylate hydroxylase) increased growth is observed (Rivas-San Vicente and Plasencia 2011). SA has well-known significance in the establishment of pathogen defenses, which is co-occurring with the expression of defense genes such as *PATHOGENESIS RELATED1* (*PR1*) and the formation of systemic acquired resistance (SAR) (Rivas-San Vicente and Plasencia 2011). The SA signaling pathway is yet lacking knowledge

on directly SA-binding receptors. Interestingly, there are few tobacco proteins capable of SA-binding such as SAPB2 involved in SA methylation (Du and Klessig 1997) and a catalase (Chen et al., 1993). SA suppresses antioxidative enzymes (CAT and APX) together with NO (Chen et al., 1993; Durner and Klessig 1995; Klessig et al., 2000). SA and ROS have a complex relationship, because ROS induce SA production, but SA has also been shown to cause ROS production; therefore a self-amplifying loop between ROS and SA has been proposed (Overmyer et al., 2003; Vlot et al., 2009). Recently SA and ROS were proposed to regulate antagonistically the O_3-responsive *CYSTEINE RICH KINASE* (*CRK*) transcripts (Wrzaczek et al., 2010). ISOCHORISMATE SYNTHASE1 (ICS1) pathway has been shown to produce SA in response to O_3 (Ogawa et al., 2007).

SA signaling is largely dependent on NPR1 (Wang et al., 2006), but especially early SA-responsive transcripts may be NPR1-independent (Uquillas et al., 2004). NPR1 is localized in cytosol as multimers prior to stress/SA treatment, and after redox change in the cell (cytosol is reduced due to antioxidant formation) NPR1 is translocated as monomers to nucleus to activate gene expression through interaction with TGA transcription factors (Mou et al., 2003). NPR1 monomerization by SA treatment was shown to be mediated by increased thioredoxin expression followed by NPR1 S-nitrosylation (Tada et al., 2008). Furthermore, degradation of (phosphorylated) NPR1 is required NPR1-mediated gene expression (Spoel et al., 2009), which further adds complexity to NPR1 as a signaling protein. NPR1 may additionally serve as a node for hormonal signal integration for instance between brassinosteroid (BR) and SA (Divi et al., 2010) and JA and SA (Dong 2004). From the point of signal integration, it is interesting that stress-induced SA is originated from the chloroplast (Fragnière et al., 2011), which is a ROS-source also involved in the biosynthesis of several other plant hormones including gibberellins (GA), auxin, BR, ABA and JA. Abundant interactions between SA and other plant hormones, perhaps most importantly in the regulation of pathogen tolerance, have been observed. In general, SA promotes resistance against biotrophs, whereas JA and ET are required for tolerance against necrotrophic pathogens (Glazebrook 2005). During immune responses, ICS1 is negatively regulated by ethylene signaling (Chen et al., 2009).

1.2.3 Jasmonic acid

The scent of jasmine flowers includes the methyl derivative of plant hormone JA (Davies 2004). JA is a lipid-derived hormone synthesized initially in chloroplasts followed by β-oxidation in peroxisomes (Schaller and Stintzi 2009). The biologically active form of JA, amino acid conjugate jasmonoyl-isoleucin (JA-Ile), is catalyzed by jasmonate-amido synthase JASMONATE RESISTANT1 (JAR1) (Staswick and Tiryaki 2004). The JA-Ile molecule structure is mimicked by the bacterial phytotoxin coronatine (Staswick and Tiryaki 2004; Katsir et al., 2008). The similarities between coronatine and methyl-jasmonate (MeJa) responses led to the isolation of *coronatine insensitive1* (*coi1*) mutant, which also was insensitive to MeJa,

male sterile and increasingly tolerant to coronatine-producing *Pseudomonas syringae* strain (Feys et al., 1994). COI1 encodes an F-box protein (Xie et al., 1998), which is part of a SKP1(S-PHASE KINASE ASSOCIATED PROTEIN1)-CULLIN-F-box (SCF) E3 ubiquitin ligase complex SCF^{COI1} (Xu et al., 2002). Binding of JA-Ile to SCF^{COI1} targets the negative regulators of jasmonate signaling, JASMONATE ZIM-domain (JAZ) proteins, to ubiquitination and subsequent degradation via 26S proteosome (Chini et al., 2007; Thines et al., 2007). This releases basic helix-loop-helix (bHLH) transcription factors MYC2, MYC3 and MYC4 to regulate expression of JA-responsive genes (Chini et al., 2007; Cheng et al., 2011; Fernández-Calvo et al., 2011). Binding of SCF^{COI1} to JAZ-proteins not only requires JA-Ile as the molecular glue, but also inositol phosphate is a co-factor in this complex (Sheard et al., 2010). Alternative splicing of the Jas domain interacting with the SCF^{COI1} complex may lead to ubiquitination-resistant JAZs proteins still able to repress *MYC2* (Yan et al., 2007; Chung et al., 2010).

Wounding or herbivore attack results in rapid increase in JA and JA-Ile levels, which induces expression of *JAZ* transcripts and JA biosynthesis genes (Chung et al., 2008). Additionally, JA-induced genes include members of ERF and MYB TF families, genes related to secondary metabolism and defense while genes related to abiotic stress responses are repressed by JA treatment (Devoto et al., 2005). JA is known to reduce growth, and mechanical wounding inducing JA has a similar effect (Yan et al. 2007). In general, JA promotes defense and reproduction (Browse 2009), while it reduces cell divisions and growth (Zhang and Turner 2008). JA and SA are well-known antagonists in the plant defense , and JA is required as a "pro-life" signal against necrotrophic pathogens, which benefit from cell death (Glazebrook 2005). Treatment with JA reduces the lesion formation triggered by O$_3$ (Örvar et al., 1997; Overmyer et al., 2000; Rao et al., 2000b; Tuominen et al., 2004) and the *jar1* mutant exhibits increased cell death in response to O$_3$ (Overmyer et al., 2000; Rao et al., 2000b; Tuominen et al., 2004; Overmyer et al., 2005). Increased JA levels are connected to simultaneous O$_3$-induced lesion occurrence in Arabidopsis (Tuominen et al., 2004; Overmyer et al., 2005) and silver birch (*Betula pendula* Roth) (Vahala et al., 2003). This might result from the release of JA precursors due to cell-death related membrane damage and subsequently synthesized JA may participate in the lesion containment (Overmyer et al., 2005).

1.2.4 Auxin

The word auxin is of Greek origin, and means "to grow". Since the initial studies of a putative mobile signal regulating the phototropic movement of grass coleoptiles (Darwin and Darwin 1880), auxin has been attributed to every aspect of plant growth and development (Benjamins and Scheres 2008). The most abundant active form of auxin is indole-3-acetic acid (IAA), which is perceived by a mechanism similar to JA. Indeed, the first auxin F-box receptor identified, *TRANSPORT INHIBITOR RESPONSE1* (*TIR1*) (Dharmasiri et al., 2005a; Kepinski and Leyser 2005), contains high similarity to COI1. Five IAA-receptors

homologous to TIR1 are named AUXIN SIGNALING F-BOX PROTEINS (AFB) 1 to 5 (Dharmasiri et al., 2005b). AUXIN/INDOLE-3-ACETIC ACID (Aux/IAA) proteins are negative regulators of IAA responses acting also as IAA co-receptors binding to $SCF^{TIR1/AFB}$ complex, which results in their ubiquitination and degradation (Gray et al., 2001; Zenser et al., 2001; Tan et al., 2007). Aux/IAAs have a high degree of functional redundancy (Overvoorde et al., 2005), but promoter-swap experiments have also revealed divergent functions of for them (Muto et al. 2007). Functional specificity of Aux/IAAs relates also to specific interaction pairs with AUXIN-RESPONSE FACTORs (ARFs), which are negatively regulated by Aux/IAAs (Weijers et al., 2005). The ARFs can be divided into positive and negative regulators of gene expression depending on the glutamine residues in their sequence (Guilfoyle and Hagen 2007). The most rapid auxin-responsive transcripts belong to *Aux/IAA* (hence forming a negative feedback loop), *SMALL AUXIN UP-REGULATED* (*SAUR*) and *GRETCHEN HAGEN3* (*GH3*) gene families (Abel and Theologis 1996). Also members of LATERAL ORGAN BOUNDARY (LOB) and some ARFs are auxin-induced (Paponov et al., 2008). Non-transcriptional auxin response occurs via AUXIN BINDING PROTEIN1 (ABP1)-mediated endocytosis of clathrin-coated vesicles (Robert et al., 2010).

Recently auxin has been shown to have a role in plant stress responses. Decreased auxin signaling in *tir1 afb* mutants improves tolerance to PQ, H_2O_2 and salt stress (Iglesias et al., 2010). The *tir1* mutant has also elevated resistance to *P. syringae* and degradation of TIR1/AFB transcripts by pathogen-induced microRNA 393 (miR393) decreases expression of auxin-responsive genes (Navarro et al., 2006). In another study, SA decreased auxin signaling, which was beneficial for pathogen tolerance, by the stabilization of Aux/IAAs (Wang et al., 2007). Auxin responses are on the other hand required for tolerance against necrotrophic fungi (Llorente et al., 2008). The relationship between ROS and auxin is complex, because some auxin-driven developmental responses such as gravitropism (Joo et al., 2001; Joo et al., 2005b) and coleoptile elongation (Schopfer et al., 2002) are ROS-dependent. In contrast, glutathione and thioredoxin triple mutants have decreased levels of auxin and auxin responsive reporter construct *DR5-uidA* expression, as well as several developmental defects such as lack of flowers and secondary roots (Bashandy et al., 2010). H_2O_2 and activation of MPKs has been shown to reduce auxin-dependent gene expression (Kovtun et al., 1998; Kovtun et al., 2000; Nakagami et al., 2006). Heat stress decreased expression of auxin marker transcript BA and growth in cultured guard cell chloroplasts independently of exogenous application of synthetic auxin NAA, H_2O_2 or H_2O_2 and $O_2^{\bullet-}$ scavengers (Dong et al., 2007). Expression of auxin-responsive genes was reduced by fungal toxin ALL downstream of H_2O_2 and ethylene production (Gechev et al., 2004). Recently oligogalacturonides, which are plant-derived damage-associated molecules, were shown to decrease auxin signaling without altered stability of Aux/IAAs, *TIR1/AFB* receptor expression or miR393 levels and independently from RBOHD-produced ROS (Savatin et al., 2011). Conjugation i.e. inactivation of another form of auxin, indole-3-butyric acid (IBA), was

recently shown to be induced by H_2O_2 and mediate tolerance to drought and salt stress (Tognetti et al., 2010).

1.2.5 Gibberellin

Gibberellins (GAs) are a vast group of diterpenoid compounds present in plants, animals and fungi. Bioactive GAs regulate plant processes such as stem elongation (cell divisions and elongation), bolting/flowering, seed germination and endosperm utilization in grains (Davies 2004). Indeed, GA_3 secreted by the pathogenic fungi *Gibberella fujikuroi* to promote stem growth in rice (*Oryza* sp.) led to the isolation of first GA which was named accordingly (Yabuta and Sumiki 1938). Somewhat later, the nuclear-localized soluble GA-receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1) of rice was identified (Ueguchi-Tanaka et al., 2005). GID1 is has three functional orthologs (AtGID1a, AtGID1b and AtGID1c) acting also as GA receptors in Arabidopsis (Nakajima et al., 2006).The basic GA signaling pathway shows functional similarity to auxin and JA perception: GA receptors are nucleus-residing F-box proteins (E3 ligases) which upon binding to GA, are activated to bind to the inhibitors of GA signaling, the DELLA proteins. This leads to the polyubiqitination of DELLAs and their subsequent degradation by the 26S proteosome, thereby allowing the expression of GA-responsive genes (reviewed in Hirano et al., 2008). There are five DELLA proteins in Arabidopsis (Dill and Sun 2001), towards which AtGIDs possess different binding affinities (Suzuki et al., 2009). Also the mere formation of the GA-GID1-DELLA complex releases DELLA targets from their inhibitory effect independently from proteolysis both in rice (Ueguchi-Tanaka et al., 2008) and Arabidopsis (Ariizumi et al., 2008). Knowledge on DELLA function and targets is crucial for the understanding of GA signaling. Several hundreds of GA-responsive genes have been identified by treating GA biosynthesis *ga1-3* mutant seeds, flowers or seedlings with GA (Ogawa et al., 2003; Cao et al., 2006; Zentella et al., 2007), and overlap between DELLA-regulated genes has been observed (Cao et al., 2006; Zentella et al., 2007). DELLA proteins increase (and GA treatment decreases) the expression of GA biosynthesis genes and also GID receptors, thereby establishing a feedback regulation loop (Zentella et al., 2007). DELLAs have been shown to directly bind and inhibit bHLH transcription factors PHYTOCHROME INTERACTING FACTOR3 (PIF3) and PIF4 (Feng et al., 2008; de et al., 2008), and DELLA and PIF target genes are partially shared (Gallego-Bartolomé et al., 2011). Transcriptomic studies have also identified genes belonging to other hormone signaling pathways (such as ethylene, auxin, JA and ABA) as GA and/or DELLA responsive transcripts (Ogawa et al., 2003; Cao et al., 2006; Zentella et al., 2007; Gallego-Bartolomé et al., 2011). DELLAs have thereby emerged as integrators of several plant hormone signaling pathways.

Additionally, GA-GID1-DELLA pathway regulates plant growth and stress tolerance in adverse environmental conditions such as salt, flooding, cold and pathogen stress (Harberd et al., 2009). Plants lacking DELLAs were susceptible to necrotrophic pathogens due to

increased sensitivity to JA, a likely explanation why necrotrophic *G. fujikuroi* produces GA (Navarro et al., 2008). Interestingly for this study, DELLAs have been shown to induce expression of genes encoding antioxidative enzymes and thereby decrease ROS levels (Achard et al., 2008). H_2O_2 was shown to induce GA biosynthetic genes in germinating Arabidopsis seeds (Liu et al., 2010). Another putative link between ROS and GA might be *GAST1 PROTEIN HOMOLOG4* (*GASA4*), transcript induced by GA, which regulates flowering time and germination likely through activation of redox-regulated cysteins (Rubinovich and Weiss 2010).

1.2.6 Abscisic acid

Abscisic acid (ABA) regulates stomatal closure, inhibits seed germination and controls dormancy, but unlike the name would allow to assume, has no direct role in organ abscission (Davies 2004). ABA biosynthesis is induced under water-limiting conditions such as drought or salinity (Nambara and Marion-Poll 2005). ABA is important also for cold tolerance (Gilmour and Thomashow 1991) and wounding responses (Hildmann et al., 1992). The transcriptional responses to these stresses are partially ABA-dependent (Yamaguchi-Shinozaki and Shinozaki 2006). Members of soluble ABA-binding receptor family have in the past few years been identified by several independent research groups with different methods and therefore named in a complex manner as PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) family (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). Binding of ABA to PYR/PYL/RCAR receptors facilitates their interaction with type 2 C protein phosphatases (PP2C), which are well-known negative regulators of ABA signaling. This relieves the SUCROSE-NONFERMENTING PROTEIN (SNF1)-RELATED KINASE 2 (SnRK2) kinases from negative regulation by PP2Cs and allows them to activate ABA responsive element (ABRE) binding factors (ABFs) by phosphorylation. ABFs belong to the family of bZIP transcription factors, which together with APETALA2 (AP2) transcription factors binding to GC-rich coupling elements regulate the expression of ABA-responsive genes. The PYR/PYL/RCAR-PP2C-SnRK2 pathway is regarded as the ABA core signaling pathway of transcriptional regulation, which may be modified for instance by other ABA-binding proteins, additional kinases phosphorylating ABFs, ABF-ABA INSENSITIVE3 (ABI3) interaction, regulation of ion channel activity by OPEN STOMATA1 (OST1/SnRK2.6), ABA-responsive transcription factors of other families than ABFs (NAC, HD-Zip, Zn-finger, WRKY) and cross-talk with other hormone signaling pathways (reviewed in Raghavendra et al., 2010; Cutler et al., 2010; Weiner et al., 2010).

Interestingly, ABA-mediated stomatal closure is dependent on ROS production by RBOHD and RBOHF (Kwak et al., 2003) and this ROS production is impaired in *ost1* mutant (Mustilli et al., 2002). This is because OST1 interacts with RBOHF and activates it by phosphorylation (Sirichandra et al., 2009). ABA amendment was shown to cause a rapid ROS production and

the H_2O_2 scavenger dimethylthiourea (DMTU) altered ABA-responses of a subset of 3494 ABA-regulated genes in Arabidopsis suspension cells (Böhmer and Schroeder 2011). The ABA-regulated genes overlap largely with drought and salinity responses, but also with H_2O_2 treatment (Seki et al., 2002; Takahashi et al., 2004), thereby validating ABA's importance in abiotic stress tolerance and the close connection between ABA and ROS signaling. The role of ABA in pathogen responses is complex: it has been presented that ABA has either a positive or negative effect depending on the pathogen life style (Robert-Seilaniantz et al., 2007). For instance, ABA signaling antagonizes SA-mediated defenses independently of the ethylene/JA pathway (Yasuda et al., 2008).

1.2.7 Brassinosteroid

First plant steroid hormone, brassinolide was isolated from *Brassica napus* flowers (Grove et al., 1978), after which approximately 70 additional brassinosteroids (BRs) have been identified. BRs are required for normal plant growth, development and reproduction, and the lack of the main BR receptor BRASSINOSTEROID INSENSITIVE1 (BRI1) results in severe dwarfism and male sterility (Clouse et al., 1996; Li and Chory 1997). *BR1* belongs to a family of plasma-membrane localized leucine-rich repeat receptor kinases which can also bind BR (Kinoshita et al., 2005). The BR signal is transferred, by protein-protein interaction, to another plasma membrane localized leucine rich repeat-receptor like kinase (LRR-RLK), BRASSINOSTEROID ASSOCIATED KINASE1 (BAK1) and its homologs (Li et al., 2002; Karlova et al., 2006). Further downstream, BR signal leads to dephosphorylation of kinase BRASSINOSTEROID INSENSITIVE2 (BIN2) and subsequent activation of BRASSINOZOL RESISTANT1 (BZR1) and BZR2 (also known as BES1) transcription factors regulating expression of BR-responsive genes (Kim et al., 2009). The direct targets of these two transciption factors have been studied with chromatin immunoprecipitation microrrays (Sun et al., 2010; Yu et al., 2011), but only approximately 25% of them overlap with BR-regulated genes (Gudesblat and Russinova 2011), which suggests that the mechanical understanding of BR-mediated transcriptional changes is yet incomplete. Indeed, the number of BR-regulated genes identified from microarray experiments from BR-treated plants and BR mutants may be several thousands (Yu et al., 2011). BR signaling and responses are entwined with other hormones such as ABA, ethylene, auxin, CK, GA and JA (Zhang et al., 2009; Bajguz and Hayat 2009; Sun et al., 2010). The especially close synergistic connection between BR and auxin may be explained by shared components of the auxin signaling cascade (Nakamura et al., 2006; Vert et al., 2008). BRs are involved also in responses to extreme temperatures, osmotic and oxidative stress, and pathogens (Bajguz and Hayat 2009). At the molecular level, BAK1 and its paralog BAK1-LIKE1 (BKK1) may directly act as co-receptors in the perception of PAMPs and further establishment of PTI (Roux et al., 2011).

1.2.8 Cytokinin

Cytokinins (CKs) are a group of adenine derivates, which are named after their classical role in promoting cell divisions. Cytokinins also regulate cell elongation and differentiation as well as delay senescence. Cytokinin is sensed by two-component histidine kinases AHK2, AHK3 and AHK4/CRE1 (AHKs) located on the plasma membrane and/or endoplasmic reticulum. Cytokinin binding triggers a phosphorelay cascade similar to animal two-component systems and ethylene signaling (Schaller et al., 2011). AHKs phosphorylate histidine phosphotransferase proteins (AHPs), which in the nucleus phosphorylate (and activate) response regulators (ARRs). Based on the domain structure, ARRS can be divided to types A, B and C. Types A and C lack DNA binding domain present in type-B ARRs, which are predominant positive regulators of cytokinin-mediated transcription (Argueso et al., 2010). In addition to type-B ARRs, several other transcription factors respond to cytokinin stimulus, such as CYTOKININ RESPONSE FACTORS (CRFs) (Rashotte et al., 2006). Recently CK has also been associated with abiotic stress responses, as plants deficient in CK were more tolerant to salt and drought (Nishiyama et al., 2011). Arabidopsis histidine kinase AHK1 has been identified as an osmotic sensor acting likely via ARRs (Tran et al., 2007; Wohlbach et al., 2008). It has been suggested that osmotic stress affects CK signaling pathway and that ARRs promote cytokinin signaling but inhibit stress/ABA responsive genes (Tran et al., 2010). CK and auxin have a complex interaction in the regulation of plant development and examples of this relationship are for instance ARF-ARR interactions in the shoot apical meristem (Zhao et al., 2010), cytokinin-induced auxin biosynthesis (Jones et al., 2010) and cytokinin-transport regulated radial auxin distribution in roots (Bishopp et al., 2011). Pathogen-produced CK delays senescence which is apparent as "green islands" remaining nutrient-rich in otherwise senescing tissue; however, plant-produced CK promotes defense responses in concert with SA by interaction between ARR and TGA transcription factors (Choi et al., 2011).

As a conclusion, the boundary between so called stress hormones (ABA, SA, ethylene and JA) and developmental hormones (GA, auxin, BR and CK) has become more and more elusive. Both in plant stress and development, hormonal signals originating from deceivingly simplistic, linear pathways are integrated at multiple levels in order to achieve appropriate gene expression and responses. This output depends not only on the environmental stress but also the tissue type and developmental stage of the plant.

1.3 RADICAL-INDUCED CELL DEATH 1: a tip of an ice berg?

1.3.1 Mutation in RCD1 causes altered development and stress responses

Forward genetics approach utilizes mutagenized plant populations in the screen for mutants with altered phenotypes of interest, such as development or stress responses. *RADICAL-*

INDUCED CELL DEATH1 (*RCD1*) mutant sensitive to O_3 treatment was isolated from one of such pools of ethylmethylsulfonate (EMS)-mutagenized Arabidopsis seeds (Overmyer et al., 2000). The *rcd1* mutant is sensitive to apoplastic $O_2^{•-}$ but not to apoplastic H_2O_2 (Overmyer et al., 2000) and tolerant to chloroplastic $O_2^{•-}$ resulting from PQ treatment (Ahlfors et al., 2004a; Fujibe et al., 2004). Sensitivity to H_2O_2 in the growth media was detected together with salt sensitivity of *rcd1* (Katiyar-Agarwal et al., 2006). Indeed, these *rcd1* phenotypes again demonstrate the specificity of different ROS as well as the role of subcellular localization in ROS signaling. Later on the lesions forming in *rcd1* in response to O_3 were confirmed to fulfill the hallmarks of PCD (Overmyer et al., 2005). Additional reported stress phenotypes of *rcd1* include UV-B tolerance (Fujibe et al., 2004; Jiang et al., 2009), glucose tolerance (Ahlfors et al., 2004a; Teotia and Lamb 2009), mannitol tolerance (Teotia and Lamb 2009) and freezing tolerance of unacclimated plants (Fujibe et al., 2004). The *rcd1* mutant is also characterized by developmental phenotypes such as altered rosette morphology with more erected, curled leaves, premature flowering and increased stomatal conductance (Overmyer et al., 2000; Ahlfors et al., 2004a). Marker genes for ABA, ethylene and JA responses (*RAB18*, *CHIB* and *VSP1*, respectively) were all expressed at lower levels in *rcd1* under normal growth conditions suggesting that RCD1 is an integrative node of hormone signaling (Ahlfors et al., 2004a). In clean air conditions, *rcd1* also slightly overproduces ethylene (Overmyer et al., 2000) and NO (Ahlfors et al., 2009). Overall, *rcd1* displays pleiotropic phenotypes regarding stress responses, hormone signaling and development.

1.3.2 SRO protein family

RCD1 belongs to a small gene family with five homologs named SIMILAR TO RCD-ONE (SRO) 1-5 in *A. thaliana* (Ahlfors et al., 2004a). The protein function of RCD1 and SROs is unknown, but conserved domains have been assigned to them. RCD1 and its closest paralog SRO1 contain nuclear localization signals and a WWE domain (PS50918) predicted to mediate protein-protein interactions involved in ubiquination and poly-ADP-ribosylation reactions (Aravind 2001). The WWE domain is absent from other SROs. Common to the whole RCD1-SRO protein family is the domain of the catalytic core of POLY-ADP-RIBOSE POLYMERASE (PARP) (PS51059) and a C-terminal domain participating in protein-protein interactions named RCD1-SRO-TAF4 (RST) domain (Belles-Boix et al., 2000; Citarelli et al., 2010; Jaspers et al., 2010; I; II). Interestingly, several interaction partners of RCD1 were either known or putative transcription factors (Belles-Boix et al., 2000; I). None of the SROs 1 to 5 had been identified through the forward genetics approach, until Borsani et al. (2005) isolated *sro5-1* as a salt sensitive mutant. However, the SRO5 function was assigned to endogenous RNA silencing mechanism due to sequence overlap with neighboring P5CDH-gene involved in proline catabolism: induction of *SRO5* by oxidative stress (salt/H_2O_2) results in degradation

of *P5CDH* transcript and therefore proline accumulates to protect the plant from the stress (Borsani et al., 2005).

2 Aims of the study

The aim of this study was to obtain novel information regarding ROS signaling and responses. For this purpose, several Arabidopsis mutants representing SRO gene family were isolated from T-DNA insertion mutant collections available from stock centers. Gene expression of respective plants was studied by microarrays in clean air conditions and in response to O_3 treatment.

1) To characterize the functional redundancy within the SRO gene family in the regulation of plant development and stress responses (I, II, IV)

2) To identify novel signaling pathways and biological processes regulated by apoplastic ROS (III)

3) To identify genes and signaling pathways responsible for O_3-triggered PCD in *rcd1* mutant (IV)

3 Materials and methods

The methods used in this study are described in the respective publications I, II, III and IV (Table II). Plant material used is listed in Table III.

Table II. Methods used in publications I-IV. Parenthesis indicates that the method was used only by the co-authors in that publication.

Method	Publication
Auxin quantification	(III)
Floral dip transformation	I
Flowering time determination	(I)
GUS activity staining	(I), III
Ion leakage	III, IV
Leaf shape determination	III
Microarray hybridizations	I, II, III, IV
O_3 treatment	III, IV
Paraquat treatment	(I)
PARP-activity assay	(II)
Plant line genotyping and cloning	I, II, (III), (IV)
Quantitative real-time PCR	(I), II, III, (IV)
Rosette diameter measurement	III
Subcellular localization	(I), (II)
Western hybridization	(I), (II)
Yeast two hybrid	(I), (II)

Table III. Arabidopsis mutants and transgenic lines used in this study. Additional double mutants were created as described in I, III and IV.

Genotype	Ecotype	Description	Publication
pRCD1:uidA	Col-0	*RCD1* promoter fused to *uidA β*-glucuronidase	I
pSRO1:uidA	Col-0	*SRO1* promoter fused to *uidA β*-glucuronidase	I
pRCD1:RCD1	Col-0	*RCD1* promoter fused to genomic *RCD1* gene	I
pSRO1:RCD1	Col-0	*RCD1* promoter fused to genomic *SRO1* gene	I
sro1-1	Col-0	*similar to rcd-one1-1*	I
rcd1-2	Col-0	*radical induced cell death1-2*	I
rcd1-3	Col-0	*radical induced cell death1-3*	I
rcd1-4	Col-0	*radical induced cell death1-4*	I
rcd1-1	Col-0	*radical induced cell death1-1*	I, III, IV
sro5-2	Col-0	*similar to rcd-one5-2*	II
axr1-3	Col-0	*auxin resistant1-3*	III
DR5:uidA	Col-0	synthetic auxin responsive promoter fused to uidA	III
ein2	Col-0	*ethylene insensitive2*	III
NahG	Col-0	transgene encoding bacterial salicylate hydroxylase	III
npr1	Col-0	*non-expressor of pathogenesis related genes1*	III
sid2	Col-0	*salicylic acid induction deficient2*	III
aux1-7	Col-0	*auxin resistant1-7*	III
nit1-3	Col-0	*nitrilase1-3*	III
coi1-16	Col-0	*coronatine insensitive1-16*	III
iaa28-2	Col-0	*indole-3-acetic acid inducible28-2*	III
iaa28-1	Ws-0	*indole-3-acetic acid inducible28-1*	III
tir1-1 afb2-3	Col-0	*transport inhibitor response1-1/ auxin signaling F-box2-3*	III
mpk3	Col-0	*mitogen activated protein kinase3*	III
mpk6	Col-0	*mitogen activated protein kinase6*	III
wrky70	Col-0	*wrky DNA-binding protein70*	IV
lsd1	Col-0	*lesions simulating disease resistance1*	IV
acd2	Col-0	*accelerated cell death2*	IV
acd5	Col-0	*accelerated cell death5*	IV

4 Results and Discussion

4.1 Redundancy within SRO gene family

4.1.1 Complementation of *rcd1*

In this study, T-DNA insertion alleles *rcd1-3* and *rcd1-4* were isolated in order to dissect the role of a putative truncated *rcd1* protein in *rcd1-1* (I). The *rcd1-1* mutation is a guanine to adenosine transition at the third exon-intron junction, in the middle of the conserved PARP catalytic domain, which leads to two different sizes of misspliced transcripts each with premature stop codons (Ahlfors et al., 2004a). Also the *rcd1-2* mutation causes a premature stop codon in exon III and *rcd1-2* plants contain a mutant transcript (Fujibe et al., 2006). The *rcd1-3* mutant harbors a T-DNA insertion in exon IV also within the putative PARP domain and contains a mutant transcript (I) approximately the same size as in wild type Col-0 (data not shown). The *rcd1-4* line is mutated within the WWE domain (I) and no *rcd1* transcript has been detected (I). All these *rcd1* alleles result in a similar mutant habitus in clean air growth conditions. Comparison of gene expression in *rcd1-1*, *rcd1-3* and *rcd1-4* mutants studied with a full-genome microarray yielded in similar results and enabled pooling of the data (I). Because the T-DNA insertion in *rcd1-4* is disrupting the WWE-domain and neither *rcd1* transcript nor truncated protein is present in *rcd1-4* plants, this result suggests that also *rcd1-1* is a loss-of-function mutant. The premature flowering, plant habitus, PQ tolerance and expression of ANAC087 in both *rcd1-2* and *rcd1-4* were complemented by a RCD1 genomic construct *pRCD1:RCD1* (I), which supports the conclusion of a-loss-of function mutant. Ahlfors et al. (2004a) suggested a gain-of-function by a truncated protein in *rcd1-1* based on a gene dosage experiment and due to the O_3 sensitivity and PQ tolerance of *35S::rcd1* plants. However, Fujibe et al. (2006) observed that overexpression of a genomic RCD1 fragment caused a weak *rcd1* phenotype quantified as smaller plant size and slight increase in O_3 sensitivity in Col-0 background, but also complemented the *rcd1-2* phenotype. During this study, several transgenic *RCD1* cDNA constructs under cauliflower mosaic virus-derived 35S promoter were cloned, but neither *rcd1* growth habitus complementation nor reporter gene/epitope tag expression in Col-0 background was achieved (data not shown). Altogether the complex results from *35S::rcd1* and *35S::RCD1* plants point towards the *RCD1* expression level being under tight control.

The establishment of *rcd1* complementation was necessary for testing functional redundancy between RCD1 and SRO1 *in planta* (I). Whereas the *pRCD1:RCD1* construct fully complemented *rcd1* mutation, *RCD1* promoter fused to *SRO1* coding sequence provided partial complementation regarding flowering time while ANAC087 gene expression and no complementation of the PQ tolerance was observed (I). This established that paralogous RCD1 and SRO1 proteins are functionally partially overlapping, which may be expected due to the strong sequence similarity. The phenotype specific degree of *rcd1* complementation by SRO1 may suggest that the common function of RCD1 and SRO1 is mostly conserved in plant development (flowering, gene expression in clean air) whereas RCD1 has unique roles

in stress responses (such as PQ tolerance). However, because the recessive *rcd1* growth habitus was not complemented by *pRCD1:SRO1* in the T1 plants (data not shown), RCD1 is responsible for some unique functions also regarding plant development. The reason for only partial complementation of pleiotropic *rcd1* phenotypes by SRO1 is perhaps due to differences in protein catalytic activities, post-translational modifications or protein-protein interactions (discussed later in chapter 4.1.3).

4.1.2 Single and double mutants reveal *in planta* functions of SROs

Based on complementation studies, RCD1 and SRO1 display partial functional redundancy. A T-DNA insertion allele of *SRO1*, *sro1-1* was isolated and crossed to *rcd1-3* to study this redundancy further. The stunted, hardly viable *rcd1-3 sro1-1* double mutant phenotype suggested that *sro1-1* plants are deficient in a major developmental function shared between RCD1 and SRO1 (I). The *rcd1 sro1* plants were only recovered when grown *in vitro*, and after transfer to soil the number of seeds produced was miniscule. Abnormal development in *rcd1 sro1* double mutants is apparent already during embryonic development (Teotia and Lamb 2009). The *rcd1/rcd1 SRO1/sro1* plants are larger than the homozygous double mutant, which states that even a single functional SRO1 allele is able to partially rescue the severe *rcd1 sro1* double mutant (I).

Comparison of GUS activity in *pRCD1:uidA* and *pSRO1:uidA* plants show that *RCD1* and *SRO1* are expressed in the same tissues in leaves, roots and flowers (I). However, only *RCD1* was expressed in the stomatal guard cells (Katiyar-Agarwal et al., 2006; I). Transient expression analysis of 35S::RCD1-YFP and 35S::SRO1-YFP showed that both RCD1 and SRO1 are localized to nucleus, which is in accordance with the nuclear localization sequences in both proteins (I). RCD1 has previously been reported to localize to nucleus (Fujibe et al., 2006) but also to cytosol under salt stress (Katiyar-Agarwal et al., 2006).

Both developmental and stress-induced phenotypes of *sro1-1* plants characterized so far have been absent or non-severe (I; Teotia and Lamb 2009). Gene expression of *sro1-1* under normal growth conditions is very similar to Col-0, and the only gene with robustly altered expression was thylakoidal ascorbate peroxidase (tAPX; At1g77490) with two-fold decreased transcript level (I). The lowered amount of tAPX in *sro1* was confirmed with a western blot using a tAPX-specific antibody (Dr. S. Kangasjärvi, unpublished results). However, *rcd1 tapx* plants were indistinguishable from *rcd1* according to their growth phenotype (data not shown), which suggests that decrease in tAPX in *sro1* was not responsible for the severe developmental defects in *rcd1 sro1* plants. The *sro1-1* mutation resides in the C-terminal RST domain and a transcript is present upstream of this T-DNA insertion site (I). It is possible that the transcript present in *sro1* could yield a yet partially functional *sro1* protein with WWE and the PARP catalytic core domains that may explain the lack of a more marked single mutant phenotype. However, the severe phenotype of *rcd1*

sro1 double mutant and the partial complementation of *rcd1* mutation with *pRCD1:SRO1* construct point towards a severe loss-of-function of *sro1-1* allele lacking the C-terminus.

Like *sro1* mutants, *sro5* plants are indistinguishable from Col-0 under clean air growth conditions and show only minor alterations in gene expression (II). *SRO5* transcript itself was over 500-fold induced in *sro5-2* plants suggesting that *SRO5* transcript levels are negatively regulated by SRO5 (II). Because *SRO5* is consistently induced by a range of stresses (Gadjev et al., 2006; II), this may provide a necessary negative feedback loop. This feed-back regulation could possibly be associated with SRO5 being able to interact with transcription factors (II). The *sro5* plants were not more O_3-sensitive than Col-0 and *rcd1 sro5* mutants have the same degree of cell death as *rcd1* (Fig. 3), thereby reinforcing the view that *SRO5* does not regulate sensitivity to apoplastic ROS. Instead the *sro5* plants are salt sensitive, which has been linked to inadequate proline accumulation (Borsani et al., 2005; Babajani et al., 2009). Also the *rcd1* mutant is salt sensitive, which may be caused by the lack of RCD1 interaction with plasma membrane Na^+/H^+ antiporter SALT OVERLY SENSITIVE1 (SOS1) (Katiyar-Agarwal et al., 2006). Interestingly, *rcd1* has a constitutively higher level of proline (N. Sipari, unpublished results), which might buffer the lack of proline accumulation caused by *sro5* mutation in *rcd1 sro5* plants. Proline is considered to protect plants from salt and cold stress, and perhaps also scavenge ROS directly (Szabados and Savouré 2010). However, proline degradation by proline dehydrogenase (ProDH) causes hypersensitive cell death in response to pathogens (Cecchini et al., 2011). Elevated proline levels resulted in heat sensitivity and increased mitochondrial ROS formation (Lv et al., 2011). More studies are needed in order to evaluate whether proline metabolism is involved in any of the stress-related phenotypes of *rcd1*.

The phenotypes of *sro2*, *sro3* or *sro4* mutants are currently not characterized. Expression of *SRO2*, *SRO3* and *SRO5*, but not *SRO1*, is elevated in *rcd1* in clean air (II). All the SROs with detectable expression levels (all apart from SRO4) are induced by O_3 and salt stress (II). The biological roles of SRO2, SRO3 and SRO4 will remain to be determined in future studies. *SRO2* was induced by high light (II) and its expression is elevated in *tapx sapx* plants that lack chloroplast and mitochondria localized ascorbate peroxidases (Kangasjärvi et al., 2008), which suggests a connection to ROS signaling.
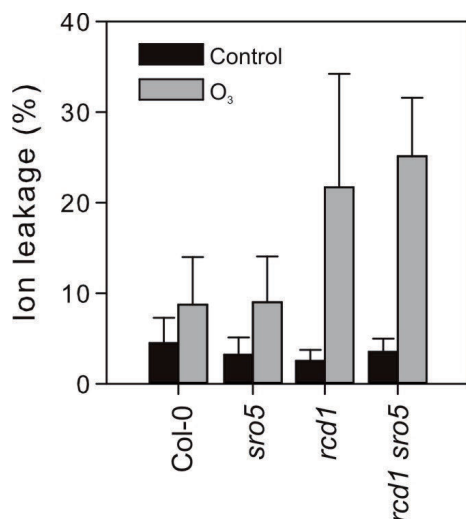
Figure 3. SRO5 does not regulate cell death triggered by apoplastic ROS in Col-0 or *rcd1*. Col-0, *rcd1*, *sro5* and *rcd1 sro5* were grown in environmentally controlled growth rooms and moved to growth chambers the day before the O_3 treatment. Three-week-old plants were treated with 400 nL L-1 for 6h and after 2h recovery in clean air plants harvested for ion leakage quantification. The means of three biological repeats are shown with standard deviation (n=14-15).

4.1.3 SRO conservation within plant kingdom: why?

Several plant genomes were analyzed for the presence of SROs (II). Two structural groups of SROs, type A and type B, could be identified based on the domains present in *Arabidopsis thaliana* SROs (II). Type A contains the longer WWE-PARP-RST structure, and type B consists of only PARP-RST domains. Interestingly, cryptogams (*Physcomitrella patens* and *Selaginella moellendorffii*) and monocots (*Oryza sativa* ssp. *japonica* and *Brachypodium distachyon*) contained only the type A, whereas eudicots (*Arabidopsis thaliana*, *Arabidopsis lyrata*, *Populus trichocarpa*, *Ricinus communis* and *Vitis vinifera*) contained both type A and type B SROs (II). SROs were absent from green algae, photosynthetic cyanobacteria, yeast and plant pathogen genomes (II), which suggests that SROs have evolved and diversified along the complexity and size of plants. The number of SROs falling into different structural types varied between the species studied: for instance, only in *Brassicaceae* RCD1 and SRO1 (both type A SROs) could be clearly defined. The Populus genome contained three type A SROs, which were equally related to both RCD1 and SRO1 of *A. thaliana*. Therefore, a unified nomenclature based on the SRO type was introduced, according to which only *A. thaliana* contains RCD1 (AtRCD1) (II).

What is the function that SROs perform? Biochemical data together with sequence analysis showed that RCD1 does not bind NAD^+ nor can function as a PARP (II). Still, the PARP domain is highly conserved within the SRO family. RCD1, SRO1 and SRO5 all interact with transcription factors and these interaction partners may be identical or represent different

members of the same protein family (I, II). These partially unique but also overlapping protein-protein interactions might explain the partially redundant functions of RCD1 and SRO1. However, these interactions should be verified with further *in planta* experiments. The putative role of SROs in transcriptional regulation is also suggested by the presence of the RST-domain, which is found in plants only in SROs and in the TBP-ASSOCIATED FACTOR4 (TAF4) protein (I; II). TAF4 belongs to the core promoter binding transcription initiation complex TFIID with TATA-BOX BINDING PROTEIN (TBP) and several other TAF proteins, and is essential for the TFIID complex stability (Wright et al., 2006). The RST domain of RCD1 is required for interactions with DREB2A and COL10 (II). On the contrary, the lack of WWE domain did not abolish any protein-protein interactions but resulted in even more interaction partners for RCD1 (I; II). This suggests that the WWE-domain is not necessary to the protein-protein interactions of RCD1, but it rather may modulate their specificity. WWE-domain may interact with another WWE domain (Zweifel et al., 2005), ankyrin domain (Matsuno et al., 1995) or with a poly-ADP-ribose moiety attached to another protein by PARPs (Zhang et al., 2011). The effect of poly-ADP-ribosylation on individual Arabidopsis proteins is largely unknown, but PARP activity has been connected to both abiotic (Vanderauwera et al., 2007) and biotic (Adams-Phillips et al., 2010) stress responses. The only defined Arabidopsis proteins with WWE domains and thus the only candidates for WWE-poly-ADP-ribose interaction are currently RCD1 and SRO1 (I; II).

The *rcd1* mutant has pronounced changes in gene expression under clean air conditions (I; IV), but only few genes were regulated in *sro1* and *sro5* plants, which suggests that SROs have obtained unique roles as putative transcriptional co-regulators. The role of RCD1 in transcriptional regulation is further discussed in the section "4.2.4 Cell death and gene expression". In addition to regulating *P5CDH* expression, no role of SRO5 protein itself has been verified yet. However, the overlapping *SRO5-P5CDH* transcript pair is only present in *A. thaliana*, so this particular function is absent from other species analyzed so far (II).

4.2 Apoplastic ROS and transcriptomics

4.2.1 Apoplastic ROS regulate thousands of stress-responsive transcripts

Apoplastic ROS are formed in response to a variety of environmental stresses, and O_3 treatment has been established as a sophisticated method to trigger apoplastic ROS production. To gain a comprehensive view on the cellular signaling cascade and responses, including cell death, downstream of O_3, we performed a time-series experiment with a nearly full-genome (21K) microarray platform with the Arabidopsis ecotype Col-0 and the *rcd1* mutant. Samples were harvested 0, 1, 2, 4, 8 and 24h from the start of the O_3 treatment (6h 350 nL L-1) (III; IV). Altogether, expression of 3635 genes (log ratio ±1; q<0.05) was changed in response to apoplastic ROS in Col-0 (III). This was clearly a larger number (2211) of O_3-responsive genes than in previously reported publically available full-

genome array experiments analyzed with the same criteria (III). This result is mainly due to the number of technical and biological replicates in this study yielding in statistically significant results, because the discovery of only approximately five hundred new O_3-regulated genes resulted from the use of also late time points (8h and 24h) and different array platform (MWG oligoarray instead of ATH1) (III). All subsequent qPCR analysis from independent biological repeats were largely in accordance with the obtained array results (III; IV), and therefore provided evidence that the used experimental conditions and analysis methods were reproducible. Clustering showed distinct expression patterns of O_3-responsive genes which allowed the detection of time-dependent, transient changes: expression profiles could be divided into early increased, late increased and decreased profiles (III). Mahalingam et al. (2005) identified similar expression patterns in a time series experiment with approximately 200 O_3-regulated genes. A larger number of O_3-induced versus decreased transcripts in early time points (III) is also in concordance with a previous study by Mahalingam et al. (2006).

To gain understanding of the biological relevance of this vast amount of O_3-responsive genes, we analyzed the Gene Ontology (GO) enrichments of O_3-regulated genes at each time point. In total this gene set represented 2219 biological processes, of which 502 were significantly enriched among genes with increased expression and 301 among genes with decreased expression (time point specific analysis) (III). The large number of enriched biological processes may be partially explained by the hierarchical overlap of GO categories. More importantly, there was also a large range of different biological processes among the O_3-regulated genes, such as abiotic and biotic stress responses, photosynthesis and secondary metabolism (III). A snapshot of this is presented in Fig. 4 showing gene expression at 4 h after the start of O_3 treatment, the time point with the largest number of O_3-regulated genes. This result is as expected according to the reported role of ROS as ubiquitous signaling molecules. Altogether one hundred biological processes were enriched among both increased and decreased transcripts, whereas individual O_3-regulated transcripts were generally only responsive in one of the two directions (up or down) (III). In general, stress treatments may simultaneously both activate and repress gene expression (Gadjev et al., 2006). The GO classifications of biological processes are based on different data sources: experimental data of microarray experiments (IEP; Inferred from Expression Pattern), mutant analysis (IMP; Inferred from Mutant Phenotype), but also computational predictions based on for instance sequence similarity (ISS; Inferred from Sequence Similarity) (http://www.geneontology.org/GO.evidence.shtml). These data sources bring heterogeneity to the GO groups, so a variety of responses may also be expected. Albeit there is constant updating of the annotations, the "response to ozone" classification (GO:0010193) contains merely 29 loci (TAIR10 annotation), which in light of the results obtained in this study (III) is an underestimate.
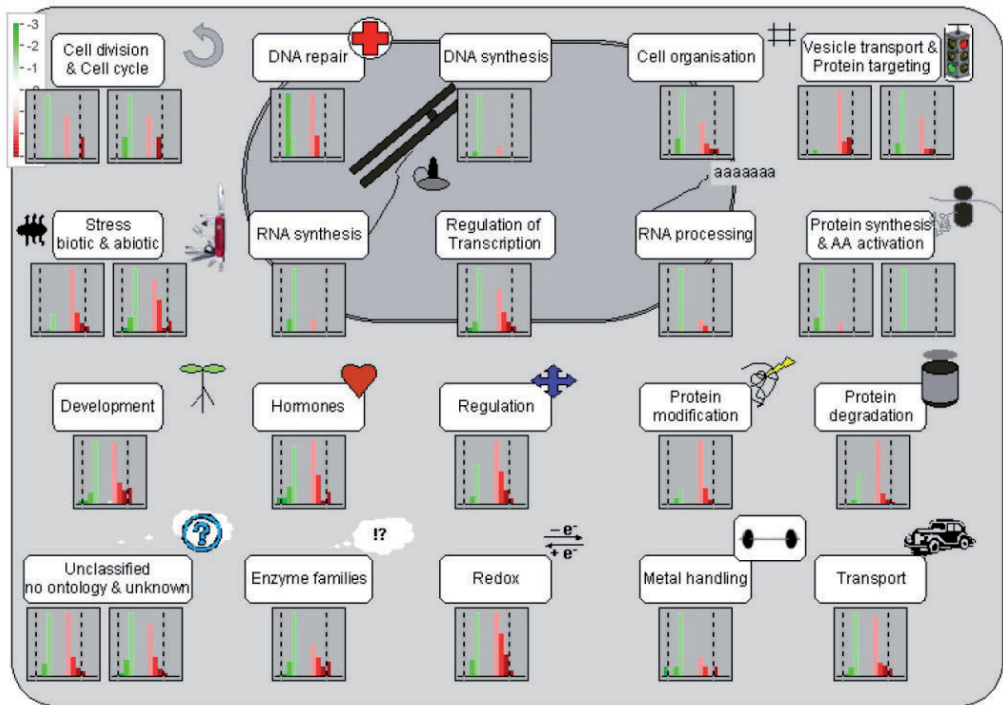
Figure 4. Apoplastic ROS may cause bidirectional changes in gene expression at the level of biological processes. The multitude of biological processes regulated by apoplastic ROS is exemplified with gene expression in Col-0 4h after the start of the O_3 treatment. 2516 O_3-regulated genes (log ratio ±1, q<0.05) were imported into the "cellular functions overview" pathway of MapMan 3.0.0 with TAIR8 Arabidopsis genome annotation (Thimm et al., 2004). The bars indicate the number of genes with increased (red) and decreased (green) expression annotated to different cellular functions.

4.2.2 Apoplastic ROS alter auxin signaling

From the GO analysis of O_3-responsive transcripts, it was apparent that apoplastic ROS affected the signaling of several plant hormones (III). Ethylene, SA and JA have all well characterized roles in responses to apoplastic ROS (Kangasjärvi et al., 2005). The biological process "response to ABA" was activated at all time points (III).Previous study by Overmyer et al. (2008) has shown that ABA concentration increases by O_3-treatment. In addition, auxin responsive transcripts were regulated by apoplastic ROS: response to auxin stimulus was enriched and comparison with transcripts regulated by IAA-treatment yielded in 60 overlapping transcripts, which comprised approximately 1/3 of the auxin responsive transcripts (III). In contrast to classical stress hormones ethylene, SA, JA and ABA, to which responses were almost exclusively induced, auxin-related transcriptional response was also

decreased. This decrease was especially abundant among *Aux/IAA* transcripts in O_3-treated plants and a similar response was observed for instance in UV-B, PQ, flagellin fragment flg22, SA-analog BTH and H_2O_2 experiments (III). Because Aux/IAAs negatively regulate their own expression via repressing ARF activity, this decrease may suggest a stabilization of Aux/IAA proteins as previously reported in response to flg22 (Navarro et al., 2006) and BTH (Wang et al., 2007). Because ARFs binding to AuxRE elements can be either positive or negative transcriptional regulators, simultaneous increased repression of both types of ARFs may explain partially reduced and increased auxin responses in O_3-treated plants. A rapid and transient decrease was observed in the expression of auxin reporter construct *DR5-uidA* and an auxin marker gene, transcription factor *HAT2* (III). This could be concomitant with transiently increased Aux/IAA protein stability: Increased stability of Aux/IAAs leads to decreased auxin-dependent gene expression, including Aux/IAA transcripts themselves, which would eventually reset the amount of Aux/IAA proteins (Fig. 5). This model about apoplastic ROS-auxin interaction involving Aux/IAA stability could be in future addressed for instance with the DII-VENUS reporter construct (Vernoux et al., 2011). The Aux/IAA stability is known to be regulated at the SCF$^{TIR/AFB}$ complex level: auxin-mediated interaction with SCF$^{TIR/AFB}$ complex leads to Aux/IAA ubiquitination and targets them for proteosomal degradation (Fig. 5, described in detail in 1.2.4).

Apoplastic ROS decreased the expression of auxin F-box receptors *TIR1*, *AFB1*, *AFB3* and *AFB5* (III), while no change was observed in the expression levels of *AFB2* or *FBX14/AFB4* (III). This could explain a decrease of SCF$^{TIR/AFB}$ interactions with Aux/IAA proteins leading to higher levels of Aux/IAA proteins. The TIR1, AFB1, AFB2 and AFB3 interact with Aux/IAA proteins in an auxin-dependent manner, are expressed in most cells and have redundant functions in auxin signaling (Dharmasiri et al., 2005b). However, *TIR1* and *AFB2* exhibit the strongest auxin-mediated Aux/IAA binding which suggests that they have more prominent roles in auxin signaling than *AFB1* or *AFB3* (Parry et al., 2009). Concordantly, *tir1 afb2* mutants performed better than *tir1 afb1* and *tir1 afb3* plants in response to salt, PQ and H_2O_2 (Iglesias et al., 2010), which may be due to a larger loss of auxin responsiveness contributing to stress tolerance. FBX14/AFB4 and AFB5 also interact with IAA3 in an auxin-dependent manner which is indicative of an auxin receptor function (Greenham et al., 2011). Unlike *AFB5*, *FBX14/AFB4* was unresponsive to apoplastic ROS (III) and was recently shown to have a negative effect on auxin responses in the hypocotyl (Greenham et al., 2011). The role of *AFB5* in mediating auxin responses is yet poorly known, because *afb5* mutants are resistant to synthetic auxins picolinate and dicamba but not to IAA or 2,4-D (Walsh et al., 2006; Greenham et al., 2011; Gleason et al., 2011a). *AFB2* transcript is decreased in response to flg22 by miRNA393-mediated degradation (Navarro et al., 2006). Neither decrease of *AFB2* transcript nor increase in miR393 in response to apoplastic ROS was observed (III), suggesting that the members of auxin F-box receptors are differentially regulated by stresses and that there are both miR393-dependent and independent mechanisms for their transcriptional regulation. It remains to be elucidated, whether stress-
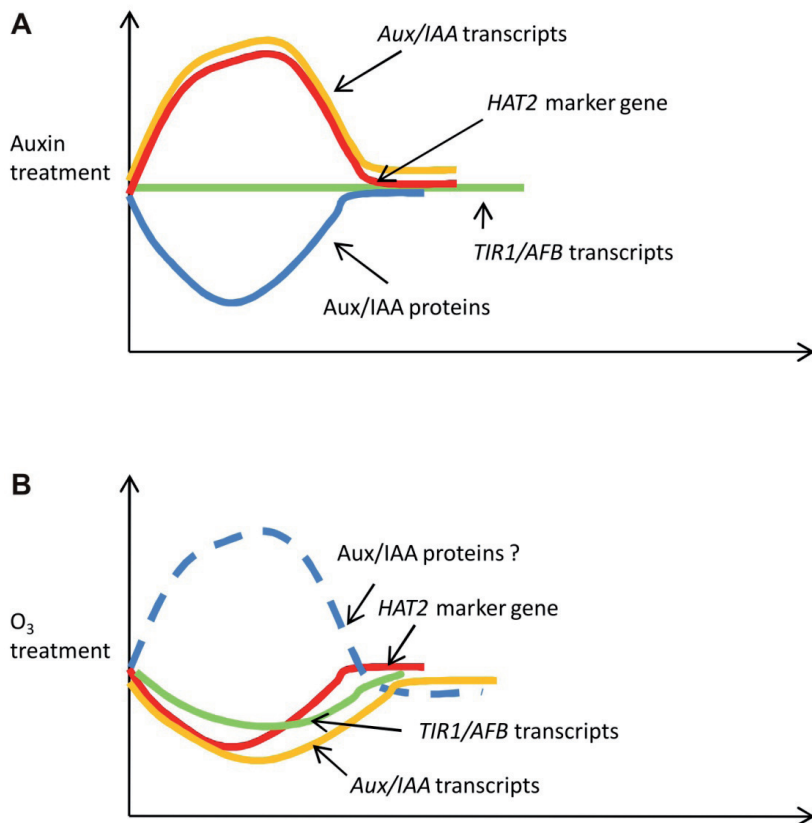
Figure 5. A model describing the expression levels of *TIR1/AFB* transcripts (green), *HAT2* (red) and *Aux/IAA*s (yellow) in response to auxin treatment and apoplastic ROS (O_3) . A) In auxin treatment, the degradation of Aux/IAAs is enhanced in a TIR1/AFB dependent manner. Auxin treatment is known to transiently decrease the levels of Aux/IAA proteins (blue). B) Aux/IAA protein levels may be negatively correlated with the *Aux/IAA* transcript expression also in O_3-treated plants (blue, dashed line). Under O_3, the expression of several *TIR1/AFB* auxin receptors is decreased and thereby Aux/IAAs may become stabilized and have a negative effect on auxin responses, such as *HAT2* expression.

triggered reduction of TIR1/AFBs is required for the stabilization of AuX/IAAs. No role for ethylene, SA or MPK signaling was found in the rapid decrease of auxin-regulated gene expression in O_3-treated plants (III). MPK3 and MPK6 are rapidly and transiently activated by O_3 (Ahlfors et al., 2004b), but no effect of MPK3 or MPK6 on repression of *HAT2*, *SAUR68* or *TIR1* was observed (III). This might be caused by functional redundancy between MPK3 and MPK6, but it is also possible that for instance MPK4 is responsible for the MPK-dependent decrease of auxin-responsive transcripts *in planta* (Nakagami et al., 2006).

Because auxin-responsive genes are regulated by protein-protein interactions between TIR1/AFBs, Aux/IAAs and ARFs, these protein families with 6, 29 and 22 functional members, respectively, offer a wide range of possible output scenarios of developmental responses and cross-talk with other signaling pathways (Weijers et al., 2005; Teale et al., 2006; Vernoux et al., 2011). ARFs may interact with other transcription factors such as MYB77, which affects auxin responses (Shin et al., 2007). Also Aux/IAAs have additional interaction partners such as co-repressor TOPLESS (Szemenyei et al., 2008), and surprisingly, RCD1 (I). Aux/IAA transcripts are known to differ in their auxin responsiveness, and *IAA28* is atypically decreased by auxin treatment in the roots (Paponov et al., 2008). *IAA10* and *IAA28* expression increased in response to apoplastic ROS, which indicates a unique role in stress responses (III). No changes in the ARF expression or free IAA concentration by apoplastic ROS were observed in this study (III), which may be interpreted that ARF activity was post-transcriptionally regulated by apoplastic ROS. ARF activity may be also suppressed by phosphorylation (Vert et al., 2008), and phosphorylation cascades are known to be activated in response to O_3 (III).

4.2.3 Stress-induced morphogenic response

Comparison between auxin-responsive genes regulated by O_3 and other stresses such as UV-B, flg22, H_2O_2, PQ and BTH revealed both common and specific expression patterns (III). More generally, this may reflect how plant stress responses affect development and growth. Indeed, in case of auxin, plant stress and development are perhaps more entwined than in regard of any other plant hormone. It has been long established that chronic stress alters plant morphology, which in a wider sense may be interpreted as classical allocation between growth and defense. However, the molecular mechanisms governing the stress-induced morphogenic response (SIMR) are not yet established. SIMR can be defined as "growing out of trouble", which means redistribution of growth away from apical meristems into lateral organs (Potters et al., 2007). SIMR includes decrease in cell elongation, localized stimulation in cell division and alterations in cell differentiation (Potters et al., 2007). In roots, this is apparent as inhibition of root elongation and increased number of lateral roots, whereas in shoots inhibition of shoot elongation and increased axillary branching occurs (Potters et al., 2007). These could also be classical symptoms of increased and decreased auxin effects in roots and shoots, respectively. Interestingly, auxin has opposing effects in

shoots and roots: in the shoot, auxin promotes cell elongation and in roots inhibits it. Auxin responsive HD-Zip transcription factor *HAT2* is a positive regulator of auxin signaling in shoots and negative regulator in roots (Sawa et al. 2002). The reason for such opposite effects in different tissues is not known.

Considering the longevity of auxin research, the knowledge about the detailed signaling mechanism is relatively new (Leyser 2010), and the molecular mechanisms of auxin in regulation of stress adaptation are beginning to be unraveled. Long-term O_3 exposure decreased leaf area, leaf length and fresh weight of Arabidopsis (III), and also induced epinastic leaf curling at leaf margins as previously described by Sharma and Davis (1994) and Booker et al. (2004). This may be viewed as a SIMR response, which was exaggerated in *tir1 afb2* plants exhibiting curling also at the leaf tips marked by a larger decrease in leaf length and increased serrations compared to Col-0 (III). Therefore, auxin was concluded to be a negative regulator of SIMR in shoots in response to chronic apoplastic ROS (III). The altered leaf curling pattern in *tir1 afb2* might take place due to yet uncharacterized, cell-type specific auxin signaling events in leaves, for instance due to presence of cell-specific Aux/IAA-ARF protein-protein interaction pairs (Weijers et al., 2005). Auxin homeostasis is controlled by a multi-level protein network consisting of biosynthesis, transport, signaling, conjugation, hydrolysis and inactivation by oxidation. During chronic stress, one or several of these processes may be altered for stress adaptation, which makes it challenging to accomplish a comprehensive view. Auxin signaling may be decreased by several mechanisms depending on the stress signal and tissue type. Lack of glutathione has been shown to decrease auxin efflux carriers, PINs (Koprivova et al., 2010; Bashandy et al., 2010). These results relate plant redox status to auxin responses and development (Tognetti et al., 2011). Improved antioxidant status of *tir1 afb2* mutant was suggested as the reason for increased oxidative stress tolerance (Iglesias et al. 2010), but the precise mechanism how this is achieved is unknown. Increased apoplastic oxidation by ascorbate oxidase overexpression decreases responses to auxin treatment but simultaneously increases growth in tobacco, which might be due to constitutive auxin signaling taking place (Pignocchi et al., 2006). This may be related to the increased expression of several auxin-induced genes observed in O_3-treated plants (III). It might be that ROS have also auxin-independent effects on plant development (Tsukagoshi et al., 2010). Because some pathogens actively produce auxin (Kazan and Manners 2009), pathogen-related growth phenotypes may be due to complex effects on the plants auxin status. Detailed studies on auxin homeostasis mutants are required to assess the role of auxin network components in regulating SIMR in response to biotic and abiotic stress. Mutant studies should also take into consideration both the redundancy and divergence within the gene families of this network. Due to the importance of auxin for plant development, these studies are also complicated by putative developmental defects of these mutant genotypes, which may have a significant impact on the stress responses.

4.2.4 Cell death and gene expression

4.2.4.1 Are *rcd1* stress phenotypes pre-determined by clean air gene expression?

The oxidative signaling in *rcd1* was studied with microarrays to gain insight in the transcriptional regulation involved in PCD (IV). Additionally, the role of RCD1 as transcriptional co-regulator was addressed. The overall transcriptional responses of *rcd1* and Col-0 to O_3 treatment were very similar: of the 3635 and 4102 genes regulated by apoplastic ROS in Col-0 and in *rcd1*, respectively, large majority (2897 genes) were similarly regulated in both genotypes in response to apoplastic ROS (IV). Expression levels of several hundreds of genes classified O_3-responsive only in a particular genotype (419 genes in Col-0 and 845 in *rcd1*) showed less than two-fold differences between the genotypes (IV). The larger number of O_3-regulated genes in *rcd1* at every individual time point indicated that O_3–induced responses are exaggerated in *rcd1* (IV). More detailed analysis revealed that the majority of the O_3-responsive genes were more strongly regulated in *rcd1* than in Col-0 at all time points, however, with less than two-fold difference in the level of expression (IV). This suggested that the differences between these genotypes in response to apoplastic ROS may be mostly quantitative. Similarly, changes in Arabidopsis gene expression evoked by incompatible and compatible pathogen, causing PCD and not, respectively, are largely also quantitative (Tao et al. 2003). Therefore, it has been suggested that when oxidative stress exceeds a certain (yet unidentified) threshold, PCD occurs (Van Breusegem and Dat 2006; Mullineaux and Baker 2010). More open stomata might cause a higher initial O_3 dose before stomata close in response to O_3 (Ahlfors et al., 2004a; Vahisalu et al., 2008; Vahisalu et al., 2010). This effect alone could be sufficient to render *rcd1* O_3 sensitive, as increasing the O_3 concentration also induces lesion formation in otherwise O_3-tolerant Col-0 (IV). However, the sensitivity of *rcd1* to apoplastic $O_2^{\bullet -}$ produced by infiltration of xanthine and xanthine oxidase (Overmyer et al., 2000), which is independent of stomatal opening, suggests that there are additional components involved in PCD in *rcd1* mutant. Furthermore, NO production is increased by O_3 treatment and *rcd1* mutant has a constitutively higher NO level in clean air (Ahlfors et al., 2009), which might contribute to *rcd1* O_3 sensitivity due to its proposed role in cell death and synergistic interaction with ROS (Zago et al., 2006; Ahlfors et al., 2009).

As most of the genes exhibited similar O_3-responses in both genotypes, only 361 genes had more than two-fold differences between the genotypes (IV). In addition to a stronger initial response, the O_3 response in *rcd1* was prolonged in comparison to Col-0 (III). This is apparent as higher numbers of O_3-regulated genes still at 8 h and 24 h. The identities of these genes largely overlapped with earlier time points. This might be due to several reasons: stronger initial response, lack of negative regulation and/or prolonged induction of gene expression. Degradation of defense proteins may be involved in the recovery of stress, and proteosome function is enriched among O_3-responsive transcripts in the late time points (III). Albeit there seems to be no difference between the genotypes in the

proteosome-related transcripts, accumulating data hints towards proteosomal malfunction in *rcd1* (IV; Dr. J. Vainonen, unpublished data). ROS formation along the progressing lesions in *rcd1* has also been demonstrated (Overmyer et al., 2000), and this ROS source might also contribute to the prolonged expression of O_3-responsive genes in *rcd1* (IV).

Is *rcd1* primed for stress? And if so, to which one? A new, unexpected answer to these questions was discovered from the similarity between *rcd1* clean air gene expression and heat stress (IV). Furthermore, a mitochondrial double mutant *msh1 recA3* tolerant to heat has constitutively higher expression of heat-induced genes such as *AOX1a*, *UPOX1*, *NFXL1*, *ANAC013*, *UNIVERSAL STRESS PROTEIN* (*At3g03270*), mitochondrial *HSP70*, *CRF6* and an ACC oxidase (Shedge et al., 2010), which are also more highly expressed in *rcd1* in clean air (I; IV). Intriguingly, *rcd1* may possess tolerance to heat shock (Dr. J. Vainonen, unpublished data). However, no role for mitochondrial genes *UPOX1* and *AOX1* were found governing the O_3 tolerance in *rcd1* suggesting that mitochondrial ROS do not regulate rapid PCD in Arabidopsis (IV). *AOX1* has been interpreted to offer an alternative route for electrons in plants therefore relieving oxidative stress in mitochondria. The *aox1a* mutants have increased anthocyanin and $O_2^{\bullet-}$ concentrations and decreased plant size in abiotic stress responses (Giraud et al., 2008). Surprisingly, overexpression of *AOX1* mediated O_3 sensitivity in tobacco (Pasqualini et al., 2007) but neither lack nor constitutive activation of *AOX1a* altered O_3 sensitivity in our experimental conditions (IV). Genes with constitutively elevated expression in *rcd1* were also shown to be less expressed during PQ treatment (IV). Whether *AOX1* and/or *UPOX1* have a role in heat and PQ responses remains to be elucidated in future studies.

4.2.4.2 Marker genes of PCD

An exaggerated, prolonged expression of O_3-responsive genes was present in *rcd1* mutant (IV). However, the PCD in *rcd1* has been shown to be regulated by plant hormones (SA, JA and ethylene) and AtCNGC2, a cyclic-nucleotide-gated cation channel (Overmyer et al., 2005), which tells that in *rcd1* PCD is an active process controlled by specific signaling pathways. Furthermore, inhibitor studies suggested a role for ATPases, kinases, Ca^{2+}, proteolytic activity and, most importantly, transcription in this cell death regulation (Overmyer et al., 2005). To explore these signaling pathways, several double mutants with *rcd1* defective in various defense and signaling pathways were created, and this led to the identification of *WRKY70* transcription factor as a positive regulator of O_3-induced PCD (IV). This result is in accordance with WRKY70 acting as a positive and negative regulator of SA and JA signaling, respectively (Li et al., 2004). To connect the transcriptional events of PCD to decreased cell death in *wrky70* and *rcd1 wrky70*, ten marker genes differentially expressed in *rcd1* mutant were selected for qPCR analysis of O_3-treated Col-0, *wrky70*, *rcd1* and *rcd1 wrky70* (IV): *RAP2.6*, *SAP12*, *RAP12* and *WRKY75* transcript levels were higher and *WRKY62* expression lower in O_3-treated *rcd1*. *FMO1*, *ALD1*, *WRKY70*, *WRKY38* and *NUDX6*

had lower expression in *rcd1* in clean air according to the array data (IV). The expression of the same genes was studied also in several lesion mimic mutants (*acd2*, *acd5* and *lsd1*) to get an overview of the PCD transcriptomics. All the marker transcripts were induced by O_3 and most of them had elevated expression in the lesion-containing leaves of lesion mimic mutants, most notably in the *acd2* background (IV). Unlike other marker genes studied, *WRKY70* and *WRKY38* were not regulated in the any of the lesion mimic mutants (IV). Interestingly, *WRKY62* and *NUDX6* expression was decreased by lesion occurrence in O_3-treated plants and no increased expression was present in *acd2* either, which would be consistent with either a role of negative regulator of cell death or, alternatively, lesion formation may directly decrease their expression (IV). *ALD1* and *FMO1* have been shown to be positive regulators of cell death (Zhang et al., 2008), but their expression was increased in O_3-treated *wrky70* and *rcd1 wrky70* exhibiting reduced cell death (IV). One possible explanation might be that *wrky70* plants could be defective in *ALD1* and *FMO1* signaling. Future studies with *rcd1 ald1* and *rcd1 fmo1* plants will provide insight into the role of *ALD1* and *FMO1* in the regulation of PCD induced by apoplastic ROS.

RAP2.6 is an ERF transcription factor with increased expression during biotic stress (He et al., 2004) and abiotic stress involving ABA signaling (Zhu et al., 2010). Interestingly, the highest *RAP2.6* expression was detected in *rcd1* after lesion formation at 8 h, which may indicate a role for RAP2.6 in the cell death process together with the markedly elevated expression in *acd2* (IV). However, increased ABA levels are observed in plants 8 hours after the O_3-treatment start and O_3-sensitive mutants accumulate ABA more (Overmyer et al., 2008), so this late, high *RAP2.6* expression in O_3-treated *rcd1* may also be at least partially ABA-stimulated. *SAP12* and *ZAT12* were both induced early by O_3, and an increased response was observed in *rcd1* and *rcd1 wrky70* plants (IV). Transcription factor ZAT12 is a well-known marker for systemic ROS signaling (Davletova et al., 2005b; Miller et al., 2009). SAP12 is a stress-associated, redox-regulated protein with a rapid increase in expression levels in response to abiotic stress, which is co-expressed with *ZAT12* and *RAP2.6* (Ströher et al., 2009).

Stress, defense and cell death signaling in plants are largely overlapping, which has complicated the search for cell death marker genes. Methodological limitations also play a role in this complexity, as the transcriptomic analysis of whole rosettes provides a mixture of both dead tissues, cells about to die as well as parts remaining alive. O_3-responsive genes classified into the GO category "programmed cell death" (GO:0012501) had similar expression patterns in Col-0 and *rcd1* (Table IV), which may be due to this type of dilution effect. Cell-type specific gene expression analysis utilizing either microdissection of developing lesions or cellular sorting of dying tissues may resolve this limitation (Long 2011; Taylor-Teeples et al., 2011), but these approaches may also be dependent on cell death marker genes transgenically expressed *in planta*. Also protoplasts have successfully provided a more simple experimental system for cell death studies (Asai et al., 2000). However, gene expression of O_3-treated *rcd1* at late time points clustered together with

Pseudomonas syringae pv. *maculicola* ES4326 experiments and the *accelerated cell death11* (*acd11*) lesion mimic mutant under cell death inducing conditions, which suggests the presence of a cell-death specific gene expression signature. Genes directly related to lesion occurrence and PCD progress may include for instance *AUTOPHAGY 8E* (*At2g45170*), which belonged to cluster IIIa of genes misregulated in *rcd1* (IV). Autophagy, a process for nutrient cycling and controlled cellular debris management, may also regulate PCD (Hofius et al., 2009).

Table IV. O_3-responsive genes belonging to the GO category "Programmed cell death" (GO:0012501) have similar expression patterns in Col-0 and *rcd1*. Values depict $\log_2$ ratios.

AGI	Description	Protein	Col-0 O_3 vs. Col-0 control						*rcd1* O_3 vs. *rcd1* control						*rcd1* O_3 vs. Col-0 O_3					
			0h	1h	2h	4h	8h	24h	0h	1h	2h	4h	8h	24h	0h	1h	2h	4h	8h	24h
At1g02170	Metacaspase 1	MC1	-0.2	0.7	2.1	1.7	1.0	0.4	-0.2	0.8	1.8	1.9	1.1	0.7	-0.1	0.2	0.1	0.2	0.0	0.2
At1g08450	Calreticulin 3	CRT3	0.1	1.6	1.8	1.2	1.3	1.2	0.1	1.7	2.0	1.5	1.5	2.0	-1.0	-0.5	-0.4	-0.4	-0.2	-0.2
At1g12060	Bcl-2-associated athanogene 5	BAG5	0.0	0.9	1.1	0.2	-0.3	-0.2	0.0	0.9	0.2	-0.1	0.1	0.2	-0.3	0.1	-0.7	0.2	0.1	0.1
At1g15890	Disease resistance protein (CC-NBS-LRR class)		0.0	0.2	1.1	0.6	0.0	-0.2	0.1	0.8	1.0	0.5	0.1	0.4	0.2	0.4	0.2	-0.2	0.2	0.2
At1g16420	Metacaspase 8	MC8	0.0	3.4	3.9	2.3	1.3	1.0	-0.2	2.7	2.6	2.5	1.5	1.0	-0.5	0.0	-0.7	0.1	0.3	-0.2
At1g17600	Disease resistance protein (TIR-NBS-LRR class)		-0.2	0.1	0.0	-0.4	-1.2	-0.2	-0.1	0.0	-0.3	-1.1	-1.9	-0.7	-0.1	-0.1	-0.3	-0.8	-0.7	-0.4
At1g19250	Flavin-containing monooxygenase	FMO1	-0.6	-0.4	2.0	3.1	2.7	0.8	-0.2	-1.3	2.6	4.3	2.9	1.1	-0.7	-0.4	0.6	0.8	0.1	0.7
At1g20850	Xylem cysteine peptidase 2	XCP2	0.0	0.0	-0.1	-0.7	-1.0	-0.4	-0.1	0.0	-0.2	-0.7	-1.1	-0.5	-0.3	-0.2	-0.3	-0.2	-0.2	-0.2
At1g21750	Protein disulfide isomerase 5	PDI5	0.0	0.1	0.3	1.3	1.8	0.9	-0.2	0.0	0.0	1.0	1.7	1.0	-0.5	-0.3	-0.3	-0.3	-0.1	-0.4
At1g27170	Disease resistance protein (TIR-NBS-LRR class)		-0.3	-0.5	0.0	1.2	0.0	-0.2	0.8	0.2	-0.9	-0.6	0.1	-0.9	0.7	-0.5	0.1	-0.4	0.2	0.0
At1g28380	Necrotic spotted lesions 1	NSL1	-0.3	2.1	2.8	1.1	0.1	0.4	-0.1	2.5	3.2	1.9	0.2	0.6	-0.1	0.3	0.6	0.4	0.2	0.1
At1g29340	Plant U-box 17	PUB17	-0.1	0.8	1.3	0.8	0.3	0.2	0.0	1.1	1.8	1.3	0.3	0.4	0.0	0.3	0.3	0.0	0.0	0.0
At1g32230	Radical-induded cell death 1	RCD1	0.1	0.4	1.0	1.1	0.8	0.5	0.0	0.1	0.6	0.3	0.1	0.3	0.2	0.2	-0.1	0.0	0.1	0.0
At1g56520	Disease resistance protein (TIR-NBS-LRR class)		0.5	0.6	0.8	0.2	0.3	0.1	-0.1	0.9	1.7	0.1	0.2	0.2	-0.3	0.0	0.6	-0.1	0.0	0.0
At1g56540	Disease resistance protein (TIR-NBS-LRR class)		0.2	0.3	0.3	0.1	-0.5	-0.2	0.2	0.8	1.5	0.0	-0.6	-0.3	-0.2	0.3	0.8	0.1	-0.3	-0.3
At1g61300	Disease resistance protein (NBS-LRR class)		0.0	-0.1	-0.6	-0.9	-0.9	-0.2	0.1	-0.1	-0.5	-0.7	-1.4	-0.4	-0.3	-0.1	-0.2	0.2	-0.7	-0.3
At1g63750	Disease resistance protein (TIR-NBS-LRR class)		0.0	0.5	0.9	0.0	0.6	0.1	-0.1	1.4	1.8	0.1	-0.1	-0.1	0.0	0.6	0.9	-0.2	-0.3	0.1
At1g66090	Disease resistance protein (TIR-NBS class)		-0.2	2.2	2.8	1.9	1.0	0.2	0.0	2.7	2.4	2.7	1.3	0.6	-0.1	0.1	-0.2	0.4	0.2	0.4
At1g71220	UDP-glucose:glycoprotein glucosyltransferase	UGGT	0.0	0.0	-0.1	0.4	1.0	0.7	0.1	0.0	0.0	0.4	1.2	0.6	0.0	-0.1	0.0	-0.1	0.2	-0.1
At1g72900	Disease resistance protein (TIR-NBS class)		-0.1	1.8	2.4	2.5	1.4	0.9	-0.3	2.0	2.2	2.3	1.2	1.6	-0.5	-0.3	-0.5	-0.6	0.1	0.3
At1g72910	Disease resistance protein (TIR-NBS class)		0.0	1.9	0.2	0.7	-0.5	0.3	0.0	2.5	-0.1	0.5	-0.4	0.7	-0.7	-0.5	-0.9	-0.8	-0.2	-0.7
At1g72940	Disease resistance protein (TIR-NBS class)		-0.3	1.5	0.8	1.0	0.3	0.2	-0.2	1.2	0.4	0.5	0.4	0.4	0.0	-0.2	-0.3	-0.2	0.4	0.1
At1g73260	Kunitz trypsin inhibitor 1	KTI1	-0.8	-0.2	0.7	0.3	3.5	4.2	-0.1	0.4	0.1	1.9	4.5	5.0	0.4	0.0	-0.3	1.4	1.1	0.5
At2g19860	Hexokinase 2	HXK2	0.0	-0.5	-0.9	-0.8	-0.4	-0.4	-0.1	-0.4	-0.8	-1.1	-0.5	-0.3	0.1	0.1	0.0	-0.2	0.1	0.1
At2g26560	Phospholipase A 2A	PLA2A	-0.5	4.3	4.8	2.9	0.5	1.4	0.9	4.0	3.7	3.6	1.3	2.5	0.9	0.2	-0.4	0.0	-0.2	1.0
At2g41560	Calcium-transporting ATPase 4	ACA4	0.0	-0.1	-1.0	-1.8	-1.2	-0.4	-0.2	0.0	-0.8	-1.9	-1.5	-0.6	-0.4	-0.3	0.0	-0.2	-0.5	-0.4
At2g46240	Bcl-2-associated athanogene 6	BAG6	-0.1	-0.1	0.9	1.3	0.3	0.6	-0.1	-0.3	1.2	2.0	0.2	1.1	-0.2	-0.3	0.5	0.5	-0.1	0.4
At3g04210	Disease resistance protein (TIR-NBS class)		-0.1	1.7	1.6	1.7	0.8	1.3	-0.1	2.7	1.5	1.6	0.3	1.9	-1.3	0.0	-0.9	-0.6	-0.3	-0.3
At3g04220	Disease resistance protein (TIR-NBS-LRR class)		-0.3	1.6	2.6	1.7	1.3	0.9	-0.4	1.6	3.1	1.9	0.7	0.7	0.1	0.6	0.7	0.1	-0.2	-0.2
At3g11820	Syntaxin 121	SYP121	0.0	3.1	3.6	2.5	1.3	0.7	0.2	3.4	3.5	3.3	1.5	1.2	-0.4	0.0	-0.4	0.1	-0.1	-0.1
At3g13610	Oxoglutarate and Fe(II)-dependent oxygenase		-0.4	0.1	1.8	3.3	3.7	0.5	0.9	-0.5	0.4	4.2	3.5	1.9	0.0	-0.1	0.1	0.3	-0.1	0.2
At3g14470	Disease resistance protein (NBS-LRR class)		-0.3	0.4	1.7	1.4	0.9	0.3	-0.2	0.4	2.1	1.8	1.1	0.4	-0.3	-0.1	0.5	0.3	0.1	-0.4
At3g27060	Ribonucleotide reductase	TSO2	0.0	-0.1	-0.3	-0.8	-1.0	0.4	0.0	0.1	-0.7	-1.1	-1.8	-0.3	0.8	0.8	0.4	0.7	0.1	0.2
At3g44400	Disease resistance protein (TIR-NBS-LRR class)		0.6	0.3	1.5	1.0	0.6	0.2	0.3	1.2	2.2	1.6	0.8	0.5	-0.3	0.7	0.6	0.3	0.3	0.2
At3g45860	Cysteine-rich receptor-like protein kinase 4	CRK4	0.4	0.9	0.6	-0.1	-0.1	0.4	0.1	1.2	0.0	-0.5	-0.5	0.0	-1.4	-0.6	-1.3	-1.0	-1.4	-1.7
At3g48090	Enhanced disease susceptibility 1	EDS1	0.0	1.7	1.7	0.9	1.0	0.6	0.1	1.8	2.5	1.5	1.4	1.0	-0.6	-0.2	0.3	0.0	0.0	-0.6
At3g50950	Disease resistance protein (CC-NBS-LRR class)		0.0	1.9	1.8	1.4	0.4	0.7	-0.2	2.1	2.2	2.0	0.8	1.2	-0.7	-0.2	0.0	-0.2	0.1	-0.1
At3g52400	Syntaxin 122	SYP122	-0.1	2.5	4.3	2.2	1.3	1.4	0.4	2.3	4.1	3.0	1.2	2.2	0.6	0.8	0.9	0.1	-0.2	0.1

Table IV continues

AGI	Description	Protein	Col-0 O₃ vs. Col-0 control						rcd1 O₃ vs. rcd1 control						rcd1 O₃ vs. Col-0 O₃					
			0h	1h	2h	4h	8h	24h	0h	1h	2h	4h	8h	24h	0h	1h	2h	4h	8h	24h
At3g54420	Class IV chitinase	EP3	-0,1	0,5	1,1	0,2	-0,3	-0,1	0,0	0,7	1,1	0,5	0,0	0,2	-0,1	0,0	-0,2	0,2	0,2	0,1
At3g57330	Calcium-transporting ATPase 11	ACA11	0,1	1,6	2,1	1,0	0,6	0,7	0,2	2,0	2,3	1,2	0,9	0,7	-0,2	0,0	-0,3	-0,2	0,0	-0,3
At4g08920	Crytochrome 1	CRY1	-0,1	-0,3	-1,0	-1,0	-0,2	-0,1	-0,2	-0,3	-1,0	-1,4	0,0	-0,1	0,0	-0,1	-0,2	-0,6	0,0	-0,1
At4g11280	ACC synthase 6	ACS6	0,1	2,3	2,7	1,5	0,7	0,4	-0,3	2,5	3,2	2,1	1,0	1,3	-0,2	0,0	-0,2	0,4	0,3	0,6
At4g12720	Nudix hydrolase 7	NUDX7	0,1	2,6	3,4	2,5	2,6	0,8	0,3	2,9	2,8	3,5	2,8	1,6	-0,6	0,2	-0,4	0,1	0,1	0,1
At4g17580	Bax inhibitor-1 family protein		0,1	-0,4	-0,5	-1,2	-0,2	-0,4	0,3	-0,1	-0,7	-0,8	-0,4	0,0	0,1	0,0	-0,4	0,1	-0,3	0,0
At4g20380	Lesions simulating disease	LSD1	0,0	0,3	0,9	0,4	0,5	0,2	0,1	0,5	1,6	0,9	0,8	0,3	0,1	0,2	0,7	0,5	0,4	0,2
At4g20870	Fatty acid hydroxylase 2	FAH2	0,1	0,1	-0,3	-1,0	-0,6	-0,6	0,0	0,1	-0,5	-0,7	-0,5	-0,6	-0,2	0,0	-0,2	0,2	-0,1	-0,2
At4g23280	Cysteine-rich receptor-like protein kinase 20	CRK20	0,0	1,1	0,6	0,4	0,1	0,3	-0,1	0,0	1,8	0,3	-0,3	1,1	-0,6	-0,5	0,6	0,3	-0,3	0,2
At4g25230	RPM1 interacting protein 2	RIN2	0,0	0,1	1,3	1,2	0,7	0,4	-0,1	0,0	1,5	1,9	0,9	0,7	0,1	-0,1	0,1	0,4	0,3	0,3
At4g26090	Disease resistance protein RPS2 (CC-NBS-LRR class)	RPS2	0,0	1,1	0,5	1,1	0,4	0,5	0,3	0,9	0,4	1,0	0,9	1,2	-0,4	-0,3	-0,1	-0,6	0,0	0,1
At4g33300	Disease resistance protein RPS2 (CC-NBS-LRR class)	ADR1-L1	0,0	1,7	1,8	1,7	1,6	1,1	-0,3	2,6	2,7	1,3	1,8	1,4	-0,9	0,1	0,6	-0,7	0,0	-0,4
At4g36150	Disease resistance protein (TIR-NBS-LRR class)		-0,3	0,9	0,7	0,9	0,3	0,1	-0,1	1,2	0,6	0,5	0,1	0,4	0,0	-0,2	0,1	-0,3	-0,4	-0,2
At4g37000	Accelerated cell death2	ACD2	0,0	-0,2	-0,7	-1,0	-0,8	-0,4	-0,1	-0,2	-0,9	-1,2	-0,9	-0,2	-0,3	-0,2	-0,3	-0,4	-0,4	-0,2
At4g37980	Mannitol dehydrogenase	ELI3-1/CAD7	0,1	-0,4	-0,8	0,1	0,6	-0,4	0,0	-0,3	-0,7	-0,7	1,3	-0,4	-0,5	0,0	-0,1	0,2	0,8	0,1
At5g04720	Disease resistance protein (CC-NBS-LRR class)	ADR1-L2	0,1	1,4	0,8	1,4	1,2	0,5	0,0	1,7	1,4	1,1	1,3	0,7	-0,5	0,0	0,2	0,2	0,0	-0,1
At5g10380	Zinc finger (C3HC4-type RING) protein	RING1	0,0	0,9	0,6	0,1	1,8	1,4	0,1	1,7	0,7	0,7	1,8	1,8	-1,2	-0,1	-0,6	-0,9	-0,8	-0,7
At5g11250	Disease resistance protein (TIR-NBS-LRR class)		0,2	0,3	0,0	0,3	-0,4	0,1	-0,4	1,1	0,0	-0,1	-0,1	0,4	-0,8	0,1	-0,3	-0,6	-0,1	-0,1
At5g15410	Cyclic nucleotide-regulated ion channel 2	DND1	0,1	-0,4	-1,5	-1,3	-0,8	0,0	0,1	-0,5	-1,8	-2,0	-0,9	-0,3	-0,1	-0,2	-0,5	-0,9	-0,3	-0,5
At5g20480	LRR transmembrane protein kinase		0,5	0,9	1,6	1,3	0,4	0,3	-0,3	1,3	2,2	1,3	1,0	1,4	-0,1	0,3	0,7	0,0	0,2	0,2
At5g22690	Disease resistance protein (TIR-NBS-LRR class)		-0,6	1,7	1,4	0,0	-0,2	0,2	-0,3	2,4	1,6	0,7	0,0	0,8	0,0	0,7	0,4	0,2	0,0	0,7
At5g44870	Disease resistance protein (TIR-NBS-LRR class)		-0,1	1,1	1,3	0,7	0,2	0,1	0,1	0,9	1,6	1,0	0,2	0,4	-0,1	-0,1	0,1	-0,1	-0,2	0,0
At5g45060	Disease resistance protein (TIR-NBS-LRR class)		-0,1	0,7	1,2	0,6	-0,4	0,3	0,1	0,7	1,5	0,7	-0,3	0,1	-0,1	-0,1	0,2	0,1	0,2	-0,3
At5g45250	Disease resistance protein (TIR-NBS-LRR class)	RPS4	-0,1	0,7	1,2	1,1	0,4	0,3	0,0	0,6	1,5	1,4	0,4	0,5	0,1	-0,1	0,1	0,2	0,1	0,2
At5g46450	Disease resistance protein (TIR-NBS-LRR class)		-0,1	1,1	0,5	0,1	0,2	-0,1	0,0	1,2	0,8	-0,2	-0,3	0,2	-0,1	0,2	-0,1	-0,3	-0,1	0,0
At5g46510	Disease resistance protein (TIR-NBS-LRR class)		0,0	1,0	0,2	0,3	-0,2	-0,4	0,1	1,1	0,4	-0,2	-0,3	-0,4	0,1	0,2	-0,1	-0,2	-0,1	0,0
At5g46520	Disease resistance protein (TIR-NBS-LRR class)		0,1	1,2	1,0	0,8	0,6	0,5	0,4	1,6	2,0	0,8	0,5	0,1	-0,2	0,1	0,5	-0,3	0,0	-0,3
At5g47120	Bax inhibitor-1	BI-1	0,1	0,3	2,0	2,2	2,3	1,2	-0,1	0,1	1,9	2,5	2,5	1,9	-0,5	-0,2	0,2	-0,1	0,2	0,2
At5g47910	Respiratory burst oxidase protein D	RBOHD	-0,2	2,3	2,8	1,6	0,8	1,1	0,1	2,2	2,7	2,4	0,8	1,2	-0,1	-0,4	-0,3	0,1	-0,3	-0,2
At5g51630	Disease resistance protein (TIR-NBS-LRR class)		0,1	0,3	1,1	0,9	0,6	0,6	0,0	0,3	1,5	1,4	1,0	0,7	-0,2	0,0	1,0	0,3	0,1	0,1
At5g52060	Bcl-2-associated athanogene 1	BAG1	0,1	-0,8	-0,9	-0,3	0,0	-0,5	0,0	-0,8	-1,1	-0,2	0,0	-0,9	0,0	-0,1	-0,3	0,0	-0,1	-0,3
At5g54250	Cyclic nucleotide-regulated ion channel 4	DND2	0,1	-0,2	-0,8	-0,7	0,0	-0,5	0,2	-0,2	-1,0	-1,0	-0,4	-0,5	-0,1	-0,3	-0,3	0,0	-0,3	0,0
At5g58120	Disease resistance protein (TIR-NBS-LRR class)		-0,3	1,3	2,1	0,7	0,7	0,0	-0,2	2,2	3,0	0,9	1,0	0,3	-0,4	0,4	0,7	0,0	-0,1	-0,2
At5g61900	Calcium-dependent phospholipid binding	BON1	0,1	1,4	2,1	0,8	0,9	0,4	0,1	2,0	3,6	1,6	1,1	0,8	-0,2	0,3	1,0	0,2	0,1	0,2
At5g62100	Bcl-2-associated athanogene 2	BAG2	0,1	-1,0	-0,1	-0,2	-0,2	-0,5	0,1	0,0	0,1	-0,4	-0,3	0,1	0,1	0,6	0,2	-0,2	-0,1	0,1
At5g66630	DA1-related protein 5	DAR5	-1,1	0,4	0,5	0,3	0,8	0,0	0,5	0,3	1,2	0,3	0,1	0,1	1,0	-0,3	0,2	-0,2	-0,3	-0,3
At5g66900	Disease resistance protein (CC-NBS-LRR class)		0,2	0,7	1,3	0,4	0,0	0,3	-0,2	0,9	1,4	0,8	0,4	0,3	-0,2	0,1	0,2	0,5	0,3	-0,1
At5g66910	Disease resistance protein (CC-NBS-LRR class)		-0,3	0,3	0,5	1,1	0,6	0,3	0,1	0,1	1,0	1,2	0,9	0,3	-0,1	-0,3	0,3	0,0	0,0	-0,2

5 Conclusions and future perspectives

Plants adapt to their environment by adjusting their cellular metabolism. Different stresses activate or repress partially overlapping signaling networks, in which plant hormones and ROS participate and interact with each other. In this work, auxin signaling was shown to be transiently decreased by apoplastic ROS in a manner independent from SA and ethylene signaling. Decreased expression of several auxin F-box receptor transcripts was observed, which may have resulted in stabilized Aux/IAA proteins. Approximately one third of auxin-responsive transcripts were regulated (either increased or decreased) by apoplastic ROS and similar expressional responses were observed in BTH, H_2O_2, UV-B, auxin transport inhibitor TIBA and PQ treatments. Altogether, these stresses share a common effect on auxin-regulated gene expression, which may result in similar adaptations in plant development. Indeed, stress-induced morphogenic responses have similar features in common albeit the initiating stresses and signals may vary.

Plants have also a wealth of gene families, which have arisen from genome duplications and later diversified to fulfill various important tasks. However, certain functions have remained shared (Briggs et al., 2006; Wang et al., 2011). The plant-specific SRO protein family is an example of unequal genetic redundancy, in which RCD1 and SRO1 together regulate plant development. RCD1 appears as a unique member of this gene family, because *sro1* or *sro5* do not have altered growth habitus or altered sensitivity to apoplastic ROS. Additionally, the pleiotropic *rcd1* mutant phenotype is also accompanied by changes of gene expression in the clean air whereas *sro1* and *sro5* showed only few genes misregulated under the same growth conditions. The conservation of the C-terminal RST-domain in the SRO proteins is detected as the capability of RCD1, SRO1 and SRO5 to interact with the same proteins or proteins belonging to the same transcription factor families. The WWE domain present in RCD1 and SRO1 but lacking from SRO5 (and SROs 2 to 4) may be involved in the fine-tuning of some protein-protein interactions, but altogether these interactions and their role in plant development and stress responses will need to be verified *in planta*. Yet unpublished and ongoing work with RCD1 protein has gained very interesting and promising results: It was long unsure, whether RCD1 even exists in adult plants, but epitope-tagged RCD1 is detected in *rcd1* complementation lines at very low levels in three-week- old plants and it has also been shown that the RCD1 protein levels are stress-regulated (Dr. J. Vainonen, submitted). Further challenge for RCD1 work will be the low amount of the protein for the verification of interaction partners and post-translational modifications. However, this work is well on its way and may provide novel aspects in plants stress responses and for the analysis of gene expression data.

Transcriptomic analysis of oxidative signaling in O_3-treated *rcd1* revealed elevated expression in the late time points of some target genes of DREB2A, which is an ERF transcription factor interacting with RCD1 in the yeast-2-hybrid system. Because there are no published studies with DREB2A and apoplastic ROS, studies with DREB2A knock-out and

constitutively active DREB2A in the *rcd1* background are needed to determine whether lack of DREB2A interaction causes sensitivity to apoplastic ROS. The expression of O_3-responsive targets of RCD1-interacting TGA2 and AS1 was not significantly altered in *rcd1*. It remains a possibility that O_3 sensitivity in *rcd1* is caused by a prestressed condition rather than the lack of protein-protein interactions during stress. These alternatives may also coexist. A suitable tool in the future studies to investigate these alternatives could an inducible RNAi line of *RCD1*, of which transgenic plants already exist (T. Blomster, unpublished). Cell death has been proposed to be regulated by a stress threshold, but clearly this threshold is adjusted in the case of *rcd1 wrky70* mutant by signaling pathways conveying the information to the cell death machinery. For the *rcd1* signaling leading to PCD, double mutants in several pathogen-related pathways are being analyzed. Future work with PCD may also try to narrow down the "cell death signature" of O_3-treated *rcd1* with microarray data clustering and data mining, and compare that to cell-death specific tissue samples.

Analysis of full genome microarray experiments has provided valuable information about the biological processes during stress. As the amount of such data continues to pile up, new methods to analyze the results are being developed. Current understanding of the stress-regulated transcriptome could benefit from more time-series in microarray studies. Also next-generation sequencing tools will increase the amount of transcripts detected and phenomena like miRNA and alternative splicing in stress responses can also be monitored. Arabidopsis genome contains approximately two thousand transcription factors, and a similar number is found in the human genome. The function of Arabidopsis transcription factors is still largely unknown: even within the best-characterized TF families the individual transcription factors may be either transcriptional regulators or activators, or even both, by mechanisms not well defined. Also the DNA-binding sites and their specificity will need more studies with chromatin immunoprecipitation. Therefore, the novel role for SRO proteins as transcriptional co-regulators may yet be masked under functional redundancy between SROs and their interacting partners. Solving the mystery of ROS perception will address mechanisms of both local and systemic stress responses, and direct regulation of protein (transcription factor) activity by redox changes will be of great importance for future plant stress and development research at the transcriptional level.

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