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APOPLASTIC ROS AND TRANSCRIPTIONAL RESPONSE IN PLANT STRESS SIGNALING

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ACADEMIC DISSERTATION

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“Anyone who uses more than two chords is just showing off.”

Woody Guthrie

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ORIGINAL PUBLICATIONS

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I. LV wrote the manuscript together with MB.

II. LV performed the RNAseq data analysis and participated in writing of the manuscript.

III. LV participated in experimental design, performed the RNAseq data analysis and visualization, participated in quantification of H_2O_2 accumulation and cell death, and writing of the manuscript.

IV. LV conceived the project together with MB, participated in analysis and visualization of RNAseq data, analysis of DNA-binding specificities, protein-protein interactions, and protein localization. LV wrote the manuscript together with MB.

LIST OF ABBREVIATIONS

ABA	Abscisic acid
ANOVA	Analysis of variance
ARF	AUXIN RESPONSE FACTOR
CAMTA	*CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR*
COI1	*CORONATINE INSENSITIVE 1*
Col-0	Columbia-0
CPK5	*CALMODULIN-DOMAIN PROTEIN KINASE 5*
DAB	3,3'-Diaminobenzidine
DIC2	*DICARBOXYLATE CARRIER 2*
EIN2	*ETHYLENE INSENSITIVE 2*
EIN3	*ETHYLENE INSENSITIVE 3*
GO	Gene ontology
GOE	Great oxygenation event
HK1	HISTIDINE KINASE 1
JA	Jasmonic acid
JA-Ile	Jasmonic acid-isoleucine
K_d	Dissociation constant
LC	Liquid chromatography
MAPKKK19	*MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 19*
MKK9	*MAP KINASE KINASE 9*
MS	Mass spectrometry
O_3	Ozone
PAMP	Pathogen-associated molecular pattern
PCC1	*PATHOGEN AND CIRCADIAN CONTROLLED 1*
PCD	Programmed cell death
PIF	*PHYTOCHROME INTERACTING FACTOR*
PP2C	PROTEIN PHOSPHATASE 2C
PR1	*PATHOGENESIS-RELATED GENE 1*
PR2	*PATHOGENESIS-RELATED GENE 2*
qPCR	Quantitative polymerase chain reaction
QTL	Quantitative trait locus
RBOH	*RESPIRATORY BURST OXIDASE HOMOLOG*
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Systemic acquired resistance
SCF	SKP1-CULLIN-F-BOX protein
SELEX	Systematic evolution of ligands by exponential enrichment
SID2	*SALICYLIC ACID INDUCTION DEFICIENT 2*
SLAC1	SLOW ANION CHANNEL 1
SnRK2	*SNF1-RELATED PROTEIN KINASE 2*
TGA	*TGACG (TGA) MOTIF-BINDING PROTEIN*
TF	Transcription factor
TIR1	*TRANSPORT INHIBITOR RESPONSE 1*

ABSTRACT

The air pollutant ozone (O_3) enters plant leaves through stomata and activates apolastic reactive oxygen species (ROS) signaling. Depending on growth conditions and genotype, this results in large transcriptional reprogramming, closure of stomatal pores and activation of cell death programs. These responses are also regulated through plant stress hormones. This thesis sheds light on how stress hormone signaling is connected with apoplastic ROS signaling in the model plant *Arabidopsis thaliana*, and investigates regulatory mechanisms which generate specificity among sequence-specific transcription factors (TFs), the executers of apoplastic ROS -induced transcriptional reprogramming. The essential methods of the thesis include O_3 exposures of *Arabidopsis* wild type and mutant plants followed by quantification of cell death and characterization of transcriptional responses supplemented with several protein-level analyses of selected WRKY family TFs. The O_3-induced cell death was found to be inhibited by plant hormone salicylic acid, and genes *RESPIRATORY BURST OXIDASE HOMOLOG F* (*RBOHF*) and *WRKY70* were found to be required for O_3-induced cell death in jasmonic acid insensitive genetic background. Even though stress hormones were verified to play important roles in the regulation of cell death, the transcriptional response to apoplastic ROS in a hormone deficient/insensitive mutant was highly similar to wild type, suggesting that much of the signaling involved is independent of the studied hormones jasmonic acid, salicylic acid, and ethylene. The potential major executers of transcriptional response to apoplastic ROS, WRKY family TFs, were studied for their transcriptional regulation, DNA-binding preferences, protein-protein interactions, subcellular localization, and effects on transcriptome. The results showed that the DNA-binding preferences of WRKYs vary substantially between phylogenetic groups, implying that the specificity in signaling between different WRKYs can be partly achieved through DNA binding preferences. Transcriptomic analyses of mutants with altered expression levels of the strongly ROS-inducible *WRKY75* implicate this TF as a positive regulator of well-known pathogen-responsive genes, such as *PATHOGENESIS-RELATED GENE 1* (*PR1*) and *PATHOGENESIS-RELATED GENE 2* (*PR2*), and as a negative regulator of several hormone signaling pathways and TFs.

1. INTRODUCTION

1.1 APOPLASTIC ROS SIGNALING IN *ARABIDOPSIS THALIANA*

1.1.1 ROOTS OF ROS SIGNALING

Oxygenic photosynthesis of mainly cyanobacterial origin began to elevate atmospheric oxygen levels leading to the Great oxygenation event (GOE) about 2.3 billion years ago, possibly causing one of the greatest mass extinctions of all time [1]. Ever since GOE, there has been a pressure for most organisms to shield their biomolecules, such as nucleic acids and proteins, from oxidation. To balance this antioxidant defense optimally with growth, a sensor mechanisms for oxidant load probably also emerged shortly. When organisms compete for nutrients through speed of growth, there is a pressure to reduce defenses to a minimum to enable optimal growth speed. In this kind of competition, it would be easy to envisage an invention to use ROS to slow down the growth of the faster-growing organisms, a scenario similar to the use of antibiotics in nature. Once the machinery for ROS production, sensing, and quenching had evolved, an organism, or colony or organisms, could have adopted the components for internal communication. For example, when one of the members of the colony experienced stress, it would have been possible to produce ROS into the growth medium and induce the defenses of the rest of the colony before the actual stressor reached the other members. This highly speculative scenario is just one of many that could explain the current situation, where most if not all aerobic organisms use ROS for signaling purposes [2]–[6].

1.1.2 PLANT APOPLAST

Plant apoplast is the compartment outside plant cell plasma membranes (PMs), where solutes can diffuse freely from cell to cell. In plants leaves, it is also the main compartment that comes in contact with air and its associates from the environment, in a sense roughly comparable to human lung epithelium. Thus, it is not surprising to find PMs covered with receptors which recognize signs of invasion, such as pathogen-associated molecular patterns (PAMPs), from the apoplast. In addition to the receptors, the PM-apoplast interphase contains enzymes capable of producing ROS into the apoplast. These ROS are not quickly quenched, because the antioxidant defense in the apoplast is weak, probably in order to allow cell wall lignification and apoplastic ROS signaling.

1.1.3 *ARABIDOPSIS THALIANA* AS MODEL ORGANISM

Even though scientists are seldom capable on agreeing on viability of a hypothesis or the music to play in a laboratory, plant scientists have been able to agree on the model plant successfully. This plant is called *Arabidopsis thaliana*, thale cress, a little weed that often grows in places where other plants have not yet had time to settle. Its better-known relatives include turnip (*Brassica rapa*), rapeseed (*Brassica napus*) and kale (*Brassica oleraceceae*). *Arabidopsis* has a fairly small genome with not too many repeats, making the genome-level analyses feasible. In addition, geneticists have found very useful *Arabidopsis*' habit of being conditionally self-pollinating. This means that a single *Arabidopsis* plant produces thousands of seeds through self-pollination within a life cycle of about two months, but with a bit of persuasion it can be crossed with another *Arabidopsis* plant. Even though *Arabidopsis* is "genetically easy", there are even stronger benefits in working with the model plant: Since there is a large community of scientists working with the same species, the resources for doing science as efficiently as possible, such as transformation methods and seed collections, are highly developed and available for the whole community. Thus, much of the basic plant research has been and will be done with this little weed.

For decades, the genetic variation between natural accessions of *Arabidopsis thaliana* has been an untapped resource, largely because of technical limitations. Since the research fields of genomics, genetics, and bioinformatics are advancing quickly, this approach gains popularity. However, most of the plant molecular biology studies are still made with the accession Columbia-0 (Col-0), the laboratory strain of choice. Just like mutagenesis-based traditional genetic screens, the use of natural variation offers an opportunity to find novel components and mechanisms of apoplastic ROS signaling. In addition, it makes it possible to find out which parts of the response are varying naturally. This information may prove valuable when applying the basic knowledge for plant breeding, for example [7].

1.1.4 APOPLASTIC ROS SIGNALING IN *ARABIDOPSIS THALIANA*

What is known about apoplastic ROS signaling in *Arabidopsis*? We know that sensing of the apoplastic ROS leads to a signal transduction cascade which can result in a massive transcriptional reprogramming, active apoplastic ROS production through NADPH oxidases and peroxidases, stomatal closure, and hypersensitive response –like programmed cell death (PCD), all of which are potential outcomes of PAMP perception as well [8]–[10]. Both ROS and PAMP perception lead to self-propagating wave of plant cell –produced apoplastic ROS spreading to distal tissues, evoking defense responses there as well. The

components of this wave include NADPH oxidase RESPIRATORY BURST OXIDASE HOMOLOGUE D (RBOHD), which produces the ROS into the apoplast, and CALMODULIN-DOMAIN PROTEIN KINASE 5 (CPK5), a cytosolic Ca^{2+}-activated kinase, which in response to ROS or PAMP perception activates RBOHD through phosphorylation [11], [12]. In addition to defense signaling, ROS are exploited in growth-related processes, such as the elongation of roots hairs, where apoplastic ROS produced by RBOHC are needed at the very tip of the root hair, probably to activate Ca^{2+} influx, which in turn can regulate ROS production through a feedback loop [13], [14]. PCD induced by apoplastic ROS requires active transcription, since the O_3-induced PCD –phenotype can be suppressed by addition of inhibitors of transcription [15]. Major executers of the transcriptional reprogramming include WRKY family TFs, since the DNA-sequence they supposedly bind, the W-box (TTGAC[C/T]), is the most highly enriched promoter motif among the genes whose transcript levels are increased in response to O_3 [16], [17]. Furthermore, this transcriptional response is very similar to responses elicited by ROS accumulation in other cellular compartments [18]. Even though the basic characteristics of the transcriptional reprogramming in response to apoplastic ROS signaling have been characterized and several essential signaling proteins are known, many of the central questions are still open:

1) How is apoplastic ROS perceived? Are there specific receptors and if so, where and how do they function?
2) Plant stress hormones are important in pathogen response. How is phytohormone signaling connected with apoplastic ROS signaling? How about other defense-related signaling?
3) How is PCD in response to apoplastic ROS initiated, how does it spread, and what makes it stop?
4) Transcriptional reprogramming is largely executed through sequence-specific TFs. Which individual TFs are important in apoplastic ROS signaling, how do they function, and how are they regulated?

1.2 CONNECTIONS OF APOPLASTIC ROS SIGNALING WITH STRESS HORMONE SIGNALING

1.2.1 SALICYLIC ACID (SA)

SA is a plant hormone best known for its importance for plant's defense against pathogens and a necessary component of systemic acquired resistance (SAR). During the decades of intense study of SA signaling, several SA-binding proteins (SABPs) have been identified. However, most of them do not seem to be required

for SAR, suggesting that these SABPs might not be true SA receptors [19]. Recently it was shown that NON-EXPRESSOR OF PR GENES 1 (NPR1) is capable of binding SA with dissociation constant (K_d) of 140 nM when Cu^{2+} was present [20]. This was an exciting finding, since NPR1 is a well-known transcriptional co-activator of SA responses. Additionally, two NPR1 homologs, NPR3 and NPR4, were found to bind SA with very different K_d's of 1000 nM and 46 nM, respectively. Furthermore, NPR3 bound to SA and NPR4 without SA bound were found to interact with NPR1 and facilitate its degradation [21]. This suggests interesting dynamics for SA signaling, where NPR1 is stabilized in response to medium levels of SA, the similar levels where NPR1 could function as an SA receptor. However, several open question in the model remain: For example, how can NPR3 and NPR4 function as SA receptors, when the binding of SA does not induce conformational change in these proteins [22]? Since apoplastic ROS perception elicits responses similar to pathogen perception, it is not surprising to find SA to be connected with both. However, the connections are not straight-forward: Regarding hypersensitive response –like PCD triggered by apoplastic ROS signaling, SA is considered to be a positive regulator [15], [23], but SA has also been found to attenuate the responses to apoplastic ROS signaling, at least at the level of gene expression [24].

1.2.2 JASMONIC ACID (JA)

JA and the intermediates of JA biosynthesis regulate diverse processes from flower development to defense responses [25], [26]. In defense responses, JA is usually considered to have an antagonistic relationship with SA: whereas SA promotes cell death and defense against biotrophic pathogens, JA promotes cell survival and defense against necrotrophic pathogens. This same antagonism has been observed regarding O_3-induced PCD [27]–[29]. The sensing of JA is achieved through an F-box protein CORONATINE INSENSITIVE 1 (COI1), which binds JA-isoleucine (JA-Ile) at nanomolar concentrations [30]. F-box proteins function in a complex called SKP1-CULLIN-F-BOX protein (SCF) ubiquitin ligase, which is an E3 ubiquitin ligase targeting specific proteins for proteasomal degradation. The binding of JA-Ile into COI1 activates SCF^{COI1} complex, leads to degradation of JASMONATE ZIM DOMAIN (JAZ) proteins, which are repressors of MYC TFs. The degradation of JAZ repressors releases MYC TFs to regulate transcription of JA-responsive genes [31]–[33]. Numerous JA-inducible genes are induced in response to apoplastic ROS signaling [16], [34], highlighting the strong connection between JA and apoplastic ROS.

1.2.3 ETHYLENE

Ethylene is a gaseous phytohormone that positively regulates apoplastic ROS –induced PCD, and apoplastic ROS signaling leads to accumulation of ethylene [28], [29], [35]. Ethylene receptors resemble prokaryotic two-component regulators, which relay the signal via transfer of phosphate group from signaling module to another. Since similar proteins have been found in cyanobacteria, it is possible that plants have gained the ability to sense ethylene during endosymbiosis with the chloroplasts [36]–[38]. Receptors at the endoplasmic reticulum membrane of can be regulated by nanomolar concentrations of ethylene, the binding event leading to inactivation of the receptor's stimulatory activity towards the downstream signaling component CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), a negative regulator of ethylene responses. Once the activity of CTR1 decreases, it can no longer inhibit the cleavage of the membrane protein ETHYLENE INSENSITIVE 2 (EIN2), whose C-terminal end is subsequently translocated into nucleus, where stabilizes ETHYLENE INSENSITIVE 3 (EIN3), a TF regulating the transcription of ethylene-responsive genes [39]–[41]. Ethylene signaling is closely connected with JA signaling, and in the case of apoplastic ROS –induced PCD the relationship appears to be mutually antagonistic: Whereas ethylene promotes cell death and attenuates JA signaling, JA promotes cell survival and attenuates ethylene signaling [28].

1.2.4 AUXIN

Auxin is best known for its role in regulation of plant growth and development. Sensing of auxin is mechanistically highly similar to JA; Auxin receptor is an F-box protein TRANSPORT INHIBITOR RESPONSE 1 (TIR1), which functions as part of SCF^{TIR1} complex. Auxin binding induces an interaction between TIR1 and Aux/IAA proteins, which are negative regulators of AUXIN RESPONSE FACTORs (ARFs), TFs mediating transcriptional response to auxin. The interaction between TIR1 and Aux/IAA leads to degradation of Aux/IAAs, and hence the activation of ARFs. Apoplastic ROS signaling transiently downregulates auxin signaling [16], and auxin has been reported to have antagonistic relationship with mitochondrial ROS accumulation [42]. These findings support the idea that the balance between growth and defense might be adjusted through interactions between auxin and ROS signaling [43].

1.2.5 ABSCISIC ACID (ABA)

ABA is best known for its role in stomatal closure and abiotic stress responses, but it is also involved in cuticle formation, pathogen defense, and wound-induced spreading cell death [44]–[46]. The debate about the identity of ABA receptors

has been lively, and several different receptors have been suggested within last 15 years. The most well-established module of ABA perception involves protein of PYR/RCAR family as ABA receptors, which upon ABA binding begin to interact with PROTEIN PHOSPHATASE 2C (PP2C) family proteins, which are negative regulators of ABA signaling. This interaction inhibits the phosphatase activity of PP2C, leading to attenuation of repression of SNF1-RELATED PROTEIN KINASE 2 (SnRK2) family proteins, which activate downstream component of ABA signaling through phosphorylation. These downstream signaling component include plasma membrane ion channel SLOW ANION CHANNEL 1 (SLAC1), plasma membrane NADPH oxidase RBOHD and several ABA-regulated TFs of the ABF/AREB family [47], [48]. Generally, hormone treatments do not significantly induce the transcription ROS-responsive genes, suggesting that ROS signaling evokes hormone signaling, but not vice versa. However, ABA appears to make an exception: ABA treatments induce the transcription of several genes considered ROS-responsive [18]. Indeed, ABA has been reported to induce oxidative burst in guard cells [49] and mitochondrial ROS accumulation in the roots [50], suggesting that ROS may be significant secondary messengers in ABA signaling.

1.3 TRANSCRIPTIONAL RESPONSE TO STRESS

1.3.1 WHY TO STUDY TRANSCRIPTIONAL RESPONSE TO STRESS?

During rapid responses to perturbations, such as acute stress, the transcriptional changes have been found to correlate poorly with changes in protein abundances [51]–[56]. Even though the conclusions of these studies have been challenged lately [57], [58], it is obvious that the transcript levels alone do not directly predict well the future of the cell; even if the correlation to protein levels would be feasible, the posttranslational regulation adds another level of complexity, making the predictions about metabolome, enzyme activities, or cell fate very challenging if not impossible. Does this mean that rapid transcriptional responses to stress are studied merely because they are easy to study, not because of their biological importance? Even though the transcriptional changes may not predict plant’s future very well, they do carry signals of plant’s immediate past, especially regarding signal transduction events leading to the transcriptional changes, thus offering means to improve our understanding of plant stress signaling [18].

1.3.2 HOW TO ACHIEVE A SPECIfiC TRANSCRIPTIONAL RESPONSE TO STRESS?

Since plants cannot escape changes in growth conditions, they need sophisticated methods to adapt. Even though it is very difficult to predict the phenotypic changes caused by the rapid transcriptional response to stress, it seems clear that the transcriptional response does play an important role in adaptation, especially during slower, long-term adaptation [59]–[61]. In the study of transcriptional responses to stresses, there are two critical questions: How much specificity is there between transcriptional responses to different stresses and how is this specificity achieved?

From yeast studies it is known that transcriptional response to stress consists of two components: Core stress response that is common to all kinds of stresses and a specific component that is distinct for a given stress [62]–[64]. In yeast, the core stress response is large, meaning that many stress-responsive genes respond to all stresses. This leads to cross-tolerance, where a single stressor leads to a response that confers tolerance to other stresses as well. In multicellular organisms with heterogeneous cell populations, the core stress response appears to be smaller [65]. However, there is a significant overlap between the transcriptional responses to different stresses in *Arabidopsis thaliana* [18], [66]–[69], implying that the specificity between different stresses might have been previously overestimated [18]. On the other hand, a study with *Arabidopsis thaliana* under 11 different stress conditions revealed that about half of the TF-encoding genes which were transcriptionally regulated in response to stress were regulated only in a single stress condition [70]. This would suggest that the stress-specific component of transcriptional response is significant, even though the general stress response cannot be neglected either. If the stress-specificity does exist, where does it arise from?

A simplified scheme of stress signaling is often reduced to a pathway, which begins with a sensing of the stress, proceeds to relay of the signal through secondary messengers such as Ca^{2+} and activation of signaling proteins such as kinases and phosphatases, and results in transcriptional response through activation of sequence-specific TFs. Even though the high degree of crosstalk between different signaling pathways is better described by a signaling network rather than a set of pathways [61], the pathway-scheme is useful for illustrative purposes.

Sensory systems can be very specific, such as HISTIDINE KINASE 1 (HK1), which can sense osmotic stress [71], but several sensors activate downstream processes that appear almost identical and use same components, such as

secondary messengers ROS or Ca^{2+}, or activate similar kinases or phosphatases. However, specificity can be retained through several mechanisms. For example, the amplitude and frequency of Ca^{2+}-bursts or the site of Ca^{2+} release retain specific information [72]. Similar mechanisms are possible for ROS as well. Co-localization or scaffolding of signaling components, such as kinases of the MPK cascade, also retains information: Even if the activated kinase could phosphorylate tens of different targets, it will probably phosphorylate the target that is located in its immediate vicinity [73].

The main executers of transcriptional reprogramming are TFs, which are regulated by the upstream signal transduction components such as kinases. The mechanisms by which the TFs can be regulated include proteolytic activation/inactivation, (selective) subcellular transport, phosphorylation/dephosphorylation, redox regulation and change of protein interactors, just mention a few. Once activated, the TF is considered to bind to the DNA in the gene's promoter area and change the rate of transcription through interactions with basal transcriptional machinery. If a specific stress signal has been relayed to the specific TF, how does this TF find its specific target genes? An obvious candidate for a source of specificity are protein-DNA interactions: TFs can recognize a specific sequence of DNA at the promoter site. This recognition guides the TF at its target gene's promoter. However, the situation is not usually so straightforward (Study I). In order for the recognition to happen, the chromatin status has to be permissive enough to allow the TF to enter the DNA; if the DNA is tightly packed in heterochromatin, the binding event is not possible. Furthermore, the bare DNA-binding preference of a TF can be modulated through protein-protein interactions ([74] and the references within): If there is another TF (or any protein) nearby which can directly interact with a TF1 but not with a TF2, the probability of TF1 binding to this site can become higher, even if the DNA binding site would match better with the preferences of TF2. This has been elegantly shown for four *Arabidopsis* PHYTOCHROME INTERACTING FACTORs (PIFs): Even though the DNA-binding preferences *per se* did not vary between the PIFs, the binding sites in the genome showed preferences for specific PIF(s) [75]. Interestingly, the binding of a TF on the promoter does not necessarily affect the rate of gene's transcription. When studying the genes whose promoter was bound by all fours PIFs, it was found that certain genes' transcript abundances did correlate with PIF occupancy positively or negatively, but in several case there was no correlation [75]. Study on the WRKY TFs in parsley cell culture revealed that most of the time the binding sites of WRKYs, the W-boxes, were bound by a WRKY even in the absence of stimulus [76]. Additionally, a ChIP-seq experiment combined with transcriptomic analysis

showed that loss-of-function mutation in *Arabidopsis WRKY33* did not have an effect on the transcript accumulation of most genes whose promoter was bound by WRKY33 in the wild type [46]. As a conclusion, it is easy to envisage several steps of regulation which could generate specificity in responses to stresses, but the contributions of these should be assessed case by case.

1.3.3 SPECIFICITY OF TF-DNA INTERACTIONS

Bacterial TFs function largely according to the established model: A TF recognizes a specific DNA sequence, binds to it and affect the rate of transcription of the gene(s) nearby. However, more complex eukaryotic organisms have more genes and a lot more DNA packed inside a nucleus. Additionally, multicellularity and elaborate cellular compartmentalization complicate the signaling networks leading to more complicated transcriptional regulation of genes. Each gene having straight-forward regulation schemes for all relevant stimuli similar to bacteria would lead to several problems, including crowding of the nucleus and immense expansion of TF gene families, which in turn would require more TFs for regulation. One of the strategies to achieve complex regulation of thousands of genes with feasible amount of regulators is combinatorial complexity: Ability to combine simple regulators with additive or emergent effects can increase the regulatory potential exponentially. For example, we take four bacterial TFs, which can each recognize their own specific 20 base pairs (bp) long response element (RE) in gene promoters. If these TFs were able to form dimers with each other and bind combinations of two consecutive REs, the number of different combinations recognized would increase from four to 20 (assuming the monomeric binding ability remains). The additional specificity gained through dimerization would allow shorter recognition motives for each TF. The shorter motives would be less prone to deleterious mutations and more agile regarding rearrangements (higher chance of successful reshuffling of motives without breaking them). Indeed, the binding sites of prokaryotic TFs are about twice as long as their eukaryotic cousins [77], and the number of TFs binding to a single promoter is significantly higher in eukaryotes [78]. Several TF families in *Arabidopsis* have tens of members with potential to form heterodimers, and heterodimers between TFs from different families are also possible. A real-life example can be drawn from *Arabidopsis* NF-Y TFs, which bind DNA as heterotrimers. The 36 TF subunits (10 NF-YA, 13 NF-YB, 13 NF-YC) have the potential to form 1690 unique combinations [79].

Regardless of the length of the RE or the stringency of TF-DNA interaction, the TF somehow has to recognize its preferred DNA sequence. There are several mechanisms for this recognition, and usually a single TF-DNA interaction benefits

from several mechanisms working in parallel. The most intuitive form of DNA recognition is direct bonding between the amino acids of the TF and the bases of the DNA. This is called direct recognition or base readout [80], and is usually mediated through hydrogen bonding. However, the direct recognition alone is seldom sufficient to confer the observed specificity. Additionally, TFs use the shape of the DNA as a readout. This is possible because the base sequence of the DNA affects the 3D structure of the DNA. For example, a stretch of thymidine (T) nucleotides leads to a more narrow minor groove, which can be efficiently recognized by correctly positioned arginine amino acids of the TF [81]. The sequence affects the shape of the DNA also in a larger scale, generating curvature or distinct forms of DNA helices which can be recognized by specific TFs [80]. In certain cases, the shape of the DNA may facilitate the formation of TF-TF interactions, highlighting the active role of DNA in TF-DNA interactions [82]. Since the mechanisms of DNA recognition are versatile, it is not possible to predict DNA-binding specificities of TFs from their amino acid sequence. The experimental in vitro methods to determine the DNA binding specificities include protein binding microarray (PBM) and systematic evolution of ligands by exponential enrichment (SELEX). Whereas PBM relies on TF protein which binds to immobilized DNA on a microarray and is later quantified using fluorescently labeled antibodies, SELEX is based on freely diffusing pool of randomized oligonucleotides which get enriched for the preferred binding site through several cycles of binding, washing, and PCR amplification. Eventually the oligonucleotides are sequenced and a model of binding preference is constructed.

2. AIMS OF THE STUDY

The central questions addressed in this thesis were:

1. What are the factors that explain the natural variation in O_3 sensitivity in *Arabidopsis thaliana*?
2. What are the roles of stress hormones SA/JA/ethylene in apoplastic ROS signaling –induced transcriptional reprogramming and PCD? What are the modulators of these responses?
3. What generates specificity in apoplastic ROS signaling between the members WRKY transcription family, important executers of transcriptional reprogramming?

3. MATERIALS AND METHODS

The materials and methods used are described in detail in publications as indicated in table 1.

Table 1. Methods used in publications II, III, and IV. Parentheses indicate that the method was applied by the co-authors of the publication.

Method	**Publication**
O_3 exposure	**II, III, IV**
Quantification of cell death by ion leakage	**II, III**
Trypan blue staining	**(II), III**
3,3'-Diaminobenzidine (DAB) staining	**(II), III**
SA treatment	**(II)**
qPCR	**(II), (III), (IV)**
Measurement of stomatal conductance	**(II)**
Microarray analysis	**(II), (III)**
RNA-seq sample preparation	**(II), (III), (IV)**
RNA-seq data analysis	**II, III, IV**
RNA-seq data visualization	**(II), III, IV**
QTL mapping	**(II)**
Statistics: ANOVA, Linear mixed model	**(II), (III)**
SELEX	**IV**
LC-MS/MS sample preparation	**IV**
LC-MS/MS	**(IV)**
LC-MS/MS data analysis	**IV**
Arabidopsis seedling transformation	**IV**
Molecular cloning	**IV**
Confocal microscopy	**IV**

Table 2. List of *Arabidopsis* mutants and natural accession used. All mutants are in Col-0 background, unless otherwise mentioned.

Genotype	Annotation	Used in publication	Comments
Col-0		II, III, IV	Natural accession
C24		II	Natural accession
CT101		II	O_3 sensitive RIL (C24/Tenela)
Cvi-0		II	Natural accession
Tenela		II	Natural accession
abi1	*aba insensitive 1*	III	Dominant ABA insensitive mutant
agb1-2	*gtp binding protein beta 1*	III	Loss-of-function mutant
anac017-1	*nac domain containing protein 17*	III	Loss-of-function mutant
anac017-2	*nac domain containing protein 17*	III	Gain-of-function mutant
anac017-3	*nac domain containing protein 17*	III	Loss-of-function mutant
aos	*allene oxide synthase*	III	Loss-of-function mutant
coi1-16	*coronatine insensitive 1*	III	Conditional loss-of-function mutant
ein2-1	*ethylene insensitive 2*	III	Loss-of-function mutant
gpa1-4	*g protein alpha subunit 1*	III	Loss-of-function mutant
NahG	salicylate hydroxylase *NahG*	III	line expressing bacterial NahG gene encoding a hydroxylase suppressing SA accumulation
rbohD	*respiratory burst oxidase homologue D*	III	Loss-of-function mutant
rbohF	*respiratory burst oxidase homologue F*	III	Loss-of-function mutant
sid2-1	*salicylic acid induction deficient 2*	III	Loss-of-function mutant
sid2-2	*salicylic acid induction deficient 2*	III	Loss-of-function mutant
wrky25		III	Loss-of-function mutant
wrky70		III	Loss-of-function mutant
aos ein2		III	
coi1-16 eds1	*enhanced disease susceptibility 1*	III	
coi1-16 ein2		III	
coi1-16 ein2 eds1		III	

coi1-16 ein2 sid2-1		III	
coi1-16 ein2 sid2-1 eds1		III	
coi1-16 rbohD		III	
coi1-16 rbohF		III	
coi1-16 sid2-1		III	
coi1-16 wrky25		III	
coi1-16 wrky70		III	
ein2 sid2		III	
tga2 tga5 tga6		III	
wrky18 wrky40 wrky60		III	
ERF6 4D-5	*ETHYLENE RESPONSE FACTOR 6*	III	Overexpression line of dominant active ERF6
ERF6 4D-7	*ETHYLENE RESPONSE FACTOR 6*	III	Overexpression line of dominant active ERF6
ERF6 EAR 65	*ETHYLENE RESPONSE FACTOR 6*	III	Overexpression line of ERF6 fused with transcription repressor domain EAR
ERF6 EAR 71	*ETHYLENE RESPONSE FACTOR 6*	III	Overexpression line of ERF6 fused with transcription repressor domain EAR
wrky25 wrky33		III, IV	SAIL_529_B11, SALK_006603
wrky75-25		IV	N121525
EST-inducible *WRKY75*		IV	(XVE)(HPT)LexA::WRKY75 N2102362
EST-inducible *WRKY75*		IV	(XVE)(HPT)LexA::WRKY75 N2102363
35S::*CRE1-HA*	*CYTOKININ RESPONSE 1*	IV	overexpression line of CRE1 fused with affinity tag HA
35S::*GBF1-HA*	*G-BOX BINDING FACTOR 1*	IV	overexpression line of GBF1 fused with affinity tag HA

35S::*TGA9-HA*	*TGACG (TGA) MOTIF-BINDING PROTEIN 9*	IV	overexpression line of TGA9 fused with affinity tag HA
35S::*WRKY25-HA*		IV	overexpression line of WRKY25 fused with affinity tag HA
35S::*WRKY33-HA*		IV	overexpression line of WRKY33 fused with affinity tag HA
35S::*WRKY38-HA*		IV	overexpression line of WRKY38 fused with affinity tag HA
35S::*WRKY50-HA*		IV	overexpression line of WRKY50 fused with affinity tag HA
35S::*WRKY53-HA*		IV	overexpression line of WRKY53 fused with affinity tag HA
35S::*WRKY60-HA*		IV	overexpression line of WRKY60 fused with affinity tag HA
35S::*WRKY75-HA*		IV	overexpression line of WRKY75 fused with affinity tag HA

Table 3. List of defense-related marker genes used for qPCR.

Gene name	AGI	Annotation	Used in publication
WRKY75	At5g13080	*ARABIDOPSIS THALIANA WRKY DNA-BINDING PROTEIN 75*	III
AOX1a	At3g22370	*ALTERNATIVE OXIDASE 1a*	III
ARGOS	At3g59900	*AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE*	III
ARR5	At3g48100	*ARABIDOPSIS THALIANA RESPONSE REGULATOR 5*	III
CRK9	At4g23170	*CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 9*	III
CRK39	At4g04540	*CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 39*	III
ERF6	At4g17490	*ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 6*	III
GRX480	At1g28480	*GLUTAREDOXIN 480*	III
IDA	At1g68765	*INFLORESCENCE DEFICIENT IN ABSCISSION*	III
LOX4	At1g72520	*LIPOXYGENASE 4*	III
ODX/DIN11	At3g49620	*DARK INDUCIBLE 11*	III
ORA59	At1g06160	*OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59*	III
RBOHD	At5g47910	*RESPIRATORY BURST OXIDASE HOMOLOGUE D*	III
CYP71A13	At2g30770	*CYTOCHROME P450, FAMILY 71 SUBFAMILY A, POLYPEPTIDE 13*	IV
PAD3	At3g26830	*PHYTOALEXIN DEFICIENT 3*	IV

4. RESULTS AND DISCUSSION

4.1 ROLES OF STRESS HORMONES JA/SA/ETHYLENE DURING ACUTE O_3 EXPOSURE

The plant hormones SA, JA, and ethylene are important regulators of stress responses. They have especially been studied in the context of plant immune responses, where they play an important role in determining the direction of defense responses. The choice of strategy is crucial for the plant, since the best strategy against biotrophic pathogens is a very poor choice against necrotrophic pathogens [83]. Previously, SA and ethylene have been considered promotors of PCD, a valid strategy against biotrophic pathogens, whereas JA has been considered to promote cell survival during the attack of necrotrophic pathogens. Indeed, JA-insensitive mutants develop visible lesions in response to O_3 exposure as well. However, the role of SA appears to be more complicated. In the case of acute O_3 exposure, SA appears to antagonize the effects of apoplastic ROS [24], suggesting a cell death –inhibitory role rather than cell death –inducing role. This was further verified in study II, where the O_3 tolerance of natural accession C24 was found to be a consequence of hyperactive SA signaling, a known feature of this accession [84], and SA pre-treatment conferred O_3 sensitive accessions Tenela and Cvi-0 O_3 tolerant (II).

Another finding that opposes the scheme of SA functioning as promotor of cell death during acute O_3 exposure was made in study III, where it was shown that impairment of SA biosynthesis by mutation in *SALICYLIC ACID INDUCTION DEFICIENT 2 (SID2*) did not decrease the amount of cell death induced by O_3 in *coronatine insensitive 1-16* (*coi1-16*)**,** a mutant insensitive to JA. Interestingly, the perturbation of ethylene signaling by mutation in *ETHYLENE INSENSITIVE 2* (*EIN2*) did decrease the amount of O_3-induced cell death in *coi1-16* background, but only when SA biosynthesis was intact. This implies that the balance between JA and ethylene signaling is important in determining the outcome of apoplastic ROS signaling, but SA is an important modulator of this response: When JA signaling is inactive, the ethylene-powered pro-death signal dominates. When both ethylene and JA signaling are silenced, SA signaling inhibits apoplastic ROS –induced PCD. If the SA levels are reduced, PCD is triggered in response to apoplastic ROS (Figure 1).

The mechanisms by which SA operates to inhibit apoplastic ROS –induced PCD are not known. The transcriptomic analyses performed in study III suggest that the mechanism could be related to SA dampening a specific branch of defense signaling triggered by apolastic ROS: Two mutant lines (*coi1 ein2 sid2* and *tga2*

tga5 tga6) impaired in different aspects of SA signaling showed increased O_3-induced expression of genes related to immune response, cell death, and transmembrane kinase activity, even though the same GO categories were enriched among genes expressed at lower levels than in the wild type under control growth conditions. Apparently the impairment of SA signaling leads to sensitization to an unknown signal produced in response to apoplastic ROS signaling. The promoter areas of the genes "hypersensitive" to O_3 would suggest that this signal is related to bursts of Ca^{2+}; element CCGCGT was enriched within these genes' promoters (unpublished). This element was previously reported to be regulated by Ca^{2+}-regulated CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR (CAMTA) family TFs [85].

Obviously, plant stress hormones do not operate alone; they are part of the signaling network including numerous mechanisms, pathways, and components. To further elucidate the signal transduction components involved in the O_3-induced cell death of JA-insensitive *coi1*, this mutant was crossed with several other mutants each deficient for a component of the known defense signaling regulators (III). From this double mutant analysis, two genes crucial for O_3-induced PCD in *coi1* emerged: *RBOHF* and *WRKY70*. Interestingly, the mutation in another respiratory burst oxidase homolog, *RBOHD*, did not affect the O_3 sensitivity of *coi1*. The same was true regarding *WRKY25*, a homolog of *WRKY70*. This points towards high degree of specificity in the components of apoplastic ROS signaling and transcriptional reprogramming.

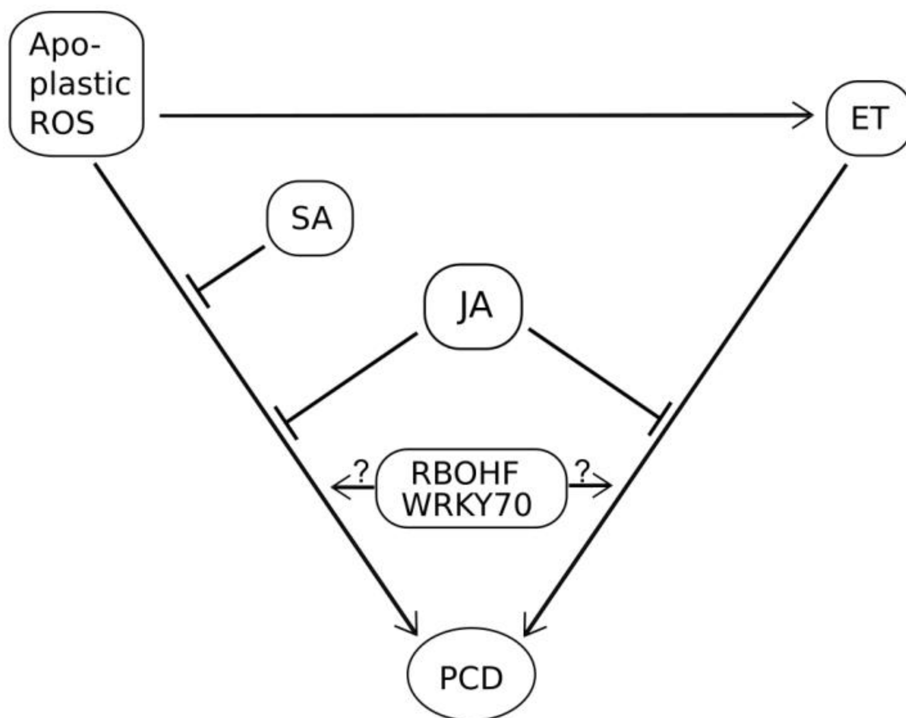

Figure 1. Interactions between apoplastic ROS signaling, plant stress hormones SA, JA, and ethylene (ET), and signaling components RBOHF and WRKY70 according to findings in study III.

4.2 SPECIFICITY IN SIGNALING AMONG WRKY TF FAMILY

WRKY TFs are a large family with 74 members in *Arabidopsis*. A majority of them were found to be transcriptionally induced in response to O_3 treatment. The induction was largely independent of stress hormones JA/SA/ethylene (Study IV). Study III already gave indications of high degree of specificity between WRKYs, but the source of this specificity was not known. Based on results from study IV, the transcript-level regulation of *WRKY*s offers little specificity regarding apoplastic signaling, since >40 WRKYs are transcriptionally induced in response to O_3 exposure. The specificity in signaling among WRKY family is likely achieved through several post-transcriptional mechanisms, including DNA-binding preferences, protein stability, protein-protein interactions, and subcellular localization.

The *in vitro* DNA binding specificities were found to vary substantially between the WRKYs from different phylogenetic subgroups (IV). WRKYs from group IIc (WRKY75, WRKY50, and WRKY51) preferred to bind very similar primary monomeric sequences TTGACTTT, but clear differences were evident: more closely related WRKYs 50 and 51 had a peculiar secondary monomeric preference: TTTTCCAC, which does not at all resemble the classical binding site reported for WRKYs, the W-box (TTGAC[C/T]). Interestingly, the same sequence has been reported to be bound *in vivo* by *Nicotiana tabacum* WRKY12, a paralog of WRKY50/51 [86]. Further differences were found by looking at dimeric binding preferences: WRKY50 and 51 showed a clear preference towards a composite site consisting of two monomeric binding sites in an immediate contact (TTGACTTCCA, TTGACTTTCCA, and TTGACTTTTTCCA), whereas WRKY75 showed co-operative binding between monomeric binding sites 5-6 nucleotides apart (such as TTGACTTTNNTTGAC), but no preference towards composite sites. WRKY25 and WRKY33, two closely related TFs from group I, which are phylogenetically more distant from the rest of the studied WRKYs (50/51/75), showed binding characteristic similar to each other but clearly different from WRKY50/51/75: Their preferred monomeric binding site was classical W-box TTGAC[C/T] with a strong potential for binding composite sites, such as TTGACTTGAC.

Protein interaction analyses performed on seven selected overexpressed and HA-tagged WRKYs revealed that certain WRKYs, namely WRKY25, WRKY33, WRKY53, and WRKY60, appeared to be stabilized on protein level in response to apoplastic ROS signaling (IV). This result does not correlate well with the transcript level regulation, highlighting the low predictive value of transcript levels regarding protein levels. For each WRKY studied, approximately 20 putative interactors were obtained, with approximately half of them shared between at least one other WRKY. Among the interactors, a significant proportion belonged to gene ontology categories “intracellular protein transport” and “chromatin organization”, suggesting that the function of WRKYs might be regulated at the levels of subcellular localization and association with chromatin (IV).

The protein localization study performed in *Arabidopsis* seedlings and with four selected YFP-tagged WRKYs showed that all studied WRKYs localized into nucleus, opposing the idea that nuclear transport would be a significant point of regulation for these TFs (IV). However, interesting patterns of subnuclear localization were observed: WRKY25 showed even YFP signal in the nucleus, but WRKY53 and WRKY75 localized into bright, fairly large speckles in most of the nuclei observed. WRKY60 appeared to localize into a lot smaller, less resolved speckles, forming structures that resemble a network.

Altogether these results suggested that there are numerous post-transcriptional steps of regulation in the function of WRKYs, but how does it reflect at the level of transcriptional regulation driven by a single WRKY or a pair of WRKYs? This was studied through transcriptomic analyses of wild type and knockout mutants *wrky75-25* and *wrky25 wrky33* in O_3-treated plants and under control growth conditions. Since the comparison to publically available data revealed 2-hour O_3 response to be transcriptionally highly similar to 14-hour *Botrytis* infection, the effects of *wrky33* during *Botrytis* infection from Liu et al. [46] were included in the comparisons. Furthermore, two estradiol-inducible overexpressor lines of *WRKY75* were analyzed.

As could be expected, a large proportion of the O_3 response was unaffected by the knockout of a single WRKY or even a pair of WRKYs, but certain clear effects were found, both in control growth conditions and after O_3 exposure (IV). In both conditions, *wrky25 wrky33* had genes related to defense against pathogens expressed at lower level than in the wild type, suggesting that the net effect of these two TFs is a positive regulation of defense genes. The enrichment of W-box in the promoter areas of the genes expressed at lower level in the mutant suggested that the regulation could go directly through WRKYs. Interestingly, the results from *wrky33* from Liu et al. are quite different: Some of the best-known target genes of WRKY33, such as camalexin synthesis–involved *PAD3* and *CYP71A13* were expressed at lower levels than in the wild type in both *wrky25 wrky33* and *wrky33,* but most of the defense-related genes that were regulated in *wrky33* vs. wild type were actually expressed at higher level in the mutant, suggesting that WRKY33 acts mainly as a negative regulator of defense-related genes. This was supported by the enrichment of W-box in the promoters of genes expressed at higher level in *wrky33* but not at lower-expressed genes. Altogether, this suggests that WRKY25 and WRKY33 might act in an antagonistic manner to regulate the balance of defense responses. Considering the similar DNA-binding preferences, the antagonism could be based on direct competition of DNA-binding sites.

Based on the results from *wrky75* under control growth conditions, WRKY75 appears to be a negative regulator of responses to several hormones, including JA, ethylene, ABA, auxin, gibberellin, and a positive regulator of pathogen defense response (response to fungus/biotic stimulus). Surprisingly, the W-box or its derivatives showed only a weak enrichment in the promoters of the genes regulated in the mutant. One probable reason for this is the enrichment of ERF and MYB family TFs among the genes regulated in the mutant: If WRKY75 regulates directly only a handful of TFs, which in turn regulate tens of target genes, the fraction of genes directly regulated by WRKY75 becomes statistically

insignificant. This hypothesis got support from the overexpression experiment: TFs were indeed enriched among the WRKY75-regulated genes. Combining the results from knockout and overexpression experiments, it was possible to construct a short list of genes most probably regulated through WRKY75. The list of 18 genes positively regulated by WRKY75 contains well-known pathogen-responsive genes PR1, PR2, PCC1, and several receptor-like kinases, highlighting the role of WRKY75 as a positive regulator of pathogen response (IV). The list of 16 genes negatively regulated by WRKY75 contains a ROS-responsive TF *ZAT10* [87], *DICARBOXYLATE CARRIER 2* (*DIC2)* probably related to redox-connection between cytosol and mitochondria [88], two genes of MPK cascade: *MITOGEN ACTIVATED PROTEIN KINASE KINASE KINASE 19* (*MAPKKK19*) and *MITOGEN ACTIVATED PROTEIN KINASE KINASE 9* (*MKK9*), suggesting that WRKY75 could function as a regulator of ROS signaling or homeostasis. Furthermore, the regulation of MPK cascade could explain why WRKY75 appears to regulate such a wide spectrum of processes. In addition, the list contains genes involved in sulfur deficiency response, suggesting that WRKY75 might play role in sulfur homeostasis, possibly through interactions with ROS, glutathione, JA, and ethylene, which all have been implicated as components of sulfur homeostasis [89].

5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Plant defense signaling pathways form a complex network, where apoplastic ROS have a significant role in signal transduction and amplification. In this thesis, O_3 was used as a tool to produce apoplastic ROS in order to find out which signaling components interact with apoplastic ROS and how the interactions work. Special emphasis was on the role of plant stress hormones and WRKY TFs. SA signaling was found to inhibit PCD induced by apoplastic ROS, and this phenomenon explained the O_3 tolerance of natural accession C24. However, impairment of SA biosynthesis by mutation in *SID2* did not significantly change the O_3 sensitivity of JA insensitive and O_3 sensitive *coi1*. On the other hand, *sid2* mutation did increase the O_3 sensitivity of *coi1 ein2*, suggesting that SA indeed does protect the plant from PCD induced by apoplastic ROS, but ethylene signaling can bypass this protection when JA signaling is impaired (Figure 1). Furthermore, the O_3 sensitivity of *coi1* was suppressed by mutations in *RBOHF* or *WRKY70*, implicating these genes as important components of PCD induced by apoplastic ROS in JA-insensitive background.

The function of WRKYs was further investigated in biochemical and transcriptomics methods to find out factors generating signaling specificity between the members of the large TF family. Several steps of regulation with potential to generate specificity were found: DNA-binding preference, protein stability, protein-protein-interactions, and subnuclear localization. Transcriptomic analyses suggested an antagonistic interaction between WRKY25 and WRKY33, and implicated WRKY75 as a regulator of well-known pathogen response genes and several TFs from different gene families. At the level of whole rosettes, more than 40 *WRKY*s were transcriptionally induced in response to apoplastic ROS. In the future, transcriptomic and chromatin-binding studies with higher spatial resolution up to the level of individual cells will probably reveal fine structure of the transcriptional regulation that helps to define the roles of individual WRKYs in stress responses.

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7. REFERENCES

[1] R. E. Kopp, J. L. Kirschvink, I. A. Hilburn, and C. Z. Nash, “The Paleoproterozoic snowball Earth: A climate disaster triggered by the evolution of oxygenic photosynthesis,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 102, no. 32, pp. 11131–11136, Aug. 2005.

[2] R. Mittler, S. Vanderauwera, N. Suzuki, G. Miller, V. B. Tognetti, K. Vandepoele, M. Gollery, V. Shulaev, and F. Van Breusegem, “ROS signaling: the new wave?,” *Trends Plant Sci.*, vol. 16, no. 6, pp. 300–309, Jun. 2011.

[3] B. D’Autréaux and M. B. Toledano, “ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis,” *Nat. Rev. Mol. Cell Biol.*, vol. 8, no. 10, pp. 813–824, Oct. 2007.

[4] J. T. Greenberg, P. Monach, J. H. Chou, P. D. Josephy, and B. Demple, “Positive control of a global antioxidant defense regulon activated by superoxide-generating agents in *Escherichia coli.*,” *Proc. Natl. Acad. Sci.*, vol. 87, no. 16, pp. 6181–6185, Aug. 1990.

[5] A. Levine, R. Tenhaken, R. Dixon, and C. Lamb, “H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response,” *Cell*, vol. 79, no. 4, pp. 583–593, Nov. 1994.

[6] S. G. Rhee, “Redox signaling: hydrogen peroxide as intracellular messenger,” *Exp. Mol. Med.*, vol. 31, no. 2, pp. 53–59, Jun. 1999.

[7] U. Krämer, “Planting molecular functions in an ecological context with *Arabidopsis thaliana*,” *eLife*, vol. 4, p. e06100, Mar. 2015.

[8] J. D. G. Jones and J. L. Dangl, “The plant immune system,” *Nature*, vol. 444, no. 7117, pp. 323–329, Nov. 2006.

[9] Y. Kadota, K. Shirasu, and C. Zipfel, “Regulation of the NADPH Oxidase RBOHD During Plant Immunity,” *Plant Cell Physiol.*, vol. 56, no. 8, pp. 1472–1480, Aug. 2015.

[10] C. Segonzac, D. Feike, S. Gimenez-Ibanez, D. R. Hann, C. Zipfel, and J. P. Rathjen, “Hierarchy and Roles of Pathogen-Associated Molecular Pattern-Induced Responses in *Nicotiana benthamiana*,” *Plant Physiol.*, vol. 156, no. 2, pp. 687–699, Jun. 2011.

[11] U. Dubiella, H. Seybold, G. Durian, E. Komander, R. Lassig, C.-P. Witte, W. X. Schulze, and T. Romeis, “Calcium-dependent protein kinase/NADPH oxidase activation circuit is required for rapid defense signal propagation,” *Proc. Natl. Acad. Sci.*, vol. 110, no. 21, pp. 8744–8749, May 2013.

[12] G. Miller, K. Schlauch, R. Tam, D. Cortes, M. A. Torres, V. Shulaev, J. L. Dangl, and R. Mittler, “The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli,” *Sci. Signal.*, vol. 2, no. 84, p. ra45, 2009.

[13] J. Foreman, V. Demidchik, J. H. F. Bothwell, P. Mylona, H. Miedema, M. A. Torres, P. Linstead, S. Costa, C. Brownlee, J. D. G. Jones, J. M. Davies, and L. Dolan, “Reactive oxygen species produced by NADPH oxidase regulate plant cell growth,” *Nature*, vol. 422, no. 6930, pp. 442–446, Mar. 2003.

[14] R. J. Carol and L. Dolan, “The role of reactive oxygen species in cell growth: lessons from root hairs,” *J. Exp. Bot.*, vol. 57, no. 8, pp. 1829–1834, May 2006.

[15] K. Overmyer, M. Brosché, R. Pellinen, T. Kuittinen, H. Tuominen, R. Ahlfors, M. Keinänen, M. Saarma, D. Scheel, and J. Kangasjärvi, “Ozone-induced programmed cell death in the Arabidopsis *radical-induced cell death1* mutant,” *Plant Physiol.*, vol. 137, no. 3, pp. 1092–1104, Mar. 2005.

[16] T. Blomster, J. Salojärvi, N. Sipari, M. Brosché, R. Ahlfors, M. Keinänen, K. Overmyer, and J. Kangasjärvi, “Apoplastic reactive oxygen species transiently decrease auxin signaling and cause stress-induced morphogenic response in Arabidopsis,” *Plant Physiol.*, vol. 157, no. 4, pp. 1866–1883, Dec. 2011.

[17] N. Tosti, S. Pasqualini, A. Borgogni, L. Ederli, E. Falistocco, S. Crispi, and F. Paolocci, “Gene expression profiles of O_3-treated *Arabidopsis* plants,” *Plant Cell Environ.*, vol. 29, no. 9, pp. 1686–1702, Sep. 2006.
[18] L. Vaahtera, M. Brosché, M. Wrzaczek, and J. Kangasjärvi, “Specificity in ROS signaling and transcript signatures,” *Antioxid. Redox Signal.*, vol. 21, no. 9, pp. 1422–1441, Sep. 2014.
[19] E. Attaran and S. Y. He, “The Long-Sought-After Salicylic Acid Receptors,” *Mol. Plant*, vol. 5, no. 5, pp. 971–973, Sep. 2012.
[20] Y. Wu, D. Zhang, J. Y. Chu, P. Boyle, Y. Wang, I. D. Brindle, V. De Luca, and C. Després, “The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid,” *Cell Rep.*, vol. 1, no. 6, pp. 639–647, Jun. 2012.
[21] Z. Q. Fu, S. Yan, A. Saleh, W. Wang, J. Ruble, N. Oka, R. Mohan, S. H. Spoel, Y. Tada, N. Zheng, and X. Dong, “NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants,” *Nature*, vol. 486, no. 7402, pp. 228–232, Jun. 2012.
[22] X. Kuai, B. J. MacLeod, and C. Després, “Integrating data on the *Arabidopsis* NPR1/NPR3/NPR4 salicylic acid receptors; a differentiating argument,” *Front. Plant Sci.*, vol. 6, p. 235, 2015.
[23] M. V. Rao and K. R. Davis, “Ozone-induced cell death occurs via two distinct mechanisms in *Arabidopsis*: the role of salicylic acid,” *Plant J.*, vol. 17, no. 6, pp. 603–614, Mar. 1999.
[24] E. Xu and M. Brosché, “Salicylic acid signaling inhibits apoplastic reactive oxygen species signaling,” *BMC Plant Biol.*, vol. 14, p. 155, 2014.
[25] A. Devoto and J. G. Turner, “Regulation of Jasmonate-mediated Plant Responses in Arabidopsis,” *Ann. Bot.*, vol. 92, no. 3, pp. 329–337, Sep. 2003.
[26] A. K. Spartz and W. M. Gray, “Plant hormone receptors: new perceptions,” *Genes Dev.*, vol. 22, no. 16, pp. 2139–2148, Aug. 2008.
[27] M. V. Rao, H. Lee, R. A. Creelman, J. E. Mullet, and K. R. Davis, “Jasmonic Acid Signaling Modulates Ozone-Induced Hypersensitive Cell Death,” *Plant Cell*, vol. 12, no. 9, pp. 1633–1647, Sep. 2000.
[28] H. Tuominen, K. Overmyer, M. Keinänen, H. Kollist, and J. Kangasjärvi, “Mutual antagonism of ethylene and jasmonic acid regulates ozone-induced spreading cell death in *Arabidopsis*,” *Plant J.*, vol. 39, no. 1, pp. 59–69, Jul. 2004.
[29] K. Overmyer, M. Brosché, and J. Kangasjärvi, “Reactive oxygen species and hormonal control of cell death,” *Trends Plant Sci.*, vol. 8, no. 7, pp. 335–342, Jul. 2003.
[30] L. B. Sheard, X. Tan, H. Mao, J. Withers, G. Ben-Nissan, T. R. Hinds, Y. Kobayashi, F.-F. Hsu, M. Sharon, J. Browse, S. Y. He, J. Rizo, G. A. Howe, and N. Zheng, “Jasmonate perception by inositol phosphate-potentiated COI1-JAZ co-receptor,” *Nature*, vol. 468, no. 7322, pp. 400–405, Nov. 2010.
[31] J. Yan, C. Zhang, M. Gu, Z. Bai, W. Zhang, T. Qi, Z. Cheng, W. Peng, H. Luo, F. Nan, Z. Wang, and D. Xie, “The Arabidopsis CORONATINE INSENSITIVE1 Protein Is a Jasmonate Receptor,” *Plant Cell*, vol. 21, no. 8, pp. 2220–2236, Aug. 2009.
[32] B. Thines, L. Katsir, M. Melotto, Y. Niu, A. Mandaokar, G. Liu, K. Nomura, S. Y. He, G. A. Howe, and J. Browse, “JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling,” *Nature*, vol. 448, no. 7154, pp. 661–665, Aug. 2007.
[33] A. Chini, S. Fonseca, G. Fernández, B. Adie, J. M. Chico, O. Lorenzo, G. García-Casado, I. López-Vidriero, F. M. Lozano, M. R. Ponce, J. L. Micol, and R. Solano, “The JAZ family of repressors is the missing link in jasmonate signalling,” *Nature*, vol. 448, no. 7154, pp. 666–671, Aug. 2007.
[34] M. Tamaoki, N. Nakajima, A. Kubo, M. Aono, T. Matsuyama, and H. Saji, “Transcriptome analysis of O_3-exposed *Arabidopsis* reveals that multiple signal pathways act mutually

antagonistically to induce gene expression," *Plant Mol. Biol.*, vol. 53, no. 4, pp. 443–456, Nov. 2003.

[35] K. Overmyer, H. Tuominen, R. Kettunen, C. Betz, C. Langebartels, H. Sandermann, and J. Kangasjärvi, "Ozone-Sensitive Arabidopsis *rcd1* Mutant Reveals Opposite Roles for Ethylene and Jasmonate Signaling Pathways in Regulating Superoxide-Dependent Cell Death," *Plant Cell Online*, vol. 12, no. 10, pp. 1849–1862, Oct. 2000.

[36] B. Chow and P. McCourt, "Plant hormone receptors: perception is everything," *Genes Dev.*, vol. 20, no. 15, pp. 1998–2008, Aug. 2006.

[37] null Bleecker, "Ethylene perception and signaling: an evolutionary perspective," *Trends Plant Sci.*, vol. 4, no. 7, pp. 269–274, Jul. 1999.

[38] S. M. Mount and C. Chang, "Evidence for a plastid origin of plant ethylene receptor genes," *Plant Physiol.*, vol. 130, no. 1, pp. 10–14, Sep. 2002.

[39] L. M. Benavente and J. M. Alonso, "Molecular mechanisms of ethylene signaling in *Arabidopsis*," *Mol. Biosyst.*, vol. 2, no. 3–4, pp. 165–173, Mar. 2006.

[40] X. Wen, C. Zhang, Y. Ji, Q. Zhao, W. He, F. An, L. Jiang, and H. Guo, "Activation of ethylene signaling is mediated by nuclear translocation of the cleaved EIN2 carboxyl terminus," *Cell Res.*, vol. 22, no. 11, pp. 1613–1616, Nov. 2012.

[41] H. Qiao, Z. Shen, S. C. Huang, R. J. Schmitz, M. A. Urich, S. P. Briggs, and J. R. Ecker, "Processing and subcellular trafficking of ER-tethered EIN2 control response to ethylene gas," *Science*, vol. 338, no. 6105, pp. 390–393, Oct. 2012.

[42] A. Ivanova, S. R. Law, R. Narsai, O. Duncan, J.-H. Lee, B. Zhang, O. Van Aken, J. D. Radomiljac, M. van der Merwe, K. Yi, and J. Whelan, "A Functional Antagonistic Relationship between Auxin and Mitochondrial Retrograde Signaling Regulates *Alternative Oxidase1a* Expression in Arabidopsis1[W][OPEN]," *Plant Physiol.*, vol. 165, no. 3, pp. 1233–1254, Jul. 2014.

[43] G. Potters, T. P. Pasternak, Y. Guisez, and M. A. K. Jansen, "Different stresses, similar morphogenic responses: integrating a plethora of pathways," *Plant Cell Environ.*, vol. 32, no. 2, pp. 158–169, Feb. 2009.

[44] F. Cui, M. Brosché, M. T. Lehtonen, A. Amiryousefi, E. Xu, M. Punkkinen, J. P. T. Valkonen, H. Fujii, and K. Overmyer, "Dissecting abscisic acid signaling pathways involved in cuticle formation," *Mol. Plant*, Apr. 2016.

[45] F. Cui, M. Brosché, N. Sipari, S. Tang, and K. Overmyer, "Regulation of ABA dependent wound induced spreading cell death by MYB108," *New Phytol.*, vol. 200, no. 3, pp. 634–640, Nov. 2013.

[46] S. Liu, B. Kracher, J. Ziegler, R. P. Birkenbihl, and I. E. Somssich, "Negative regulation of ABA signaling by WRKY33 is critical for *Arabidopsis* immunity towards *Botrytis cinerea 2100*," *eLife*, vol. 4, p. e07295, Jun. 2015.

[47] K. E. Hubbard, N. Nishimura, K. Hitomi, E. D. Getzoff, and J. I. Schroeder, "Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions," *Genes Dev.*, vol. 24, no. 16, pp. 1695–1708, Aug. 2010.

[48] K. G. Kline, M. R. Sussman, and A. M. Jones, "Abscisic Acid Receptors," *Plant Physiol.*, vol. 154, no. 2, pp. 479–482, Oct. 2010.

[49] J. M. Kwak, I. C. Mori, Z.-M. Pei, N. Leonhardt, M. A. Torres, J. L. Dangl, R. E. Bloom, S. Bodde, J. D. G. Jones, and J. I. Schroeder, "NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*," *EMBO J.*, vol. 22, no. 11, pp. 2623–2633, Jun. 2003.

[50] J. He, Y. Duan, D. Hua, G. Fan, L. Wang, Y. Liu, Z. Chen, L. Han, L.-J. Qu, and Z. Gong, "DEXH box RNA helicase-mediated mitochondrial reactive oxygen species production in *Arabidopsis* mediates crosstalk between abscisic acid and auxin signaling," *Plant Cell*, vol. 24, no. 5, pp. 1815–1833, May 2012.

[51] L. M. F. de Godoy, J. V. Olsen, J. Cox, M. L. Nielsen, N. C. Hubner, F. Fröhlich, T. C. Walther, and M. Mann, "Comprehensive mass-spectrometry-based proteome quantification of haploid versus diploid yeast," *Nature*, vol. 455, no. 7217, pp. 1251–1254, Oct. 2008.
[52] M. L. Fournier, A. Paulson, N. Pavelka, A. L. Mosley, K. Gaudenz, W. D. Bradford, E. Glynn, H. Li, M. E. Sardiu, B. Fleharty, C. Seidel, L. Florens, and M. P. Washburn, "Delayed correlation of mRNA and protein expression in rapamycin-treated cells and a role for Ggc1 in cellular sensitivity to rapamycin," *Mol. Cell. Proteomics MCP*, vol. 9, no. 2, pp. 271–284, Feb. 2010.
[53] S. Komili and P. A. Silver, "Coupling and coordination in gene expression processes: a systems biology view," *Nat. Rev. Genet.*, vol. 9, no. 1, pp. 38–48, Jan. 2008.
[54] T. Maier, M. Güell, and L. Serrano, "Correlation of mRNA and protein in complex biological samples," *FEBS Lett.*, vol. 583, no. 24, pp. 3966–3973, Dec. 2009.
[55] B. Schwanhäusser, D. Busse, N. Li, G. Dittmar, J. Schuchhardt, J. Wolf, W. Chen, and M. Selbach, "Global quantification of mammalian gene expression control," *Nature*, vol. 473, no. 7347, pp. 337–342, May 2011.
[56] C. Vogel and E. M. Marcotte, "Insights into the regulation of protein abundance from proteomic and transcriptomic analyses," *Nat. Rev. Genet.*, vol. 13, no. 4, pp. 227–232, Mar. 2012.
[57] J. J. Li and M. D. Biggin, "Statistics requantitates the central dogma," *Science*, vol. 347, no. 6226, pp. 1066–1067, Mar. 2015.
[58] J. J. Li, P. J. Bickel, and M. D. Biggin, "System wide analyses have underestimated protein abundances and the importance of transcription in mammals," *PeerJ*, vol. 2, p. e270, Feb. 2014.
[59] E. de Nadal, G. Ammerer, and F. Posas, "Controlling gene expression in response to stress," *Nat. Rev. Genet.*, vol. 12, no. 12, pp. 833–845, Dec. 2011.
[60] J. Mizoi, K. Shinozaki, and K. Yamaguchi-Shinozaki, "AP2/ERF family transcription factors in plant abiotic stress responses," *Biochim. Biophys. Acta BBA - Gene Regul. Mech.*, vol. 1819, no. 2, pp. 86–96, Feb. 2012.
[61] K. B. Singh, R. C. Foley, and L. Oñate-Sánchez, "Transcription factors in plant defense and stress responses," *Curr. Opin. Plant Biol.*, vol. 5, no. 5, pp. 430–436, Oct. 2002.
[62] A. P. Gasch, P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown, "Genomic expression programs in the response of yeast cells to environmental changes," *Mol. Biol. Cell*, vol. 11, no. 12, pp. 4241–4257, Dec. 2000.
[63] H. C. Causton, B. Ren, S. S. Koh, C. T. Harbison, E. Kanin, E. G. Jennings, T. I. Lee, H. L. True, E. S. Lander, and R. A. Young, "Remodeling of yeast genome expression in response to environmental changes," *Mol. Biol. Cell*, vol. 12, no. 2, pp. 323–337, Feb. 2001.
[64] D. Chen, W. M. Toone, J. Mata, R. Lyne, G. Burns, K. Kivinen, A. Brazma, N. Jones, and J. Bähler, "Global transcriptional responses of fission yeast to environmental stress," *Mol. Biol. Cell*, vol. 14, no. 1, pp. 214–229, Jan. 2003.
[65] L. López-Maury, S. Marguerat, and J. Bähler, "Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation," *Nat. Rev. Genet.*, vol. 9, no. 8, pp. 583–593, Aug. 2008.
[66] W. Chen, N. J. Provart, J. Glazebrook, F. Katagiri, H.-S. Chang, T. Eulgem, F. Mauch, S. Luan, G. Zou, S. A. Whitham, P. R. Budworth, Y. Tao, Z. Xie, X. Chen, S. Lam, J. A. Kreps, J. F. Harper, A. Si-Ammour, B. Mauch-Mani, M. Heinlein, K. Kobayashi, T. Hohn, J. L. Dangl, X. Wang, and T. Zhu, "Expression Profile Matrix of Arabidopsis Transcription Factor Genes Suggests Their Putative Functions in Response to Environmental Stresses," *Plant Cell*, vol. 14, no. 3, pp. 559–574, Mar. 2002.

[67] M. Seki, M. Narusaka, H. Abe, M. Kasuga, K. Yamaguchi-Shinozaki, P. Carninci, Y. Hayashizaki, and K. Shinozaki, "Monitoring the Expression Pattern of 1300 Arabidopsis Genes under Drought and Cold Stresses by Using a Full-Length cDNA Microarray," *Plant Cell*, vol. 13, no. 1, pp. 61–72, Jan. 2001.

[68] W. E. Durrant, O. Rowland, P. Piedras, K. E. Hammond-Kosack, and J. D. G. Jones, "cDNA-AFLP Reveals a Striking Overlap in Race-Specific Resistance and Wound Response Gene Expression Profiles," *Plant Cell*, vol. 12, no. 6, pp. 963–977, Jun. 2000.

[69] P. M. Schenk, K. Kazan, I. Wilson, J. P. Anderson, T. Richmond, S. C. Somerville, and J. M. Manners, "Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis," *Proc. Natl. Acad. Sci.*, vol. 97, no. 21, pp. 11655–11660, Oct. 2000.

[70] P. Barah, M. N. B N, N. D. Jayavelu, R. Sowdhamini, K. Shameer, and A. M. Bones, "Transcriptional regulatory networks in *Arabidopsis thaliana* during single and combined stresses," *Nucleic Acids Res.*, vol. 44, no. 7, pp. 3147–3164, Apr. 2016.

[71] T. Urao, B. Yakubov, R. Satoh, K. Yamaguchi-Shinozaki, M. Seki, T. Hirayama, and K. Shinozaki, "A Transmembrane Hybrid-Type Histidine Kinase in Arabidopsis Functions as an Osmosensor," *Plant Cell*, vol. 11, no. 9, pp. 1743–1754, Sep. 1999.

[72] M. J. Berridge, P. Lipp, and M. D. Bootman, "The versatility and universality of calcium signalling," *Nat. Rev. Mol. Cell Biol.*, vol. 1, no. 1, pp. 11–21, Oct. 2000.

[73] H. Knight and M. R. Knight, "Abiotic stress signalling pathways: specificity and cross-talk," *Trends Plant Sci.*, vol. 6, no. 6, pp. 262–267, Jun. 2001.

[74] F. Merino, B. Bouvier, and V. Cojocaru, "Cooperative DNA Recognition Modulated by an Interplay between Protein-Protein Interactions and DNA-Mediated Allostery," *PLOS Comput Biol*, vol. 11, no. 6, p. e1004287, Jun. 2015.

[75] A. Pfeiffer, H. Shi, J. M. Tepperman, Y. Zhang, and P. H. Quail, "Combinatorial complexity in a transcriptionally centered signaling hub in *Arabidopsis,*" *Mol. Plant*, vol. 7, no. 11, pp. 1598–1618, Nov. 2014.

[76] F. Turck, A. Zhou, and I. E. Somssich, "Stimulus-Dependent, Promoter-Specific Binding of Transcription Factor WRKY1 to Its Native Promoter and the Defense-Related Gene *PcPR1-1* in Parsley," *Plant Cell*, vol. 16, no. 10, pp. 2573–2585, Oct. 2004.

[77] A. J. Stewart, S. Hannenhalli, and J. B. Plotkin, "Why Transcription Factor Binding Sites Are Ten Nucleotides Long," *Genetics*, vol. 192, no. 3, pp. 973–985, Nov. 2012.

[78] Y. Bilu and N. Barkai, "The design of transcription-factor binding sites is affected by combinatorial regulation," *Genome Biol.*, vol. 6, p. R103, 2005.

[79] N. Siefers, K. K. Dang, R. W. Kumimoto, W. E. Bynum, G. Tayrose, and B. F. Holt, "Tissue-Specific Expression Patterns of Arabidopsis NF-Y Transcription Factors Suggest Potential for Extensive Combinatorial Complexity," *Plant Physiol.*, vol. 149, no. 2, pp. 625–641, Feb. 2009.

[80] R. Rohs, X. Jin, S. M. West, R. Joshi, B. Honig, and R. S. Mann, "Origins of specificity in protein-DNA recognition," *Annu. Rev. Biochem.*, vol. 79, pp. 233–269, 2010.

[81] R. Rohs, S. M. West, A. Sosinsky, P. Liu, R. S. Mann, and B. Honig, "The role of DNA shape in protein-DNA recognition," *Nature*, vol. 461, no. 7268, pp. 1248–1253, Oct. 2009.

[82] A. Jolma, Y. Yin, K. R. Nitta, K. Dave, A. Popov, M. Taipale, M. Enge, T. Kivioja, E. Morgunova, and J. Taipale, "DNA-dependent formation of transcription factor pairs alters their binding specificity," *Nature*, vol. 527, no. 7578, pp. 384–388, Nov. 2015.

[83] E. M. Govrin and A. Levine, "The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*," *Curr. Biol. CB*, vol. 10, no. 13, pp. 751–757, Jun. 2000.

[84] U. Bechtold, T. Lawson, J. Mejia-Carranza, R. C. Meyer, I. R. Brown, T. Altmann, J. Ton, and P. M. Mullineaux, "Constitutive salicylic acid defences do not compromise seed yield,

drought tolerance and water productivity in the *Arabidopsis* accession C24," *Plant Cell Environ.*, vol. 33, no. 11, pp. 1959–1973, Nov. 2010.
[85] T. Yang and B. W. Poovaiah, "A calmodulin-binding/CGCG box DNA-binding protein family involved in multiple signaling pathways in plants," *J. Biol. Chem.*, vol. 277, no. 47, pp. 45049–45058, Nov. 2002.
[86] M. C. van Verk, D. Pappaioannou, L. Neeleman, J. F. Bol, and H. J. M. Linthorst, "A Novel WRKY transcription factor is required for induction of *PR-1a* gene expression by salicylic acid and bacterial elicitors," *Plant Physiol.*, vol. 146, no. 4, pp. 1983–1995, Apr. 2008.
[87] S. Davletova, K. Schlauch, J. Coutu, and R. Mittler, "The Zinc-Finger Protein Zat12 Plays a Central Role in Reactive Oxygen and Abiotic Stress Signaling in Arabidopsis," *Plant Physiol.*, vol. 139, no. 2, pp. 847–856, Oct. 2005.
[88] L. Palmieri, N. Picault, R. Arrigoni, E. Besin, F. Palmieri, and M. Hodges, "Molecular identification of three *Arabidopsis thaliana* mitochondrial dicarboxylate carrier isoforms: organ distribution, bacterial expression, reconstitution into liposomes and functional characterization," *Biochem. J.*, vol. 410, no. 3, pp. 621–629, Mar. 2008.
[89] N. Iqbal, A. Masood, M. I. R. Khan, M. Asgher, M. Fatma, and N. A. Khan, "Cross-talk between sulfur assimilation and ethylene signaling in plants," *Plant Signal. Behav.*, vol. 8, no. 1, Jan. 2013.