Plants have evolved complex regulatory mechanisms in adapting to environmental stresses. A recently emerging concept is the role of mitochondria in stress signal transduction, a process termed mitochondrial retrograde regulation. We aimed to uncover one of the first regulators involved in this process.

Thesis submitted as partial fulfillment
of the requirements for the degree of
Doctor (Ph.D.) in Sciences: Biotechnology
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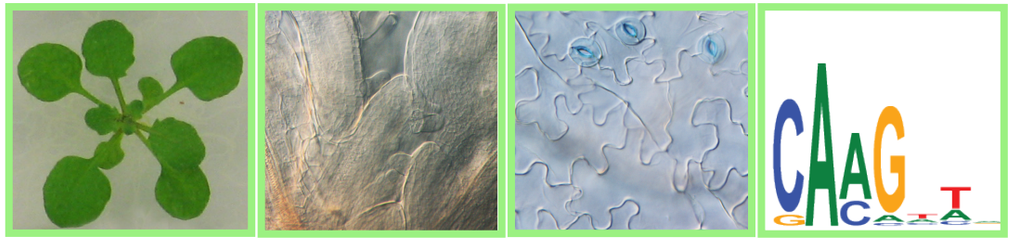

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Dit onderzoek werd uitgevoerd in het departement Planten Systeem Biologie van het Vlaams Instituut voor Biotechnologie (VIB).
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Voorwoord

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Inge

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LIST OF ABBREVIATIONS

35S	Cauliflower Mosaic Virus 35S promoter
AA	antimycin A
ABA	abscisic acid
AD	activation domain
AOX	alternative oxidase
ATP	adenosine triphosphate
3-AT	3-Amino-1,2,4-triazole
At	*Arabidopsis thaliana*
bp	base pair
CaMV35S	Cauliflower Mosaic Virus 35S promoter
CAT	catalase
cDNA	complementary DNA
CDS	coding sequence
ChIP	chromatin immunoprecipitation
Col-0	Arabidopsis thaliana Columbia-0 ecotype
CRR	chloroplast retrograde regulation
DBD	DNA binding domain
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
ETC	electron transport chain
$FADH_2$	flavin adenine dinucleotide
FC	fold change
FDR	false discovery rate
GFP	green fluorescent protein
GO	Gene Ontology
GUS	β-glucuronidase
H_2O_2	hydrogen peroxide
His3	histidinol-phosphate aminotransferase imidazole acetol phosphate transaminase
HSP	heat shock protein
HR	hypersensitive response
kb	kilobase
kDa	kilodalton
KO	knockout
LacZ	β-galactosidase
log	logarithm
LUC	luciferase
MDM	mitochondrial dysfuction motif (*cis*-regulatory element)

MDR	mitochondrial dysfunction regulon (genes)
MFA	monofluoroacetate
miR	microRNA
MRR	mitochondrial retrograde regulation
MS	Murashige and Skoog basal salt mixture
MV	methyl viologen
mRNA	messenger RNA
mtETC	mitochondrial electron transport chain
mtROS	mitochondrial reactive oxygen species
NAC	no apical meristem/Arabidopsis transcription activation factor/cup-shaped cotyledon
NADH	nicotinamide adenine dinucleotide
NTL	NAC WITH TRANSMEMBRANE MOTIF1-LIKE
$^1O_2$	single oxygen
$O_2^{\bullet-}$	superoxide radical
OE	overexpression
$OH^{\bullet}$	hydroxyl radical
ORF	open reading frame
PCR	polymerase chain reaction
PDI	protein-DNA interaction
Pro	promoter
PHB	prohibitin
PS	photosystem
qRT-PCR	quantitative reverse transcriptase PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
SE	standard error
TAIR	The Arabidopsis Information Resource
TCA	tricarboxylic acid cycle
T-DNA	transfer DNA
TF	transcription factor
WT	wild-type
Y1H	yeast one-hybrid

SCOPE AND OBJECTIVES

Due to their sessile lifestyle, plants are continuously exposed to adverse environmental conditions that negatively influence growth and productivity. Environmental stresses, including perturbations in water availability, temperature, soil composition and light intensities as well as pathogen assaults are responsible for major crop yield losses worldwide. With the rapidly growing world population and expected climate change, sufficient food has to be produced on less arable land. Therefore, one of the major objectives of plant biotechnology is the development of high-yielding crop plants that can resist adverse environmental conditions. For these reasons, understanding the molecular mechanisms underlying plant adaptation to stress is of primary importance.

Upon the perception of stress by the cell, an intracellular signal transduction cascade is activated that transmits the signal to the nucleus, where one or more transcription factors are activated that alter the expression of stress responsive genes. Due to the multigene nature of abiotic stress tolerance, manipulation of upstream signaling components including transcription factors is a powerful tool for developing tolerant plants as they tend to control multiple stress responsive genes. During the last years, several transcription factors have been identified that can enhance stress tolerance by manipulating their expression in transgenic plants.

A recently emerging concept concerns the role of mitochondria in intracellular signal transduction to the nucleus to alter gene expression, a process named mitochondrial retrograde regulation (MRR). Several stresses alter or perturb mitochondrial function, and in turn, dysfunctional mitochondria initiate and contribute to the stress response through MRR. At the beginning of this Ph.D. project, no protein signaling components of plant MRR had been discovered. Therefore, the main aim of this project was to identify transcription factors involved in MRR and evaluate their contribution to the stress response in plants.

In a second part of the Ph.D. work, we aimed to provide a better understanding of control mechanisms of abiotic stress tolerance through reverse engineering of gene regulatory networks. Several experimental studies have contributed to our understanding of the mechanisms of the stress response, but the global regulatory mechanisms are far from clear from these individual studies. Therefore, we reconstructed a model of the abiotic stress regulatory network from abiotic stress-related gene expression data using the reverse-engineering algorithm LeMoNe. Altogether,

the identification of key players together with a detailed understanding of the complex regulatory mechanisms of the stress response, will contribute to the development of stress tolerant crops.

Chapter 1

Mitochondrial retrograde regulation in plants

Inge De Clercq and Frank Van Breusegem

AUTHOR CONTRIBUTIONS

I.D.C. wrote the chapter with help of F.V.B.

Introduction

Compartmentalization of the eukaryotic cell into different organelles requires a more complex regulation of cellular function compared to prokaryotes, where all cellular processes happen within the same compartment. In addition, mitochondria, and in photosynthetic organisms chloroplasts, contain their own genome. However, these organellar genomes are small and encode only a few proteins. Most organellar proteins (93-99%) are encoded in the nucleus, synthesized in the cytosol and then imported into the organelles (Woodson and Chory, 2008). Thus, compartmentalization of the genome requires coordinated gene expression to maintain organellar as well as whole-cellular function. These genome coordination mechanisms include both anterograde (nucleus to organelle) and retrograde (organelle to nucleus) regulation (Figure 1.1). Anterograde regulation controls the flow of proteins and information from the nucleus to the organelles (mitochondria or chloroplasts) to coordinate organellar gene expression. Retrograde signaling transmits signals from the organelles to regulate nuclear gene expression.

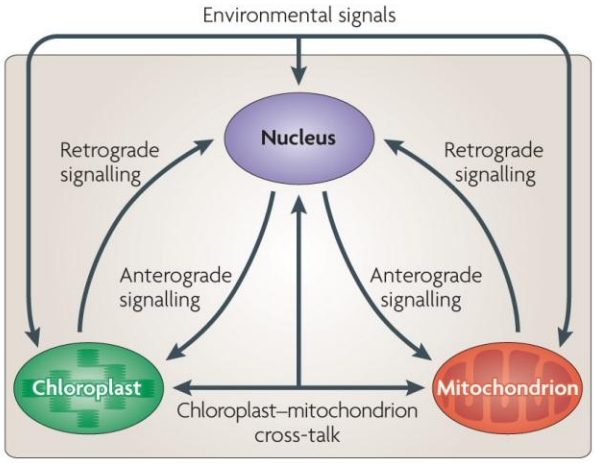

Figure 1.1. An overview of genome coordination between the nucleus and intracellular organelles (mitochondria and chloroplasts) in plants. Environmental signals, including stress, and endogenous signals, such as hormones, affect the expression of nuclear genes that encode organellar proteins. This process will, in turn, affect organelle function and gene expression through anterograde mechanisms. Chloroplasts and mitochondria are also able to sense environmental stimuli that can affect their functional activities. By means of retrograde signals, organelles communicate these received stimuli and their functional status to the nucleus, which leads to altered nuclear gene expression. From Woodson and Chory (2008).

Among the plant retrograde signaling pathways, chloroplast retrograde regulation (CRR) has been most extensively studied (Woodson and Chory, 2008). Genomes Uncoupled (GUN) proteins and the ABSCISIC ACID INSENSITIVE (ABI4) transcription factor have been identified as regulators of CRR during chloroplast biogenesis, termed ‘biogenic control’ (Koussevitzky et al.,

2007; Pogson et al., 2008). GUN proteins are divergent types of proteins with functions ranging from chlorophyll metabolism to chloroplast gene expression. Moreover, in mature chloroplasts, the communication with the nucleus is necessary to keep chloroplast functioning at optimal levels (named 'operational control') according to endogenous (e.g. fluxes in metabolites) and environmental (e.g. fluctuating light) changes (Pogson et al., 2008). The most important signals during these responses are likely to be reactive oxygen species (ROS) and redox changes. In addition to the role of CRR in regulating chloroplastic processes, it is apparent that chloroplasts play an important role in the adaptation to stresses. Chloroplastic metabolic processes are readily perturbed by and act as a sensor of stress conditions to coordinate the nuclear-encoded adaptive stress response (Fernandez and Strand, 2008).

This chapter is focused on plant mitochondrial retrograde regulation, which is further referred to as MRR. The general process of MRR is conserved across eukaryotes as it has been reported in yeast, plants, animals and human (Butow and Avadhani, 2004; Rhoads and Subbaiah, 2007). However, the molecular signaling mechanisms as well as the specific functions of MRR appear not to be conserved across species. In yeast, MRR is mainly directed towards readjustment of (mitochondrial) metabolism in nutrient depletion scenarios (Liu and Butow, 2006). MRR in animals is an important factor in various diseases associated with mitochondrial dysfunction (Butow and Avadhani, 2004). In contrast to yeast and animals, relatively little is known about the signaling mechanisms of plant MRR. Similar as in other systems, MRR in plants is important for coordination of nuclear and mitochondrial genome expression during development, as mitochondrial biogenesis is largely dependent on nuclear gene expression (Millar et al., 2008). Moreover, changing environmental conditions (e.g. abiotic and biotic stresses) cause perturbation in the mitochondrial function. Although relatively little is known about the specific consequences of mitochondrial dysfunction in plants, there is increasing evidence that mitochondria can act as sensors of adverse environmental conditions and contribute to the overall stress response by altering stress responsive nuclear gene expression (Rhoads and Subbaiah, 2007). To what extent mitochondria and MRR contribute to stress responsive gene expression and whether they act as general stress sensors or contribute to specific stress responses, are subjects of ongoing studies.

MRR in plants has been mainly studied in mitochondrial mutants and in response to chemical disruption of mitochondrial function. Mitochondrial alternative oxidase (*AOX*) was the

first gene shown to be retrograde regulated (Vanlerberghe and McIntosh, 1994), and due to its general responsiveness to various mitochondrial perturbations, its induction is used as a model for plant MRR studies. The importance of ROS has been shown in at least some MRR pathways. In addition, calcium, changes in metabolite levels and redox and energy status are likely candidates. However, no protein signaling components, such as transcription factors, kinases and phosphatases have been identified, except for the recent discovery of the involvement of the ABI4 transcription factor and Cyclin-Dependent Kinase E1 (CDKE;1) (Giraud et al., 2009; Ng et al., 2013).

The role of mitochondria in the cell

The primary role of the mitochondria is energy production by synthesis of adenosine triphosphate (ATP) through oxidative phosphorylation (Millar et al., 2011). This is mediated by the mitochondrial electron transport chain (mtETC) that consists of four large protein complexes (Complex I, II, III and IV) interacting with each other via the small lipid ubiquinone (UQ) and the small protein cytochrome *c* in the inner mitochondrial membrane (Figure 1.2A). The mtETC oxidizes high-energy organic compounds (nicotinamide adenine dinucleotide [NADH] and flavin adenine dinucleotide [$FADH_2$]) and subsequently transfers electrons to reduce oxygen to water. This electron transfer is coupled to pumping of protons to create a proton gradient across the inner mitochondrial membrane, which is utilized by ATP synthase for the production of ATP. The high-energy compounds are provided by the tricarboxylic acid (TCA) cycle that oxidizes organic acids derived from degradation of sugars, fatty acids and proteins. Besides energy production, the mitochondria provide biosynthesis precursors such as amino acids and are involved in vitamin biosynthesis, cellular calcium homeostasis and programmed cell death (Lam et al., 2001; Nicholls and Chalmers, 2004; Rebeille et al., 2007).

The plant mtETC is more complex compared to most other eukaryotes as it contains additional components (Figure 1.2B). In addition to Complex IV (cytochrome *c* oxidase), plants contain a second terminal oxidase, called the alternative oxidase (AOX). AOX accepts electrons from UQ and directly transfers them to oxygen, thereby circumventing Complex III and IV, without translocating protons. Another group of additional components are the alternative NADH dehydrogenases that function alongside Complex I, the "usual" NADH dehydrogenase, without proton pumping. AOX and the alternative NADH dehydrogenases together with UQ can form a

complete respiratory chain, called the alternative respiratory pathway that does not contribute to the proton gradient and ATP synthesis. The most likely function of the alternative respiration is preventing ROS production by removing electrons from the normal cytochrome respiratory pathway when it is over-reduced (Moller, 2001; Rhoads et al., 2006). This can be critical when the cytochrome pathway is impaired by metabolic changes or by biotic and abiotic stresses. Accordingly, AOX and alternative NADH dehydrogenase functions are induced when the mtETC is inhibited.

AOX is encoded by a small multigene family of three to five members in several plant species that have different developmental, tissue-specific and stress-responsive expression characteristics (Finnegan et al., 1997; Considine et al., 2001; Clifton et al., 2006). The AOX family can be divided in two subfamilies: AOX1, which is present in both monocot and dicot plants, and AOX2, which is only present in dicots (Considine et al., 2002). These subfamilies contain a variable number of members in different plant species. Arabidopsis thaliana contains four AOX1 members (*AOX1a, AOX1b, AOX1c* and *AOX1d*) and one AOX2 member (*AOX2*) (Clifton et al., 2006). Among the Arabidopsis AOX members, *AOX1a* is predominantly expressed both under basal and induction conditions (Clifton et al., 2005; Clifton et al., 2006). As will be discussed below, the expression of *AOX1a* is increased through MRR when mitochondrial function is altered or inhibited (Zarkovic et al., 2005).

MRR induction upon mitochondrial perturbation

The best studied plant MRR system is the response of the nuclear gene *AOX* to mitochondrial perturbations. The regulation of *AOX* is therefore used as a model for the investigation of MRR. Mitochondrial perturbations used for the study of MRR can be classified into four different categories: 1) chemical inhibition of mitochondrial function; 2) mutations or transgene expression that cause mitochondrial dysfunction; 3) mitochondrial perturbation caused by abiotic stress (see below); and 4) mitochondrial perturbation caused by biotic stress (see below).

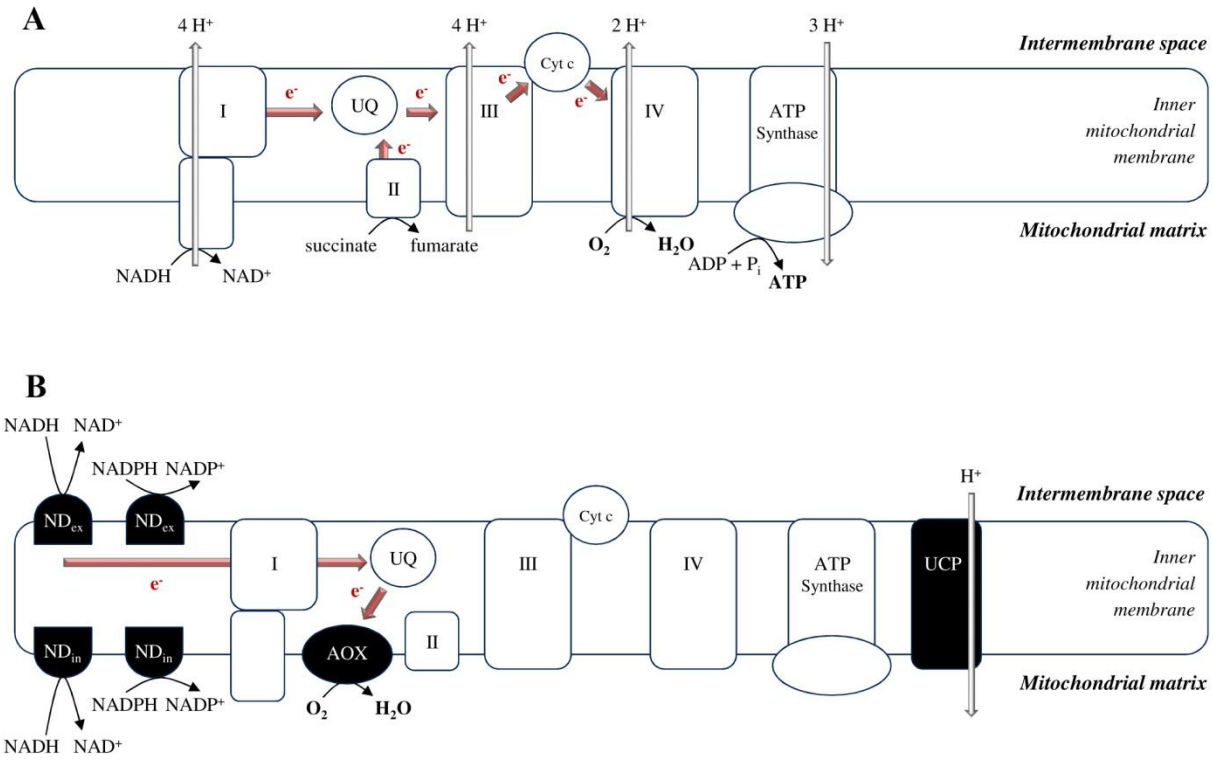

Figure 1.2. Overview of the components involved in the "standard" and alternative reactions of mitochondrial electron transport.
(A) Overview of the "standard" cytochrome pathway. Electrons pass from Complex I (NADH dehydrogenase) and II (succinate dehydrogenase) to the electron carrier ubiquinone (UQ). Via Complex III (ubiquinol-cytochrome *bc1* reductase) and cytochrome *c* (cyt *c*), O_2 is ultimately reduced to H_2O at the level of complex IV (cytochrome *c* oxidase). The ATP synthase complex catalyzes the formation of ATP driven by the proton gradient resulting from electron transfer.
(B) Overview of the alternative respiratory pathway consisting of non-proton-pumping alternative oxidase (AOX) as well as alternative NADH dehydrogenases (NDs) on either the external (NDex) or internal (NDin) side of the inner mitochondrial membrane. Electrons are passed from the alternative NDs to UQ and directly to AOX reducing O_2 to H_2O. Uncoupling proteins (UCPs) are able to dissipate the proton electrochemical gradient over the inner mitochondrial membrane.
From Keunen et al. (2011).

Chemical perturbation of mitochondrial function

Several chemicals that specifically target complexes of the mtETC have been extensively used to study the effects of mitochondrial perturbation. Antimycin A (AA), an inhibitor of mitochondrial Complex III, as well as Complex I and IV inhibition by rotenone and cyanide, respectively, induces *AOX* expression (Vanlerberghe and McIntosh, 1994; Chivasa and Carr, 1998; Clifton et

al., 2005; Ho et al., 2008; Xu et al., 2012). Oligomycin inhibits mitochondrial ATP synthase and also results in elevated *AOX* transcript levels. When the mtETC is inhibited, electron flow is disrupted leading to electron leakage to oxygen and production of ROS (Maxwell et al., 1999; Moller, 2001). Therefore, induction of the alternative respiration could serve to alleviate ROS production from mtETC inhibition (Maxwell et al., 1999). Moreover, inhibition of aconitase in the TCA cycle by monofluoroacetate (MFA) also induces *AOX*, but was not associated with increased mitochondrial ROS production (Vanlerberghe and McLntosh, 1996; Umbach et al., 2012). Besides *AOX* induction studies, whole genome transcriptome analyses have been performed upon pharmacological disruption of mitochondrial function (Clifton et al., 2005; Umbach et al., 2012). These studies revealed that specific types of mitochondrial dysfunction cause specific changes in the transcriptome that are partially overlapping (Schwarzlander et al., 2012; Umbach et al., 2012). These data suggest the existence of specific MRR pathways in response to specific mitochondrial perturbations.

Mutants with mitochondrial dysfunction

Several mitochondrial and nuclear mutants that affect mitochondrial function and exhibit altered nuclear gene expression have been discovered (Rhoads, 2011). One such family of mutants is the maize non-chromosomal stripe (ncs) mutants. These NCS mutants contain mutated mitochondrial DNA (mtDNA) resulting in defects in the mtETC, such as decreased complex I levels (*ncs2* mutant) and decreased complex IV levels (*ncs5* and *ncs6* mutants) (Newton et al., 1990; Marienfeld and Newton, 1994). Each NCS mutation causes a unique pattern of induction of maize *AOX* genes (Karpova et al., 2002). Interestingly, chemical inhibitors of mtETC complexes that correspond to each mutation cause induction of the same *AOX* genes. Thus, in both the *ncs2* mutant and upon rotenone treatment, the maize *AOX2* gene is induced and in *ncs6* and from cyanide, *AOX3* is induced. This suggests that alteration of *AOX* gene expression in these mutants is a result of MRR from specific mitochondrial dysfunction rather than from an altered metabolic shift in stable mutants and might serve as a compensatory mechanism for the disrupted mtETC. Another mitochondrial mutant, the tobacco cytoplasmic male sterility II (CMSII) that lacks functional complex I and transgenic tobacco and Arabidopsis plants expressing a mutant form of subunit 9 of ATP synthase (ATP9) also display altered nuclear gene expression (Gomez-Casati et al., 2002; Dutilleul et al., 2003). In addition, several nuclear mutations resulting in defective

mitochondria that alter nuclear gene expression have been described (Kushnir et al., 2001; Kuhn et al., 2009; Shedge et al., 2010; Gleason et al., 2011; Kuhn et al., 2011). How the status of the mitochondria is conveyed to the nucleus in these mutants, whether through direct MRR or via secondary means such as shifted metabolism and/or altered redox status is currently unclear because the mutant plants are in an altered steady state.

Function of MRR in biogenesis of mitochondria in plants

Mitochondria are semi-autonomous organelles that have a genome and contain a complete gene expression machinery. However, the vast majority of mitochondrial proteins are encoded in the nucleus and imported into mitochondria from the cytosol (Gray et al., 2001; Burger et al., 2003). Moreover, components of the respiratory chain and mitochondrial ribosomes are multiprotein complexes composed of nuclear- and mitochondrial-encoded subunits. This implies the existence of mechanisms that coordinate the nuclear and mitochondrial gene expression through both anterograde and retrograde regulation. Nevertheless, these mechanisms of genome expression coordination are largely unknown.

Evidence for the involvement MRR in mitochondrial biogenesis comes from the observation that upon rotenone treatment, nuclear transcripts encoding proteins involved in mitochondrial chaperone activity, protein degradation, respiratory chain assembly, and division were induced (Lister et al., 2004). In another example, transgenic Arabidopsis expressing a mutant form of ATP9 have an increased expression of nuclear-encoded Complex I genes (Gomez-Casati et al., 2002). Many dual targeted proteins (nuclear-organellar) are RNA and/or DNA binding proteins and are therefore good candidates for coordinating the expression of nuclear and organellar genomes (Krause et al., 2012). Pentatricopeptide repeat (PPR) proteins are involved in organellar post-transcriptional regulation (Schmitz-Linneweber and Small, 2008). Among them, PNM1 (for PPR protein localized to the nucleus and mitochondria 1) was recently shown to be dual localized to the nucleus and mitochondria in Arabidopsis (Hammani et al., 2011b; Hammani et al., 2011a). In mitochondria, PNM1 is associated with polysomes and may play a role in translation. In the nucleus it binds a member of the TEOSINTE BRANCHED1/CYCLOIDEA/PCF1 (TCP) transcription factor family that is known to control the expression of nuclear genes encoding mitochondrial proteins (Giraud et al., 2010). Furthermore, it was shown that the full length PNM1 containing both a mitochondrial targeting

signal and a nuclear localization signal is preferentially localized to mitochondria and that nuclear localization is only achieved when the MTS is absent. This indicates that it is first translocated to the mitochondria and subsequently released for nuclear localization. For these reasons, PNM1 likely serves as a retrograde signaling molecule for the fine-tuning of nuclear gene expression required for mitochondrial biogenesis.

Function of MRR in the stress response in plants

Several studies have indicated that mitochondria are an important target during environmental stresses. Perturbation of mitochondrial function alters the expression of stress responsive genes and affects plant stress tolerance. These observations indicate that plant mitochondria may function as sensors of stresses and initiate or contribute to the overall response to a given stress through MRR (Jones, 2000; Rhoads and Subbaiah, 2007). This may include both responses to abiotic stresses and biotic stresses.

MRR in response to abiotic stresses

One of the major and common effects of abiotic stress on plants is a disturbance of cellular metabolism leading to the production of ROS. Mitochondrial processes are directly inhibited by the resulting oxidative damage. For example, mitochondria are the main target for oxidative damage resulting from drought stress, as evidenced by higher contents of oxidatively modified proteins in mitochondria compared to other organelles (Bartoli et al., 2004). Metal stress causes mtETC dysfunction and over-reduction, thereby increasing mitochondrial ROS (mtROS) production (Keunen et al., 2011). However, it is not clear whether the mtETC inhibition is the result of cytosolic oxidative stress or the consequence of metals entering the mitochondria (Keunen et al., 2011). Other abiotic stresses such as high salt concentrations, cold and phosphate limitation have also been shown to impair mitochondrial respiration and increase mtROS production (Hernández et al., 1993; Prasad et al., 1994; Parsons et al., 1999). The elevated mtROS levels can further damage mitochondria but can also serve as signaling molecules (see below). The resulting mitochondrial oxidative stress upon these various abiotic stress treatments is associated with the activation of mechanisms to avoid (by the action of AOX) or detoxify (by mitochondrial antioxidants) stress-induced mtROS and maintain the functioning of the mitochondria (Hernández et al., 1993; Prasad et al., 1994; Parsons et al., 1999; Keunen et al.,

2011). Thus, MRR might be involved in the protection of mitochondria against oxidative stress induced by abiotic stresses. However, AOX does not only play a role to reduce the production of ROS in the mitochondria, but appears to have a broad role in determining the whole cell redox balance (Giraud et al., 2008).

Several studies have indicated that mitochondrial perturbation does not only regulate genes to maintain mitochondrial homeostasis during stress, but also steers the stress response at the whole cell level, regulating genes associated with any cellular location and function (Schwarzlander et al., 2012; Umbach et al., 2012). Arabidopsis mutants defective in mitochondrial prohibitin 3 (*phb3*) are more resistant to high salinity stress and display induction of stress-responsive genes encoding mitochondrial proteins, including *AOX1a*, as well as non-mitochondrial proteins, that are possibly involved in cellular hormone homeostasis and/or growth regulation during stress (Van Aken et al., 2007; Van Aken et al., 2010). In another study, an Arabidopsis mutant with defective mitochondrial complex I was isolated in a screen for impaired cold stress-responsive gene expression, implicating mitochondrial retrograde regulation of the cold stress response (Lee et al., 2002). Another complex I mutant in tobacco mediates antioxidant alterations at the whole cell level to maintain the cellular redox balance which is associated with higher tolerance to ozone and *Tobacco mosaic virus* (Dutilleul et al., 2003). Moreover, different tobacco respiratory mutants commonly induce a specific set of cytosolic and mitochondrial heat shock proteins (Kuzmin et al., 2004). In addition, several other mitochondrial mutants have been reported to alter the stress response (Meyer et al., 2009; Shedge et al., 2010). Besides mutants, chemical perturbations of mitochondrial function also appear to mimic stress responses. Cyanide (inhibitor of the terminal cytochrome *c* oxidase) preacclimates plants to salt, osmotic and cold stress probably by activating AOX (Xu et al., 2012). Respiratory inhibitors induce the expression of hypoxic genes (Nie and Hill, 1997) and hypoxia stimulates the release of calcium from the mitochondria, indicating calcium-mediated MRR is involved in the low oxygen response (see below) (Subbaiah et al., 1994, 1998; Bailey-Serres and Chang, 2005). To further address the relationship between MRR and stress responsive gene expression, whole transcriptomes of chemical mitochondrial disruption were compared to that of several abiotic and biotic stress treatments (Umbach et al., 2012). Overall, a strong correlation was observed with ozone, UV (Umbach et al., 2012) and metal stress and H_2O_2-mediated oxidative stress (Yu et al., 2001). On the other hand, mitochondrial perturbation transcriptomes were more strongly correlated with

biotic stress-related expression changes and were enriched for oxidative stress- and biotic stress-related proteins (Schwarzlander et al., 2012; Umbach et al., 2012). Taken together, these observations suggest a role for mitochondria in sensing stress and the contribution of MRR to the overall response to (specific) abiotic stresses.

MRR in response to biotic stresses

When plants are attacked by pathogens, they induce local and systemic defenses and/or programmed cell death at the site of infection (hypersensitive response, HR) to limit pathogen spread (Dangl and Jones, 2001; Lam et al., 2001). Defense and HR responses are mediated by several signaling molecules, such as salicylic acid (SA), nitric oxide (NO), and ROS (Alvarez, 2000; Laloi et al., 2004; Wendehenne et al., 2004). Although several studies have indicated the importance of plant mitochondria, including MRR, during pathogen attack, the precise roles and mechanisms have not yet been elucidated (Amirsadeghi et al., 2007; Rhoads and Subbaiah, 2007; Rhoads, 2011). Several biotic stress factors disrupt mitochondrial function and induce nuclear gene expression, including the MRR model gene *AOX*. For instance, SA can inhibit the mtETC and induce *AOX* expression in tobacco (Norman et al., 2004). Some genes induced by SA are also induced by AA while others are not, indicating common (likely mediated by ROS resulting from mtETC inhibition) and distinct pathways (Norman et al., 2004). In addition, several toxins produced by plant pathogens (called elicitors) have been shown to disrupt mitochondrial function and induce nuclear gene expression (Rhoads and Subbaiah, 2007). Harpins are elicitors produced by bacterial pathogens, including *Pseudomonas syringae*, that induce a HR. Exogenously applied harpin elicits a rapid HR-like cell death that was associated with an early ROS burst from the mitochondria, and decreased mitochondrial membrane potential and ATP levels (Krause and Durner, 2004). Moreover, harpins induce expression of *AOX*, genes encoding small heat shock proteins and genes encoding plant defense proteins, which is likely through MRR (Desikan et al., 1998; Krause and Durner, 2004). Thus, biotic stress-mediated mitochondrial perturbation, including increased mtROS production, appears to initiate HR-mediated programmed cell death and mediate expression of nuclear defense genes via MRR. However, the induction of *AOX* during HR is unexpected as it might dampen programmed cell death by preventing excess ROS production (Vanlerberghe et al., 2002). Indeed, increased *AOX* expression leads to a reduction in lesion size during HR (Ordog et al., 2002). It is postulated that AOX plays a role in orchestrating

whole-cellular ROS levels and fine-tuning the balance between defense and programmed cell death, that might depend on a certain threshold level of cellular ROS. Finally, another indication for the involvement of MRR in the biotic stress response is that inhibition of mitochondrial function (pharmacologically or by mutations) causes a transcriptome response similar to that of pathogen attack (Maxwell et al., 2002; Schwarzlander et al., 2012; Umbach et al., 2012).

Regulatory components of plant MRR

Reactive oxygen species

Reactive oxygen species are produced as byproducts of aerobic metabolism in various subcellular compartments (chloroplasts, mitochondria, and peroxisomes) and can act both as damaging agents and as signaling molecules (Dat et al., 2000; Apel and Hirt, 2004). Upon disturbance of cellular metabolism by adverse external conditions, ROS production is increased. In the mitochondria, ROS are mainly produced at Complex I and III following inhibition of the mtETC (Maxwell et al., 1999; Moller, 2001). This results in superoxide anion (O_2^-) accumulation that is subsequently dismutated to H_2O_2 (Moller, 2001). For example, chemical inhibition of respiratory Complex III by AA treatment specifically increases mtROS levels in cultured tobacco cells (Maxwell et al., 1999; Rhoads and Subbaiah, 2007). In addition, several physiological stresses, such as cold, salt stress, and phosphate starvation, cause oxidative damage in the cell and perturb the mtETC resulting in increased mtROS production (Hernández et al., 1993; Prasad et al., 1994; Parsons et al., 1999). Evidence for the importance of mtROS in transmitting the signal of mitochondrial perturbation to the nucleus came from the finding that antioxidants impair AA-mediated induction of *AOX* transcript (Maxwell et al., 1999; Vanlerberghe et al., 2002). However, mtROS is likely not required for all MRR induced by mitochondrial perturbation. A ROS-independent MRR pathway was suggested based on the ability of MFA-mediated inhibition of the TCA cycle, causing an increase in citrate levels (Vanlerberghe and McLntosh, 1996), as well as exogenously added citrate, isocitrate, malate, or 2-oxoglutarate to induce *AOX* transcript without a dramatic increase in ROS (Djajanegara et al., 2002; Gray et al., 2004; Umbach et al., 2012). Thus, metabolic changes such as TCA cycle intermediates could be the underlying signals of ROS-independent MRR.

As discussed above, mitochondrial perturbation is a source of ROS production but is also the result of increased cellular ROS levels during oxidative stress. Oxidative stress caused by exogenous hydrogen peroxide (H_2O_2) treatment has been shown to inhibit mitochondrial function by degradation of protein components of the mtETC and the TCA cycle (Sweetlove et al., 2002). Thus, oxidative stress resulting from external biotic and abiotic stress conditions could be the cause of MRR induction of the stress response. Comparison of the transcriptome response to specific chemical disruptions of mitochondrial function and non-specific oxidative stresses that act throughout the cell by exogenous ROS treatments, revealed overlapping but distinct responses (Rhoads and Subbaiah, 2007). These differences could be attributed to the specific types or combinations of ROS or the specific mitochondrial localization of ROS production by these chemical treatments (Laloi et al., 2004; Gadjev et al., 2006). Therefore, plant cells must be able to detect changes in mtROS levels independently from ROS produced in other subcellular compartments.

Although ROS are likely involved in MRR, it is not clear how this signal is transmitted to the nucleus. ROS-specific transcriptome fingerprints, however, are suggestive of local detection mechanisms present inside or in close proximity to the mitochondria (Gadjev et al., 2006). This could be mediated by secondary messengers formed locally by the interaction of ROS with specific molecules (Rhoads et al., 2006; Moller and Sweetlove, 2010). For example, lipid peroxides have been implicated in MRR as their levels increase in Arabidopsis cells upon AA-treatment (Winger et al., 2005) and have been shown to act as signaling molecules in animals (Tang et al., 2002). In addition, it is suggested that oxidized peptides derived from oxidatively damaged mitochondrial proteins could be specific ROS messengers (Sweetlove et al., 2002; Moller and Sweetlove, 2010). In turn, lipid peroxides and oxidized peptides might interact with other signaling components, such as kinases and transcription factors to transmit the ROS signal to the nucleus (Tang et al., 2002; Haynes et al., 2010). Although no protein kinases involved in MRR have been identified to date, kinases that are modulated by ROS or mitochondrial dysfunction (Takahashi et al., 2003; Pitzschke and Hirt, 2006) are particularly interesting candidates. Alternatively, ROS themselves could participate in signaling pathways. Several studies suggest that ROS such as H_2O_2 and superoxide can leave the mitochondria via permeability transition pores upon mitochondrial dysfunction (Maxwell et al., 2002; Yao et al., 2002; Han et al., 2003).

Redox and metabolite signals

Adverse environmental conditions alter the cellular metabolic state and thus the redox balance, which in turn is used by the plant as an environmental sensor to induce an appropriate defense response (Vanacker et al., 2000). Mitochondria can alter the cellular redox state because: 1) of redox metabolism in the TCA cycle; 2) of the redox state of and ROS production from the mtETC; and 3) they contain the glutathione-ascorbate cycle (Jimenez et al., 1997; Dutilleul et al., 2003; Rhoads and Subbaiah, 2007). 1) The TCA cycle reduces NAD(P) to NAD(P)H and therefore impacts the NAD(P)/NAD(P)H ratio. For this reason, it is possible that MFA-mediated MRR, that appears to act independently of ROS, is mediated by redox signals from inhibition TCA cycle metabolism. 2) Although ROS themselves can act as signaling molecules, they might also indirectly mediate signaling through changes in the cellular redox status (Foyer and Noctor, 2003). For instance, oxidative stress can increase the NADP/NADPH ratio as NADPH is required as electron donor for H_2O_2 detoxification in the glutathione-ascorbate cycle (Sweetlove and Foyer, 2004; Foyer and Noctor, 2005). 3) It is becoming increasingly clear that antioxidants themselves can fulfill signaling roles (Foyer and Noctor, 2005). For example, glutathione and ascorbate appear to be key molecules in redox signaling and mediate various stress responses in plants (Kocsy et al., 2001; Pastori et al., 2003; Gomez et al., 2004). Interestingly, ascorbate synthesis is dependent on the respiratory chain and is affected upon treatment with the respiratory inhibitors AA and cyanide (Bartoli et al., 2000). However, experimental evidence for the involvement of redox signaling in plant MRR is currently lacking. Besides redox changes, changes metabolite levels itself (e.g. of the TCA cycle) and altered energy status (e.g. ATP/ADP ratio) are also potential stimuli to trigger mitochondrial signaling (reviewed in Schwarzländer and Finkermeier, 2013).

Calcium

Mitochondria play an important role in cellular calcium storage and homeostasis (Nicholls and Chalmers, 2004). The release of Ca^{2+} from intracellular stores or the apoplast in the cytosol contributes to various signaling pathways involved in development and the stress response (Berridge et al., 1998; Stael et al., 2012). Multiple studies in mammalian cells have shown the role of calcium as a signaling component in MRR (reviewed in Butow and Avadhani (2004)).

The increased cytosolic Ca^{2+} concentrations upon mitochondrial dysfunction are attributed to the lack of Ca^{2+} uptake by dysfunctional mitochondria (due to disruption of mitochondrial membrane potential) and/or reduced efflux due to decreased ATP levels (Biswas et al., 1999; Amuthan et al., 2002). In plants, the involvement of calcium in MRR has been implicated by the observation that AA-directed *AOX* transcript induction is partially inhibited by a ruthenium red, an inhibitor of Ca^{2+} efflux from the mitochondria and other internal stores (Vanlerberghe et al., 2002). In addition, hypoxia causes an increase in cytosolic calcium that originates from the mitochondria and addition of ruthenium red suppresses hypoxia-responsive gene expression (Subbaiah et al., 1994, 1998; Bailey-Serres and Chang, 2005). Altered cytosolic and mitochondrial Ca^{2+} levels have also been observed during other environmental stress conditions, such as cold, osmotic and oxidative stress and showed stress-specific signatures (Logan and Knight, 2003; Loro et al., 2012).

How the mitochondria-induced changes in cellular Ca^{2+} signatures are directed to the nucleus to alter gene expression in plants is currently not clear. It is likely that commonly known calcium detector proteins are involved, including calcium-dependent protein kinases, calmodulins, and calcineurin B-like proteins (Luan et al., 2002; Sanders et al., 2002). In mammalian cells, calcineurin and several protein kinases arbitrate Ca^{2+}-mediated activation of transcription factors during MRR (Luo et al., 1997; Biswas et al., 1999; Amuthan et al., 2002; Arnould et al., 2002). Another possibility is that Ca^{2+} itself may propagate to the nucleus to affect gene expression. This is indicated by the observation that increases in nuclear Ca^{2+} accompany the cytosolic Ca^{2+} rise during hypoxia (Subbaiah et al., 1998). Distinct nuclear calcium signatures have also been observed upon various other stress stimuli (Pauly et al., 2001; Xiong et al., 2004). Moreover, it has been observed that altered nuclear calcium concentrations can affect nuclear gene expression, for example by modulating transcription factor DNA binding and transcriptional activity (Hardingham et al., 1997; Kim et al., 2009).

Protein signaling components

Protein regulatory components of plant MRR are only recently beginning to be discovered in contrast to the more extensively studied yeast and animal MRR pathways. The best studied retrograde pathway is the yeast RTG pathway that is mediated by the heterodimeric basis helix-loop-helix/leucine zipper transcription factors RTG1 and RTG3 and a cascade of cytoplasmic

regulators that control their nuclear translocation (through dephosphorylation) and subsequent activation (Liao and Butow, 1993; Sekito et al., 2000; Liu and Butow, 2006). In animals, one of the best studied MRR pathways involves the release and nuclear translocation of NFκB (Nuclear Factor kappa-light-chain-enhancer of activated B cells) dimers through calcineurin-mediated inactivation (dephosphorylation) of inhibitory IκBβ (inhibitor of nuclear factor-κβ) proteins following increased cytosolic Ca^{2+} levels (Biswas et al., 2003; Biswas et al., 2008). However, several other (RTG/NFκB-independent) MRR pathways have been described in yeast and animals (Butow and Avadhani, 2004; Jones et al., 2012).

The first evidence for the involvement of proteins in plant MRR signaling came from Zarkovic et al. (2005). They identified genetic mutants with impaired *AOX1a* induction in response to AA and MFA. However, the corresponding proteins have not been identified so far. Other approaches to identify MRR protein components relied on identifying *cis*-regulatory elements in the *AOX1a* promoter. Promoter activity studies indicated that AA- and MFA-mediated expression of *AOX1a* is directed at the promoter level (Dojcinovic et al., 2005; Zarkovic et al., 2005). Dojcinovic et al. (2005) identified a 93-bp region (bases -406 to -313) in the *AOX1a* promoter, that is critical for MRR. Most of this 93-bp "MRR region" is important for AA and MFA-mediated induction, indicating complex regulation involving multiple transcription factor – *cis*-regulatory element interactions. Although the identity of the DNA-binding proteins was not discovered in this study, the MRR region was shown to be bound *in vitro* by Arabidopsis proteins from whole cell extracts and this interaction was favored under oxidative conditions. Analysis of the presence of known *cis*-regulatory elements revealed putative basic leucine zipper (bZIP), WRKY, and DNA-binding with one finger (Dof) binding sites, suggesting a role for bZIP, WRKY and Dof transcription factors in MRR. An alternative study identified several abscisic acid response elements in the *AOX1a* promoter, lying outside the 93-bp MRR region (Ho et al., 2008). Among them is the B element that represses the promoter under normal conditions and allows derepression upon rotenone-mediated mitochondrial perturbation. This led to the identification of the first transcription factor mediating MRR, as ABI4 acts as a repressor of the *AOX1a* promoter activity under normal conditions by binding the B element and allows derepression during MRR (Giraud et al., 2009). A recent genomewide transcriptome analysis of AA and MFA treatment revealed enrichment of several signaling-related genes that were affected in the same way by both inhibitors, including calcium signaling components, WRKY, and NO

APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) domain transcription factors, indicating these are candidates to be involved in common MRR signaling (Umbach et al., 2012). Moreover, the protein kinase CDKE;1 was identified in a genetic screen as a positive regulator of *AOX1a* during AA treatment (Ng et al., 2013). Finally, two other kinases were identified that are activated upon mitochondrial perturbation. The potential involvement of these kinases in MRR is further supported by the observation that their activation as well as *AOX* mRNA induction was suppressed by bongkrekic acid, an inhibitor of the opening of the mitochondrial permeability transition pores (Takahashi et al., 2003). However, this pathway needs further investigation.

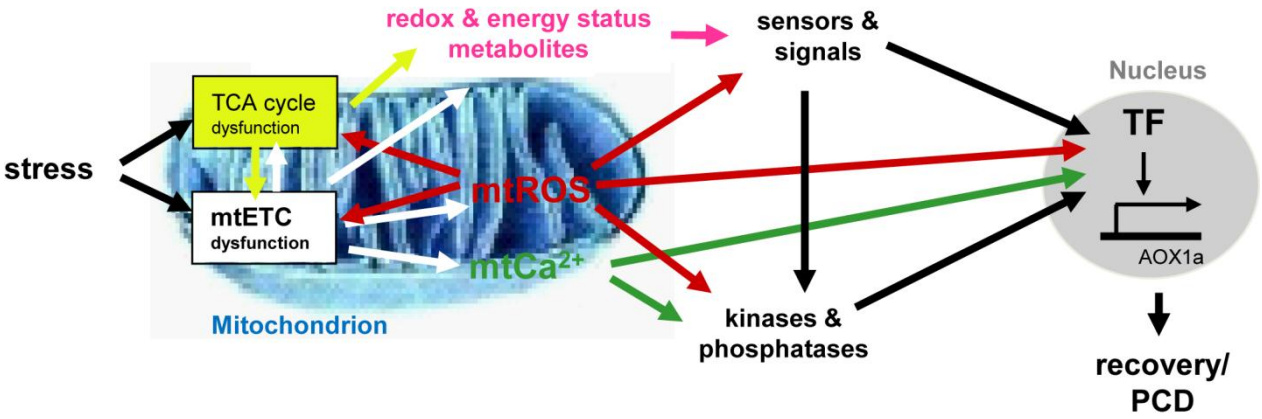

Figure 1.3. Potential signaling pathways in plant MRR.
Potential signals include mitochondrial reactive oxygen species (mtROS), mitochondrial calcium ($mtCa^{2+}$), altered redox and energy status and metabolite levels originating in the mitochondria, and protein components such as kinases, phosphatases (PPases), and transcription factors (TFs). See text for further details. mtETC, mitochondrial electron transport chain; TCA cycle, tricarboxylic acid cycle; PCD, programmed cell death.
Adapted from Rhoads and Subbaiah (2007).

Multiple MRR pathways

Several lines of evidence indicate the existence of multiple MRR pathways in plants. First, different mitochondrial perturbations cause distinct changes in the nuclear gene expression patterns. For example, temporal *AOX1a* transcript accumulation patterns vary depending on different respiratory or ATP synthesis inhibitors in Arabidopsis (Saishoa et al., 2001). Different *AOX* genes are induced depending on the mitochondrial defect in maize mitochondrial DNA mutants (Karpova et al., 2002). Moreover, genomewide expression profiling upon respiratory

(AA) and TCA cycle (MFA) inhibition revealed distinct but overlapping expression patterns, suggesting the operation of both common and distinct signaling pathways between the two treatments (Rhoads and Subbaiah, 2007; Umbach et al., 2012). Secondly, one of the isolated genetic mutants with impaired *AOX1a* induction by AA still exhibits induction by MFA, again indicating that these two perturbations initiate partially overlapping MRR pathways (Zarkovic et al., 2005). Thirdly, several studies suggest the existence of both ROS-dependent and ROS-independent MRR pathways. AA-mediated *AOX* induction is dependent on ROS, whereas *AOX* induction upon MFA and citrate treatment is not associated with detectible increases in ROS (Djajanegara et al., 2002; Gray et al., 2004; Umbach et al., 2012). Moreover, citrate-mediated *AOX* induction is inhibited by a protein kinase inhibitor, but not the induction by AA (Djajanegara et al., 2002). To conclude, these observations suggest the existence of multiple, partially overlapping MRR pathways that steer specific gene expression changes in response to specific types of mitochondrial dysfunction.

Crosstalk of MRR with other organellar signaling pathways

Mitochondria and chloroplasts are tightly connected through metabolism, energy and redox status (Raghavendra and Padmasree, 2003; van Lis and Atteia, 2004; Noguchi and Yoshida, 2008), and thus cannot be regarded as isolated compartments. For instance, photosynthesis provides substrates for mitochondrial respiration (O_2 and malate), but depends itself on mitochondrial products (CO_2 and ATP in the dark). Moreover, mitochondrial respiration protects chloroplasts against photoinhibition by dissipating excess redox equivalents from the chloroplasts. Crosstalk between mitochondria and chloroplasts has been deduced from genetic studies. For example, in the tobacco CMSII mutant, which lacks mitochondrial Complex I, the rate of photosynthesis is decreased (Sabar et al., 2000). In the barley *albostrians* mutant, the absence of chloroplast activity in an otherwise fully differentiated leaf tissue leads to an increase in mitochondrial gene copy number and elevated level of mitochondrial transcripts (Hedtke et al., 1999). Other studies suggest that mitochondrion-chloroplast crosstalk might affect retrograde regulation from either organelle (Woodson and Chory, 2008). The maize *ncs6* mutant with dysfunctional mtETC has decreased nuclear photosynthetic gene expression (Jiao et al., 2005). In the other direction, in mutant barley cells that lack chloroplast ribosomes, the nuclear-encoded mitochondrial RNA

polymerase is upregulated (Emanuel et al., 2004). However, it remains to be determined whether mitochondria and chloroplasts directly signal to each other or whether retrograde regulation from one of the organelles modulates the anterograde control of the other. The interactions between mitochondria and chloroplasts are further supported by the observation that nuclear photosynthetic genes are downregulated in a double Arabidopsis mutant that lacks both a chloroplast and a mitochondrial isoform of prolyl-tRNA, while each mutation alone does not result in this phenotype (Pesaresi et al., 2006). Moreover, there is recent evidence that retrograde signals from both organelles might interact, as the ABI4 transcription factor is involved in both chloroplast and mitochondrial retrograde regulation of nuclear gene expression (Koussevitzky et al., 2007; Giraud et al., 2009).

Close physical associations between the mitochondria and the endoplasmic reticulum (ER) have been observed in yeast and animal systems (Copeland and Dalton, 1959; Achleitner et al., 1999). A major function of these associations is the transfer of Ca^{2+} between these two organelles, thereby mediating Ca^{2+} homeostasis and interorganellar signaling (Hayashi et al., 2009; Elbaz and Schuldiner, 2011). It was recently shown that Ca^{2+}-mediated ER-mitochondria crosstalk is important for *AOX1a* induction in Arabidopsis during salt stress conditions, indicating it might be involved in plant MRR (Vanderauwera et al., 2012). Moreover, the ER also signals to the nucleus during the so-called unfolded protein response or UPR, which is an evolutionarily conserved transcriptional response to maintain ER homeostasis upon accumulation of unfolded proteins (Urade, 2007). Vanderauwera et al. (2012) suggest the involvement of the UPR in the Ca^{2+}-mediated *AOX1a* induction by regulating the activity of an ER Ca^{2+} pump. This points to a link between ER-to-nucleus communication and MRR.

Conclusion

When plants experience environmental stresses, they alter gene expression to allow cellular adjustments and adaption to the changed conditions. Stress conditions also involve perturbation of mitochondrial activity. It is becoming increasingly evident that mitochondria can sense abiotic and biotic stresses and subsequently alter nuclear gene expression through MRR, thereby contributing to the stress response. MRR in plants is likely composed of several distinct signaling pathways. In contrast to the more extensively studied yeast and animal MRR pathways, plant MRR mechanisms and components are still poorly understood. ROS, calcium, metabolic, energy,

and redox changes are likely signaling components of plant MRR. However, except for the recent discovery of the ABI4 transcription factor and the CDKE;1 kinase, no protein signaling components have been identified. Moreover, increasing evidence suggests that MRR overlaps with other signaling pathways such as retrograde regulation from the chloroplasts. Thus, further research is necessary to elucidate plant MRR signaling mechanisms, its interaction with other signaling pathways and its role in the stress response.

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Chapter 2

A membrane-bound NAC transcription factor is a regulator of mitochondrial retrograde regulation of the oxidative stress response

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AUTHOR CONTRIBUTIONS

I.D.C. and F.V.B designed the research. I.D.C. wrote the manuscript with help of F.V.B., V.V., O.V.A. and K.V. I.D.C. identified the MDM *cis*-regulatory element with help of K.V. (Fig. 2.2A; Suppl. Table 2.2). K.V. performed the evolutionary conservation analyses on the MDM (Suppl. Table 2.2 and 2.4). V.V. and I.D.C. performed the microarray meta-analyses (Fig. 2.2B; Suppl. Table 2.1; Suppl. Fig. 2.2). I.D.C. generated transgenic Arabidopsis lines, designed, performed and analyzed the promoter:LUC assays (Fig. 2.3), Y1H screens (Fig. 2.4A), ChIP (Fig. 2.5A; Suppl. Fig. 2.3), transactivation (Fig. 2.5B), qRT-PCR (Fig. 2.6; Suppl. Fig. 2.7) and autoactivation experiments (Fig. 2.7), and the abiotic and biotic stress assays (Fig. 2.9, 2.10 and 2.11; Suppl. Fig. 2.5 and 2.6) with help of D.R. and B.v.d.C. O.V.A. and I.D.C. performed the EMSA experiments (Suppl. Fig. 2.1). I.D.C. and M.M. performed the protein gel blots (Suppl. Fig. 2.4). S.L. and A.I. performed the subcellular localization assays (Fig. 2.8).

ABSTRACT

Upon disturbance of their function by stress, mitochondria can signal to the nucleus to steer the expression of responsive genes. This mitochondria-to-nucleus communication is often referred to as mitochondrial retrograde regulation (MRR). Although reactive oxygen species and calcium are likely candidates for MRR, the protein signaling components in plants remain largely unknown. Through meta-analysis of transcriptome data, we detected a set of genes that are common and robust targets of MRR and used them as a bait to identify transcriptional regulators of MRR. In the upstream regions of these mitochondrial dysfunction regulon (*MDR*) genes, a *cis*-regulatory element, the mitochondrial dysfunction motif (MDM) was found that is necessary and sufficient for gene expression under various mitochondrial perturbation conditions. Yeast one-hybrid analysis and electrophoretic mobility shift assays revealed that five transmembrane domain containing NAC transcription factors (ANAC013, ANAC016, ANAC017, ANAC053, and ANAC078) bound to the MDM *cis*-regulatory element. We demonstrate that ANAC013 is strongly regulated by various mitochondrial and environmental stress conditions at the transcript level and has expression characteristics similar to those of the *MDR*, hence pointing to its importance in MRR. By means of a detailed *in planta* functional analysis, ANAC013 was shown to mediate MRR-induced expression of the *MDR* genes by direct interaction with the MDM *cis*-regulatory element and triggers an increased oxidative stress tolerance. In conclusion, we characterized ANAC013 as a regulator of MRR upon stress in *Arabidopsis thaliana*.

A modified version of this chapter is in preparation for resubmission in The Plant Cell.

INTRODUCTION

Plants are regularly exposed to adverse environmental conditions, such as drought, extreme temperatures, lack of nutrients, and pathogen assaults. These factors negatively affect plant growth and development and are responsible for major yield losses in agriculture (Bray et al., 2000). At the cellular level, the functioning of organelles, such as chloroplasts and mitochondria, is perturbed by these environmental stresses (Huner et al., 1998; Taylor et al., 2002) and, in response, feedback mechanisms are triggered that steer changes in nuclear gene expression to sustain and/or restore the organellar and at large, cellular function (Rhoads and Subbaiah, 2007; Piñas Fernández and Strand, 2008). Mitochondria-to-nucleus signaling is referred to as mitochondrial retrograde regulation (MRR) that is a key event in eukaryotic cells during various stress situations (Butow and Avadhani, 2004).

The best studied mitochondrial retrograde pathway is the yeast (*Saccharomyces cerevisiae*) retrograde (RTG) pathway, mainly regarding its role in metabolic compensation of mitochondrial dysfunction in ageing and nutrient depletion scenarios. Mitochondrial dysfunction provokes a cascade of cytosolic events that activates heterodimeric RTG transcription factors through their nuclear translocation (Liao and Butow, 1993; Liu and Butow, 2006). A related pathway, comprising the central stress-mediating transcription factor NF-κB, has been identified in mammals (Biswas et al., 1999; Srinivasan et al., 2010). Similarly to the RTG pathway, the NF-κB retrograde system stimulates the glycolysis-derived ATP production under impaired mitochondrial respiration (Jazwinski and Kriete, 2012), but is much more complex with alternative and specialized functions during innate immune responses (Hayden et al., 2006), reflecting the higher metabolic intricacy. Other (RTG/NF-κB-independent) retrograde pathways have been reported in yeast and mammals that trigger various responses to specific mitochondrial defects (Epstein et al., 2001; Jones et al., 2012).

Research on MRR in plants has been centered mainly on the induction of components of the alternative respiratory chain, more specifically, the *ALTERNATIVE OXIDASE* (*AOX*) gene, in response to mitochondrial perturbation (Dojcinovic et al., 2005; Zarkovic et al., 2005). *AOX* is induced by various treatments with chemical inhibitors and mutations that disrupt the mitochondrial function at the respiratory chain level (mitochondrial electron transport chain [mtETC]) or the tricarboxylic acid (TCA) cycle. Therefore, *AOX* is used as a marker for the MRR

response in plants and specifically *AOX1a* in *Arabidopsis thaliana* (Vanlerberghe and McIntosh, 1996; Clifton et al., 2005; Rhoads and Subbaiah, 2007) that is also induced by various external stress treatments that might indirectly target mitochondrial function (Van Aken et al., 2009). A common component of various stresses that might result from mtETC inhibition is an increase in mitochondrial reactive oxygen species (mtROS) (Prasad et al., 1994; Maxwell et al., 1999). Induction of *AOX* serves to lower ROS formation from the impaired respiratory chain (Maxwell et al., 1999; Cvetkovska and Vanlerberghe, 2012). In addition to their damaging effects, mtROS probably act as MRR-triggering signaling molecules (Vanlerberghe et al., 2002; Rhoads et al., 2006). Calcium originating from the mitochondria might also be an MRR signal (Subbaiah et al., 1998), as seen in mammalian cells (Butow and Avadhani, 2004), as well as changes in mitochondrial redox changes and metabolites (Schwarzländer and Finkemeier, 2013).

To date, one protein has been shown to play a role in MRR in plants, namely ABSCISIC ACID INSENSITIVE4 (ABI4) that represses the basal expression of the Arabidopsis *AOX1a* gene, thereby allowing derepression by MRR (Giraud et al., 2009), and is also a common component of multiple chloroplast retrograde pathways (Koussevitzky et al., 2007). Although the effect of mitochondrial dysfunction on nuclear gene expression has been elucidated in several studies (Schwarzländer et al., 2012), the current understanding of the underlying regulatory mechanisms in plants is still limited. Although the Arabidopsis *AOX1a* promoter had been used to search for *cis*-regulatory elements involved in MRR and to isolate loss-of-function mutants impaired in its retrograde induction, the corresponding regulatory proteins have not been identified yet, except for the negative regulatory protein ABI4 (Dojcinovic et al., 2005; Zarkovic et al., 2005; Ho et al., 2008; Giraud et al., 2009).

Here, we followed a bottom-up approach to discover unknown regulatory components of MRR. Among the Arabidopsis gene promoters that are common and robust responsive targets of mitochondrial dysfunction, a *cis*-regulatory element was identified. This promoter element was necessary and sufficient to drive MRR-mediated gene expression and revealed the regulatory role of a transcription factor of the NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) family in the plant MRR.

RESULTS

Identification of a *cis*-regulatory motif in the promoters of MRR-regulated genes

To identify transcriptional regulators of MRR, we looked for shared *cis*-regulatory elements in the promoters of MRR-regulated genes. To select these genes, we assembled a compendium of 12 publicly available and microarray-derived transcriptome data sets that encompassed 22 perturbation experiments in which mitochondrial function was impaired by short-term treatments with respiratory inhibitors or by genetic mutation of mitochondrial proteins (oligomycin and rotenone (Clifton et al., 2005); *35S:AOX1a* and *aox1a* (NCBI GEO database; Edgar *et al*., 2002, accession GSE4113); *35S:PHB3, 35S:PHB4*; *phb3* and *phb4* (Van Aken et al., 2007); *aox1a* (Giraud et al., 2008); *ndufs4* and *ndufa1* (Meyer et al., 2009); *rpoTmp* (Kühn et al., 2009); *msh1 recA3* (Shedge et al., 2010); *mia40* (Carrie et al., 2010); *dsr1* (Gleason et al., 2011); *rug4* (Kühn et al., 2011); and *35S:TIM23-2* and *tim23-2* (Wang et al., 2012)) (Figure 2.1). For experimental details of the individual microarray studies see Supplemental Table 2.1. In this meta-analysis, 34 nuclear transcripts were up-regulated significantly (*P* value <0.01 and $\log_2$-fold >1) in five or more of the 22 conditions, hence representing a robust set of general mitochondrial stress-responsive genes. In these 34 genes, we assessed the presence of shared sequence elements within the first 1-kb upstream region. By various de novo motif discovery algorithms (Bailey and Elkan, 1994; Linhart et al., 2008; Thomas-Chollier et al., 2011), a common motif with the consensus sequence CTTGNNNNNCA[AC]G was identified with the corresponding position weight matrix shown in Figure 2.2A. This *cis*-regulatory motif occurred at least once in 24 out of the 34 genes (Supplemental Table 2.2) and the consensus was significantly enriched in this gene set compared to the genome (16.6-fold, hypergeometric *P* value 1.31E-18). As we hypothesized that this motif is a *cis*-regulatory element for transcriptional regulation in response to mitochondrial dysfunction, it was designated as mitochondrial dysfunction motif (MDM). Eighteen of the 24 MDM-containing genes are part of the so-called mitochondrial dysfunction regulon (MDR) (Van Aken et al., 2007; Skirycz et al., 2010). Therefore, we hereafter refer to these 24 MDM-containing genes as the *MDR* genes (Supplemental Table 2.3).

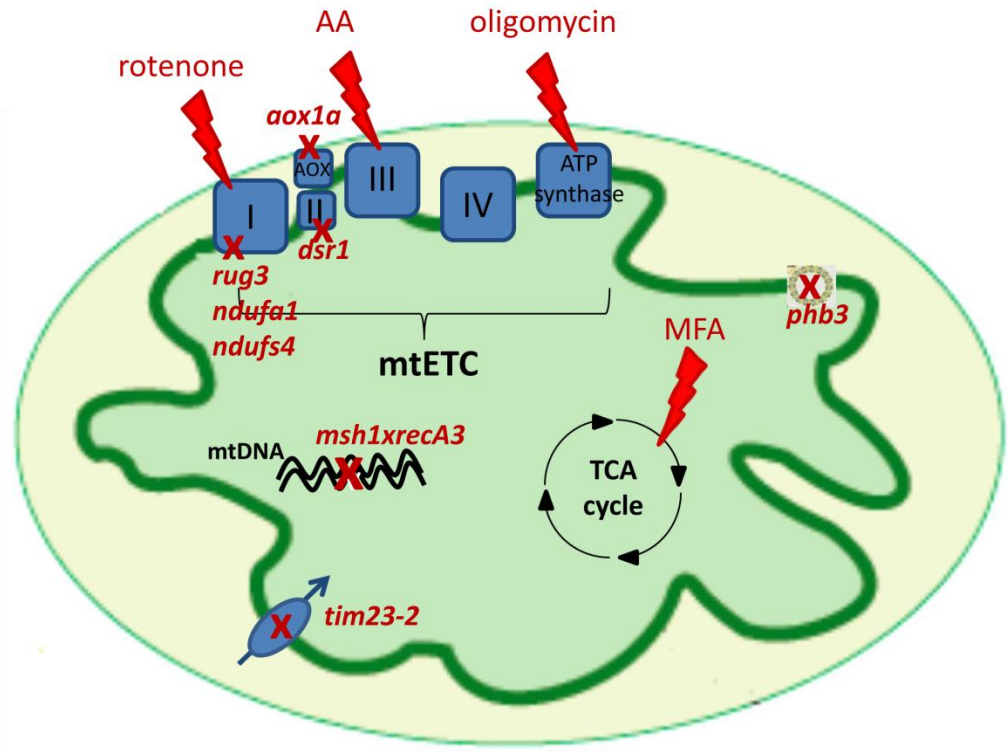

Figure 2.1. Schematic overview of the mitochondrial perturbation experiments used for the meta-analysis.
Rotenone and Antimycin A (AA) are inhibitors of Complex I and Complex III of the mitochondrial electron transport chain (mtETC), respectively. Oligomycin inhibits mitochondrial ATP synthase. Aconitase of the tricarboxylic acid (TCA) cycle is inhibited by monofluoroacetate (MFA). Mutation of *RUG3*, that is required for splicing of a Complex I subunit mRNA, and mutation of Complex I subunits *NDUFA1* and *NDUFS4* results in reduced or absent levels of Complex I, respectively (Kühn et al., 2011; Meyer et al., 2009). *dsr1* is mutated in a Complex II subunit (Gleason et al., 2010). TIM23-2 encodes a subunit of the mitochondrial inner membrane protein import complex TIM17:23, and its overexpression results in decreased Complex I levels (Wang et al., 2012). *MSH1* and *RECA3* are involved in the recombination of the mitochondrial genome and their disruption results in extensive mitochondrial genome rearrangements (Shedge et al., 2010). Prohibitins (PHB) form complexes in the inner mitochondrial membrane and they are suggested to be important for mitochondrial biogenesis and/or function (Van Aken et al., 2010). Mutation of *PHB3* results in altered mitochondrial morphology (Van Aken et al., 2007).

The 24 MDM-containing *MDR* genes were among the most responsive and up-regulated genes with respect to the number of perturbation experiments and the response magnitude, implying they are common and robust targets of MRR (Figure 2.2B). Hierarchical clustering of the 34 significantly up-regulated genes upon mitochondrial dysfunction revealed that the 24 *MDR* genes clustered in two groups with distinct transcriptional profiles: one that comprises genes affected by treatments and in mutants (cluster III) and one containing genes mainly affected in mutants and, to a much lesser extent, by treatments (cluster I). As evolutionary conservation is suggestive of functional importance (Freeling and Subramaniam, 2009), we investigated the biological

relevance of our candidate cis-regulatory element by examining its evolutionary conservation in six related dicot species (see Supplemental Methods online and Supplemental Table 2.4 online). Of the 24 *MDR* genes, nine had a significant evolutionarily conserved motif in at least two other species (Supplemental Table 2.2), suggesting a functional role of the motif in MRR.

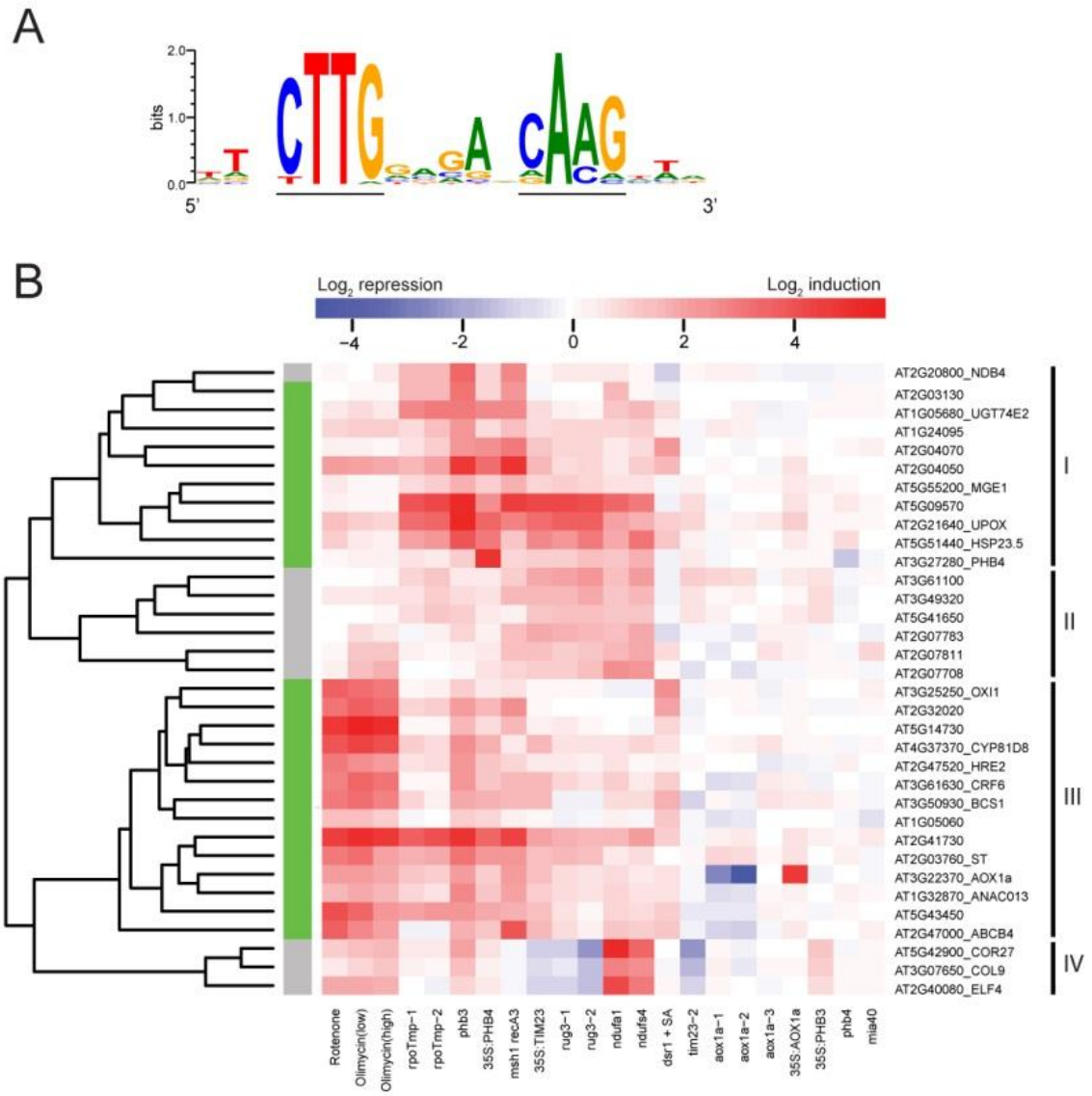

Figure 2.2 Identification of the MDM *cis*-regulatory element in MRR up-regulated genes.
(A) Position weight matrix of MDM representing the occurrence in the 24 *MDR* promoters, showing the probability of nucleotide(s) at each position. The MDM consensus (CTTGNNNNNCA[AC]G) is underlined.
(B) Hierarchical clustering of expression profiles of the 34 MRR-up-regulated genes (P <0.01, $\log_2$-fold change >1, in five or more mitochondrial dysfunction conditions). Color codes represent the actual $\log_2$-fold changes in transgenic or treated plants compared to wild-type or untreated plants, respectively. The *MDR* genes containing the *cis*-regulatory MDM in their 1-kb upstream sequence are indicated with a green bar.

MDM is a *cis*-regulatory element required for MRR-induced gene expression

To assess the functionality of this *cis*-regulatory element in mediating MRR-induced gene expression, we studied the effect of its deletion on the promoter activity of *AOX1a*, which is a model gene for MRR studies (Rhoads and Subbaiah, 2007) and contains the $MDM1_{[AOX1a]}$ and $MDM2_{[AOX1a]}$ sequences (Figure 2.3A). MDM deletions in the 1.5-kb *AOX1a* promoter were generated and fused to the luciferase (*LUC*) reporter gene. Three independent transgenic *ProAOX1a-WT:LUC*, *ProAOX1a-ΔMDM1:LUC* and *ProAOX1a-ΔMDM2:LUC* Arabidopsis plants were treated with antimycin A (AA), rotenone, monofluoroacetate (MFA) or hydrogen peroxide (H_2O_2) for 12 h and 24 h, prior to LUC activity measurements. AA and rotenone inhibit the mtETC and MFA the TCA cycle. H_2O_2 was included because components of the mtETC and TCA cycle are sensitive to oxidative stress (Sweetlove et al., 2002) and because H_2O_2 had been proposed as a signal within MRR (Vanlerberghe et al., 2002; Rhoads and Subbaiah, 2007). The absolute LUC signal in *ProAOX1a-ΔMDM1:LUC* and *ProAOX1a-ΔMDM2:LUC* was significantly lower than that of the *ProAOX1a-WT-LUC* plants after treatment with AA, rotenone, MFA, or H_2O_2 as well as the basal expression levels of the deletion constructs under nonstressed conditions (Figure 2.3Bi and 2.3Bii).

Additionally, we assessed the effect of the MDM deletions on the *AOX1a* promoter activity in *PROHIBITIN 3*-defective mutants (*phb3*) of Arabidopsis. Prohibitins play an important role in mitochondrial biogenesis and activity in plants (Ahn et al., 2006) and the Arabidopsis *phb3* mutant has an altered mitochondrial morphology and strongly induces the *MDR* genes (Figure 2.2B) (Van Aken et al., 2007). Similarly to the chemical inhibition results, the activity of *ProAOX1a-ΔMDM1* and *ProAOX1a-ΔMDM2* was significantly lower than that of *ProAOX1a-WT* in *phb3* mutants (Figure 2.3Biii). These results imply that a proficient *ProAOX1a* activity requires the contribution of both $MDM1_{[AOX1a]}$ and $MDM2_{[AOX1a]}$, either under control or mitochondrial stress conditions.

The effect of the MDM deletion was analyzed on the promoter activity of the *UDP-GLYCOSYL TRANSFERASE 74E2 (UGT74E2*) gene that is responsive to mitochondrial dysfunction (Figure 2.2B), is one of the most strongly H_2O_2-responsive genes, and is induced by various abiotic stresses (Tognetti et al., 2010). The *UGT74E2* promoter contains only one MDM consensus sequence ($MDM_{[UGT74E2]}$; Figure 2.3A), thereby avoiding potential redundancy issues.

Under nonstressed conditions, the activity of *ProUGT74E2-ΔMDM* was slightly higher than that of *ProUGT74E2-WT*, but, after AA and MFA treatments, the absolute LUC activity of the deletion construct was significantly lower than that of the wild-type construct (Figure 2.3Ci and 2.3Cii). After H_2O_2 treatment and in the *phb3* mutant, but not after rotenone treatment, the MDM deletion resulted in significantly reduced *UGT74E2* promoter activity, although to a lesser extent than after AA and MFA treatments (Figure 2.3Ci, 2.3Cii, and 2.3Ciii). These results indicate that $MDM_{[UGT74E2]}$ is necessary for *UGT74E2* promoter activation under mitochondrial stress conditions, especially during mitochondrial perturbation mediated by AA and MFA.

MDM is sufficient for MRR-mediated gene activation

To evaluate to what extent the MDM is sufficient to direct MRR-induced gene expression, we assessed the activity of the 50-bp region at -377 to -328 from the *AOX1a* promoter containing both the $MDM1_{[AOX1a]}$ and $MDM2_{[AOX1a]}$ elements by gain-of-function experiments. Therefore, reporter plasmids with a six-tandem repeat of this *ProAOX1a[-377, -328]* fused to the minimal cauliflower mosaic virus (CaMV) 35S promoter (*P35Smin*) located upstream of the *LUC* reporter gene were stably transformed in Arabidopsis plants (Figure 2.3D). Three independent transgenic *6xProAOX1a[-377, -328]-P35Smin:LUC* plants were treated with AA, rotenone, MFA, or H_2O_2 for 12 and 24 h, prior to the LUC activity measurements. All treatments activated the *6xProAOX1a[-377, -328]* (Figure 2.3Ei and 2.3Eii). After 12 h, the induction of the *LUC* gene was the highest by AA and H_2O_2 and by MFA after 24 h. To determine whether the MDM alone was sufficient to trigger the gene expression after mitochondrial perturbations, we built a similar reporter construct with a hexamer of the 23-bp *AOX1a* promoter sequence containing the 13 bp of $MDM1_{[AOX1a]}$ with neighboring 5 bp at each end (5'-TCCATCTTGGAGAGCAAGAAAAA-3'), hereafter designated $6xMDM1_{[AOX1a]}$. The $MDM1_{[AOX1a]}$ was chosen because its similarity was the highest to the position weight matrix representation of the MDM consensus motif (Figure 2.2A). Like *6xProAOX1a[-377, -328]*, the *P35Smin:LUC* reporter-driving $6xMDM1_{[AOX1a]}$ construct was strongly activated by AA, rotenone, MFA, and H_2O_2. Similar constructs with base substitutions in the $MDM1_{[AOX1a]}$ sequence (5'-TCCATAAAAAAAGGGGGGAAAAA-3' and designated $6xMDM1mut_{[AOX1a]}$) or lacking the MDM promoter fragment (designated *P35Smin*) did not display any increase in *LUC* expression after mitochondrial perturbations. Furthermore,

the responsiveness of *MDM1*$_{[AOX1a]}$ to mitochondrial dysfunction was confirmed in the *phb3* mutant (Figure 2.3Eiii). All together, these data indicate that the MDM is regulated by mitochondrial perturbations and is a regulatory unit sufficient to confer MRR-mediated gene expression.

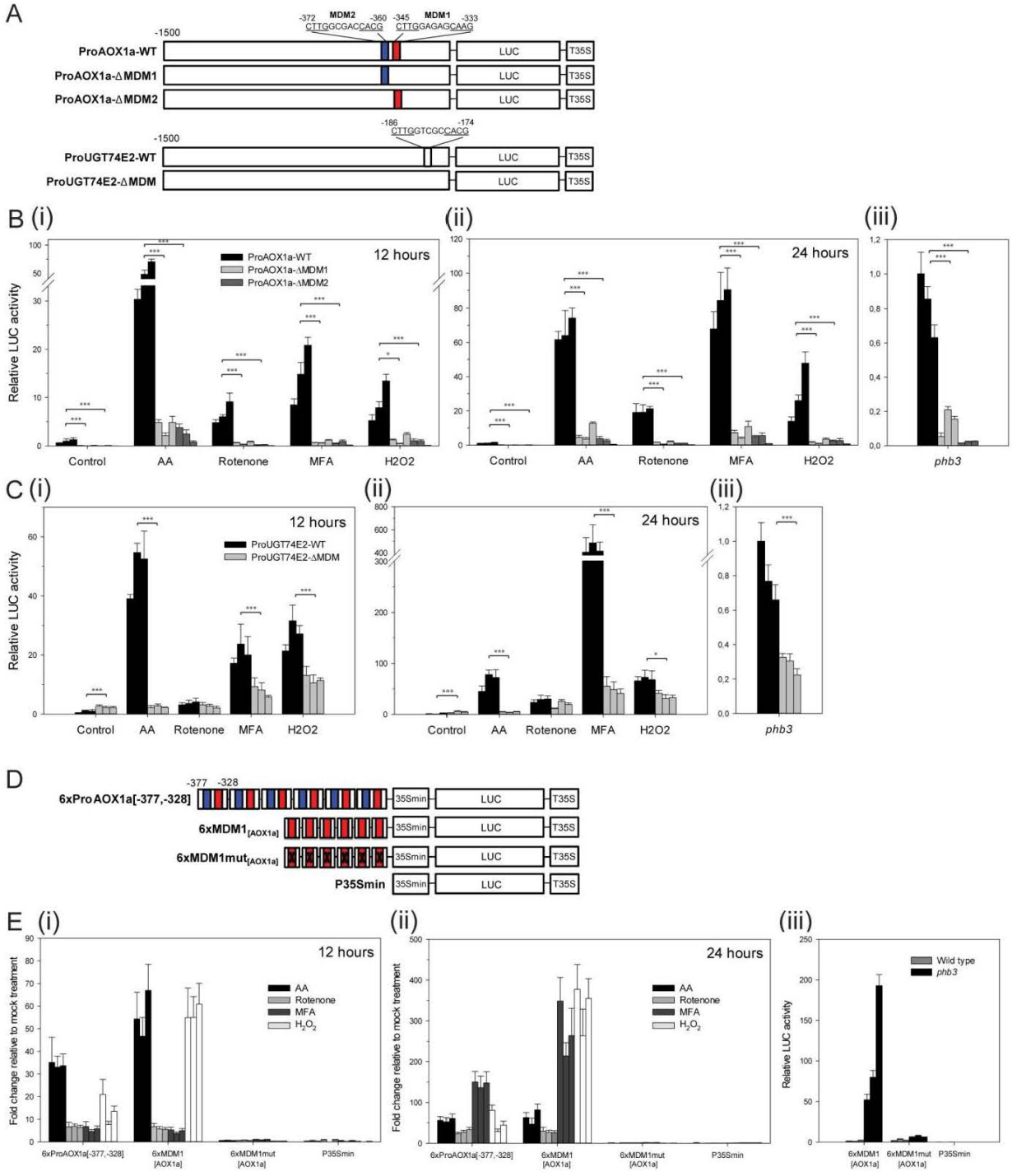

Figure 2.3. MDM is necessary and sufficient for MRR-mediated promoter activation.

Figure 2.3. MDM is necessary and sufficient for MRR-mediated promoter activation. (*Continued*).
(A) Schematic overview of *AOX1a* and *UGT74E2* promoter deletion constructs. MDM deletions were generated in the 1.5-kb promoters and fused in-frame to the *LUC* reporter gene.
(B) Regulatory characteristics of the MDM elements from the *AOX1a* promoter tested by comparison of the *LUC* expression driven by the *AOX1a* promoter (*ProAOX1a-WT*) to the same promoter construct with deletion of either $MDM1_{[AOX1a]}$ (*ProAOX1a-ΔMDM1*) or $MDM2_{[AOX1a]}$ (*ProAOX1a-ΔMDM2*) in transgenic Arabidopsis plants. Promoter activities were analyzed after mock (Control), AA, rotenone, MFA, or H_2O_2 treatment for 12 h (i) or 24 h (ii), and in *phb3* mutants (iii). Bars indicate average relative LUC activities from eight biological replicates ± SE. The relative luciferase activity of the first bar was arbitrarily set to 1. Per construct, data of three independent transgenic lines are shown. * indicates a significant difference at an overall significance level of 0.05, **0.01, and ***0.001.
(C) Regulatory characteristics of the MDM element from the *UGT74E2* promoter tested by comparing the *LUC* expression driven by the *UGT74E2* promoter (*ProUGT74E2-WT*) to the same promoter construct with deletion of $MDM_{[UGT74E2]}$ (*ProUGT74E2-ΔMDM*) under the same conditions as in panel B.
(D) Schematic overview of gain-of-function promoter constructs containing hexamers of the *AOX1a* promoter regions cloned upstream of the minimal CaMV 35S promoter (*P35Smin*) driving transcription of the *LUC* gene.
(E) Regulatory activity of the synthetic sequence containing six consecutive repeats of the 50-bp *AOX1a* promoter fragment, including two MDM elements (*6xProAOX1a[-377,-328]*) and one of the MDM sequence alone ($6xMDM1_{[AOX1a]}$) in transgenic Arabidopsis plants. Constructs mutated in the MDM sequence ($6xMDM1mut_{[AOX1a]}$) or without promoter fragment (*P35Smin*) were included as negative control. Average fold changes of LUC activity after 12 h (i) or 24 h (ii) of AA, rotenone, MFA, or H_2O_2 treatment relative to mock treatment are shown for three independent transgenic lines (± SE; n = 8 biological replicates). (iii) Average relative LUC activity of the synthetic sequences in *phb3* mutants (± SE; n = 8 biological replicates).

NAC transcription factors specifically bind to the MDM of several *MDR* promoters

To identify transcriptional regulators that interact with the MDM, yeast one-hybrid (Y1H; see Chapter 3) screening was performed with the *6xProAOX1a[-377, -328]* promoter fragment as bait against a cDNA expression library enriched for stress-responsive genes (Jaspers et al., 2009). In this effort, five NAC family transcription factors (Ooka et al., 2003; Olsen et al., 2005a) were found that bound to the *AOX1a* promoter fragment: ANAC013, ANAC016, ANAC017, ANAC053, and ANAC078 (Figure 2.4A). In addition, we demonstrated that these NAC transcription factors also interacted with a promoter fragment containing only the MDM element with 5-bp flanking sequence at each end ($6xMDM1_{[AOX1a]}$). This interaction was completely abolished when base substitutions were introduced into the MDM sequence ($6xMDM1mut_{[AOX1a]}$). These data indicate that the five NAC proteins specifically interact with $MDM1_{[AOX1a]}$ in the Y1H system and not with its 5'- or 3'-flanking ends.

To confirm the binding of the NAC transcription factors to the MDM, we performed electrophoretic mobility shift assays with five different *MDR* promoters. Radioactively labeled DNA probes containing 30- to 50-bp regions of the *AOX1a*, *UGT74E2*, *UPREGULATED BY OXIDATIVE STRESS* (*UPOX*), *At5g09570,* and *At2g04050* promoters surrounding MDM were synthesized (Supplemental Table 2.5). Recombinant NAC-glutathione-*S*-transferase (GST) fusion proteins for ANAC013, ANAC017, ANAC053, and ANAC078 were successfully produced and purified in *Escherichia coli*. For the four tested NAC proteins, specific shifts with the *AOX1a*, *UGT74E2*, *UPOX*, *At5g09570,* and *At2g04050* probes were observed that could be abolished with a non-labeled competitor against these probes, whereas the MDM-mutated labeled probes did not gave retardation complexes (Supplemental Figure 2.1). Hence, ANAC013, ANAC017, ANAC053, and ANAC078 bind specifically to promoter fragments carrying the MDM element from several *MDR* genes.

The five MDM-binding NAC transcription factors are all putative membrane-associated NAC proteins, containing a C-terminal transmembrane (TM) motif and designated NAC WITH TRANSMEMBRANE MOTIF 1-LIKE (NTL) (Kim et al., 2007, 2010b). NTL transcription factors are proteolytically cleaved at the membrane to release an active transcription factor that can enter the nucleus. As *ANAC013* itself belongs to the *MDR* regulon, it displayed expression characteristics similar to those of genes that contain the MDM element in their promoters, including *AOX1a* (Pearson correlation coefficient 0.7, Figure 2.2B). This coexpression occurred not only under mitochondrial, but also under various environmental stress conditions (Supplemental Figure 2.2). Of the five isolated NTL genes, *ANAC013* and, to a lesser extent, *ANAC053*, were transcriptionally regulated under mitochondrial dysfunction conditions (Figure 2.4B), as also evidenced by their induction after AA treatments (Figure 2.4C), whereas *ANAC016*, *ANAC078*, and *ANAC017* showed only minor transcriptional changes.

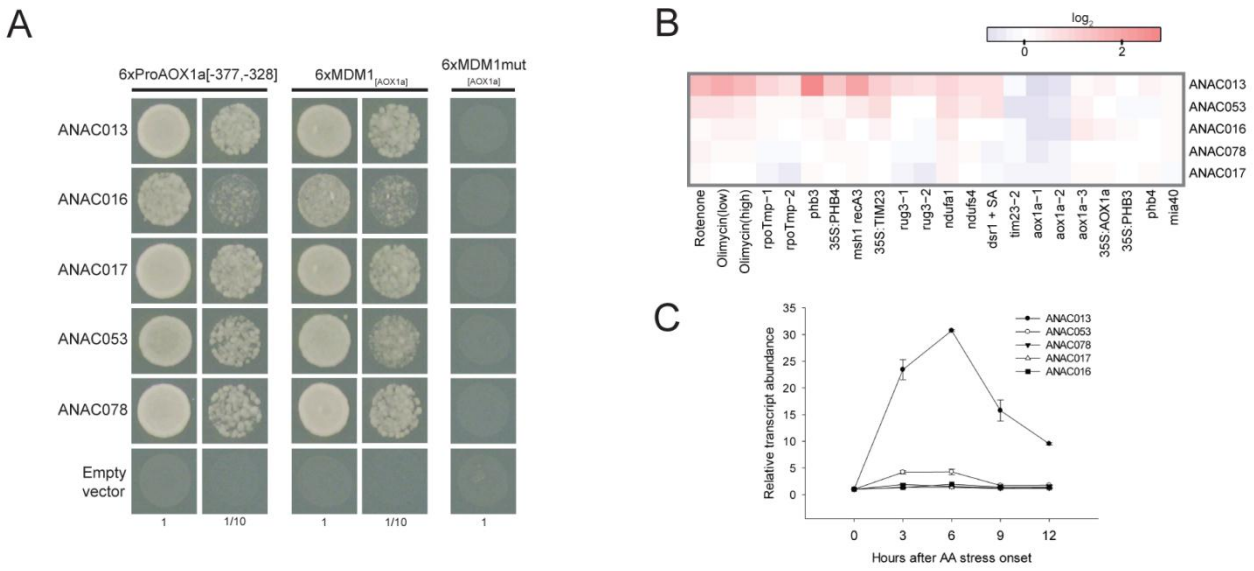

Figure 2.4. Binding of NAC transcription factors to the MDM.
(A) Interaction of NAC transcription factors with the MDM in yeast as shown by Y1H assays. The promoter sequences of interest were fused to histidinol-phosphate aminotransferase imidazole acetol phosphate transaminase (HIS3). The interaction was positive upon growth on 20 mM 3-AT, a competitive inhibitor of HIS3, and was observed with synthetic sequences containing six consecutive repeats of the 50-bp *AOX1a* promoter fragment, including two MDM elements (*6xProAOX1a[-377,-328]*) and one of the MDM sequence alone (*6xMDM1*$_{[AOX1a]}$), but was abolished when the MDM sequence was mutated (*6xMDM1mut*$_{[AOX1a]}$).
(B) and **(C)** Expression pattern of the isolated NAC transcription factors under mitochondrial dysfunction conditions. Expression data were obtained from publicly available microarray data (B) or from own qRT-PCR analyses of AA (50 µM) time series (± SE; n = 2 biological replicates) (C).

ANAC013 binds and transactivates MDM *in planta*

For a more detailed *in planta* functional analysis, we focused on ANAC013. By means of chromatin immunoprecipitation (ChIP), we examined whether ANAC013 was able to bind in Arabidopsis to 13 of the 24 *MDR* promoters that contained an MDM sequence with strong similarity to its position weight matrix representation (Figure 2.2A; Supplemental Figure 2.3) for further analysis. ChIP experiments were done on transgenic Arabidopsis seedlings overexpressing a green fluorescent protein (GFP)-tagged version of *ANAC013* (*35S:GFP-ANAC013*). The MDM-containing promoter regions of *AOX1a*, *UPOX*, *At5g09570*, *At2g04050*, *At2g41730*, *HEAT SHOCK PROTEIN 23.5* (*HSP23.5*), *CYTOKININ RESPONSE FACTOR 6* (*CRF6*) relative to *ACTIN2*, *CYCLIN-DEPENDENT KINASE A;1* (*CDKA;1*), and *UBIQUITIN 10* (*UBQ10*) were more than 10-fold enriched after precipitation with an anti-GFP antibody, with the strongest enrichment for *AOX1a*, *UPOX*, and *At5g09570* (Figure 2.5A). The enrichment was

smaller (~ 9.5-fold, 7-fold, and 4-fold) for *HYPOXIA RESPONSIVE ETHYLENE RESPONSE FACTOR 2* (*HRE2*), *ATP-BINDING CASSETTE B4* (*ABCB4*), and *SULFOTRANSFERASE* (*ST*), respectively, and only minor (~ 2.5-fold, 2-fold, and 1.5-fold) for *UGT74E2*, *At2g04070*, and *CYTOCHROME P450-FAMILY 81-SUBFAMILY D-POLYPEPTIDE 8* (*CYP81D8*), respectively. These results confirm the in vivo binding of ANAC013 to several *MDR* promoter regions.

To determine whether ANAC013 could transactivate a MDM-containing promoter fragment *in planta*, the *6xMDM1*$_{[AOX1a]}$*-P35Smin:LUC*, *6xMDM1mut*$_{[AOX1a]}$*-P35Smin:LUC* and *P35Smin:LUC* reporter constructs were transformed into Arabidopsis plants overexpressing *ANAC013* (*35S:ANAC013-6*). On average, the *6xMDM1*$_{[AOX1a]}$-containing reporter constructs in *35S:ANAC013-6* plants were 100-fold more transactivated by ANAC013 than in the wild-type background, whereas the mutated and control reporter constructs were not activated (Figure 2.5B).

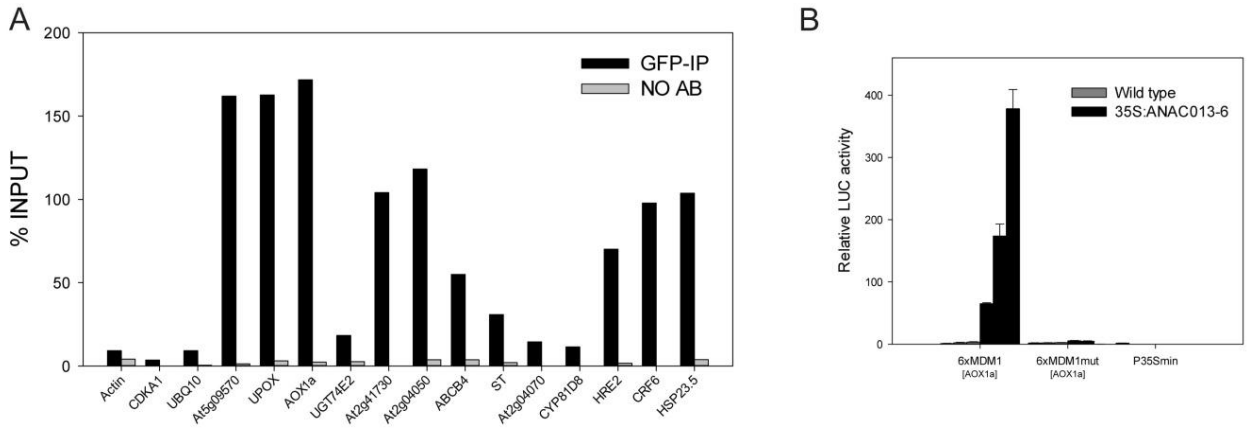

Figure 2.5. Interaction of ANAC013 with the MDM from several *MDR* genes in Arabidopsis.
(A) Interaction of ANAC013 with *MDR* promoters *in planta* as shown by ChIP. Enrichment of promoter fragments surrounding the MDM after ChIP on *35S:GFP-ANAC013* seedlings with anti-GFP antibody (GFP-IP) and without antibody (NO AB). *ACTIN2*, *CDKA;1*, and *UBQ10* fragments were used as negative controls. Bars represent fold enrichment relative to the total genomic DNA from one biological sample. Similar data were obtained in at least one other biological repeat experiment.
(B) Transactivation of the MDM from the *AOX1a* promoter by ANAC013 in Arabidopsis. ANAC013 activated the *6xMDM1*$_{[AOX1a]}$-driven *LUC* reporter gene in *ANAC013*-overexpressing plants (*35S:ANAC013-6*) when compared to wild-type plants. The induction was abolished when the MDM was mutated (*6xMDM1mut*$_{[AOX1a]}$) or in the absence of the promoter fragment (*P35Smin*). Bars indicate average relative LUC activities ± SE (n = 8 biological replicates).

ANAC013 mediates MRR-induced gene expression

To assess the role of ANAC013 in MRR-mediated gene expression, we determined the level of *MDR* transcripts in *ANAC013* gain-of-function plants (*35S:ANAC013-6*). For the above-mentioned 13 *MDR* genes, including *AOX1a*, the transcript levels were induced in *35S:ANAC013-6* and most strongly for *At5g09570*, *At2g04050*, and *UPOX* (Figure 2.6A). Next, the AA-mediated induction of the *MDR* genes in *ANAC013* loss-of-function plants was tested. As no true loss-of-function T-DNA knockout mutants were available for *ANAC013*, transgenic lines were generated containing artificial *ANAC013*-targeting microRNA (miR) constructs. In these *ANAC013-miR* plants, the *ANAC013* transcript levels were 67% lower than those of the wild type. *ANAC013-miR* and wild-type plants were either mock-treated or treated with AA for 3 or 6 h. Analysis of the expression pattern of the 13 *MDR* genes revealed that under nonstressed conditions (mock treatment), the *MDR* expression levels were very low and did not significantly differ between *ANAC013-miR* and wild-type plants (Figure 2.6B). After 3 h of AA treatment, up-regulation of the *MDR* transcripts in *ANAC013-miR* plants was slightly, albeit not significantly, lower than in the wild-type plants, but significantly reduced after 6 h of AA stress, except for *AOX1a*, *CRF6*, and *CYP81D8*, with the most dramatic effect on the *At5g09570* and *UPOX* genes. All together, these data indicate that ANAC013, together with ANAC017 (Ng et al., unpublished results), are positive MRR regulators, necessary for AA-mediated induction of the *MDR*.

To verify that the transcriptional enhancement of *AOX1a* and *UPOX* in *35S:ANAC013-6* plants also resulted in increased protein production, their protein levels were examined by protein gel blot analysis on isolated mitochondria. Wild-type, *35S:ANAC013-6*, and *ANAC013-miR* plants were either mock treated or treated with AA for 6 h. Under nonstressed conditions, the AOX1a and UPOX protein levels were very low in wild-type plants, but highly induced by *ANAC013* overexpression, accumulating to levels higher than those detected in AA-treated wild-type plants (Supplemental Figure 2.4). After treatment with AA, the AOX1a and UPOX protein levels were induced in wild-type plants and remained higher in *35S:ANAC013-6* than those in wild-type plants. In *ANAC013-miR* lines, the AOX1a and UPOX protein levels did not significantly differ from those of wild-type plants both under nonstressed and AA conditions.

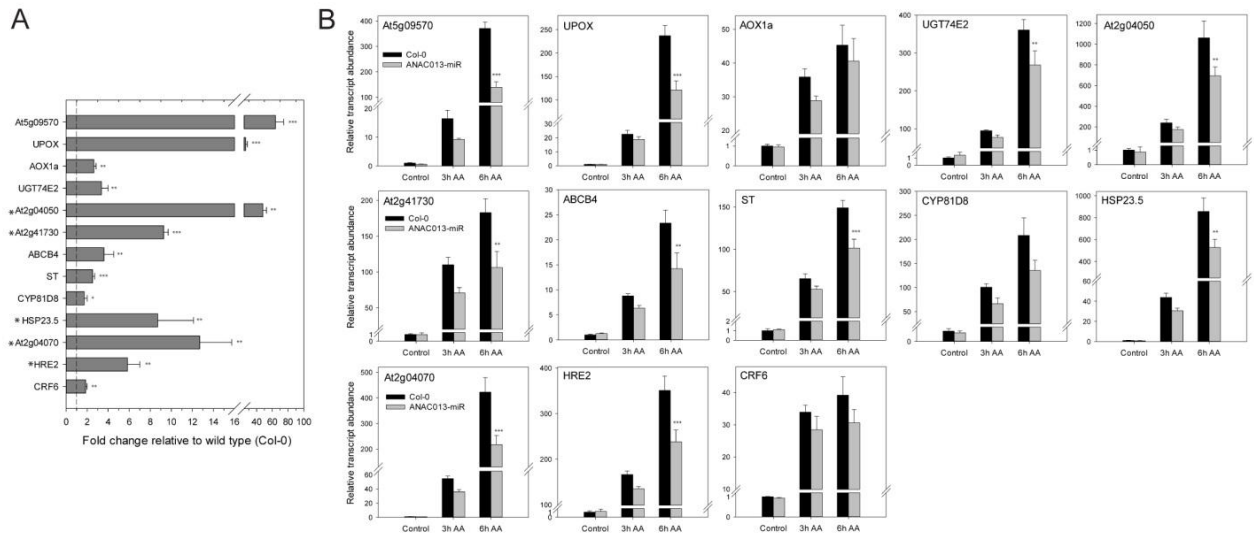

Figure 2.6. MRR-mediated gene expression of the *MDR* regulated by ANAC013 in Arabidopsis.
(A) *ANAC013*-overexpressing plants (*35S:ANAC013-6*) induce *MDR* gene expression under nonstressed conditions. Transcript abundance was analyzed with the Nanostring nCounter system, except for five genes (indicated with asterisk) for which the probes were not present on the CodSet en hence were analyzed with qRT-PCR. Bars represent average fold changes relative to wild-type (Col-0) plants from three biological replicates (± SE). Asterisk indicates significant differences to Col-0 (Student's *t* test; * $P<0.05$, ** $P<0.01$, and *** $P<0.001$).
(B) Failure of *ANAC013-miR* to fully induce *MDR* gene expression in response to AA-mediated mitochondrial perturbation. *MDR* transcript levels were analyzed in wild-type (Col-0) and *ANAC013-miR* plants that were either mock-treated (Control) or treated with AA for 3 h (3h AA) or 6 h (6h AA). Expression was analyzed by qRT-PCR and data are average expression values (± SE) obtained from three biological replicates. The transcript level of wild-type (Col-0) under nonstressed conditions was arbitrarily set to 1. * indicates a significant difference between *ANAC013-miR* and Col-0 at an overall significance level of 0.05, **0.01, and ***0.001.

ANAC013 autoregulates its promoter activity

As the expression profile of *ANAC013* was similar to that of its target *MDR* genes (Figure 2.2B; Supplemental Figure 2.2), we hypothesized that it could autoregulate its own expression. The *ANAC013* promoter also contained the MDM *cis*-regulatory element (CTTGgagaaGAAG; Supplemental Table 2.2) that overlapped with two *ANAC013* promoter elements shown previously to be required for UV-B induction of *ANAC013* (Safrany et al., 2008). To examine the effect of ANAC013 on its own promoter activity, *ProANAC013* fused to β-glucuronidase (GUS) constructs (*ProANAC013:GUS)* were transformed in *35S:ANAC013*-6 plants. Whereas in wild-type *ProANAC013:GUS* plants, GUS staining was visible only in the shoot apical meristem (Skirycz et al., 2010) and hydathodes of 2-week-old seedlings, the *ANAC013* promoter activity was strongly induced throughout the whole plant in the *35S:ANAC013-6* background (Figure

2.7A). ChIP experiments on *35S:GFP-ANAC013* plants also revealed an enrichment of an *ANAC013* promoter fragment surrounding the MDM (Supplemental Figure 2.3; Figure 2.7B). Hence, both results indicate that ANAC013 positively regulates its own promoter-mediated expression through binding with the MDM.

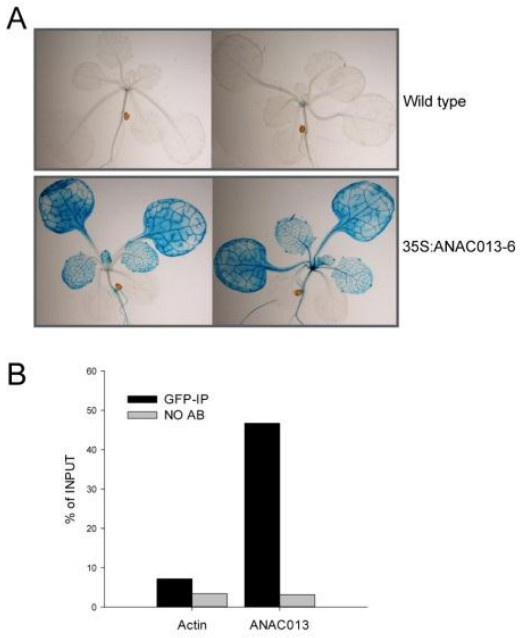

Figure 2.7. Autoregulation of *ANAC013* expression.
(A) Activation by ANAC013 of its own promoter-driven *GUS* reporter gene in *ANAC013*-overexpressing plants. These results were confirmed in four independent transgenic lines, of which one is shown here.
(B) Interaction of ANAC013 with its own promoter *in planta*, as shown by ChIP. Enrichment of the *ANAC013* promoter fragment surrounding the MDM and an *ACTIN2* fragment, as a negative control, after ChIP on *35S:GFP-ANAC013* with anti-GFP antibody (GFP-IP) and no antibody (NO AB). Bars represent fold enrichment relative to the total genomic DNA from one biological sample. Similar data were obtained in another biological repeat experiment.

ANAC013 colocalizes with the endoplasmic reticulum

Overexpression of *ANAC013* translationally fused to GFP had been shown previously to have a nucleocytosolic subcellular localization in transiently transformed tobacco (*Nicotiana benthamiana*) cells (Inzé et al., 2012). To further analyze the subcellular localization, we constructed stable transgenic *35S:GFP-ANAC013* Arabidopsis lines. However, none of the *35S:GFP-ANAC013* lines showed any detectable GFP fluorescence. Also immune electron microscopy studies on these stable GFP fusion lines were unsuccessful, possibly due to the low GFP-ANAC013 protein abundance and consistent with the low protein stability potentially due to proteasomal degradation observed for other NTL transcription factors (Kim et al., 2006; Seo et al., 2008). Protein gel blot analysis of the *35S:GFP-ANAC013* lines with an anti-GFP antibody revealed weak GFP-ANAC013 protein levels and showed that the overproduced GFP-ANAC013 was partially processed (Figure 2.8A). Based on their molecular mass, the cross-reactive protein

bands reflected most probably the full-length GFP-ANAC013 and the processed version lacking the C-terminal TM motif (Kim et al., 2010a), in agreement with the nucleocytosolic localization reported in Inzé et al. (2012).

We analyzed the subcellular localization pattern of GFP-ANAC013 in more detail by *Agrobacterium tumefaciens*-mediated transient transformation of *35S:GFP-ANAC013* constructs in tobacco, hinting at an endoplasmic reticulum (ER) localization, in addition to a nuclear localization (Figure 2.8B). Comparison of the GFP-ANAC013 localization pattern to that of an ER marker indicated a putative ER localization for ANAC013 (Figure 2.8C). The GFP did not overlap with the mCherry labeled mitochondrial marker (data not shown). In another approach, epidermal cells of onion (*Allium cepa*) were transformed biolistically with C-terminal GFP fusions to the full-length ANAC013 (*ANAC013-GFP*), the full-length ANAC013 minus the TM domain (*ANAC013ΔTM-GFP*), and the isolated TM domain ($TM_{ANAC013}$-*GFP*) under the control of the *35S* promoter. For the full-length and TM-deleted constructs, no GFP signal could be detected, whereas $TM_{ANAC013}$-*GFP* colocalized with the ER marker (Figure 2.8D), but not with mitochondrial and peroxisomal marker proteins (data not shown). Thus, these data indicate that ANAC013 is potentially targeted to the ER.

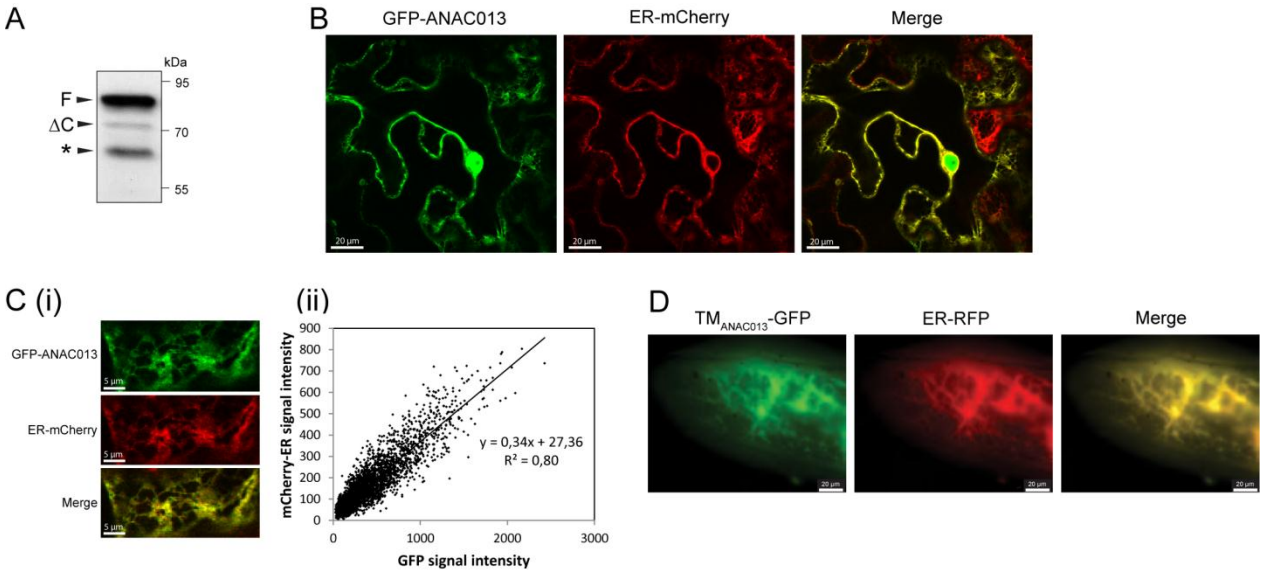

Figure 2.8. ANAC013 localizes to the ER and the nucleus.

Figure 2.8. ANAC013 localizes to the ER and the nucleus. (*Continued*).
(A) Overexpression of GFP-tagged *ANAC013* in transgenic Arabidopsis plants (*35S:GFP-ANAC013*) resulting in premature processing. Different forms of GFP-ANAC013 were immunologically detected and most probably correspond to the full-length GFP-ANAC013 (F, ~86 kDa), the processed form without the C-terminal TM domain (ΔC, ~74 kDa), and a lower band that is probably a degradation product (*).
(B) Localization of GFP-ANAC013 after transient transformation in tobacco epidermal cells. Besides a nuclear localization, GFP-ANAC013 co-localizes with the mCherry-labeled ER marker.
(C) A magnified view of the ER-co-localization of GFP-ANAC013 in tobacco (i) and scatterplot displaying the signal intensity of GFP and mCherry for each pixel and showing substantial co-localization (ii).
(D) Localization of the isolated TM domain of ANAC013 fused to GFP ($TM_{ANAC013}$-GFP) in onion epidermal cells. $TM_{ANAC013}$-GFP targeted to the ER, as confirmed by its co-localization with ER-RFP.

ANAC013-overexpressing plants exhibit increased methyl viologen-mediated oxidative stress tolerance

As the production of ROS is a common component of cellular stresses that might result from inhibition of mitochondrial function, we assessed the effect of alterations in *ANAC013* expression on oxidative stress tolerance. Methyl viologen (MV) and AA that block chloroplast and mitochondrial electron transport, respectively, were used to increase cellular ROS levels. For the MV stress assays, wild-type, *ANAC013*-overexpressing (*35S:ANAC013-4* and *35S:ANAC013-6*) and miR (*ANAC013-miR-3* and *ANAC013-miR-5*) plants were germinated and grown on Murashige and Skoog (MS) medium supplemented with 50 or 100 nM MV. Postgermination growth inhibition by MV was significantly lower for *35S:ANAC013-4* and *35S:ANAC013-6* than for wild-type seedlings (Figure 2.9A). When grown on 100 nM MV, wild-type plants showed a severely retarded development, whereas *35S:ANAC013* seedlings developed with milder symptoms. When grown on 50 nM MV, the rosette area of the overexpressing plants was visibly larger than that of wild-type plants. The fresh biomass of *35S:ANAC013* seedlings grown for 3 weeks on 50 or 100 nM MV was approximately 3-fold higher than that of wild-type plants (Figure 2.9B). Furthermore, root growth was improved in *35S:ANAC013* seedlings at both MV concentrations tested (Figure 2.9C). The fresh weight and root length of *ANAC013-miR* seedlings were significantly lower than that of wild-type plants in the presence of MV. Furthermore, altered *ANAC013* expression did not change the plant growth performance in the presence of externally applied H_2O_2 (see Methods). We are currently assaying the performance of *35S:ANAC013* and *ANAC013-miR* seedlings under mitochondrial stress conditions (AA, rotenone and MFA; see

Methods), and obtained preliminary results showing a better performance of *35S:ANAC013* in the presence of rotenone (Supplemental Figure 2.5).

To assess whether the MV resistance of *ANAC013*-overexpressing plants during postgermination and early development occurred also when plants were treated at a later developmental stage, 2-week-old seedlings grown on a nylon mesh on MS medium were transferred to 2-µM MV-containing medium. The MV stress tolerance was monitored by quantification of photosystem II (PSII) maximum efficiency (F_v'/F_m') (Baker, 2008). Young leaves were examined for decrease in F_v'/F_m' as an indication of stress sensitivity, because they were more affected by MV treatment than the older leaves. The maximum quantum efficiency of PSII was similar in transgenic and wild-type plants before transfer to MV, but after 7 days and later, the decrease in F_v'/F_m' of the overexpressing plants was significantly lower, implying an increase in MV tolerance (Supplemental Figure 2.6). Furthermore, prolonged exposure to MV (6 weeks) was fatal to wild-type plants, whereas overexpressing plants still remained partially green. These results show that ANAC013 positively affects tolerance to chloroplast-initiated oxidative stress and that this phenotype is most pronounced during the early developmental stages. Further experiments are required to reveal the effect of ANAC013 on tolerance to mitochondrial stress conditions.

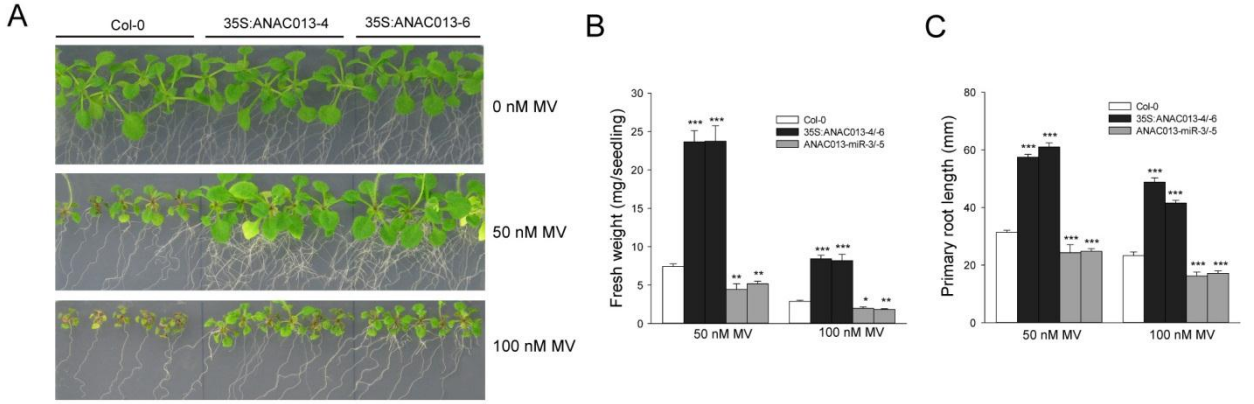

Figure 2.9. Increased MV tolerance of *ANAC013*-overexpressing plants.
(A) Three-week-old wild-type (Col-0) and *ANAC013*-overexpressing seedlings germinated and grown on MS medium supplemented with 50 or 100 nM MV.
(B) and **(C)** Fresh weight (B) and primary root length (C) of 3-week-old wild-type (Col-0), *ANAC013*-overexpressing (*35S:ANAC013-4* and *35S:ANAC013-6*, respectively), and *ANAC013-miR* (*ANAC013-miR-3* and *ANAC013-miR-5*, respectively) seedlings germinated and grown in the presence of 50 or 100 mM MV. Data represent average ± SE (n = 20 to 25 plants).

ANAC013 overexpression increases sensitivity to salt and osmotic stress

Next, we assessed whether ANAC013 is involved in abiotic stress tolerance. *35S:ANAC013* and *ANAC013-miR* plants did not display any significant phenotype under UV, high light and heat stress (data not shown). However, *35S:ANAC013* seedlings were more sensitive to salt stress when germinated and grown on 100 mM NaCl (Figure 2.10A), showing severely stunted development. This phenotype was not completely penetrant, but consistent with the transgene expression levels of the overexpression lines (Figure 2.10B; *35S:ANAC013-4*, 46-fold; and *35S:ANAC013-6*, 67-fold). However, when 3-day-old control grown *35S:ANAC013* seedlings were exposed to salt stress, no significant effect on seedling growth was observed compared to the wild-type situation (data not shown), indicating that ANAC013 functions in the stress response during early developmental stages. Similarly, *ANAC013* overexpressing seedlings appeared more sensitive to mild osmotic stress when germinated and grown on 25 mM mannitol. By visual observation, *35S:ANAC013* showed a smaller rosette area compared to wild-type plants. After 3 weeks of growth on mannitol, *35S:ANAC013* leaves displayed increased rolling, whereas the wild-type leaves maintained stretched, indicating that the overexpression plants suffer more from water deficit (Figure 2.10A). Under more severe osmotic stress conditions (100 and 150 mM mannitol), no phenotype was observed for the overexpression lines when compared to wild-type plants (data not shown).

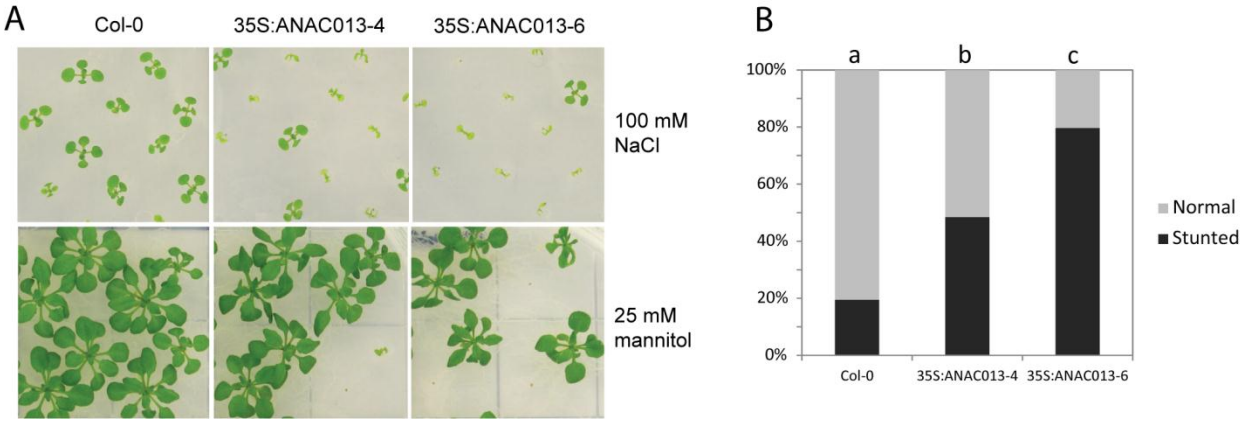

Figure 2.10. *ANAC013* overexpressing plants are more sensitive to salt and mild osmotic stress.
(A) Wild-type and *ANAC013* overexpressing plants were germinated and grown on 100 mM NaCl and 25 mM mannitol, and pictures were taken after 2 and 3 weeks, respectively.
(B) Percentage of seedlings with stunted growth under salt stress (n = 128 plants for wild type and n = 64 plants for *35S:ANAC013*). The data were statistically analyzed through binary logistic regression using the SPSS software, taking *P* value < 0.05 as significant.

Altered *ANAC013* levels affect sensitivity to the necrotrophic fungus *Botrytis cinerea*

Besides its responsiveness to abiotic stress conditions, *ANAC013* is induced after infection with various pathogens, such as the nectrotrophic fungus *Botrytic cinerea* and the hemibiotrophic bacterium *Pseudomonas syringae* (Zimmermann et al., 2004). To assess whether ANAC013 is involved in the biotic stress response, we tested the performance of transgenic lines with altered *ANAC013* expression during *B. cinerea* infection. Both independent overexpressing lines showed substantially increased sensitivity to the fungus, as revealed by the increased lesion size (Figure 2.11A). A slight reduction in lesion size was observed for the *ANAC013* knockdown line. However, these results will need to be consolidated in a repeat experiment. Moreover, further experiments will be necessary to elucidate whether ANAC013 also affects resistance to biotrophic pathogens. In addition, another member of the MDR regulon, the *BCS1* gene that was defined as one of the most widely stress-responsive mitochondrial proteins (Van Aken et al., 2009) is strongly induced in various biotic stress-related microarray studies, such as *B. cinerea*, *Blumeria graminis*, *Phytophthora parasitica*, and *P. syringae* infection and treatment with bacterial elicitors, such as flagellin (Flg22) and harpinZ (HrpZ), and salicylic acid (Zimmermann et al., 2004). Consistent with these expression data hinting at a function of BCS1 in the response to biotrophic pathogens, elevated *BCS1* expression increased resistance to *P. syringae* (Van Aken, unpublished results; data not shown). However, similarly to ANAC013, *BCS1*-overexpressing plants displayed increased susceptibility to *B. cinerea* (Figure 2.11B).

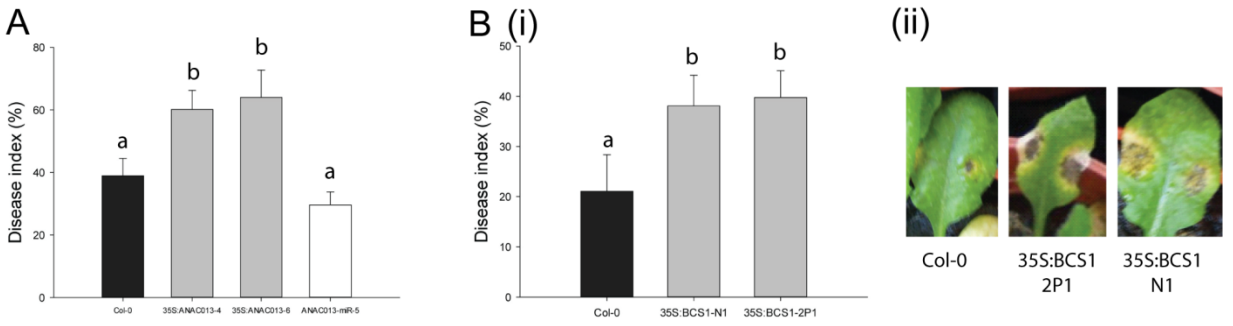

Figure 2.11. Altered *ANAC013* and *BCS1* levels affect sensitivity to *Botrytis cinerea*.
(A) Disease severity of *35:ANAC013* and *miR* lines at 10 days post inoculation (dpi) assessed using a disease index score that takes into account the spreading of the lesions. Disease index was calculated on five leaves of 3-week-old plants and was evaluated using four scoring categories (0, resistant; 1, slightly spreading lesion; 2, moderately spreading lesion; 3, severely spreading lesion). Data represent means ± SE of seven different plants. Data were

Figure 2.11. Altered *ANAC013* and *BCS1* levels affect sensitivity to *Botrytis cinerea*. (*Continued*).
statistically analyzed using one-way ANOVA and Duncan multiple comparison tests, taking *P* value < 0.05 as signficant.
(B) Disease severity of *35S:BCS1* plants at 7 dpi (n = 7 plants) **(i)** and pictures of representative disease symptoms for each genotype **(ii)**.

DISCUSSION

When eukaryotic cells are exposed to environmental stresses, mitochondrial function can be disturbed and transmit the stress-induced signals to the nucleus to activate a transcriptional defense response with the aim to restore cellular function, a process often designated MMR. In plants, MRR has been shown to be involved in signal transduction events during biotic and various abiotic stresses, such as cold, oxygen deprivation, heat, and heavy metals (Lee et al., 2002; Bailey-Serres and Chang, 2005; Rhoads et al., 2005; Keunen et al., 2011). In contrast to yeast and mammalian systems, signal transducing proteins and transcriptional regulators of MRR in plants remain largely unknown. Here, we report on the discovery of a *cis*-regulatory motif in the promoters of genes that are under MRR control and on the identification of transcription factors that bind to this element, hereby steering mitochondrial retrograde-induced gene expression.

The MDM *cis*-regulatory element steers mitochondrial retrograde-induced gene expression

To identify cellular components of the mitochondrial retrograde signal transduction pathways in plants, we compiled a set of genes induced in response to perturbations of mitochondrial function, the MDR. By selecting genes that were significantly modified under multiple MRR-related conditions, we wanted to avoid potential off-target effects of chemical inhibitors and acclimation responses in stable mutants. The MDR encompasses, in addition to well-established general mitochondrial stress markers, such as *AOX1a* and *UPOX* (Ho et al., 2008; Schwarzländer et al., 2012), several other mitochondrial proteins, transmembrane transporters, UDP-glucosyltransferase, *N*-acetyltransferase, cytochrome P450, steroid sulfotranferase, and three transcription factors (Supplemental Table 2.3). Based on the *MDR* coexpression under mitochondrial dysfunction conditions, we aimed at identifying transcription factor-binding sites involved in MRR via the presence of common sequence motifs in the *MDR* promoters and

discovered an unknown DNA motif. This *cis*-regulatory motif is necessary and sufficient for mitochondrial stress-responsive gene expression during inhibition of the cytochrome respiratory pathway and TCA cycle, during oxidative stress, and in the mitochondria-defective *phb3* mutant; hence, its designation as the MDM. Accordingly, the MDM element is present in the 93-bp promoter region of *AOX1a* that, upon deletion and mutation impairs induction by AA and MFA (Dojcinovic et al., 2005). However, further experiments will be needed to elucidate whether MDM exclusively regulates responses to mitochondrial stress conditions or whether it is also mediates transcriptional responses triggered by stresses that do not involve mitochondrial events, but rather signals originating from other subcellular compartments. As the *MDR* genes (except for *At5g09570*) also respond to various chloroplast perturbations (Van Aken and Whelan, 2012), it would be interesting to test whether these signals are also mediated via the MDM. The responsiveness of MDM to various pharmacological and genetic perturbations that act on different mitochondrial components has also been demonstrated for other promoter elements responsive to mitochondrial stress provoked by rotenone and H_2O_2 application (Ho et al., 2008). Different mitochondrial perturbations act via distinct, but overlapping, pathways (Zarkovic et al., 2005; Ng et al., unpublished results), indicating a convergence of different MRR-transducing events at the promoter level or, more plausibly, more upstream by provoking the production and/or release of (a) common signal(s) shortly after triggering mitochondrial dysfunction. A perturbation in mtROS homeostasis is a common component of stress conditions as consequence of mitochondrial damage (Prasad et al., 1994; Maxwell et al., 1999). As ROS can act as signaling molecules in plant stress responses, they are potential candidates for the common signals produced upon mitochondrial dysfunction (Gechev et al., 2006; Rhoads et al., 2006). However, mtROS are probably not required for all plant MRR (Gray et al., 2004). Although the strong MDM responsiveness to H_2O_2 supports ROS as early MRR signals, we cannot exclude that external application of H_2O_2 in our experiments caused mitochondrial damage and dysfunction, such as inhibition of the cytochrome respiratory pathway and TCA cycle components (Sweetlove et al., 2002), thereby initiating MRR through another unidentified mechanism.

The MDM consensus sequence (CTTGNNNNNCA[AC]G) is also present in the promoters of other Arabidopsis genes that are not responsive to mitochondrial perturbations (data not shown). Therefore, additional factors might be required for its specificity, such as the flanking sequence, position in the promoter, or location within the chromatin. In this regard, the

spacer and the surrounding sequence of the MDM have some degree of conservation within the *MDR*, suggesting they account for specificity. Additionally, MDM preferentially occurs within 400 bases of the translational start site, and this positional bias was also observed for the genomewide conserved occurrences (data not shown). Nine of the *MDR* promoters also contain a perfect G-box/ABA-RESPONSIVE ELEMENT (ABRE) (CACGTG; data not shown), although not adjacent to the MDM sequence. This G-box/ABRE from the *AOX1a* promoter was previously shown to have a role in MRR upon rotenone treatment (Ho et al., 2008).

Transmembrane domain containing NAC transcription factors bind the MDM

We identified five closely related NAC transcription factors that interact with the MDM *cis*-regulatory element. Several NAC proteins have been reported to bind the so-called NAC-binding site (NACBS) CGT[GA] or its reverse complement [TC]ACG (Olsen et al., 2005b; Jensen et al., 2010; Lee et al., 2012). NAC transcription factors bind DNA as dimers with two palindromically oriented NACBS repeats as preferred binding site (Olsen et al., 2005b), but with some degree of flexibility because a NAC dimer can bind to a high-affinity NACBS with one monomer and to a lower-affinity (suboptimal) binding site in a nonpalindromic sequence with a second monomer (Welner et al., 2012). Accordingly, the MDM contains the imperfect inverted-repeat structure CTTGN_5CA[AC]G. Moreover, the flexibility in dimer binding might explain the degeneracy of the identified consensus. Furthermore, our data are in agreement with the previously determined recognition sequence of ANAC078, sharing the MDM core sequence CA[AC]G (Yabuta et al., 2010) but slightly diverge from a reported binding site for ANAC053 (TACG[AC]CA) in the AtrbohC and AtrbohE gene promoters (Lee et al., 2012).

The MDM binding NAC proteins belong to the subclass of putative membrane-bound NTL transcription factors (Kim et al., 2007, 2010b) and represent a complete phylogenetic subgroup (NAC2) in this NTL family. Phylogenetic analysis of all Arabidopsis NAC domains revealed that the sequence similarity among the isolated NTL proteins was higher than that to other NAC subfamilies (Ooka et al., 2003; Kim et al., 2010b), reflecting their common DNA-binding capacities to the MDM.

ANAC013 regulates MMR

Previous functional studies indicated that ANAC078 regulates light stress-dependent flavonoid biosynthesis (Morishita et al., 2009). In transgenic Arabidopsis plants overexpressing *ANAC053*, ROS production and leaf senescence was promoted during drought stress (Lee et al., 2012). We showed that individual overexpression of *ANAC013*, *ANAC053*, and *ANAC078* (Figure 2.6A; Supplemental Figure 2.7), or expression of a truncated *ANAC017* form devoid of the TM domain (Ng et al., unpublished results) constitutively activates the *MDR* genes, regardless of stress conditions, hinting at functional redundancy in the *MDR* regulation. However, the distinct spatiotemporal expression patterns of the five NTL proteins (Figure 2.4B and 2.4C; Kim et al., 2007) most probably suggest specialized subfunctionalization. From the five MDM-binding NTL proteins identified, *ANAC013* is strongly controlled by MRR at the transcript level, and its transcript profile highly resembles the expression characteristics of the *MDR* genes under mitochondrial perturbation as well as during environmental stress conditions (Figure 2.2B; Supplemental Figure 2.2). In the *ANAC013* knockdown transgenic plants, the *MDR* induction is reduced in response to the mitochondrial complex-III inhibitor AA. However, to some degree, these plants could still transduce the MRR signal for *MDR* induction, possibly due to the residual *ANAC013* transcript masking the knock-down readout and/or partial redundancy with respect to other (MDM-binding) transcription factors, such as ANAC017 (Ng et al., unpublished results). For example, induction of *AOX1a* upon AA treatment was not significantly impaired in the *ANAC013* knockdown plants, but was strongly reduced in the *ANAC017* knockout. However, the most drastically affected transcript in the *ANAC013* overexpression and knockdown plants, respectively, *At5g09570* was recently defined as an MRR marker gene that uniquely responds to mitochondrial perturbations and no chloroplast perturbations in contrast to the other *MDR* genes, including *AOX1a* (Van Aken and Whelan, 2012). Taken together, our results indicate that ANAC013 positively regulates the *MDR* gene expression upon mitochondrial dysfunction by direct interaction with the MDM *cis*-regulatory element and, hence, can be considered as a signal-transducing component of MRR. Previously, transcriptional regulation of MRR had been suggested to be controlled by ABI4 that had been identified as a negative regulator of basal *AOX1a* expression, thereby allowing derepression by MRR initiated through respiratory complex-I inhibition (Giraud et al., 2009). To our knowledge, ANAC013, together with

ANAC017 (Ng et al., unpublished results), are the first identified transcriptional activators of MRR in plants.

Transmembrane transcription factors are anchored to intracellular membranes, from which they are, in response to various stresses released via proteolysis and subsequently translocated to the nucleus, allowing a prompt activation of the downstream transcriptional responses (Kim et al., 2007; Seo et al., 2008). Transient expression of GFP-tagged *ANAC013* suggests that ANAC013 is localized in the ER under nonstressed conditions (Figure 2.8). The ER represents a dynamical network throughout the cell, that is continuously rearranged, and physically associates at some points with the mitochondria, allowing local communication between the two organelles (Hayashi et al., 2009). This physical interaction could facilitate the proteolytic activation of ANAC013 by mitochondrial signals, such as ROS or calcium that have previously been implicated in MRR (Rhoads and Subbaiah, 2007; Vanderauwera et al., 2012). Moreover, ANAC013 is seemingly strongly regulated at the gene transcription level by mitochondrial perturbations, as revealed by its early and strong responsiveness and its very low expression levels in the absence of stress. In addition, DNA binding and gain-of-function studies (Figure 2.7) indicate that a potential posttranslational ANAC013 activation can initiate a positive feedback loop, autoamplifying its own transcription and driving the expression of its target genes. Moreover, ANAC017 and ANAC053 also contain the MDM *cis*-regulatory element in their promoter, indicative of regulatory interactions with other NTL proteins that are also involved in MRR regulation (Ng et al., unpublished results).

In accordance with the MRR function in stress signal transduction, overexpression of *ANAC013* leads to a stress phenotype in transgenic Arabidopsis plants (Figure 2.9, 2.10 and 2.11). The tolerance of *ANAC013*-overexpressing lines to MV-induced oxidative stress was enhanced (Figure 2.9), most probably due to the constitutive production of MDR proteins, among which AOX1a. The AOX1a function is best studied with respect to mitochondria-initiated oxidative stress responses (Maxwell et al., 1999; Umbach et al., 2005), but is not limited to mitochondrial stress responses as evidenced by its importance in chloroplast protection during oxidative stress-promoting conditions (Yoshida et al., 2007; Giraud et al., 2008). To reveal whether MV tolerance of *ANAC013* overexpression lines could be attributed to increased AOX1a levels, *AOX1a* overexpression and knockout lines will be analyzed under MV stress conditions. Furthermore, the MDR is enriched for members of multigene families associated with

detoxification pathways (cytochrome P450 monooxygenase, *N*-acetyltransferase, glycosyltransferase, ATP-binding cassette [ABC] and multi antimicrobial extrusion protein transporters) (Manabe et al., 2007), together with two mitochondrial proteins (HSP23.5 and MITOCHONDRIAL GRPE1 [MGE1]) that might have chaperone-like activities (Visioli et al., 1997; Hu et al., 2012). Other Arabidopsis genes that contain evolutionarily conserved MDM instances are also enriched for components of the alternative respiratory chain, proteins involved in multidrug/xenobiotic transport, mitochondrial HSPs and subunits of the 20S proteasome (Supplemental Tables 4 and 6). The potential involvement of the MDM in proteasome regulation is consistent with a previous report on ANAC078 regulating 20S and 26S proteasome levels (Yabuta et al., 2011). Thus, from these data and previous observations, we suggest that ANAC013 and the MDR integrate oxidative stress responses, xenobiotic stress resistance, and protein quality control. This coordinated response presumably allows the cell to prevent ROS formation, inactivate and eliminate the offending agent or toxic byproducts, and rapidly control and repair the damage under adverse conditions. On the other hand, *ANAC013*-overexpressing plants are more sensitive to the necrotrophic fungus *B. cinerea* (Figure 2.11). This could potentially be explained by the importance of ROS production in the mitochondria during pathogen attack, consistent with a previous report on the increased disease susceptibility of a mitochondrial mtETC mutant due to lowered mtROS production (Gleason et al., 2011). Mitochondrial ROS may contribute to plant defense by either directly acting against the pathogen or by acting as a signaling molecule in plant defenses (Amirsadeghi et al., 2007).

In yeast, a highly interconnected transcriptional network links the oxidative stress, multidrug resistance, protein degradation, and protein folding responses through the coordinated action of several transcription factors (Salin et al., 2008; Teixeira et al., 2008). Interestingly, one of them (Pleiotropic Drug Resistance Protein 3 [Pdr3]) is involved in a MRR response in which mitochondrial dysfunction triggers the expression of an ABC transporter involved in multidrug resistance (Hallstrom and Moye-Rowley, 2000; Traven et al., 2001; Devaux et al., 2002). Similarly to yeast, multidrug resistance transporter, mitochondrial chaperone, and oxidative stress genes are induced by mitochondrial dysfunction in mammalian cells (Martinus et al., 1996; Park et al., 2004; Ferraresi et al., 2008), indicating that this process is conserved in eukaryotes. Furthermore, the basic leucine zipper (bZIP) transcription factor AP-1-like transcription factor 1 (Yap1), which is a central node in the above-mentioned stress transcriptional network in yeast, is

redox-activated by the glutathione peroxidase 3 (Gpx3) in response to elevated H_2O_2 levels and xenobiotics (Delaunay et al., 2002; Azevedo et al., 2003). Although plants lack orthologs of Yap1-like transcription factors, a previously reported ANAC013-interacting protein, RADICAL-INDUCED CELL DEATH1 (RCD1), was hypothesized to be the plant equivalent of Yap1 (Miao et al., 2006; Jaspers et al., 2009). RCD1 complemented the oxidative stress-sensitive phenotype of the Yap1-deficient yeast strain and interacted with the Arabidopsis GPX3 protein that functions in H_2O_2 sensing and signal transduction (Belles-Boix et al., 2000; Miao et al., 2006). A similar redox-sensing regulatory system in mammals contains the bZIP transcription factor Nuclear factor erythroid 2-Related Factor 2 (Nrf2) that mediates oxidative and xenobiotic stress responses (Kobayashi and Yamamoto, 2006). Unlike Yap1, Nrf2 does not directly senses the stress, but is activated by redox regulation of its interaction partner Kelch-like epichlorohydrin-associated protein 1 (Keap1) (Itoh et al., 1999). Under unstressed conditions, Keap1 negatively regulates Nrf2 by proteosomal degradation and cytoplasmic sequestration, but, upon stimulation by oxidative or chemical stresses, Keap1 releases Nrf2 to escape proteosomal degradation and translocate to the nucleus (Kang et al., 2004; Kobayashi et al., 2004). Nrf2 induction and nuclear translocation have also been shown in response to an inhibitor of specific mitochondrial proteins (such as HSPs, and aconitase and α-ketoglutarate dehydrogenase of the TCA cycle), although this activation probably does not involve increased ROS levels, but rather ER stress signaling induced by dysfunctional mitochondria (Ho et al., 2005). Taking the above described similarities in yeast, plants, and mammals into account, we can hypothesize that the Arabidopsis MDR might be functionally equivalent to the above-mentioned yeast and mammalian oxidative and xenobiotic stress responses.

Besides its association with RCD1, ANAC013 also interacts with another member of the SIMILAR TO RCD-ONE (SRO) family, SRO5 (Jaspers et al., 2009). Interestingly, SRO5 has been suggested to function in mitochondria-nucleus communication, based on its reported localization in mitochondria or nucleus (Borsani et al., 2005; Jaspers et al., 2010). The interaction of RCD1 and SRO5 with transcription factors through the conserved RCD-SRO-TATA box binding protein (TBP)-associated factor 4 **(**TAF4) domain implies a role in the regulation of the transcription factor activity (Jaspers et al., 2009, 2010). As RCD1 is localized in the nucleus under nonstressed conditions and *rcd1* mutants exhibit an elevated expression of ANAC013 target genes, RCD1 might negatively affect the ANAC013 function in the nucleus in the absence

of stress (Jaspers et al., 2009). Similarly, RCD1 negatively regulates the stability of the DEHYDRATION-RESPONSE ELEMENT-BINDING PROTEIN 2A (DREB2A) transcription factor, but is rapidly degraded upon stress, promoting the proper DREB2A function under these conditions (Vainonen et al., 2012). Similar to *ANAC013*-overexpressing plants, *rcd1* mutants display an increased MV tolerance and salt stress susceptibility (Fujibe et al., 2004; Katiyar-Agarwal et al., 2006). RCD1 has also been reported to be necessary for proper meristem function, presumably by controlling the redox/ROS balance (Teotia and Lamb, 2011). *ANAC013* and *MDR* genes are predominantly expressed in meristematic tissues (Skirycz et al., 2010) and the stress phenotype of *ANAC013*-overexpressing plants is the most pronounced when plants are stressed during early seedling development (Figure 2.9 and 2.10), suggesting that ANAC013 is important for the stress response in young tissues. Accordingly, maintenance of an optimal mitochondrial function has been postulated to be required for proper meristem development under stress conditions (Skirycz et al., 2010; Van Aken et al., 2010; Addendum), because energy is required by dividing cells and ROS can damage the mitochondrial function.

METHODS

Microarray analysis, motif detection, and evolutionary conservation analysis

For the meta-analysis of the transcriptome data of the MRR compendium, raw CEL files (http://www.ncbi.nlm.nih.gov/geo/) were preprocessed per data set in Bioconductor (http://www.bioconductor.org/), comprising normalization by Robust Multi-array Average (RMA) with a custom Computer Document Format (CDF) based on The Arabidopsis Information Resource (TAIR10) and provided by Brainarray (TAIR10 genes –v14; brainarray.mbni.med.umich.edu). Differential gene expression was analyzed by the *limma* package, *P* values were adjusted for multiple hypothesis testing by the false discovery rate and the significance cut-off was stringently set at a corrected *P* value <0.01 and a $\log_2$-fold change >1. Genes that were up-regulated in five or more conditions, were hierarchically clustered after gene centering and scaling of the $\log_2$ expression ratios, with Euclidian distance as distance measure and average linkage. For the meta-analysis of the biotic and abiotic stress compendium,

the raw CEL files were obtained from the stress data set in CORNET (De Bodt et al., 2012) and preprocessed together in Bioconductor and clustered as described above.

For the de novo motif discovery, Multiple Expection-Maximization for Motif Elicitation (MEME) version 4.8.1 (Bailey and Elkan, 1994), Amadeus (Linhart et al., 2008) and the *Regulatory Sequence Analysis Tools* (RSAT)-spaced dyad tool (Thomas-Chollier et al., 2011) were used with default parameter settings. The MDM consensus CTTGNNNNNCA[AC]G was commonly identified by the three algorithms. MDM matches in the promoters of the input genes were defined based on the MEME output that provided motif occurrences significantly similar to the identified position weight matrix T[CGA]CTTG[GA][AC]GA[GAC]CAAG and, additionally, by mapping of the consensus sequence by means of DNA pattern (Thomas-Chollier et al., 2011). The position weight matrix of all MDM matches in the *MDR* promoters (Supplemental Table 2.2) was visualized by generating a sequence logo by means of WebLogo 3 (http://weblogo.berkeley.edu/) (Crooks et al., 2004).

To evaluate the evolutionary conservation of an individual motif instance, for each Arabidopsis gene the orthologous genes from six other dicot species: *A. lyrata*, papaya (*Carica papaya*), soybean (*Glycine max*), apple tree (*Malus domestica*), black cottonwood (*Populus trichocarpa*), and common grape vine (*Vitis vinifera*) were retrieved with the PLAZA 2.0 Integrative Orthology method that combines orthology information from phylogenetic trees, OrthoMCL families, and Best-Hits-and-Inparalogs families (Van Bel et al., 2012). Integrative orthologous genes supported by at least one orthology prediction method were retained for the conservation analysis. Based on the 1-kb orthologous upstream intergenic sequences, a test motif was mapped with DNA pattern (Thomas-Chollier et al., 2011) and the number of conserved motif matches was determined for each Arabidopsis gene (and its orthologs). Finally, the significance of the observed motif conservation per Arabidopsis gene was tested by random sampling of 1,000 nonorthologous gene sets, maintaining the gene and species composition as observed in the real orthologous data set, and scoring the number of random gene sets with a similar or improved motif conservation level. As *A. lyrata* is closely related to *A. thaliana*, only genes with conserved motif matches in at least two other species (not including *A. lyrata*) and conservation *P* value <0.05 were defined as significant evolutionarily conserved. Gene Ontology (GO) enrichment was analyzed with the PLAZA Workbench (Van Bel et al., 2012).

Plant growth conditions and stress treatments

Arabidopsis thaliana (L.) Heyhn. plants were grown on half-strength (½) MS medium (Duchefa) supplemented with 1% (w/v) sucrose, 0.75% (w/v) agar, and B5 vitamins, pH 5.7 at 21°C and 100 μE m^{-2} s^{-1} light intensity in a 16-h/8-h light/dark photoperiod. Unless stated otherwise, seedlings were grown for 2 weeks until stage 1.04 (Boyes et al., 2001). For the AA induction experiments, seedlings were sprayed with 50 μM AA (Sigma-Aldrich) (with 0.1% (v/v) dimethyl sulfoxide (DMSO)) containing 0.01% (v/v) Tween 20, or with 0.1% (v/v) DMSO containing 0.01% (v/v) Tween 20 (mock). For AA stress assays, plants were germinated and grown on ½MS medium supplemented with 10, 50 or 100 μM AA or grown for 2 weeks on standard ½MS, and subsequently sprayed with 50 μM AA. For rotenone and MFA stress assays, plants were grown on 10 and 50 μM rotenone or on 10 mM MFA, respectively. For the H_2O_2 stress assays, plants were germinated and grown on 1, 2, 4, and 8 mM H_2O_2 (Merck) or on 1, 2, 4, and 8 μM of 3-amino-1,2,4-triazole (3-AT) (Acros Organics). The maximum efficiency of the PSII photochemistry (F'_v/F'_m) was determined with a PAM-2000 chlorophyll fluorometer and ImagingWin software application (Walz; Effeltrich, Germany) on light-adapted plants. Two or more independent experiments were carried out for all stress assays.

Generation of transgenic Arabidopsis plants

Overexpressing plants were generated by cloning the open reading frame of *ANAC013*, *ANAC053*, and *ANAC078* into pK7WG2D (Supplemental Table 2.7) (Karimi et al., 2002). To generate artificial miR plants, *ANAC013*-specific sequences were identified with the Web MicroRNA Designer (WMD) (www.weigelworld.org). The miR precursors were constructed according to Schwab et al. (2006) and cloned into pK7WG2D. Constructs were transformed into Arabidopsis Columbia-0 (Col-0) by *Agrobacterium*-mediated floral dipping (Clough and Bent, 1998).

Promoter-LUC constructs

The 1.5-kb upstream region from the translational start site of *AOX1a* and *UGT74E2* was cloned into the pDONRP4-P1r vector (Invitrogen). The MDM sequence was deleted according to the PCR-fusion/Gateway cloning procedure (Atanassov et al., 2009) (Supplemental Table 2.7). Promoter-LUC fusion constructs were created by recombining the above promoter plasmids with pEN-L1-LUC+-L2 into the destination vector pB7m24GW by means of the MultiSite Gateway technology (Invitrogen) (Karimi et al., 2005).

The artificial promoter constructs were synthesized by DNA2.0, provided in a pJ244 vector backbone (*6xP-AOX1a[-377,-328]* and *6xMDM1*$_{[AOX1a]}$) or as an oligonucleotide (Invitrogen) (*6xMDM1mut*$_{[AOX1a]}$), annealed by heating followed by gradual cooling, and subsequently cloned into pDONRP4-P1r. The -46 bp CaMV 35S minimal promoter (*P35Smin*) was synthesized as an oligonucleotide (Invitrogen) and subsequently cloned into pDONR221 and pDONRP4-P2. Promoter-LUC constructs were created by recombining the synthetic promoter plasmids (pEN-L4-promoter-R1), pEN-L1-P35Smin-L2, and pEN-R2-LUC^{+}-L3 into the multisite destination vector pB7m34GW. The *P35Smin:LUC* reporter construct was generated by recombining pEN-L4-P35Smin-L2 and pEN-R2-LUC^{+}-L3 into pB7m34GW (Karimi et al., 2005).

LUC assay

Plants were grown for 10 days in a 96-well white CulturPlate-96 (PerkinElmer) as sample holder. Five seedlings were grown per well containing 150 µL of medium (½MS with 0.5% (w/v) sucrose). The promoter activity was detected in the T2 generation of promoter-LUC mutants. As stress treatments, AA (Sigma-Aldrich) was added at a final concentration of 50 µM (0.1% (v/v) DMSO), rotenone (Sigma-Aldrich) at 100 µM (0.1% (v/v) DMSO), MFA (Sigma-Aldrich) at 25 mM, or H_2O_2 at 10 mM. Control plants were mock-treated with 0.1% (v/v) DMSO. After 12 or 24 h of treatment, 100 µL luciferin (One Glo; Promega) was added to each well, followed by a 10-min dark incubation. Luminescence was measured with a LUMIstar Galaxy luminometer (BMG labtechnologies, Offenburg, Germany).

Y1H screening

Yeast strain YM4271 and destination vector pMW#2 were obtained from Dr. M. Walhout (University of Massachusetts Medical School, Worcester, MA, USA). Design of the yeast reporter strains and cDNA library screening were done as described (Deplancke et al., 2006). From the approximately $6x10^6$ screened transformants obtained from three independent cDNA library transformation experiments, 106 potential positives were selected to confirm their growth on 20 mM 3-AT. From the positive yeast clones, plasmids were isolated and retransformed in the reporter yeast strain for growth confirmation on 20 mM 3-AT. The pYESTrp2 empty vector containing only the transactivation domain was used as a negative control.

Chromatin immunoprecipitation

The ChIP experiments were done as described (Bowler et al., 2004; Berckmans et al., 2011) with minor modifications. 35S:GFP-ANAC013 plants were grown for 8 days and 1.5 g material of whole seedlings was harvested for crosslinking in 1% (v/v) formaldehyde. Chromatin was isolated and fragmented by sonication with a Bioruptor sonicator (Diagenode). The chromatin was precleared with 80 µl of Protein A Agarose/Salmon Sperm DNA (Millipore). Ten µL was used as INPUT, whereas the remainder was split into three samples, of which two were treated with 30 µL of anti-GFP antibody coupled to agarose beads (GFP-Trap_A; Chromotek) and the third one with no antibody. The samples were incubated overnight at 4°C and subsequently eluted from the beads. Proteins were de-cross-linked and DNA was purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. qPCRs were analyzed on a LightCycler 480 apparatus (Roche Diagnostics) with the SYBR Green I Master kit (Roche Diagnostics) and each reaction was done in triplicate. ACTIN2, CDKA;1 and UBQ10 were used as negative controls. ChIP-qPCR data were normalized against the amount of chromatin used in the ChIP (INPUT) and were represented as %INPUT. For the quantitative PCR analysis, specific primers were designed for the MDM-containing promoter regions by means of primer-BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Rozen and Skaletsky, 2000) and tested for amplification specificity by melt-curve analysis before use (see Supplemental

Table 2.7 online). Data were obtained from single experiments, but similar data were obtained in independent runs and with independent 35S:GFP-ANAC013 lines.

Electrophoretic mobility shift assays

Oligonucleotide probes of 30 to 50 bp (Supplemental Table 2.5) with wild-type or mutated MDM promoter sequences were annealed by heating at 99°C, followed by gradual cooling. Annealed probes were radiolabeled with ^{32}P γ-ATP (PerkinElmer) and polynucleotide kinase (Roche) and purified with Sephadex G-25-radiolabeled DNA Quick Spin columns (Roche). *ANAC013*, *ANAC016*, *ANAC017*, *ANAC053*, and *ANAC078* were cloned into the GST-tag expression vector pDEST15 (Invitrogen) and transformed into *Escherichia coli* Rosetta 2 (DE3) pLysS-competent expression cells. Proteins were produced in 500 mL of culture overnight with 0.2 mM isopropyl-β-D-thio-galactoside at 18°C and shaking at 250 rpm. Cells were harvested, resuspended in extraction buffer (5× extraction buffer: 250 mM Tris-HCl (pH 8.5), 500 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol), and lysed by digestion with 1 mg/mL lysozyme and sonication. Lysate was clarified by centrifugation at 16,000×*g* for 20 min. The filtered lysate was incubated with glutathione-agarose beads for 1 h at 4°C (ThermoFisher) and washed with 10 volumes of extraction buffer containing the Complete EDTA-free protease inhibitor cocktail (Roche). Purified proteins were eluted with the extraction buffer containing 10 mM reduced glutathione. For gel shift assays, 20-μL reactions were setup with 4 μL 5× binding buffer (100 mM HEPES (pH 7.8), 0.5 M KCl, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 5 mM EDTA, 0.25 mg/mL poly dI-dC, 50% (v/v) glycerol), 1 fmol radiolabeled probe, 500 fmol unlabeled probe for competitor reactions, and 1.5 μg purified protein extract. Reactions were incubated for 20 min and separated on polyacrylamide gels (0.5× Tris/Borate/EDTA, 2.5% (v/v) glycerol, and 6 % (w/v) acrylamide) for 2 h at 200 V on a 16×20 cm^2 Protean II gel system (Bio-Rad). Gels were dried on Whatman paper in a gel dryer, exposed overnight (or longer), and visualized with PhosphorImager detection plates.

Quantitative reverse-transcription (qRT)-PCR

Total RNA and first-strand cDNA were prepared with TRIzol Reagent (Invitrogen) and iScript cDNA Synthesis Kit (Bio-Rad), respectively according to the manufacturer's instructions. As a

template in the subsequent PCR, 5 µL of a 1:8 diluted first-strand cDNA was run on the iCycler iQ (Bio-Rad) with the SYBR Green I Master kit (Roche Diagnostics) according to the manufacturer's instructions. All individual reactions were done in triplicate. Primers were designed with the Universal ProbeLibrary Assay Design center ProbeFinder software (Roche; http://www.roche-applied-science.com/; Supplemental Table 2.7). For the expression analysis, values were normalized against *ACTIN-RELATED PROTEIN 7* (*ARP7*), whereas two reference genes, *ARP7* and *At2g28390* (Czechowski et al., 2005), were used to normalize data from the *ANAC013-miR* experiments. The Δ cycle threshold method (Livak and Schmittgen, 2001) was applied for relative quantification of transcripts.

Nanostring nCounter assays

The raw nCounter data were rescaled by dividing each experiment by two factors: (i) the sum of the positive controls per experiment divided by the median of the sums of the positive controls over all experiments to correct for technical errors and (ii) the geometric mean of at least three stable household genes, selected by geNorm (Vandesompele et al., 2002), per experiment divided by the total geometric mean of these four household genes over all experiments to correct for differences in mRNA content of the samples. Transcripts differentially expressed between wild type and *35S:ANAC013-6* were identified through the DESeq package in R (Anders and Huber, 2010), feeding the raw nCounter data and writing the above rescaling factors in the sizeFactors slot of the package.

Promoter-GUS analysis

The 1.5-kb upstream region of the translational start site of *ANAC013* was amplified by PCR from Arabidopsis Col-0 genomic DNA with primers (Supplemental Table 2.7) and cloned into pBGWFS7 (Karimi et al., 2002), generating an in-frame GFP-GUS fusion. The construct was transformed into Arabidopsis wild-type (Col-0) and *35S:ANAC013-6* plants and GUS assays were performed as described (Beeckman and Engler, 1994). Samples were photographed with a stereomicroscope (Stemi SV11; Zeiss) or with a Nomarski differential interference contrast microscope (Olympus BX51; Leica).

UPOX antibody production

For antibodies against UPOX, the full-length cDNA was cloned into pDEST15 with the Gateway technology (Invitrogen). GST-tagged recombinant proteins were expressed in BL21 (DE3) pLys cells. Proteins were purified with GST-Sepharose (Scientifix) and inoculated in rabbits with Freund's Adjuvant according to the standard protocol (Cooper and Paterson, 2008).

Protein gel blots

For the ANAC013 processing analysis, total protein extracts were prepared from *35S:GFP-ANAC013* seedlings by grinding leaf material in extraction buffer (phosphate buffered saline, 1% (v/v) Nonidet 40, 0.5% (w/v) deoxycholate, 0.1% (w/v) sodium dodecyl sulfate (SDS), 10% (v/v) glycerol), and a protease inhibitor cocktail (Complete, EDTA-free; Roche). Insoluble debris was removed by centrifugation at 20,800×*g* for 15 min at 4°C. Proteins (30 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon P; Millipore), and immunodetected with anti-GFP monoclonal antibody (Clontech Laboratories) and Western Lightning *Plus* enhanced chemiluminescence (PerkinElmer).

For the analysis of AOX1a and UPOX protein levels, mitochondria were isolated as described previously (Lister et al., 2007) from wild-type, *35S:ANAC013-6*, and *ANAC013-miR* seedlings that had been treated with 50 μM AA or mock-treated for 6 h. Twenty and 40 μg of purified mitochondria were separated on a 16% SDS-PAGE gel, transferred to Hybond-C extra nitrocellulose membranes (GE-Healthcare), and immunodetected with antibodies raised against AOX (Elthon et al., 1989), TOM40 (Carrie et al., 2009) and UPOX with the BM Chemiluminescence Western Blotting Kit (Roche) and visualized with the Image Quant RT ECL Imager (GE Healthcare). The band intensity was quantitated with Quantitiy One (Bio-Rad) imaging software with the pixel density set to 100 for the highest band detected and that of the remaining bands relative to it. The average and standard error of band intensities were calculated from three biological repeat experiments.

Determination of subcellular localization

The localization in tobacco (*Nicotiana benthamiana*) was analyzed as described (Inzé et al., 2012). For the localization analyses in epidermal cells of onion (*Allium cepa*), the full-length open reading frame (ORF), the ORF minus the TM domain, and isolated TM domain of *ANAC013* was cloned in-frame with GFP by Gateway cloning (Invitrogen) as described (Carrie et al., 2009) (Supplemental Table 2.7). Constructs were transiently transformed into onion epidermal cells by biolistic transformation as described (Carrie et al., 2007). In addition to the GFP constructs, a series of organelle markers were employed, including an ER-targeted red fluorescent protein (RFP), a mitochondria-targeted mCherry, and a peroxisome-targeted RFP (Nelson et al., 2007). The fluorescent proteins were visualized with an BX61 fluorescence microscope (Olympus) with excitation wavelengths of 460/480 nm for GFP and 535/555 nm for RFP, whereas emission wavelengths were measured at 495/540 nm for GFP and 570/625 nm for RFP. Micrographs were captured and processed with the Cell imaging software as described (Carrie et al., 2007).

***Botrytis cinerea* infections and disease scoring**

Botrytis cinerea strain *Arabidopsis* was grown on potato dextrose agar (PDA) for 10 days. On the day of infection, spores were washed from the plate with distilled water containing 0.01% Tween20. After filtration over nylon and centrifugation, spores were contained and the inoculation solution containing $5x10^5$ in ½ potato dextrose broth (PDB) was prepared. Arabidopsis plants were grown *in vitro* for 2 weeks on ½MS under long day conditions and subsequently transferred to soil and grown till the stage just before flowering. Four leaves per plant were infected by the drop (10 µL) inoculation method. Infected plants were kept at high humidity by means of transparent containers containing a bottom of water. Growth conditions during infection consisted of long day conditions and at room temperature. Symptoms were evaluated per plant at 7 days post inoculation (dpi) by calculating a disease index with four different scores (0, non-spreading lesion; 1, slightly spreading lesion; 2, moderately spreading lesion; 3, severely spreading lesion) using the formula $[(0 \times a) + (1 \times b) + (2 \times c) + (3 \times d)/(a + b + c + d)] \times 100/3$ where a, b, c, and d are the number of leaves examined with scores 0, 1, 2, and 3, respectively (Curvers et al., 2010). For each transgenic line, leaves of 7 plants were scored (n =

7). Data were statistically analyzed using one-way ANOVA and Duncan multiple comparison tests with the SPSS software.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: ANAC013, At1g32870; ANAC016, At1g34180; ANAC017, At1g34190; ANAC053, At3g10500; ANAC078, At5g04410; PHB3, At5g40770; ABCB4, At2g47000; AOX1a, At3g22370; CRF6, At3g61630; CYP81D8, At4g37370; HRE2, At2g47520; HSP23.5, At5g51440; ST, At1g32870; UGT74E2, At1g05680; UPOX, At2g21640; MGE1, At5g55200; ACT2, At3g18780; CDKA;1, At3g48750; UBQ10, At4g05320; TOM40, At3g2000; GPX3, At2g43350; RCD1, At1g32230; SRO5, At5g62520; and DREB2A, AT5G05410.

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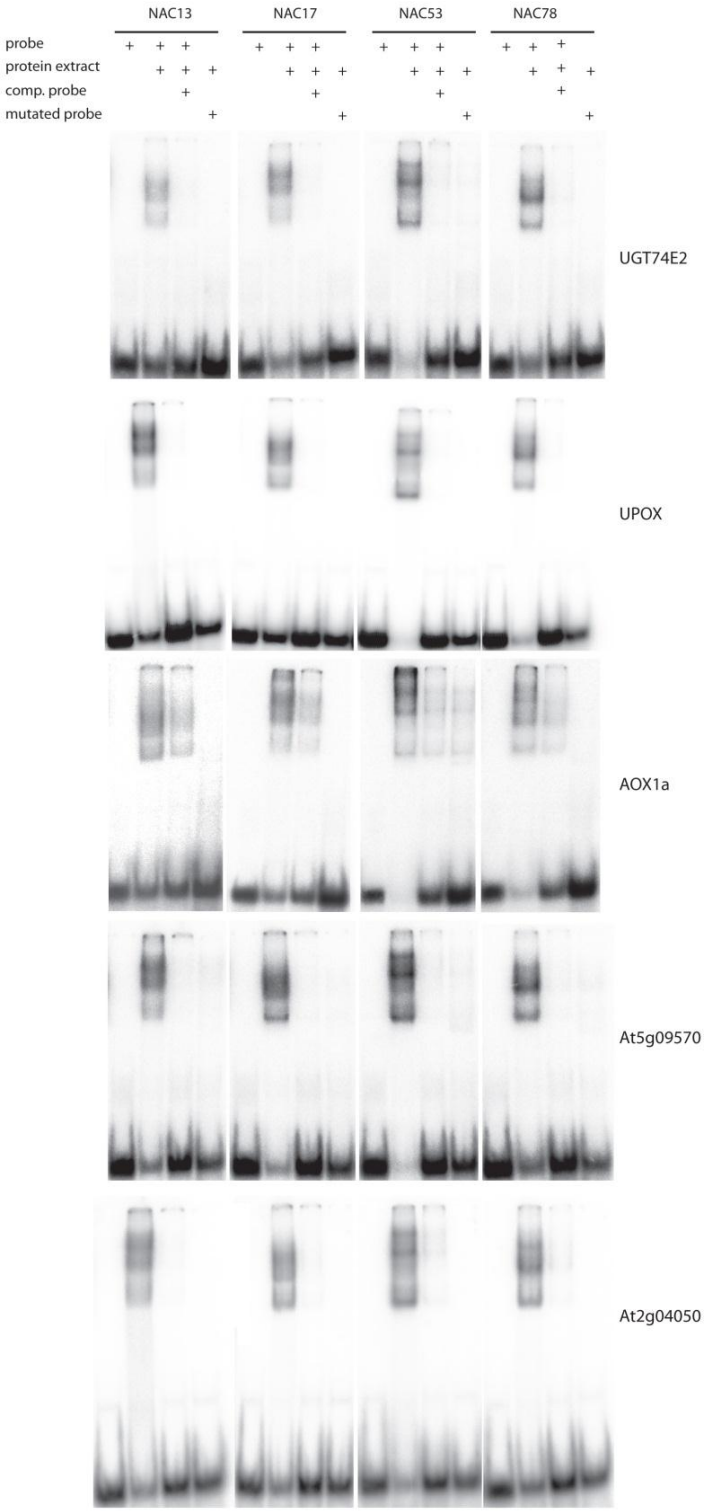
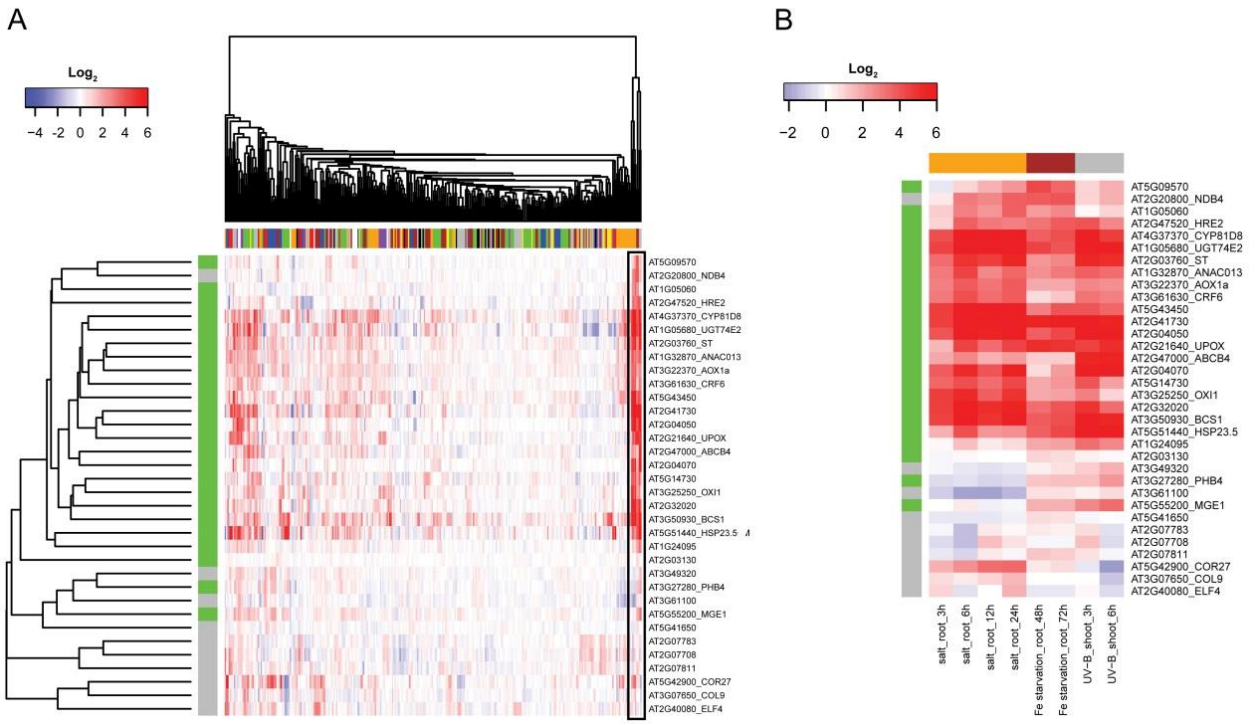
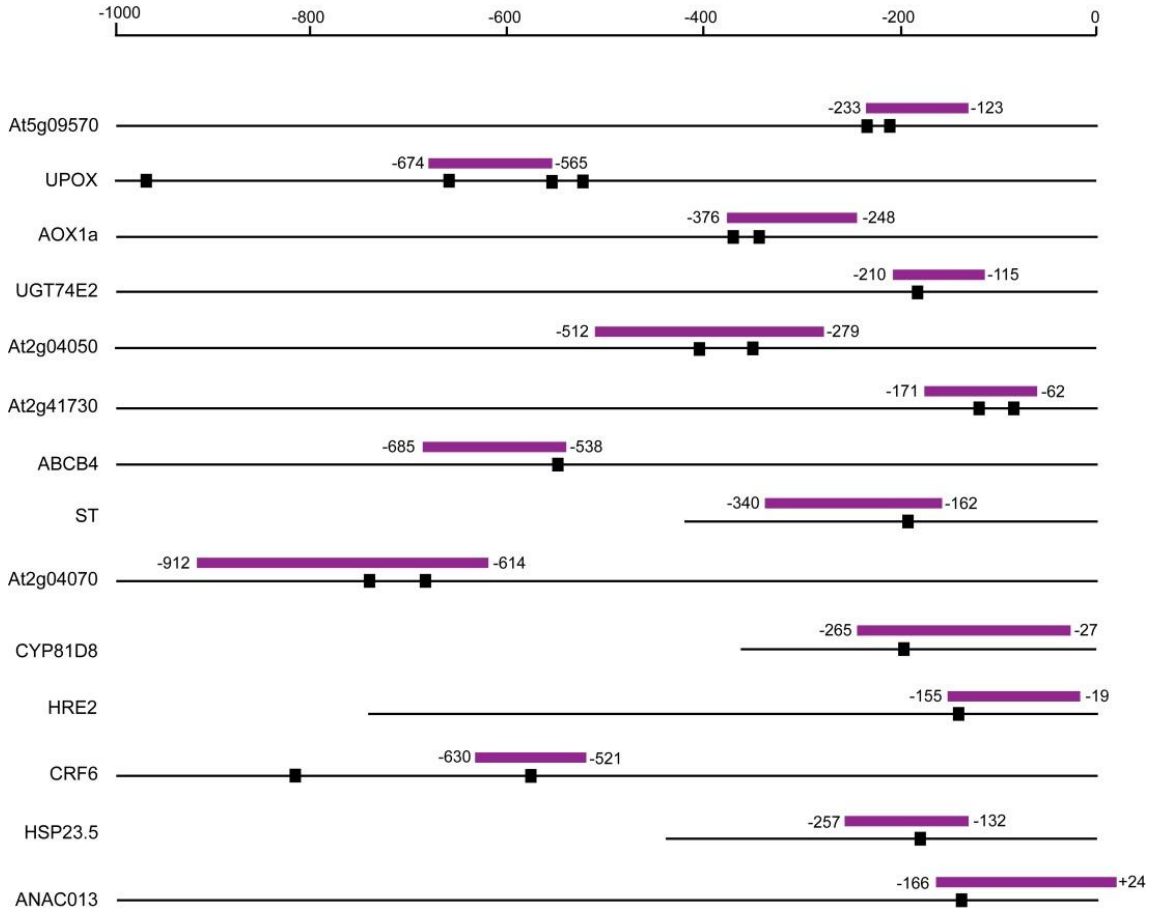
SUPPLEMENTAL DATA



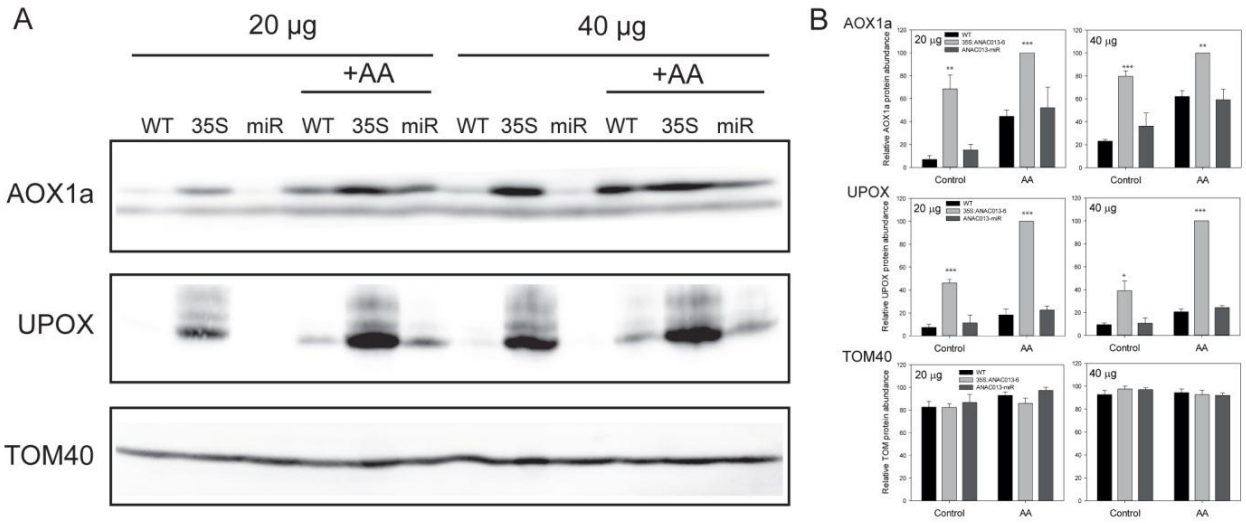
Supplemental Figure 2.1. Binding of NAC transcription factors with the MDM *in vitro* as shown by electrophoretic mobility shift assays. Purified NAC-GST proteins interact with radioactively-labeled probes of promoter regions of *UGT74E2*, *UPOX*, *AOX1a*, *At5g09570* and *At2G04050* containing the MDM element. Interactions were abolished in the presence of excess unlabeled competitor probes or when the MDM sequence was mutated.



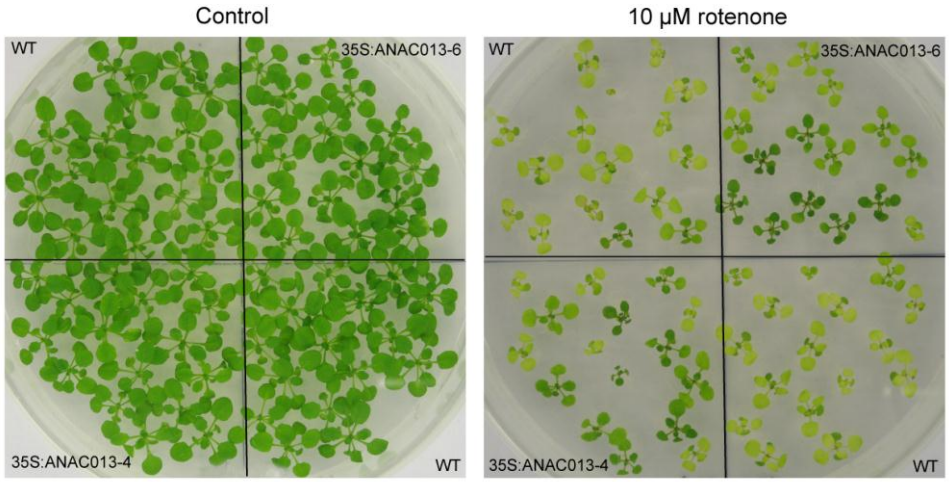
Supplemental Figure 2.2. Hierarchical clustering of the MRR up-regulated genes in the stress data set of CORNET, a compilation of biotic and abiotic stress conditions. Color codes in the heat map represent the actual $\log_2$-fold changes in treated plants relative to untreated plants. Conditions are indicated with a color code: cold (blue), heat (red), salt and osmotic (orange), drought (yellow), light and oxidative (white), chemical (purple), nutrient deprivation (brown), mechanical (black), pathogen (green), and other stresses and control conditions (white). The *MDR* genes containing the *cis*-regulatory MDM in their 1-kb upstream sequence are indicated with a green bar.
(A) Heatmap showing all selected stress conditions.
(B) Enlargement of a part of the heat map, indicated by a rectangle in panel A, containing the conditions under which the *MDR* genes are highly up-regulated and co-expressed.



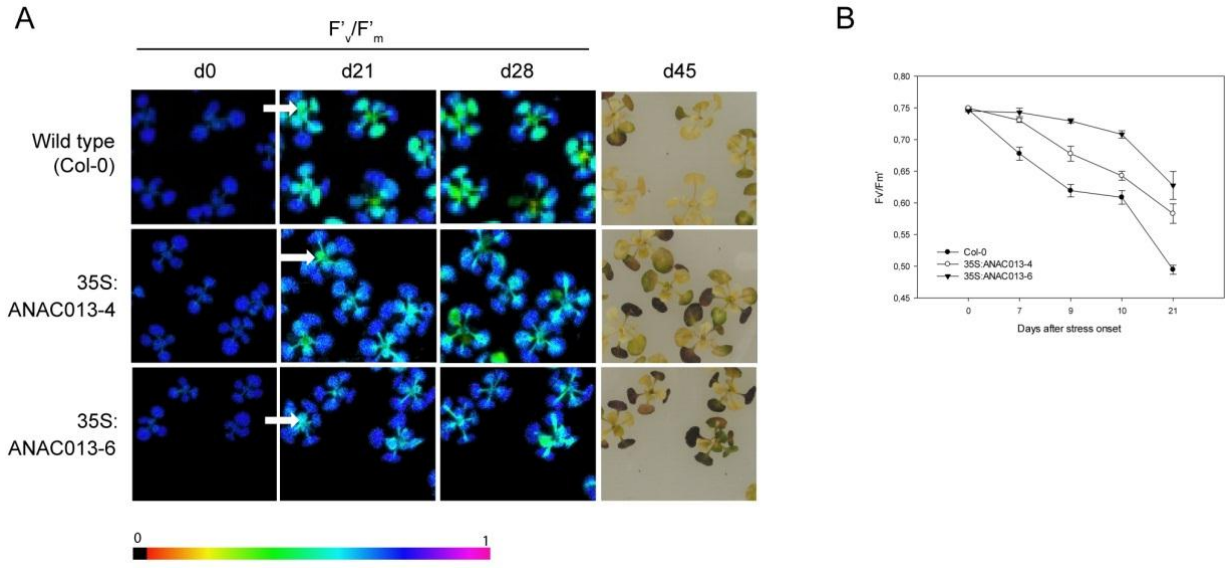
Supplemental Figure 2.3. Schematic overview of the amplicons used in ChIP-qPCR analyses. The MDM (black box) and the amplicons used in ChIP analyses (purple box) are displayed in the 1-kb upstream intergenic regions of the *MDR* genes.



Supplemental Figure 2.4. Increase in AOX1a and UPOX protein abundance by *ANAC013* overexpression.
(A) Protein gel blot analysis of isolated mitochondria from wild-type (WT), *35S:ANAC013-6*, and *ANAC013-miR* plants exposed to AA or mock-treated for 6 h. Mitochondrial proteins were detected with antibodies raised against AOX and UPOX, and against TRANSLOCASE OF THE OUTER MITOCHONDRIAL MEMBRANE 40 (TOM40) to show equal protein loading between the samples.
(B) Quantified protein abundance. Bars represent average band intensities of three biological repeat experiments ± SE (Student's *t* test; * $P<0.05$, ** $P<0.01$, and *** $P<0.001$).



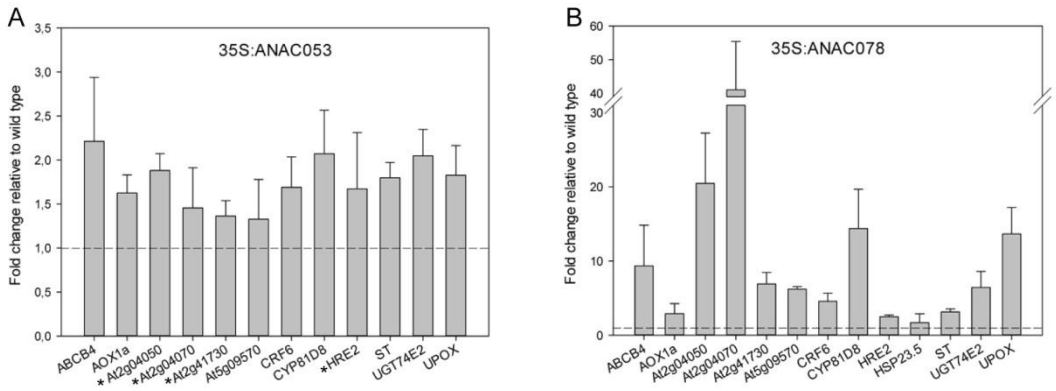
Supplemental Figure 2.5. Phenotype of *ANAC013*-overexpressing plants in the presence of the mitochondrial complex I inhibitor, rotenone. Three-week-old wild-type (WT) and ANAC013-overexpressing seedlings germinated and grown on MS medium supplemented with or without 10 µM rotenone.



Supplemental Figure 2.6. Increased photosynthetic performance of *ANAC013*-overexpressing plants after exposure to MV-mediated oxidative stress.

(A) Maximum quantum efficiency of PSII (F'_v/F'_m) in leaves (left) and photos (right) of wild-type (Col-0) and *35S:ANAC013* plants at different time points after transfer to 2 µM MV. The youngest leaves are the most affected by MV compared to the older leaves (arrows). d, days.

(B) Quantification of F'_v/F'_m of youngest leaves in wild-type and *35S:ANAC013* plants. Data indicate average ± SE (n = 5 plants for *35S:ANAC013*, n = 10 plants for Col-0).



Supplemental Figure 2.7. *MDR* gene expression regulated by ANAC053 and ANAC078 in Arabidopsis.
(A) Accumulation of *MDR* transcripts in (weak; 4-fold) ANAC053-overexpressing (*35S:ANAC053*) plants. Transcript abundance was analyzed with the Nanostring nCounter system, except for four genes (indicated with asterisk) for which the probes were not present on the CodSet and, hence, were analyzed with qRT-PCR. Bars represent average fold changes relative to wild-type plants from three biological replicates (± SE).
(B) Accumulation of *MDR* transcripts in *ANAC078*-overexpressing (*35S:ANAC078*; 57-fold) lines. Transcript abundance was analyzed with qRT-PCR. Bars represent average fold changes relative to wild-type plants ± SE (n = 2 biological replicates).

Supplemental Table 2.1. Overview of MRR microarray data sets used in the meta-analysis
[1], Clifton et al. (2005); [2], Van Aken et al. (2007); [3], Shedge et al. (2010); [4], Kühn et al. (2009); [5], Wang et al. (2012); [6], Kühn et al. (2011); [7], Meyer et al. (2009); [8], Giraud et al. (2008); [9], NCBI GEO database (Edgar et al., 2002), accession GSE4113; [10], Carrie et al. (2010); [11], Gleason et al. (2011).

Experiment	Mitochondrial target	Treatment	Mutation	Up[a]	Down[b]	Reference
Rotenone	Inhibition of complex I	40 μM rotenone (3 h)	-	430	391	[1]
Oligomycin	Inhibition ATP-synthase	0.125 μM oligomycin (3 h)	-	677	551	[1]
		1.25 μM oligomycin (3 h)	-	551	438	
Prohibitin	Loss or overexpression of prohibitin	-	*phb3*	141	112	[2]
		-	*phb4*	2	0	
		-	*35S:PHB3*	17	50	
		-	*35S:PHB4*	22	2	
MSH1 and RECA3	Mitochondrial genome rearrangement	-	*msh1 recA3*	34	1	[3]
RPOTmp	Loss of mitochondrial RNA polymerase	-	*rpoTmp-1*	22	36	[4]
			rpoTmp-2	67	136	
TIM23-2	Loss or overexpression of translocase of the inner membrane subunit 23-2	-	*tim23-2*	3	0	[5]
		-	*35S:TIM23-2*	70	26	
RUG3	Inhibition of complex I biogenesis	-	*rug3-1*	87	24	[6]
			rug3-2	214	111	
NDUFA1 and NDUFS4	Loss of complex I	-	*ndufa1*	98	48	[7]
			ndufs4	2	0	
AOX1a(1)	Loss of alternative oxidase	-	*aox1a-1*	0	0	[8]
			aox1a-2	0	0	
AOX1a(2)	Loss or overexpression of alternative oxidase		*35S:AOX1a*	0	0	[9]
			aox1a-3			
MIA40	Loss of mitochondrial intermembrane assembly 40	-	*mia40*	0	0	[10]
DSR1	Loss of complex II subunit succinate dehydrogenase 1-1 (SDH1-1)	1 mM salicylic acid (40 minutes)	*dsr1*	181	271	[11]

[a] Total number of significantly (log_2-fold >1; $P < 0.01$) up-regulated genes.
[b] Total number of significantly (log_2-fold <1; $P < 0.01$) down-regulated genes.

Supplemental Table 2.2. Presence of the MDM in the *MDR* gene set and its conservation in orthologous genes of related dicot species.

					Conservation		
Gene	Start[a]	End[b]	Strand[c]	Sequence (5'-3')[d]	Sequence	*P* value[f]	Species[g]
AT1G05060	-151	-139	-	aacCTTGgagatCAAGgtt	**CTTGNNNNNCA[AC]G**	<0.001***	Pt, Vv, Gm, Al
	-241	-229	+	caaCTTGtcaagAAAGtaa			
	-555	-543	+	ggaCTTGgaagaCAAAagg			
UGT74E2	-186	-174	+	catCTTGgtcgcCACGgaa	**CTTGNNNNNCA[AC]G**	0.172	Al
AT1G24095	-115	-103	+	ggaCTTGgagctCAAGcaa	NA		
ANAC013	-143	-131	-	ttcCTTGgagaaGAAGgtt	CTTGNNNNNGA[AC]G	0.359	Al
AT2G03130	-170	-158	+	ttgCTTGgagagCAAGttg	**CTTGNNNNNCA[AC]G**	0.259	Al
ST	-202	-190	-	ttaCTTGaagagCACGtag	CTTGNNNNNCACG	0.009	Gm, Al
	-122	-110	+	ttaCTTGtagaaCAACctt	CTTGNNNNNCAAC	0.043*	Pt, Cp
AT2G04050	-413	-401	-	ttaCTTGggaatCAAGttc	**CTTGNNNNNCA[AC]G**	0.044	Al
	-354	-342	-	ataCTTGgggacCAAGttc			
AT2G04070	-746	-734	-	ttaCTTGggaatCAAGttc	**CTTGNNNNNCA[AC]G**	0.038	Al
	-688	-676	-	acaCTTGgggatCACGttc			
UPOX	-658	-646	-	ctcCTTGgagagCAAGtta	**CTTGNNNNNCA[AC]G**	0.28	Al
	-968	-956	+	aacCTTGatgaaGAAGgaa			
	-569	-557	+	gtgCTTGcagagAAAGata			
	-532	-520	+	gaaCTTGcagagAAAGcaa			
AT2G32020	-247	-235	+	ttgTTTGgcaacCAAGtaa	TTTGNNNNNCA[AC]G	0.452	Al
AT2G41730	-127	-115	-	atgCTTGtccagCAAGata	**CTTGNNNNNCA[AC]G**	<0.001***	Pt, Cp, Vv, Gm, Al, Md
	-92	-80	-	atgTTTGgggtaCAAGaaa			
ABCB4	-551	-539	-	tttCTTGacgaaCAAGctg	**CTTGNNNNNCA[AC]G**	0.002**	Pt, Gm, Al, Md
HRE2	-154	-142	-	ttgCTTGacgacCAAGttc	**CTTGNNNNNCA[AC]G**	<0.001***	Vv, Gm, Al, Md
AOX1a	-372	-360	+	gctCTTGgcgacCACGcaa	**CTTGNNNNNCA[AC]G**	<0.001***	Pt, Vv, Al, Md
	-345	-333	-	tttCTTGgagagCAAGatg			
OXI1	-167	-155	-	tgcCTTGccgggGAAGatt	CTTGNNNNNGA[AC]G	0.031	Cp, Al
	-280	-268	-	agcCTTGtttaaAAAGtgt	CTTGNNNNNAA[AC]G	nd	
PHB4	-724	-712	-	taaCTTGgaaacCAAGtaa	CTTGNNNNNCAAG	0.035*	Pt, Vv
	-356	-344	-	cttCTTAgagagCAAGatg			
BCS1	-806	-794	-	atcCTTGaacaaCACCctt	CTTGNNNNNCA[AC]C	0.246	Md
CRF6	-574	-562	+	ttgCTTGgagacCAAGcta	**CTTGNNNNNCA[AC]G**	<0.001***	Pt, Cp, Al, Md
	-818	-806	-	ttgCTTGgagacCAAGtct			
CYP81D8	-205	-193	+	tttCTTGaggaaCAAGact	**CTTGNNNNNCA[AC]G**	nd	
AT5G09570	-222	-210	-	cccCTTGcccgtCACGttt	CTTGNNNNNCACG	0.011	Al, Md
	-238	-226	+	gggTTTGgagcgCAAGaaa			
AT5G14730	-729	-717	-	attCTTGattgaCAAGttg	**CTTGNNNNNCA[AC]G**	nd	
	-180	-168	-	ttaCTTGtgcagCAAGagg			

Supplemental Table 2.2. Presence of the MDM in the *MDR* gene set and its conservation in orthologous genes of related dicot species. (*Continued*).

AT5G43450	-306	-294	+	ttcCTTGcacaaCAC<u>A</u>aca	CTTGNNNNNCA[AC]<u>A</u>	nd	
	-155	-143	+	ttcCTTGcacaaCAC<u>A</u>aca			
HSP23.5	-189	-177	+	ttaCTTGgagacCACGcag	**CTTGNNNNNCA[AC]G**	<0.001***	Pt, Cp, Vv, Gm, Al
MGE1	-128	-116	-	gttCTTGgggag<u>G</u>AAGcaa			

[a, b] Upstream nucleotide position relative to the translational start codon of the respective gene.

[c] Forward strand (+), reverse strand (-).

[d] MDM sequence including 3-bp surrounding sequence. Capital letters represent the MDM consensus (CTTGNNNNNCA[AC]G) and nucleotides underlined differ from the consensus.

[e] For each Arabidopsis gene containing the MDM consensus sequence (CTTGNNNNNCA[AC]G), indicated in bold, the conservation data for this sequence are displayed. If the consensus is not significantly conserved but a (mismatch) variant of the consensus (not in bold) is conserved, conservation data of the latter are displayed.

[f] Sequences were defined as conserved if present in orthologs of at least two other species (not including *Arabidopsis lyrata* (Al)) with conservation *P* value less than 0.05, and are indicated with asterisk (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). nd, not detected.

[g] Species containing the motif in orthologues genes. Al, *Arabidopsis lyrata*; Cp: *Carica papaya*; Pt, *Populus trichocarpa*; Md, *Malus domestica*; Gm, *Glycine max*; Vv, *Vitis vinifera*.

Supplemental Table 2.3. Functional annotations of the *MDR* genes.

Gene ID	Other name	Description[a]
AT1G05060		
AT1G05680	*UGT74E2*	Uridine diphosphate glycosyltransferase 74E2 | Encodes a UDP-glucosyltransferase, UGT74E2, that acts on IBA (indole-3-butyric acid) and affects auxin homeostasis. The transcript and protein levels of this enzyme are strongly induced by H2O2 and may allow integration of ROS (reactive oxygen species) and auxin signaling. This enzyme can also transfer glycosyl groups to several compounds related to the explosive TNT when this synthetic compound is taken up from the environment.
AT1G24095		Putative thiol-disulfide oxidoreductase DCC
AT1G32870	*ANAC013*	
AT2G03130		Ribosomal protein L12/ATP-dependent Clp protease adaptor protein ClpS family protein
AT2G03760	*ST*	Sulfotransferase 12 | Encodes a brassinosteroid sulfotransferase. *In vitro* experiements show that this enzyme has a preference for 24-epibrassinosteroids, particularly 24-epicathasterone, but does not act on castasterone and brassinolide. It is differentially expressed during development, being more abundant in young seedlings and actively growing cell cultures. Expression is induced in response to salicylic acid and methyl jasmonate and bacterial pathogens.
AT2G04050		MATE efflux family protein
AT2G04070		MATE efflux family protein
AT2G21640	*UPOX*	Encodes a protein of unknown function that is a marker for oxidative stress response.
AT2G32020		Acyl-CoA N-acyltransferases (NAT) superfamily protein
AT2G41730		
AT2G47000	*ABCB4*	ATP binding cassette subfamily B4 | Multidrug resistance P-glycoprotein (MDR/PGP) subfamily of ABC transporters. Functions in the basipetal redirection of auxin from the root tip. Exhibits apolar plasma membrane localization in the root cap and polar localization in tissues above.
AT2G47520	*HRE2*	Integrase-type DNA-binding superfamily protein | encodes a member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 5 members in this subfamily including RAP2.2 AND RAP2.12.
AT3G22370	*AOX1a*	Alternative oxidase 1A | Encodes AOX1a, an isoform of alternative oxidase that is expressed in rosettes, flowers, and root. The alternative oxidase of plant mitochondria transfers electrons from the ubiquinone pool to oxygen without energy conservations. It is regulated through transcriptional control and by pyruvate. Plays a role in shoot acclimation to low temperature. Also is capable of ameliorating reactive oxygen species production when the cytochrome pathway is inhibited. AOX1a also functions as a marker for mitochondrial retrograde response.
AT3G25250	*OXI1*	AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein | Arabidopsis protein kinase
AT3G27280	*PHB4*	Part of protein complexes that are necessary for proficient mitochondrial function or biogenesis, thereby supporting cell division and differentiation in apical tissues
AT3G50930	*BCS1*	Cytochrome BC1 synthesis

Supplemental Table 2.3. Functional annotations of the *MDR* genes. (*Continued*).

AT3G61630	*CRF6*	Cytokinin response factor 6 | CRF6 encodes one of the six cytokinin response factors. CRF5 belongs to the AP2/ERF superfamily of the transcriptional factors. CRF proteins rapidly relocalize to the nucleus in response to cytokinin. Analysis of loos-of-function mutants revealed that the CRFs function redundantly to regulate the development of embryos, cotyledons and leaves.
AT4G37370	*CYP81D8*	Cytochrome P450, family 81, subfamily D, polypeptide 8 | member of CYP81D
AT5G09570		Cox19-like CHCH family protein
AT5G14730		
AT5G43450		2-Oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein | encodes a protein whose sequence is similar to ACC oxidase
AT5G51440	*HSP23.5*	HSP20-like chaperones superfamily protein
AT5G55200	*MGE1*	Co-chaperone GrpE family protein

[a]Gene functional annotations were obtained from TAIR10.

Supplemental Table 2.4. Arabidopsis genes containing conserved MDM motif instances.

Gene ID	Conservation *P* value[a]	Species[b]	Other name	Gene description
AT1G02520	<0.001	Pt, Gm, Al, Md	PGP11	P-glycoprotein 11
AT1G02530	<0.001	Pt, Gm, Al, Md	PGP12	P-glycoprotein 12
AT1G05060	<0.001	Pt, Vv, Gm, Aly		
AT1G07510	<0.001	Pt, Gm, Al, Md	FTSH10	FTSH protease 10 | encodes an FtsH protease that is localized to the mitochondrion
AT1G10800	<0.001	Pt, Gm, Aly		
AT1G28470	<0.001	Pt, Vv, Gm, Aly	SND3	NAC domain containing protein 10
AT1G32030	<0.001	Cp, Md		Domain of unknown function (DUF313)
AT1G49630	<0.001	Vv, Al, Md	PREP2	Zinc metalloprotease pitrilysin subfamily A. Signal peptide degrading enzyme targeted to mitochondria and chloroplasts. Expressed in flower, leaf and root. Not expressed in silique and shoot.
AT2G05360	<0.001	Pt, Md		
AT2G21830	<0.001	Cp, Al, Md		Cysteine/Histidine-rich C1 domain family protein
AT2G23660	<0.001	Pt, Cp, Aly	LBD10	
AT2G40370	<0.001	Pt, Vv, Gm, Aly	LAC5	Laccase 5 | putative laccase, a member of laccase family of genes (17 members in Arabidopsis).
AT2G41730	<0.001	Pt, Cp, Vv, Gm, Al, Md		
AT2G47520	<0.001	Vv, Gm, Al, Md	HRE2	Integrase-type DNA-binding superfamily protein | encodes a member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 5 members in this subfamily including RAP2.2 AND RAP2.12.
AT3G01600	<0.001	Pt, Vv, Gm, Aly	NAC044	NAC domain containing protein 44
AT3G03700	<0.001	Pt, Cp, Vv, Al, Md		Plasma-membrane choline transporter family protein
AT3G04440	<0.001	Pt, Cp, Vv, Al, Md		Plasma-membrane choline transporter family protein
AT3G09690	<0.001	Pt, Al, Md		
AT3G11020	<0.001	Pt, Cp, Vv, Gm, Al, Md	DREB2B	DRE/CRT-binding protein 2B | encodes a member of the DREB subfamily A-2 of ERF/AP2 transcription factor family (DREB2B). The protein contains one AP2 domain. There are eight members in this subfamily including DREB2A.
AT3G22360	<0.001	Pt, Al, Md	AOX1B	Alternative oxidase 1B | encodes an alternative oxidase whose expression is limited to flowers and floral buds.
AT3G22370	<0.001	Pt, Vv, Al, Md	AOX1A	Alternative oxidase 1A | Encodes AOX1a, an isoform of alternative oxidase that is expressed in rosettes, flowers, and root. The alternative oxidase of plant mitochondria transfers electrons from the ubiquinone pool to oxygen without energy conservations. It is regulated through transcriptional control and by pyruvate. Plays a role in shoot acclimation to low temperature. Also is capable of ameliorating reactive oxygen species production when the cytochrome pathway is inhibited. AOX1a also functions as a marker for mitochondrial retrograde response.
AT3G22630	<0.001	Pt, Vv, Al, Md	PRCGB	20S proteasome beta subunit D1 | Encodes 20S proteasome beta subunit PBD1 (PBD1).
AT3G24780	<0.001	Pt, Cp, Vv, Gm, Al, Md		Uncharacterised conserved protein UCP015417, vWA
AT3G24850	<0.001	Cp, Al, Md		Domain of unknown function (DUF313)

Supplemental Table 2.4. Arabidopsis genes containing conserved MDM motif instances. (*Continued*).

AT3G27880	<0.001	Pt, Gm, Al, Md		Protein of unknown function (DUF1645)
AT3G30210	<0.001	Pt, Gm, Aly	MYB121	Myb domain protein 121 | Encodes a putative transcription factor, member of the R2R3 factor gene family (MYB121).
AT3G59820	<0.001	Pt, Al, Md		
AT3G61630	<0.001	Pt, Cp, Al, Md	CRF6	Cytokinin response factor 6 | CRF6 encodes one of the six cytokinin response factors. CRF5 belongs to the AP2/ERF superfamily of the transcriptional factors. CRF proteins rapidly relocalize to the nucleus in response to cytokinin. Analysis of loos-of-function mutants revealed that the CRFs function redundantly to regulate the development of embryos, cotyledons and leaves.
AT3G62260	<0.001	Vv, Gm, Md		
AT4G05020	<0.001	Pt, Gm, Al, Md	NDB2	NAD(P)H dehydrogenase B2 (NDB2)
AT4G14800	<0.001	Pt, Vv, Al, Md	PBD2	Encodes 20S proteasome beta subunit PBD2 (PBD2).
AT4G18990	<0.001	Pt, Vv, Aly	XTH29	Xyloglucan endotransglucosylase/hydrolase 29
AT4G21490	<0.001	Pt, Gm, Al, Md	NDB3	NAD(P)H dehydrogenase B3
AT4G25200	<0.001	Pt, Cp, Vv, Gm, Aly	HSP23.6-M	Mitochondrion-localized small heat shock protein 23.6 | AtHSP23.6-mito mRNA, nuclear gene encoding mitochondrial
AT4G27110	<0.001	Pt, Cp, Vv, Al, Md	COBL11	COBRA-like protein 11 precursor
AT4G35460	<0.001	Vv, Al, Md	NTRB	NADPH-dependent thioredoxin reductase B | NADPH-dependent thioredoxin reductase 1 (NTR1. Similar to E.coli NTR and has conserved NADPH binding domains.
AT4G37030	<0.001	Cp, Vv, Gm, Md		
AT4G39740	<0.001	Vv, Gm, Al, Md	HCC2	Thioredoxin superfamily protein
AT5G02970	<0.001	Pt, Al, Md		α/β-Hydrolases superfamily protein
AT5G05390	<0.001	Pt, Vv, Gm, Aly	LAC12	Laccase 12 | putative laccase, a member of laccase family of genes (17 members in Arabidopsis).
AT5G13210	<0.001	Pt, Cp, Vv, Gm, Al, Md		Uncharacterised conserved protein UCP015417, vWA
AT5G17300	<0.001	Pt, Vv, Gm, Aly	RVE1	Homeodomain-like superfamily protein | Myb-like transcription factor that regulates hypocotyl growth by regulating free auxin levels in a time-of-day specific manner.
AT5G24050	<0.001	Cp, Al, Md		Domain of unknown function (DUF313)
AT5G24640	<0.001	Pt, Cp, Vv, Gm, Al, Md		
AT5G37140	<0.001	Vv, Gm, Al, Md		P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT5G40690	<0.001	Pt, Cp, Vv, Gm, Al, Md		
AT5G43390	<0.001	Pt, Cp, Vv, Gm, Al, Md		Uncharacterized conserved protein UCP015417, vWA
AT5G51430	<0.001	Cp, Al, Md	EYE	Conserved oligomeric Golgi complex component-related / COG complex component-related | Encodes a protein that is homologous to Cog7, a subunit of the conserved oligomeric Golgi (COG) complex, which is required for the normal morphology and function of the Golgi apparatus. It is likely to be involved in transport or retention of Golgi-localized proteins and in maintenance of Golgi morphology.
AT5G51440	<0.001	Pt, Cp, Vv, Gm, Aly	HSP23.5-M	HSP20-like chaperones superfamily protein
AT5G52090	<0.001	Vv, Gm, Al, Md		P-loop containing nucleoside triphosphate hydrolases superfamily protein

Supplemental Table 2.4. Arabidopsis genes containing conserved MDM motif instances. (*Continued*).

AT5G53290	<0.001	Pt, Al, Md	CRF3	Cytokinin response factor 3 | encodes a member of the ERF (ethylene response factor) subfamily B-5 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 7 members in this subfamily.
AT5G55490	<0.001	Pt, Cp, Vv, Gm, Al, Md	GEX1	Gamete expressed protein 1 | Encodes a transmembrane domain containing protein that is expressed in pollen germ cells.
AT1G05370	0.002	Cp, Al, Md		Sec14p-like phosphatidylinositol transfer family protein
AT1G49900	0.002	Pt, Gm, Md		C2H2 type zinc finger transcription factor family
AT2G47000	0.002	Pt, Gm, Al, Md	PGP4	ATP binding cassette subfamily B4 | Multidrug resistance P-glycoprotein (MDR/PGP) subfamily of ABC transporters. Functions in the basipetal redirection of auxin from the root tip. Exhibits apolar plasma membrane localization in the root cap and polar localization in tissues above.
AT5G16930	0.002	Pt, Al, Md		AAA-type ATPase family protein
AT5G37160	0.002	Vv, Gm, Al, Md		P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT3G13080	0.003	Pt, Vv, Gm, Al, Md	MRP3	Encodes an ATP-dependent MRP-like ABC transporter able to transport glutathione-conjugates as well as chlorophyll catabolites. The expression of this gene is upregulated by herbicide safeners such as benoxacor and fenclorim.
AT5G24920	0.003	Pt, Md	GDU5	Glutamine dumper 5 | Encodes a member of the GDU (glutamine dumper) family proteins involved in amino acid export: At4g31730 (GDU1), At4g25760 (GDU2), At5g57685 (GDU3), At2g24762 (GDU4), At5g24920 (GDU5), At3g30725 (GDU6) and At5g38770 (GDU7).
AT1G06330	0.004	Pt, Vv, Aly		Heavy metal transport/detoxification superfamily protein
AT5G09590	0.004	Cp, Gm, Aly	MTHSC70-2	Mitochondrial HSO70 2 | heat shock protein 70 (Hsc70-5); nuclear
AT2G16060	0.005	Vv, Gm, Aly	NSHB1	Hemoglobin 1 | Encodes a class 1 nonsymbiotic hemoglobin induced by low oxygen levels with very high oxygen affinity. It is not likely to be a hemoglobin transporter because of its extremely high affinity for oxygen.
AT3G18610	0.005	Pt, Al, Md	PARLL1	Nucleolin like 2 | Encodes ATNUC-L2 (NUCLEOLIN LIKE 2).
AT4G37910	0.005	Cp, Gm, Aly	MTHSC70-1	Mitochondrial heat shock protein 70-1
AT1G74870	0.006	Pt, Al, Md		RING/U-box superfamily protein
AT5G38800	0.006	Pt, Al, Md	BZIP43	Basic leucine-zipper 43
AT3G28580	0.007	Pt, Vv, Md		P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT5G25840	0.007	Cp, Al, Md		Protein of unknown function (DUF1677)
AT1G64930	0.009	Gm, Al, Md	CYP89A7	Cytochrome P450, family 87, subfamily A, polypeptide 7 | member of CYP89A
AT5G14980	0.009	Pt, Vvi		α/β-Hydrolases superfamily protein
AT1G64900	0.01	Gm, Al, Md	CYP89A2	Cytochrome P450, family 89, subfamily A, polypeptide 2 | Encodes cytochrome P450 (CYP89A2).
AT3G23570	0.013	Gm, Al, Md		α/β-Hydrolases superfamily protein
AT2G37280	0.014	Pt, Cp, Vv, Gm, Md	PDR5	Pleiotropic drug resistance 5
AT2G29930	0.017	Gm, Al, Md		
AT2G31460	0.018	Cp, Md		Domain of unknown function (DUF313)
AT1G09400	0.019	Gm, Al, Md		FMN-linked oxidoreductases superfamily protein
AT1G48400	0.019	Cp, Al, Md		F-box/RNI-like/FBD-like domains-containing protein

Supplemental Table 2.4. Arabidopsis genes containing conserved MDM motif instances. (*Continued*).

AT4G27740	0.021	Cp, Vvi		Yippee family putative zinc-binding protein
AT2G07714	0.023	Vv, Al, Md		Transcription factor-related
AT3G21180	0.044	Pt, Cp, Gm, Md	ATACA9	Autoinhibited Ca(2+)-ATPase 9 | one of the type IIB calcium pump isoforms. encodes an autoinhibited Ca(2+)-ATPase that contains an N-terminal calmodulin binding autoinhibitory domain.

[a]Evolutionary conservation of the MDM consensus (CTTGNNNNNCA[AC]G) was examined in six related dicot species. The MDM consensus was defined as conserved if present in orthologs of at least two other species (not including *Arabidopsis lyrata* (Aly)) with conservation *P* value less than 0.05.

[b]Species containing the MDM consensus in orthologues genes. Al, *Arabidopsis lyrata*; Cp, *Carica papaya*; Pt, *Populus trichocarpa*; Md, *Malus domestica*; Gm, *Glycine max*; Vv, *Vitis vinifera*.

Supplemental Table 2.5. Probes used for electrophoretic mobility shift assays.

Probe	Sequence[a]
AOX1a_MDM1+2_FWD	CTAGCTCTTGGCGACCACGCAAGTATCTTCCATCTTGCTCTCCAAGAAAA
AOX1a_MDM1+2_REV	TTTTCTTGGAGAGCAAGATGGAAGATACTTGCGTGGTCGCCAAGAGCTAG
AOX1a_MDM1+2_mut12_FWD	CTAGCT**GC**T**A**GCGAC**T**A**GC**G**G**AGTATCTTCCAT**GCTT**CTCTC**TC**AGAAAA
AOX1a_MDM1+2_mut12_REV	TTTT**C**T**GA**GAGAGA**A**A**GC**ATGGAAGATACT**CC**G**C**T**A**GTCGC**T**A**GC**AGCTAG
At2g04050_MDM_FWD	GATTATACGAACTTGGTCCCCAAGTATATTTCTTG
At2g04050_MDM_REV	CAAGAAATATACTTGGGGACCAAGTTCGTATAATC
At2g04050_MDM_mut_FWD	GATTATACGAA**GT**A**C**GTCCC**TTC**GTATATTTCTTG
At2g04050_MDM_mut_REV	CAAGAAATATA**C**G**AA**GGGAC**GT**A**C**TTCGTATAATC
UGT74E2_MDM_FWD	GGTCACTGTTCCGCACATCTTGGTCGCCACGGAACATAGACAATTTTTGG
UGT74E2_MDM_REV	CCAAAAATTGTCTATGTTCCGTGGCGACCAAGATGTGCGGAACAGTGACC
UGT74E2_MDM_mut_FWD	GGTCACTGTTCCGCACAT**TGAC**GTCGC**GCAT**GAACATAGACAATTTTTGG
UGT74E2_MDM_mut_REV	CCAAAAATTGTCTATGTTC**ATGC**GCGAC**GTCA**ATGTGCGGAACAGTGACC
UPOX_MDM_FWD	GCGTTTAGTAACTTGCTCTCCAAGGAGATCTAGCTT
UPOX_MDM_REV	AAGCTAGATCTCCTTGGAGAGCAAGTTACTAAACGC
UPOX_MDM_mut_FWD	GCGTTTAGTAA**GT**T**A**CTCTC**TG**A**C**GAGATCTAGCTT
UPOX_MDM_mut_REV	AAGCTAGATCTC**GT**C**A**GAGAG**T**AA**C**TTACTAAACGC
At5g09570_MDM_FWD	GTTTGGAGCGCAAGAAACGTGACGGGCAAGGGGAATATTCTCATCTAGAG
At5g09570_MDM_REV	CTCTAGATGAGAATATTCCCCTTGCCCGTCACGTTTCTTGCGCTCCAAAC
At5g09570_MDM_mut_FWD	GTTTGGAGCGCAAGAAA**TCGT**ACGGG**TGCT**GGGAATATTCTCATCTAGAG
At5g09570_MDM_mut_REV	CTCTAGATGAGAATATTCCC**AGCA**CCCGT**ACGA**TTTCTTGCGCTCCAAAC

[a]MDM is underlined in the probe sequences. Mutations in MDM are indicated in bold.

Supplemental Table 2.6. Overrepresented Gene Ontology terms in the *MDR* gene set and in the gene set containing all Arabidopsis genes with conserved MDM motif instances.

GO category		GO enrichment		
GO label	GO description	*P* value	Fold	%
MDR (24 genes)				
GO:0015238	Drug transmembrane transporter activity	0.0026	49.9	14
GO:0042221	Response to chemical stimulus	0.0024	4.3	50
GO:0051238	Sequestering of metal ion	0.0014	498.0	9
GO:0050896	Response to stimulus	0.01	3.1	59
GO:0015691	Cadmium ion transport	0.02	131,6	9
All Arabidopsis genes with conserved MDM motif instances (80 genes)[a]				
GO:0008559	Xenobiotic-transporting ATPase activity	6.22E-5	165.4	5
GO:0015239	Multidrug transporter activity	6.22E-5	165.4	5
GO:0042910	Xenobiotic transporter activity	6.22E-5	165.4	5
GO:0016682	Oxidoreductase activity, acting on diphenols and related substances as donors, oxygen as acceptor	6.27E-5	58.9	6
GO:0016887	ATPase activity	1.11E-4	8.3	15
GO:0030554	Adenyl nucleotide binding	0.01	2.9	26
GO:0009916	Alternative oxidase activity	0.01	147.0	3
GO:0003677	DNA binding	0.02	2.7	24
GO:0071365	Cellular response to auxin stimulus	5.15E-5	39.4	8
GO:0010315	Auxin efflux	1.14E-4	190.0	5
GO:0010038	Response to metal ion	7.51E-4	6.5	16
GO:0010540	Basipetal auxin transport	9.22E-4	101.8	5
GO:0042221	Response to chemical stimulus	0.0036	2.8	32
GO:0046686	Response to cadmium ion	0.01	6.6	13
GO:0007585	Respiratory gaseous exchange	0.02	147.0	3
GO:0031930	Mitochondrial signaling pathway	0.02	147.0	3
GO:0042991	Transcription factor import into nucleus	0.02	147.0	3
GO:0045333	Cellular respiration	0.03	15.9	6
GO:0044429	Mitochondrial part	3.19E-4	10.3	11
GO:0031314	Extrinsic to mitochondrial inner membrane	0.0020	221.3	3
GO:0048046	Apoplast	0.0041	6.9	11
GO:0005739	Mitochondrion	0.01	3.6	18
GO:0005759	Mitochondrial matrix	0.02	24.1	5
GO:0031980	Mitochondrial lumen	0.02	24.1	5

[a]All Arabidopsis genes with significantly ($P<0.05$) conserved instances of the MDM consensus CTTGNNNNNCA[AC]G in at least two other dicot species (not including *Arabidopsis lyrata*).

Supplemental Table 2.7. PCR primers used.

Primer	Sequence
Cloning	
Promoters	
attB4_ProUGT74E2_FWD	GGGGACAACTTTGTATAGAAAAGTTGATTTCACCCATGATATACTGTC
attB1r_ProUGT74E2_REV	GGGGACTGCTTTTTTGTACAAACTTGTTTCTCCTTCTTTTTAATCTTGTG
ProUGT74E2_ΔMDM_FWD	CGGTCACTGTTCCGCACATGAACATAGACAATTTTTGG
ProUGT74E2_ΔMDM_REV	CCAAAAATTGTCTATGTTCATGTGCGGAACAGTGACCG
attB4_ProAOX1a_FWD	ATAGAAAAGTTGATCTGAAGAGCTTCTAGC
attB1r_ProAOX1a_REV	TGTACAAACTTGTGTTTCAAATCGGAAAAAGTG
ProAOX1a_ΔMDM1_FWD	GCGACCACGCAAGTATCTTCCATAAAAATCTACACCGGCTTTAAATTTAC
ProAOX1a_ΔMDM1_REV	GTAAATTTAAAGCCGGTGTAGATTTTTATGGAAGATACTTGCGTGGTCGC
ProAOX1a_ΔMDM2_FWD	GACCAATAAGAATCTAGCTCAAGTATCTTCCATCTTGC
ProAOX1a_ΔMDM2_REV	GCAAGATGGAAGATACTTGAGCTAGATTCTTATTGGTC
attB1_ProANAC013_FWD	AAAAAGCAGGCTGTAAATTTTTCAGATGAAAGTATT
attB2_ProANAC013_REV	AGAAAGCTGGGTCACTTTTTTCTCTCTCGTTATC
Electrophoretic mobility shift assays and biolistic transformations	
ANAC013_FWD	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGACTTGTCGGTTGAGAACGG
ANAC013_NOSTOP_REV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATCCCCATAACAAAGGCCTCCCTGAAC
ANAC013_ΔTM_NOSTOP_REV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATCCTTTGTTCTTCTTCTTCTTCTTATC
ANAC013_TM_FWD	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGCTGGAATCCATTCCTGCAAAGCC
ANAC013_ΔTM_REV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATCCTCATTTGTTCTTCTTCTTCTTCTTATC
ANAC017_FWD	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGCGGATTCTTCACCCGA
ANAC017_short_REV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATCMTCTGTTTCTAATACCCTTCTTTGC
ANAC053_FWD	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGGTCGTGGCTCAGTAACATC
ANAC053_ΔTM_REV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATCCTCATGCTGAACTATTCCCTGATTTT
ANAC078_FWD	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGGGTCGTGGCTCAGTGACGTC
ANAC078_ΔTM_REV	GGGGACCACTTTGTACAAGAAAGCTGGGTCTCCACCTCCGGATCCTCATCTTGTCGCCGTAATTCCTGTCT
Overexpressing and -miR lines	
ANAC013_35S_FWD	AAAAAGCAGGCTCCACCATGGACTTGTCGGTTGAGAA
ANAC013_35S_REV	AGAAAGCTGGGTCTCACCATAACAAAGGCCTCC
ANAC053_35S_FWD	AAAAAGCAGGCTCCACCATGGGTCGTGGCTCAGTAAC
ANAC053_35S_REV	AGAAAGCTGGGTCTCACCTGGAAGAGACCAAAATG
ANAC078_35S_FWD	AAAAAGCAGGCTCCACCATGGGTCGTGGCTCAGTG
ANAC078_35S_REV	AGAAAGCTGGGTCTTACCGAGCAGACACCATG
ANAC013-I miR-s	gaTGTAACGGTAAGTCCGTCGTTtctctctttgtattcc
ANAC013-II miR-a	gaAACGACGGACTTACCGTTACAtcaaagagaatcaatga
ANAC013-III miR*s	gaAAAGACGGACTTAGCGTTACTtcacaggtcgtgatatg
ANAC013-IV miR*a	gaAGTAACGCTAAGTCCGTCTTTtctacatatatattcct
qRT-PCR	
ANAC013_RT_FWD	ACCAGACAGATAAACAATGGATCA
ANAC013_RT_REV	CAGAAGGAACAGGGTTTAGGAA
ANAC016_RT_L	ATTCACTTCACAGTCAACAGGTG
ANAC016_RT_R	GCTGATGAGAACTGGCTCCT
ANAC017_RT_L	CATTTGCTTCACCCTCATCA
ANAC017_RT_R	CCTTGGGCATTCACACTCAT
ANAC053_RT_155_L	GCAACAGAGTTTGAGCCAGA
ANAC053_RT_155_R	GCAGGAATAGCACCCAACAT
ANAC078_RT_L	CGATATTGATGACATTGACGAGA
ANAC078_RT_R	CTTGATTCCCCATGACAATAGTT
ABCB4_RT_FWD	CAAAGTCTCCAAAGTTGCTCTG
ABCB4_RT_REV	CTCGCTGCTTGTCTCTCTCC
AOX1a_RT_FWD	TGGTTGTTCGTGCTGACG
AOX1a_RT_REV	CACGACCTTGGTAGTGAATATCAG
ARP7_RT_FWD	ACTCTTCCTGATGGACAGGTG
ARP7_RT_REV	CTCAACGATTCCATGCTCCT
at2g04050_RT_FWD	CCACAATGGTGAGCTCCAG
at2g04050_RT_REV	CACCCGCTAACCCAAACA
at2g04070_RT_FWD	CTCCAGCTCTCCGGTGTC
at2g04070_RT_REV	GTGAACCCACTAACCCAAACA
AT2G28390_FWD	AACTCTATGCAGCATTTGATCCACT
AT2G28390_REV	TGATTGCATATCTTTATCGCCATC
at2g41730_RT_FWD	GTCACCAAGGCATCGTAAGG
at2g41730_RT_REV	AAAGCTGGTGGTGAATCGAG

Supplemental Table 2.7. PCR primers used. (*Continued*).

at5g09570_RT_FWD	GAAACCGTTGTTTCTCAGGTTC
at5g09570_RT_REV	CCAAAATGGTTGACGCAAT
CRF6_RT_FWD	TGGCTTGGGACTTTTGTCA
CRF6_RT_REV	GAGATGAATCGCGGCTCTA
CYP81D8_RT_L	CGTCTTTCTCGGAACTTTTCA
CYP81D8_RT_R	AACACCGTCTCCGTAGTAACG
HRE2_RT_FWD	GAAGCGTAAACCCGTCTCAGT
HRE2_RT_REV	AATCTCCGCTGCCCATTT
HSP23.5_RT_FWD	TCAAACCGACATGTTTCTCG
HSP23.5_RT_REV	AAGCTTCTCGTTGGAGTAAACG
ST_RT_FWD	GGTCACCAATCCACACCTTC
ST_RT_REV	CGAAATCTGGGGACTCGTAG
UGT74E2_RT_FWD	TAACTTCTTCCACACTTCTCATAATCT
UGT74E2_RT_REV	ACAACAAAAACTAGAGTCAGTAACAAC
UPOX_RT_FWD	TTCAAAAACACCATGGACAAGA
UPOX_RT_REV	GCCTCAATTTGCTTCTCTGC
ChIP-qPCR	
ABCB4_ChIP_FWD	ACGCCAATTGTGCTATGGTTACA
ABCB4_ChIP_REV	TCTTGACGAACAAGCTGTGACTTG
ANAC013_ChIP_FWD	CGTGTCGTCTCTGTAAGCCAAACCT
ANAC013_ChIP_REV	TCCGTTCTCAACCGACAAGTCCATC
AOX1a_ChIP_FWD	AGCTCTTGGCGACCACGCAA
AOX1a_ChIP_REV	CCCTTGTGGTCATGAGAGAGACT
AT2g04050_ChIP_FWD	GTGAGTCACGTGTTTGCACTGTGT
AT2g04050_ChIP_REV	GTGGACGTTGTGGTCAACAGTCAT
AT2G04070_ChIP_FWD	CAGCCTCAGCTGCACGTTTGA
AT2G04070_ChIP_REV	ACGTCGTCGTCAACACTCAACA
AT2G41730_ChIP_FWD	TGCCGACAAAAACAAAAGGTAGACA
AT2G41730_ChIP_REV	TGGGGCTTACTAAATATGTTTGGGG
AT5G09570_ChIP_FWD	AGCGCAAGAAACGTGACGGG
AT5G09570_ChIP_REV	GCGGTGAAGACCACGGGAAGA
CRF6_ChIP_FWD	TAGCCGGCGCGTGTAGCAAA
CRF6_ChIP_REV	GTTACCGTACCGAAAGTAGAGCCAA
CYP81D8_ChIP_FWD	TGTTGAACACGTGGAGGGCATT
CYP81D8_ChIP_REV	GCAGTTTTGGAAACTTTTGTGGGGT
HRE2_ChIP_FWD	ACTTGGTCGTCAAGCAAAAACAGC
HRE2_ChIP_REV	ACGCTTCCAACTCCAAACTAGAACA
HSP23.5_ChIP_FWD	GCCCCAAAGTGATGGGCCG
HSP23.5_ChIP_FWD	AGCTGCTAGAGGCTAGAGAGTCG
ST_ChIP_FWD	AGGTTGTTGACTTGTTGTGATGGAC
ST_ChIP_REV	GGTGGTGAAATGGTCAACACATCCT
UGT74E2_ChIP_FWD	GACCTCGGTCACTGTTCCGCA
UGT74E2_ChIP_REV	ACTACGGCTTTGGTGGGGGA
UPOX_ChIP_FWD	ACGCTGCGTTTAGTAACTTGCTC
UPOX_ChIP_REV	GCAAGCACAAGGACGACG
ACTIN2_ChIP_FWD	ACTACGAGCAGGAGATGGAAACCT
ACTIN2_ChIP_REV	GCAGCTTCCATTCCCACAAACGAG
CDKA1_ChIP_FWD	CTTACTCTCAATTAGTCAATCCCC
CDKA1_ChIP_REV	GATGTAGTGTCAGTGAGACTAGC
UBQ10_ChIP_FWD	CTATTGCTTCACCGCCTTAGC
UBQ10_ChIP_REV	GATCACGGTAGAGAGAATTGAGAG

SUPPLEMENTAL METHODS

Statistical analyses

Analysis of wild-type and MDM-deleted promoter activities

Log_{10}-transformed luminescence values were analyzed according to the following model:

$$y_{ijkl} = \mu + G_i + C_j + G_i * C_j + L_k(G_i) + C_j * L_k(G_i) + \varepsilon_{ijkl}$$

Where y_{ijkl} is the log_{10}-transformed luminescence value of biological repeat l of independent transgenic line k of genotype i under condition j. μ represents an overall mean, G is the main genotype effect, C is the main condition effect, G*C is the interaction effect between genotype and condition, L is the random effect for the independent transgenic line nested under genotype, C*L is the random interaction term between condition and independent transgenic line, and ε is the error term. $L_k(G_i)$, $C_j*L_k(G_i)$, and ε_{ijkl} were all assumed to be normally distributed random variables with zero means and variance components $\sigma^2_{L(G)}$, $\sigma^2_{C*L(G),}$ and σ^2_{ε}, respectively. These random effects were assumed to be independent with each other. For the random effects, those models with the lowest Akaike's Information Criterion values were chosen. Significance of the fixed interaction term was tested with a Wald test. Residual analysis was performed to verify the model assumptions. Post hoc comparisons were corrected for multiple testing with Bonferroni. Within each condition, two-sided comparisons were made between the genotypes (promoter deletion line and wild-type line). All analyses were performed with the mixed model procedure in SAS Enterprise Guide 5.1 (SAS Institute Inc., 2012, Cary, North Carolina).

Transcript profiling of wild-type and 35S:ANAC013-6 lines under nonstressed conditions

Prior to the analysis, average expression values were taken of the three qRT-PCR technical repeats. To the expression values that also contained values between 0 and 1, the value 1 was added prior to transformation (Osborne, 2010). Significant differences in log_2 expression values between the *35S:ANAC013-6* and the wild type were assessed with a two-sample *t* test for each gene. Equality of variance was assessed with the Folded F-statistic. In the case of equal variances, the pooled method was used, in the case of unequal variances the Welch-Sattherthwaite method was used. The analyses were performed with the *t* test procedure in SAS Enterprise Guide 5.1 (SAS Institute Inc., 2012, Cary, North Carolina).

Transcript profiling of wild-type and ANAC013-miR lines under nonstressed and AA stress conditions

The log_2-transformed average values of the three technical repeats (see above) were further analyzed according to following general linear model for each gene separately:

$$y_{ijk} = \mu + G_i + C_j + G_i * C_j + \varepsilon_{ijk}$$

Where y_{ijk} is the log_2-transformed expression value of biological repeat k of genotype i under condition j. μ represents an overall mean, G is the main genotype effect, C is the main condition effect, G*C is the interaction term between genotype and condition and ε is the error term. ε_{ijk} was assumed to be a normally distributed random variable with zero mean and variance component σ^2_ε. Residual analysis was performed to verify the model assumptions. For each condition, two-sided comparisons were made between the two genotypes (wild type (Col-0) and *ANAC013-miR*). The analyses were performed with the glm procedure in SAS Enterprise Guide 5.1 (SAS Institute Inc., 2012, Cary, North Carolina).

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ADDENDUM

The *MDR* is potentially required for maintenance of mitochondrial function and cell proliferation under stress in young developing leaves

The complete version of this chapter has been published in **Plant Physiology, 152:** 226-244, January 2010 by **Skirycz, A., De Bodt, S., Obata, T., De Clercq, I., Claeys, H., De Rycke, R., Andriankaja, M., Van Aken, O., Van Breusgem, F., Fernie, A.R., and Inzé, D**. under the title "Developmental stage specificity and the role of mitochondrial metabolism in the response of Arabidopsis leaves to prolonged mild osmotic stress".

I.D.C. selected the genes, generated the transgenic promoter:GUS Arabidopsis lines and performed the spatiotemporal analysis of the *MDR* expression by means of histochemical GUS assays.

The *MDR* is specifically induced in proliferating tissues during mild osmotic stress

When subjected to adverse environmental conditions, plants induce numerous adaptation responses to minimize the harmful effects, such as the production of protective proteins or antioxidants. Another important adaptation mechanism is the reprogramming of growth. After the onset of stress, growth rates decrease rapidly, followed by growth recovery and adaptation to the new condition (Skirycz and Inze, 2010). These growth changes allow plants to save and redistribute resources that can become limited.

To gain insight into the process of growth adaptation during stress, a mild osmotic stress assay was developed in which plants were germinated and grown on 25 mM of mannitol, that decreases the water potential of the growth medium. In this experimental setup, plant growth was reduced (50% reduced leaf size) (Figure 2.12A and 2.12B), but other stress parameters such as seed germination, leaf morphology and the operating efficiency of photosystem II (PSII) were not affected (data not shown). To identify the mechanisms underlying the 50% reduction in the final leaf size, the third leaf that initiates and subsequently develops under stress conditions was used for kinematic analysis. Early during development, cell number and cell size were reduced by the stress, but cell division and expansion rates reached that of nonstressed plants within a few days of leaf initiation (Figure 2.12C and 2.12D).

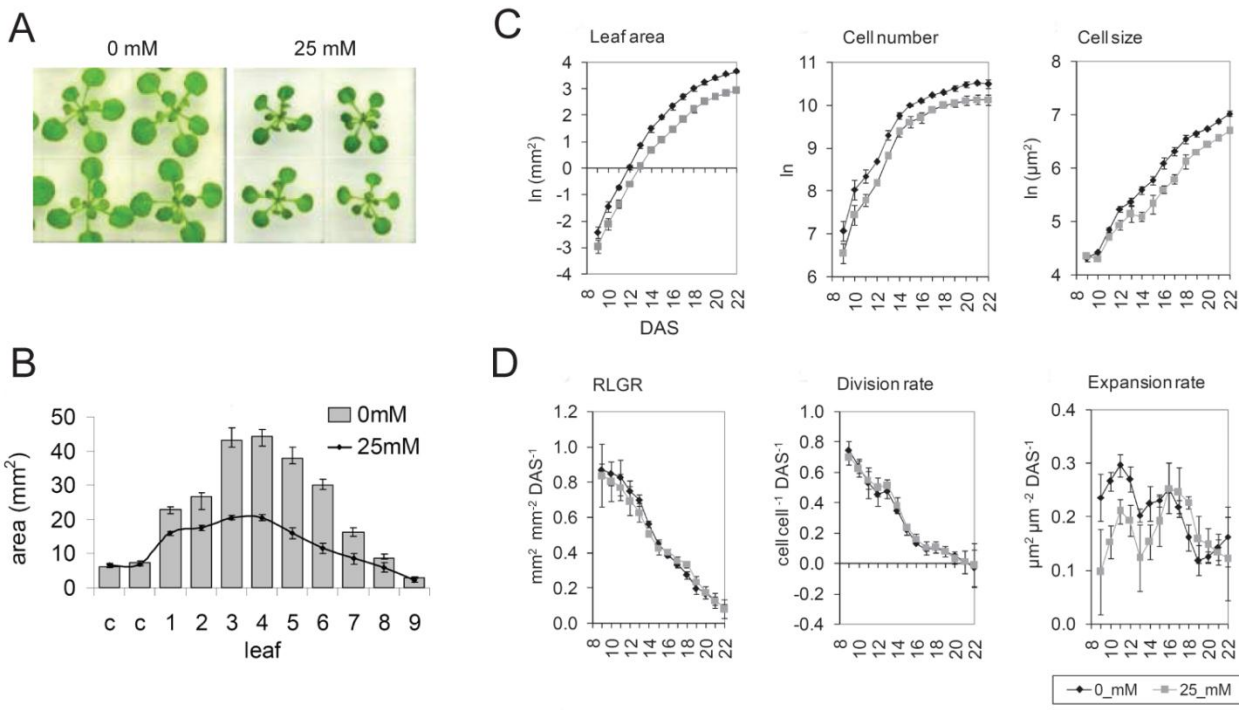

Figure 2.12. Mild osmotic stress experimental setup.
(A) Plants at 22 days after stratification (DAS) grown in the absence (left) or presence (right) of 25 mM mannitol.
(B) Leaf area calculation at 22 DAS grown on 0 and 25 mM mannitol.
(C) and **(D)** Kinematic analysis of leaf 3 from plants grown with or without 25 mM mannitol, showing leaf area, cell number and cell size (C) and relative leaf growth rate (RLGR), relative cell division rate and relative cell expansion rate (D).

To investigate the molecular basis of the growth adaptation, the transcriptional response to mild osmotic stress was analyzed during different developmental stages: exclusively proliferating leaf primordia (P) and expanding (E) and mature (M) leaves (Figure 2.13A). Mild osmotic stress significantly altered the transcriptome, with hundreds of transcripts affected exclusively in one leaf stage and only very few genes that were differentially expressed across the three stages (Figure 2.13B). Overall, the stress response in P and E leaves was more similar, while in fully grown M leaves it was more distinct, especially when compared with P leaves.

Among the 170 genes that were induced by mannitol exclusively in the P leaves (Supplemental Table 2.7), 13 of the *MDR* (mitochondrial dysfunction regulon) genes were among the most strongly up-regulated genes (Figure 2.14A). To confirm these changes and to provide further spatial resolution, promoter:GUS reporter lines were constructed for eight of the 13 *MDR* genes (*At2g03760* [*ST*], *At2g04050*, *At3g61630* [*CRF6*], *At2g21640* [*UPOX*], *At4g37370* [*CYP81D8*], *At2g41730*, *At5g51440* [*HSP23.5*] and *At1g32870* [*ANAC013*]). The obtained GUS

staining agreed very well with the microarray data, confirming induction for seven out of the eight genes in the P or P and E leaves (Figure 2.14B and 2.14C). At the spatial level, GUS induction was not just observed in fully P leaves but also in leaves that were partially P (i.e. that had already started to differentiate at their tip). Moreover, this analysis allowed the response of other leaves to be studied. Induction was not restricted to leaf 3 but could be observed in young leaves throughout plant development.

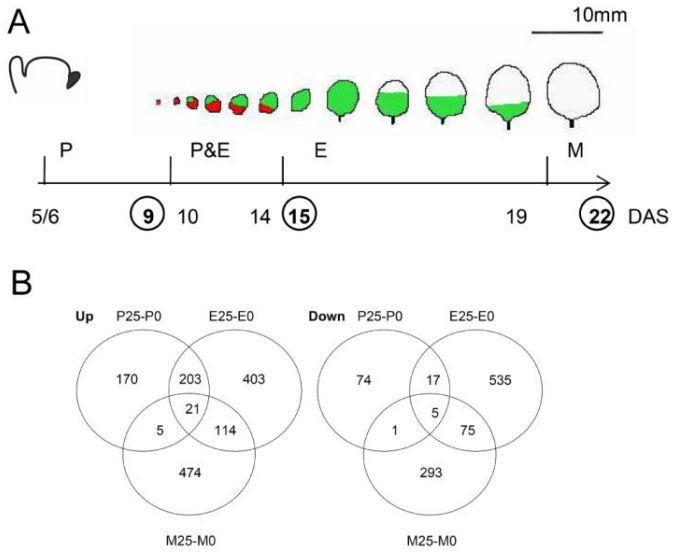

Figure 2.13 Transcript changes by mild osmotic stress during different developmental stages.
(A) Experimental setup. Schematic representation of Arabidopsis leaf development. Proliferating (P) cells, red; expanding (E) cells, green; and mature (M) cells, white. The scale bar only applies to the leaves, not the representation of the shoot apical meristem. Leaf 3 initiates at approximately 5 days after stratification (DAS); all cells proliferate at 9 DAS, expand exclusively around 15 DAS, and approach maturity at 22 DAS, both under control and stress conditions. Samples for transcript profiling analysis were dissected at 9, 15 and 22 DAS.
(B) Venn diagram grouping of genes differentially regulated by osmotic stress in P, E and M leaves.

Alternative respiration is important for cell division under stress conditions

As transcripts of the mitochondrial dysfunction regulon (*MDR*) were affected in the proliferating (P) leaves, the mitochondria of the shoot apical meristem (SAM) and P leaves were examined for phenotypic changes by means of transmission electron microscopy. A significant difference in the SAM sections of the mannitol-grown seedlings was the presence of large mitochondria. In addition, in both SAM and P leaf sections, mitochondria were rounder, which could be expressed as increased circularity index (Figure 2.15A and 2.15B).

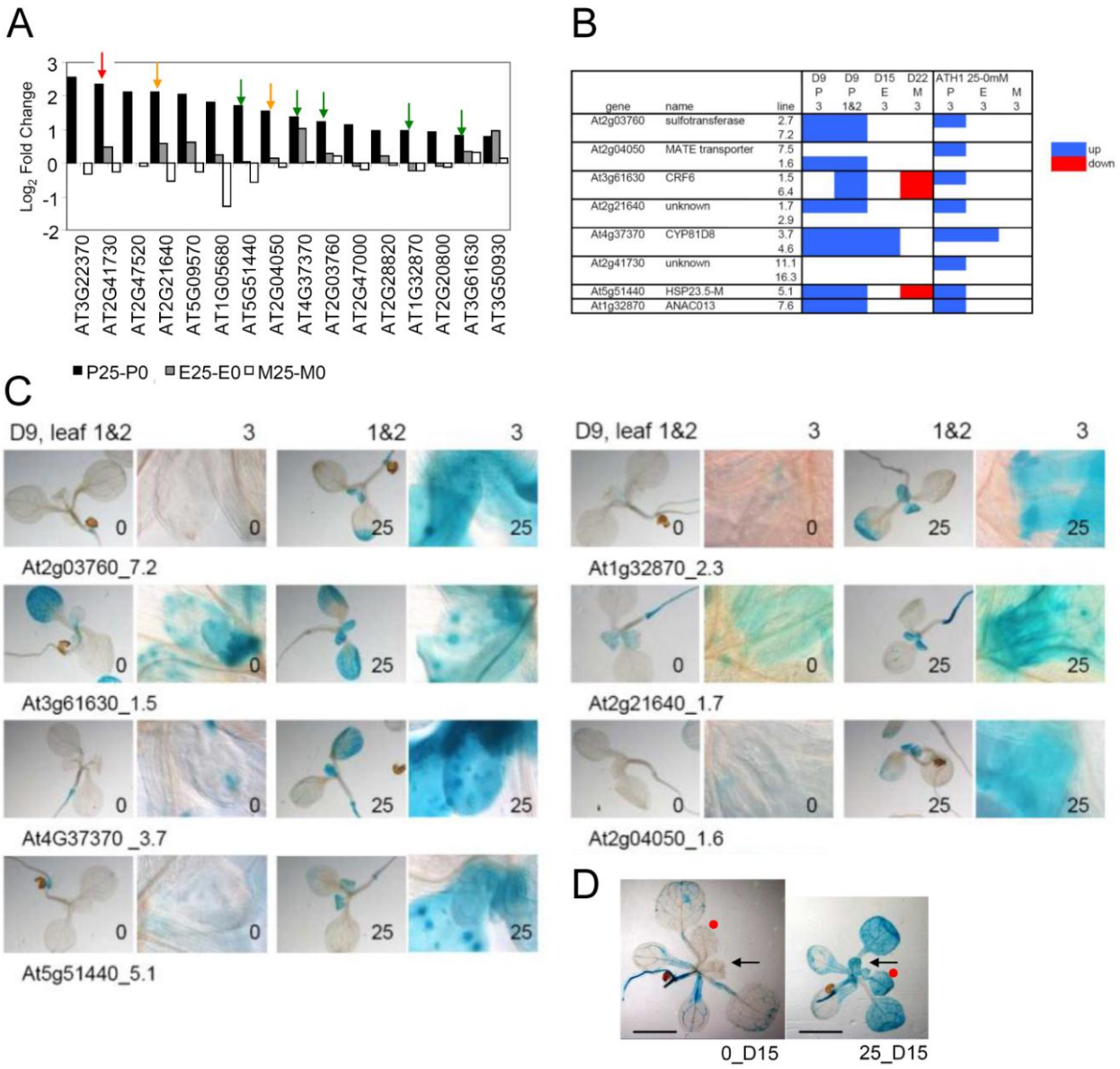

Figure 2.14. The *MDR* is induced by mild osmotic stress in young, proliferating leaves.
(A) Log_2-fold change of *MDR* genes in long-term mannitol experiments measured in P, E and M leaves. Arrows indicate genes used for promoter:GUS analysis; green, orange, and red arrows indicate expression validated by GUS staining in two independent GUS lines, in one of two independent GUS lines, and not validated, respectively.
(B) Promoter:GUS analysis to score the activation of eight *MDR* promoters in leaves of mannitol-grown plants. GUS staining was assessed in proliferating, expanding and mature leaf 3 from 9-, 15- and 22-DAS-old-plants, respectively. In addition to leaf 3, leaves 1 and 2 from 9-DAS-old plants were used to score staining at the proliferating stage. Induction of the *MDR* genes in proliferating leaves was confirmed, except for *At2g41730*. For *CYP81D8*, induction was also observed in expanding leaf 3 (see panel D).
(C) Representative photos are presented. 25, mannitol; 0, control.
(D) Photographs of 15-DAS-old plants of the *ProCYP81D8:GUS* line. The arrows mark the young leaves. The red dots indicate the expanding leaf 3.

Among the *MDR* genes, the alternative oxidase *AOX1a* gene was strongly induced in the P leaves, together with the alternative NADH dehydrogenases *NDB4* and *NDB2* that are also part of the alternative respiratory chain (Van Aken et al., 2009). Alternative oxidases bypass the normal mitochondrial electron transport chain (mtETC) by transferring electrons directly to oxygen and are mainly employed under stress conditions that impair the mtETC, thereby preventing mtROS formation. Moreover, together with alternative NADH dehydrogenases, alternative oxidase provides a path to recycle cytosolic NAD^+, supporting glycolysis and ATP synthesis via substrate phosphorylation, or to recycle NAD^+ required for the tricarboxylic acid (TCA) cycle and, hence, nucleotide and amino acid synthesis, respectively (Fernie et al., 2004). To determine whether the alternative respiration plays a role in growth adaptation, the growth of *AOX1a*-overexpressing plants (*35S:AOX1a*) was measured under normal and mild osmotic stress (25 mM mannitol) conditions. Leaf areas (of leaf number 3) were recorded daily between 9 and 23 days after stratification (DAS), and the obtained data were used to calculate the percentage of reduction of leaf area caused by the stress. Under nonstressed conditions, the leaf area of *35S:AOX1a* plants was reduced by 20% during the early developmental stages, but caught up with wild-type plants by 19 DAS (Figure 2.15C). However, during stress, growth of *35S:AOX1a* plants was less reduced very early during leaf development (Figure 2.15D).

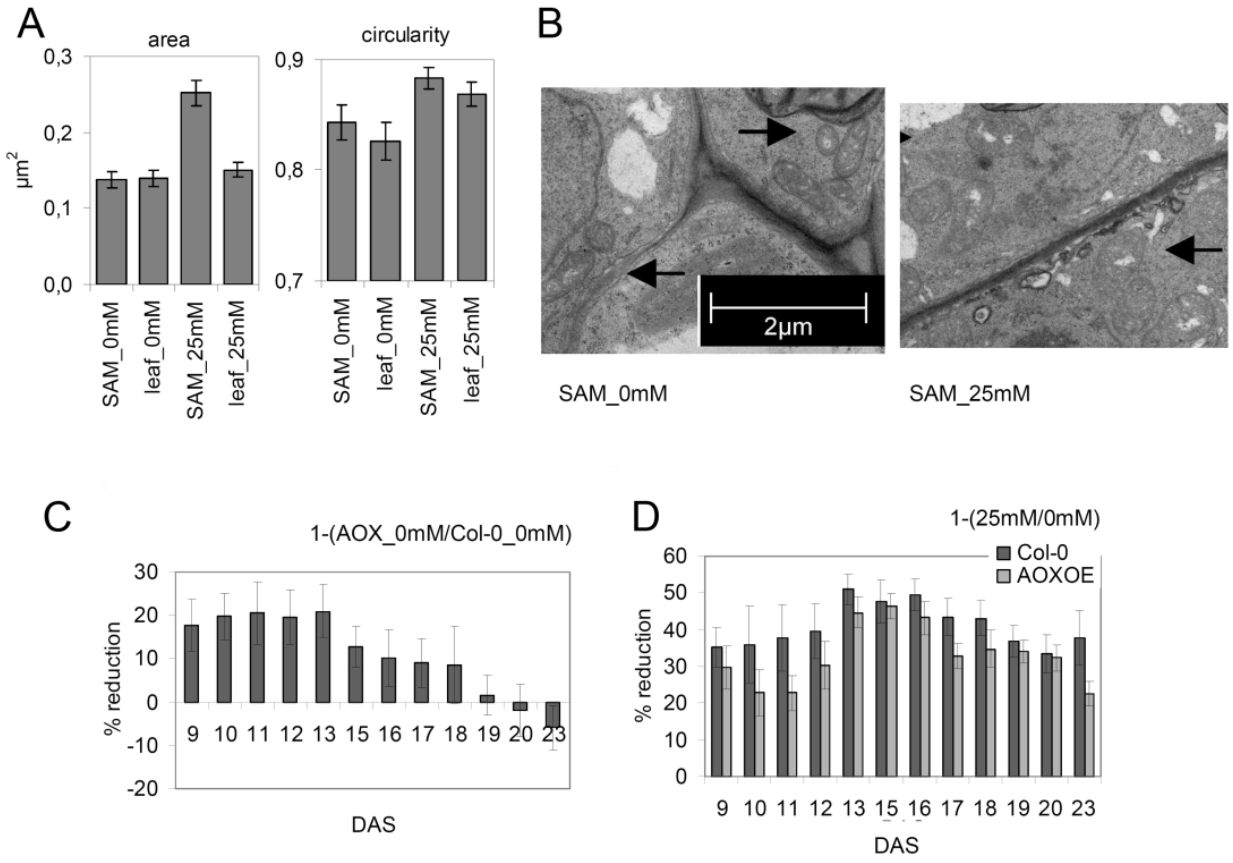

Figure 2.15. Importance of alternative respiration in P leaves during stress.
(A) Transmission electron micrographs of control- and mannitol-grown plants (P leaf 3 and SAM) were used to calculate area and circularity of mitochondria. Data are means ± SE of 40 to 60 mitochondria.
(B) Transmission electron micrographs of SAM from control- and mannitol-grown seedlings. Arrows point to mitochondria clusters.
(C) and **(D)** Leaf area measured for leaf 3 dissected from wild-type (Col-0) and *35S:AOX1a* seedlings grown without or with 25 mM mannitol from 9 DAS until 23 DAS. Obtained data were used to calculate percentage reduction under control conditions (C) and stress conditions (D). Data are means ± SE of eight to 10 leaves.

Taken together, we observed the induction of the mitochondrial dysfunction regulon as well as an altered mitochondrial morphology in proliferating leaves during stress. This, together with the decreased stress-mediated growth inhibition of *35S:AOX1a* plants during early development support the role of the alternative respiration in maintenance of mitochondrial function and cell division rates in young leaves during stress. As energy, nucleotides and amino acids are indispensable for cell division and ROS can cause serious damage to replicating DNA, alternative respiration would certainly be of central importance for growth maintenance in proliferating leaves under stress. Smaller and larger rosettes of *35S:AOX1a* and *aox1a* (data not shown) under

control conditions, respectively, indicate that the mtETC is a preferable energy source under favorable conditions and alternative oxidation under stress conditions. Although the function of the alternative oxidase in the stress response is becoming increasingly clear (Fiorani et al., 2005; Giraud et al., 2008), further experiments will be needed to elucidate the function of the other *MDR* genes during adverse conditions.

METHODS

Plant growth

Seedlings of Arabidopsis (*Arabidopsis thaliana* ecotype Columbia-0 [Col-0]) were grown *in vitro* in half-strength Murashige and Skoog (1962) medium supplemented with 1% sucrose under a 16-h-day (110 μmol m^{-2} s^{-1}) and 8-h-night regime. Before autoclaving, 25 mM mannitol (Sigma) was added to the agar medium.

Growth analysis

Leaf 3 was harvested daily from 9 to 22 DAS from eight to 10 plants in three independent experiments. After clearing with 70% ethanol, leaves were mounted in lactic acid on microscope slides. Epidermal cells (40–100 cells) were drawn for four leaves with a DMLB microscope (Leica) fitted with a drawing tubus and a differential interference contrast objective, while leaves were photographed under a binocular. Photographs of leaves and drawings were used to measure the leaf area and the cell size, respectively, with the ImageJ software. Leaf area and cell size were subsequently used to calculate cell numbers. Means of leaf area, cell size, and cell number were transformed logarithmically and locally fitted to a quadratic function of which the first derivative was taken as the relative growth rate (De Veylder et al., 2001). Mean values of the three biological experiments were used for statistical analysis.

Sampling for profiling analysis

Leaf 3 was harvested from plants at 9, 15, and 22 DAS. All samples were from three independent experiments and from multiple plates within the experiment. Complete harvest was done in growth chambers starting at 2 h into the day and took less than 15 min. As leaf initiation and developmental timing were not affected, samples were harvested simultaneously from both control and mannitol-treated plants. Because of their small size, leaves from plants at 9 DAS were dissected under a binocular microscope. Briefly, whole seedlings were harvested in an excess of RNAlater solution (Ambion) and, after overnight in 4°C, dissected on the cooling plate using the binocular microscope with precision microscissors. Dissected leaves were transferred to a new tube, frozen in liquid nitrogen,

and ground with a Retsch machine and 3-mm metal balls. Microarray analysis, qRT-PCR confirmation, and metabolite measurements were carried out on material harvested from separate experiments.

RNA extraction

RNA was extracted with Trizol according to the manufacturer's protocol (Invitrogen) and 4 μg of glycogen as carrier during the precipitation step. RNA samples were subjected to DNA digestion (Roche) and subsequently to the RNeasy clean-up kit (Qiagen).

ATH1 expression profiling and data analysis

RNA samples (three biological replicates for each treatment and stage) were hybridized to single Affymetrix ATH1 genome arrays at the Flanders Institute for Biotechnology Microarray Facility in Leuven, Belgium. Expression data were processed with Robust Multichip Average background correction, normalization, summarization, and BioConductor (Irizarry et al., 2003a; Irizarry et al., 2003b; Gentleman et al., 2004). An alternative cdf (tinesath1cdf) was used, in which each probe is uniquely assigned to one transcript (Casneuf et al., 2007); http://www.bioconductor.org/packages/release/data/experiment/html/tinesath1cdf.html). BioConductor package Limma was used to identify differentially expressed genes (Smyth, 2004). A factorial design (mannitol treatment 25–0 mm and developmental stage P-E-M) was applied to analyze the data. For comparisons of interest, moderated t statistics with the eBayes method were used, and P values were corrected for multiple testing (for each contrast separately using topTable) (Hochberg and Benjamini, 1990). In addition, we applied a more stringent correction for multiple testing across genes and across contrasts with decideTest. Importantly, lists of significant genes obtained with both methods were very similar, leading to identical conclusions. Besides the moderated t statistics for each pairwise contrast, we calculated global F statistics to identify the genes that were affected in at least one contrast. Two-fold changes in expression (only for 0–0 mm comparisons) and decideTest (global) cutoffs of less than 0.05 were used to delineate gene lists of interest. Further subsets of these differentially expressed gene lists were identified and subjected to overrepresentation analysis.

GUS staining

Whole plantlets were harvested after 9, 15, and 22 d and incubated in 90% acetone (4°C) for 30 min, washed in 100 mM Tris-HCl/50 mM NaCl (pH 7.0), and subsequently incubated in 5-bromo-4-chloro-3-indolyl-β-glucuronide (X-gluc) buffer (100 mM Tris-HCl/50 mM NaCl buffer [pH 7.0], 2 mM $K_3[Fe(CN)_6]$, and 4 mM X-gluc) at 37°C for 2.5 h. Seedlings were washed in 100 mM Tris-HCl/50 mM NaCl (pH 7.0) and cleared overnight in 90% lactic acid. Samples were photographed under a differential interference contrast microscope (Leica).

Transmission electron microscopy

Young leaves and SAM of 9-DAS seedlings were excised, immersed in a fixative solution of 2% paraformaldehyde and 2.5% glutaraldehyde, and postfixed in 1% OsO_4 with 1.5% $K_3Fe(CN)_6$ in 0.1 m Na-cacodylate buffer (pH 7.2) for 1 h under vacuum infiltration at room temperature and 4 h of rotation at room temperature, followed by overnight

fixation at 4°C. After washing three times for 20 min with the buffer, samples were dehydrated through a graded ethanol series, including a bulk staining with 2% uranyl acetate at the 50% ethanol step, followed by embedding in Spurr's resin. Ultrathin sections of a gold interference color were cut with an ultramicrotome (Leica EM UC6), poststained with uranyl acetate and lead citrate (Leica EM AC20), collected on formvar-coated copper slot grids, and viewed with a transmission electron microscope (1010; JEOL).

Transgenic Lines and Mutants

Seeds of *35S:AOX1a* lines were kindly provided by Prof. James E. Siedow (Duke University; Fiorani et al., 2005). For generation of promoter:GFP-GUS lines, genomic DNA was isolated from Arabidopsis (Col-0) with DNeasy plant kits (Qiagen) according to the manufacturer's instructions. The 1.5-kb genomic regions (or the intergenic region in the case of nearby coding sequences) upstream of the specific start codon were amplified by PCR with the Platinum Taq High Fidelity DNA polymerase (Invitrogen) and the forward and reverse primers (Supplemental Table 2.8). The PCR products were cloned into pDONR221 and cloned by recombination to pBGWFS7, generating a transcriptional GFP-GUS fusion. The constructs were transformed into Arabidopsis Col-0 by *Agrobacterium tumefaciens*-mediated floral dipping. Transformants with the bar resistance gene were selected by spraying with 40 mg L^{-1} Pestanal (glufosinate ammonium; Sigma-Aldrich). Transformants with a single insertion locus, and subsequently homozygous lines, were selected by segregation analysis on MS medium containing 5 mg L^{-1} glufosinate ammonium. All analyses were performed with nonsegregating homozygous T3 transgenic lines. *aox1a* mutants were obtained from the Arabidopsis Seed Stock Center (N584897).

SUPPLEMENTAL DATA

Supplemental Table 2.7. Genes specifically up-regulated by mild osmotic stress in proliferating leaves.
P, proliferating leaves; 25, 25 mM mannitol; 0, control condition; FC, fold change; FDR, false discovery rate.

Gene ID[a]	Other name	Log_2 FC P25-P0	FDR *P* value	Gene description
AT3G22370	AOX1a	2,57	4,22E-06	ATAOX1A, AOX1A | AOX1A (alternative oxidase 1A); alternative oxidase
AT2G41230	ORS1	2,45	1,01E-03	similar to ARL (ARGOS-LIKE) [*Arabidopsis thaliana*] (TAIR:AT2G44080)
AT2G41730		2,34	2,81E-04	
AT3G10930		2,28	2,40E-10	
AT1G65490		2,27	1,19E-06	
AT2G47520	HRE2	2,11	2,04E-04	AP2 domain-containing transcription factor, putative
AT2G21640	UPOX	2,11	2,53E-04	
AT5G09570		2,04	2,26E-04	
AT1G05680	UGT74E2	1,81	6,41E-03	UDP-glucoronosyl/UDP-glucosyl transferase family protein
AT3G14990		1,76	7,09E-04	4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein, putative
AT5G51440	HSP23.5	1,70	1,93E-02	23.5 kDa mitochondrial small heat shock protein (HSP23.5-M)
AT4G35110		1,66	4,20E-06	
AT2G44080	ARL	1,61	1,55E-03	ARL | ARL (ARGOS-LIKE)
AT1G22890		1,57	2,70E-05	
AT2G04050		1,54	8,97E-04	MATE efflux family protein
AT5G52760		1,52	3,56E-03	heavy-metal-associated domain-containing protein
AT4G23270		1,50	5,31E-04	protein kinase family protein
AT5G64905	PROPEP3	1,49	7,40E-03	PROPEP3 | PROPEP3 (Elicitor peptide 3 precursor)
AT3G51920	CAM9	1,47	4,15E-09	CAM9 | CAM9 (CALMODULIN 9); calcium ion binding
AT5G13330	RAP2.6L	1,33	5,31E-04	RAP2.6L | RAP2.6L (related to AP2 6L); DNA binding / transcription factor
AT5G44580		1,30	7,53E-04	
AT3G29970		1,29	4,22E-04	germination protein-related
AT2G03760	ST	1,25	2,10E-03	RAR047, ST | ST (steroid sulfotransferase); sulfotransferase
AT2G47000	ABCB4	1,13	4,47E-03	PGP4 | PGP4 (P-GLYCOPROTEIN 4, P-GLYCOPROTEIN4); ATPase, coupled to transmembrane movement of substances / xenobiotic-transporting ATPase
AT3G23250	MYB15	1,13	5,48E-06	MYB15, AtY19, AtMYB15 | AtMYB15/AtY19/MYB15 (myb domain protein 15); DNA binding / transcription factor
AT4G28460		1,09	5,73E-04	
AT2G43610		1,08	3,45E-03	glycoside hydrolase family 19 protein
AT2G16060	GLB1	1,06	3,61E-02	ARATH GLB1, GLB1, NSHB1, ATGLB1, AHB1 | AHB1 (ARABIDOPSIS HEMOGLOBIN 1)
AT5G17330	GAD1	1,00	5,68E-02	GAD1, GAD | GAD (Glutamate decarboxylase 1); calmodulin binding
AT1G36180		0,99	1,82E-02	
ATMG01370		0,97	6,70E-03	ORF111D | Identical to Hypothetical mitochondrial protein AtMg01370 (ORF111d) [*Arabidopsis Thaliana*] (GB:P92565)
AT2G28820		0,97	5,61E-03	structural constituent of ribosome
AT1G32870	ANAC013	0,96	1,09E-03	ANAC013 | ANAC013 (Arabidopsis NAC domain containing protein 13); transcription factor
AT3G54150		0,96	1,12E-04	embryo-abundant protein-related
AT4G18170	WRKY28	0,95	1,50E-02	ATWRKY28, WRKY28 | WRKY28 (WRKY DNA-binding protein 28); transcription factor
AT2G20800	NDB4	0,93	2,25E-02	NDB4 | NDB4 (NAD(P)H DEHYDROGENASE B4); NADH dehydrogenase

Supplemental Table 2.7. Genes specifically up-regulated by mild osmotic stress in proliferating leaves. (*Continued*).

AT2G22470	AGP2	0,92	7,09E-04	ATAGP2, AGP2 | AGP2 (ARABINOGALACTAN-PROTEIN 2)
AT1G31580	ECS1	0,91	8,37E-05	CXC750, ECS1 | ECS1
AT5G24280		0,90	8,11E-03	ATP binding
AT4G13510	ATAMT1	0,90	6,85E-04	ATAMT1, ATAMT1;1, AMT1;1 | AMT1;1 (AMMONIUM TRANSPORT 1); ammonium transporter
AT1G63840		0,89	7,92E-03	zinc finger (C3HC4-type RING finger) family protein
AT4G05020	NDB2	0,87	7,92E-03	NDB2 | NDB2 (NAD(P)H DEHYDROGENASE B2); disulfide oxidoreductase
AT4G36040		0,86	8,98E-03	DNAJ heat shock N-terminal domain-containing protein (J11)
AT5G04340		0,85	5,61E-03	C2H2 | C2H2; nucleic acid binding / transcription factor/ zinc ion binding
AT2G03130		0,85	3,94E-02	ribosomal protein L12 family protein
AT2G26650	AKT1	0,84	6,24E-03	AKT1 | AKT1 (ARABIDOPSIS K TRANSPORTER 1); cyclic nucleotide binding / inward rectifier potassium channel
AT3G61630	CRF6	0,83	3,00E-02	CRF6 | CRF6 (CYTOKININ RESPONSE FACTOR 6); DNA binding / transcription factor
AT5G53550	YSL3	0,82	1,82E-02	YSL3 | YSL3 (YELLOW STRIPE LIKE 3); oligopeptide transporter
AT2G32190		0,81	5,23E-04	
AT1G71697	ATCK1	0,79	4,76E-04	CK, ATCK1 | ATCK1 (CHOLINE KINASE)
AT1G26930		0,78	3,23E-02	kelch repeat-containing F-box family protein
AT2G15480	UGT73B5	0,78	4,66E-02	UGT73B5 | UGT73B5 (UDP-glucosyl transferase 73B5); UDP-glycosyltransferase/ transferase, transferring glycosyl groups
AT1G23390		0,77	1,49E-02	kelch repeat-containing F-box family protein
AT3G60520		0,76	1,31E-02	zinc ion binding
ATCG00590	ORF31	0,74	6,18E-03	ORF31 | similar to cytochrome b6/f complex subunit VI [Gossypium hirsutum] (GB:YP_538953); contains InterPro domain Cytochrome B6-F complex subunit VI; (InterPro:IPR007802)
AT5G65640	BHLH093	0,74	3,53E-02	BHLH093 | BHLH093 (BETA HLH PROTEIN 93); DNA binding / transcription factor
AT1G01560	ATMPK11	0,74	8,11E-03	ATMPK11 | ATMPK11 (*Arabidopsis thaliana* MAP kinase 11); MAP kinase/ kinase
AT2G36460		0,73	1,22E-02	fructose-bisphosphate aldolase, putative
AT4G28290		0,72	1,46E-03	
AT1G04310	ERS2	0,71	3,13E-02	ERS2 | ERS2 (ETHYLENE RESPONSE SENSOR 2); receptor
AT5G47220	ERF2	0,71	3,00E-02	ATERF2, ATERF-2, ERF2 | ATERF-2/ATERF2/ERF2 (ETHYLENE RESPONSE FACTOR 2); DNA binding / transcription factor/ transcriptional activator
AT2G33710		0,71	2,47E-02	AP2 domain-containing transcription factor family protein
AT1G21010		0,70	1,61E-02	
AT2G30140		0,70	5,39E-02	UDP-glucoronosyl/UDP-glucosyl transferase family protein
AT1G53345		0,69	1,00E-03	
AT5G14780	FDH	0,69	4,37E-02	FDH | FDH (FORMATE DEHYDROGENASE); NAD binding / cofactor binding / oxidoreductase, acting on the CH-OH group of donors, NAD or NADP as acceptor
AT1G70410		0,68	3,79E-03	carbonic anhydrase, putative / carbonate dehydratase, putative
AT5G05140		0,68	7,24E-02	transcription elongation factor-related
AT1G13260	RAV1	0,67	4,52E-02	RAV1 | RAV1 (Related to ABI3/VP1 1); DNA binding / transcription factor
AT1G24090		0,66	5,49E-02	RNase H domain-containing protein
AT1G58420		0,65	2,36E-03	
AT4G23880		0,64	3,07E-03	
AT3G02840		0,64	1,11E-02	immediate-early fungal elicitor family protein
AT1G71040		0,64	6,57E-03	multi-copper oxidase type I family protein
AT1G11330		0,64	1,19E-02	S-locus lectin protein kinase family protein

Supplemental Table 2.7. Genes specifically up-regulated by mild osmotic stress in proliferating leaves. (*Continued*).

AT5G38310		0,64	1,29E-02	
AT4G30530		0,64	7,92E-03	defense-related protein, putative
AT2G39980		0,64	7,58E-02	transferase family protein
AT2G45170	ATG8E	0,64	6,13E-02	ATG8E, AtATG8e | AtATG8e (AUTOPHAGY 8E); microtubule binding
AT5G53870		0,63	8,62E-02	plastocyanin-like domain-containing protein
ATCG00130	ATPF	0,63	5,74E-02	ATPF | ATPase F subunit.
AT4G39260	CCR1	0,63	7,24E-02	GR-RBP8, ATGRP8, CCR1 | ATGRP8/GR-RBP8 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 1, GLYCINE-RICH PROTEIN 8); RNA binding
AT2G22970	SCPL11	0,62	9,17E-02	SCPL11 | SCPL11; serine carboxypeptidase
AT4G02450		0,62	3,80E-02	glycine-rich protein
AT1G11260	STP1	0,61	5,34E-02	STP1 | STP1 (SUGAR TRANSPORTER 1); carbohydrate transporter/ sugar porter
AT3G59080		0,60	3,92E-02	aspartyl protease family protein
AT1G51805		0,60	8,11E-03	leucine-rich repeat protein kinase, putative
AT2G21660	CRR2	0,60	2,50E-02	GR-RBP7, CCR2, ATGRP7 | ATGRP7 (COLD, CIRCADIAN RHYTHM, AND RNA BINDING 2); RNA binding / double-stranded DNA binding / single-stranded DNA binding
AT3G57330		0,59	8,11E-03	calcium-transporting ATPase, plasma membrane-type, putative / Ca2+-ATPase, putative (ACA11)
AT1G76040	CPK29	0,59	7,00E-02	CPK29 | CPK29 (calcium-dependent protein kinase 29); calcium- and calmodulin-dependent protein kinase/ kinase
AT5G45510		0,58	7,92E-03	leucine-rich repeat family protein
AT2G41010	ATCAMBP25	0,57	2,50E-02	ATCAMBP25 | ATCAMBP25 (*ARABIDOPSIS THALIANA* CALMODULIN (CAM)-BINDING PROTEIN OF 25 KDA); calmodulin binding
AT1G32170	XTR4	0,57	1,34E-02	XTR4 | XTR4 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 4); hydrolase, acting on glycosyl bonds
AT1G10140		0,57	3,44E-02	
ATMG00060	NAD5	0,57	7,05E-02	NAD5, NAD5C | Mitochondrial NADH dehydrogenase subunit 5. The gene is trans-spliced from the three different pre-cursors, NAD5a, NAD5b and NAD5c.
AT4G39640	GGT1	0,56	1,01E-03	GGT1 | GGT1; gamma-glutamyltransferase/ glutathione gamma-glutamylcysteinyltransferase
AT1G67470		0,56	4,34E-02	protein kinase family protein
AT3G15420		0,56	8,44E-04	
AT1G66970		0,56	4,97E-03	glycerophosphoryl diester phosphodiesterase family protein
AT2G15490	UGT73B4	0,56	2,36E-03	UGT73B4 | UGT73B4; UDP-glycosyltransferase/ transferase, transferring glycosyl groups
AT5G59820	ZAT12	0,56	4,77E-02	ZAT12, RHL41 | RHL41 (RESPONSIVE TO HIGH LIGHT 41); nucleic acid binding / transcription factor/ zinc ion binding
AT4G28390	AAC3	0,55	3,53E-02	ATAAC3, AAC3 | AAC3 (ADP/ATP CARRIER 3); ATP:ADP antiporter/ binding
AT3G10720		0,55	6,24E-03	pectinesterase, putative
AT1G09415	NIMIN-3	0,55	8,92E-02	NIMIN-3 | NIMIN-3 (NIM1-INTERACTING 3)
AT1G74490		0,54	1,06E-02	protein kinase, putative
AT5G63690		0,54	4,03E-02	
AT4G28703		0,54	6,33E-02	
AT5G03240	UBQ3	0,54	6,15E-02	UBQ3 | UBQ3 (POLYUBIQUITIN 3); protein binding
AT1G55850	CSLE1	0,54	6,49E-02	CSLE1, ATCSLE1 | ATCSLE1 (Cellulose synthase-like E1); cellulose synthase/ transferase, transferring glycosyl groups
AT5G58120		0,54	2,89E-02	disease resistance protein (TIR-NBS-LRR class), putative

Supplemental Table 2.7. Genes specifically up-regulated by mild osmotic stress in proliferating leaves. (*Continued*).

AT2G22300		0,53	5,77E-02	ethylene-responsive calmodulin-binding protein, putative (SR1)
AT1G13330		0,53	5,31E-04	
AT5G09790	ATXR5	0,53	5,39E-03	ATXR5 | ATXR5 (Trithorax- related protein 5); DNA binding
AT1G56220		0,53	9,55E-02	dormancy/auxin associated family protein
AT3G25600		0,53	7,05E-02	calmodulin, putative
AT1G49500		0,53	5,90E-02	
AT5G44290		0,52	6,83E-03	protein kinase family protein
AT3G02550	LBD41	0,51	9,07E-02	LBD41 | LOB domain protein 41 / lateral organ boundaries domain protein 41 (LBD41)
AT1G22280		0,50	1,72E-02	protein phosphatase 2C, putative / PP2C, putative
AT2G40940	ERS1	0,50	4,94E-02	ERS, ERS1 | ERS1 (ETHYLENE RESPONSE SENSOR 1); receptor
AT5G61010	ATEXO70E2	0,49	6,44E-02	ATEXO70E2 | ATEXO70E2 (EXOCYST SUBUNIT EXO70 FAMILY PROTEIN E2); protein binding
AT1G67100	LBD40	0,49	5,49E-02	LBD40 | LOB domain protein 40 / lateral organ boundaries domain protein 40 (LBD40)
AT3G27220		0,49	4,21E-02	kelch repeat-containing protein
AT1G51700	ADOF1	0,49	5,71E-03	ADOF1 | ADOF1 (Arabidopsis dof zinc finger protein 1); DNA binding / transcription factor
AT4G16990		0,48	4,52E-02	disease resistance protein (TIR-NBS class), putative
AT2G45290		0,48	2,20E-03	transketolase, putative
ATMG00650	NADL4	0,48	1,93E-02	NAD4L | Encodes NADH dehydrogenase subunit 4L.
AT1G68300		0,48	1,77E-02	universal stress protein (USP) family protein
AT4G13850	GR-RBP2	0,48	8,48E-02	GR-RBP2, ATGRP2 | ATGRP2 (GLYCINE-RICH RNA-BINDING PROTEIN 2); RNA binding / double-stranded DNA binding / single-stranded DNA binding
AT2G01750	ATMAP70-3	0,48	4,52E-02	ATMAP70-3 | ATMAP70-3 (microtubule-associated proteins 70-3); microtubule binding
AT2G22770	NAI1	0,47	4,36E-02	NAI1 | NAI1; DNA binding / transcription factor
AT2G15580		0,47	6,13E-02	zinc finger (C3HC4-type RING finger) family protein
AT4G18120	AML3	0,47	2,53E-02	AML3 | AML3 (ARABIDOPSIS MEI2-LIKE); RNA binding
AT5G03380		0,46	9,50E-02	heavy-metal-associated domain-containing protein
AT2G47730	GST6	0,46	2,19E-02	GST6, ATGSTF5, GSTF8, ATGSTF8 | ATGSTF8 (GLUTATHIONE S-TRANSFERASE 8); glutathione transferase
AT1G55920	SAT5	0,46	9,48E-02	SAT5, SAT1, AtSerat2;1 | AtSerat2;1 (SERINE ACETYLTRANSFERASE 1)
AT2G37430		0,46	9,13E-02	zinc finger (C2H2 type) family protein (ZAT11)
AT5G54940		0,44	4,37E-02	eukaryotic translation initiation factor SUI1, putative
AT5G56980		0,44	7,97E-02	
AT2G39725		0,44	7,28E-02	complex 1 family protein / LVR family protein
AT2G43330	ATINT1	0,43	4,63E-03	ATINT1 | ATINT1 (INOSITOL TRANSPORTER 1); carbohydrate transporter/ sugar porter
AT4G11600	ATGPX6	0,43	8,91E-02	PHGPX, LSC803, ATGPX6 | ATGPX6 (GLUTATHIONE PEROXIDASE 6); glutathione peroxidase
AT1G66200	ATGSR2	0,43	6,10E-02	ATGSR2 | ATGSR2 (*Arabidopsis thaliana* glutamine synthase clone R2); glutamate-ammonia ligase
AT2G40270		0,42	6,75E-02	protein kinase family protein
AT5G61560		0,41	7,40E-02	protein kinase family protein
AT1G08570		0,41	5,68E-02	thioredoxin family protein
AT1G63980		0,41	3,57E-02	D111/G-patch domain-containing protein
AT3G27870		0,40	8,59E-02	haloacid dehalogenase-like hydrolase family protein
AT2G37970		0,40	2,79E-02	SOUL heme-binding family protein

Supplemental Table 2.7. Genes specifically up-regulated by mild osmotic stress in proliferating leaves. (*Continued*).

AT1G27090		0,40	9,55E-02	glycine-rich protein
AT1G11950		0,40	1,67E-02	transcription factor jumonji (jmjC) domain-containing protein
AT5G66500		0,40	2,08E-02	pentatricopeptide (PPR) repeat-containing protein
AT5G21090		0,39	3,65E-02	leucine-rich repeat protein, putative
AT5G54170		0,39	2,33E-02	similar to CP5 [*Arabidopsis thaliana*] (TAIR:AT1G64720); similar to putative nodule membrane protein [Medicago sativa] (GB:AAL57201); contains InterPro domain Lipid-binding START; (InterPro:IPR002913)
AT4G26120		0,39	8,15E-02	ankyrin repeat family protein / BTB/POZ domain-containing protein
AT2G43350	ATGPX3	0,39	4,90E-02	ATGPX3 | ATGPX3 (GLUTATHIONE PEROXIDASE 3); glutathione peroxidase
AT5G04930		0,37	7,05E-02	ALA1 | ALA1 (AMINOPHOSPHOLIPID ATPASE1); ATPase, coupled to transmembrane movement of ions, phosphorylative mechanism
AT3G57360		0,36	9,51E-02	
AT3G26300	CYP71B34	0,36	7,05E-02	CYP71B34 | CYP71B34 (cytochrome P450, family 71, subfamily B, polypeptide 34); oxygen binding
AT2G30990		0,35	9,51E-02	
AT1G78890		0,35	2,40E-02	
AT3G11460		0,35	4,52E-02	pentatricopeptide (PPR) repeat-containing protein
AT2G47720		0,33	4,92E-02	similar to AtATG18a (*Arabidopsis thaliana* homolog of yeast autophagy 18 (ATG18) a) [*Arabidopsis thaliana*] (TAIR:AT3G62770.2); contains domain WIPI-RELATED (PTHR11227:SF1); contains domain WD-REPEAT PROTEIN INTERACTING WITH PHOSPHOINOSIDES (WIPI)-RELATED (PTHR11227)
AT1G71060		0,32	8,31E-02	pentatricopeptide (PPR) repeat-containing protein
AT1G02305		0,32	6,83E-02	cathepsin B-like cysteine protease, putative
AT1G64720	CP5	0,32	4,12E-02	CP5 | CP5
AT1G19680		0,32	3,94E-02	protein binding / zinc ion binding
AT4G31950	CYP82C3	0,32	9,17E-02	CYP82C3 | CYP82C3 (cytochrome P450, family 82, subfamily C, polypeptide 3); oxygen binding
AT2G47960		0,30	8,29E-02	
AT4G31080		0,29	9,16E-02	

[a]Genes of the *MDR* are underlined. Genes in bold encode mitochondrial proteins.

Supplemental Table 2.8. Primers used for cloning of the *MDR* promoters.

Primer	Sequence
AT2G21640_FW	AAAAAGCAGGCTTGAGAAAAAGATTTAGGCAAGA
AT2G21640_RV	AGAAAGCTGGGTCTGAAAACAGAAAGAAATCTCA
AT2G04050_FW	AAAAAGCAGGCTTTAAAGGTTCACCCGACTCT
AT2G04050_RV	AGAAAGCTGGGTTGTTGTCCTTCCTAATGTTGA
AT2G41730_FW	AAAAAGCAGGCTCAAAGTTAAACATCAAAGCAAAG
AT2G41730_RV	AGAAAGCTGGGTGTTTGCTTATTTTGATTTGAGAC
AT2G03760_FW	AAAAAGCAGGCTACTTAGTTCTCAATTTCTTGAG
AT2G03760_RV	AGAAAGCTGGGTTGTTGAGACTTGAGAGATCG
AT5G51440_FW	AAAAAGCAGGCTCGATGATGATGTTGAAGAGAAG
AT5G51440_RV	AGAAAGCTGGGTTTTTGGAAAGAGAAGAAGCTTA
AT4G37370_FW	AAAAAGCAGGCTCACATTGTAATTTGTACGTGAA
AT4G37370_RV	AGAAAGCTGGGTGTTTTAGATCTATTTTGGTGAG
AT1G32870_FW	AAAAAGCAGGCTGTAAATTTTTCAGATGAAAGTATT
AT1G32870_RV	AGAAAGCTGGGTCACTTTTTTCTCTCTCGTTATC
AT3G61630_FW	AAAAAGCAGGCTGAAATCCAGTTACAAAATAGGT
AT3G61630_RV	AGAAAGCTGGGTAGAGAGAGAGAGAGAGAGG

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Chapter 3

Identification of MRR regulators through yeast one-hybrid screening

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AUTHOR CONTRIBUTIONS

I.D.C. designed, performed and analyzed the Y1H screening experiments with help of T.V.G. and V.V.

ABSTRACT

To identify transcriptional regulators of plant mitochondrial retrograde regulation, a set of *Arabidopsis thaliana* genes that respond to multiple mitochondrial perturbation conditions was assembled, referred to as the mitochondrial dysfunction regulon (MDR; Chapter 2). Using a large-scale pairwise yeast one-hybrid screening approach against the REGIA transcription factor collection, 50 transcription factors (TF) were identified that bind to one or more of the *MDR* promoters. These TFs represent different transcription factor families, with the strongest enrichment for the APETALA2-ETHYLENE-RESPONSE ELEMENT BINDING PROTEIN (AP2-EREBP) and the NO APICAL MERISTEM/*ARABIDOPSIS* TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) family. Gene ontology enrichment analysis showed overrepresentation of the "response to stress" GO term and 16 of the isolated TFs have been previously characterized to have a function in the stress response. Moreover, meta-analysis showed that several of the isolated TFs have an expression pattern similar to that of the *MDR* genes during stress conditions, indicating they could be candidate regulators of the *MDR*. However, further experiments will be required to assess the biological relevance of these novel regulatory interactions *in planta*.

INTRODUCTION

Several studies have indicated that mitochondrial function is disturbed under adverse environmental conditions, and, in turn, alteration of mitochondrial function can affect plant stress tolerance (Giraud et al., 2008; Meyer et al., 2009; Shedge et al., 2010; Van Aken et al., 2010). One of the major effects of stress on plants is a disturbance of cellular metabolism that leads to the production of reactive oxygen species (ROS) (Dat et al., 2000). Mitochondrial processes are directly inhibited by the resulting oxidative damage (Sweetlove et al., 2002). Therefore, it is not surprising that a large part of plant stress responses is sensed in mitochondria, triggering retrograde feedback mechanisms to the nucleus to activate a defense response. Although it is known that mitochondria are involved in mediating and executing stress responses in plants, very little is understood about how this is regulated (Rhoads and Subbaiah, 2007). Likely signals that transmit the information to the nucleus are thought to be ROS and calcium originating from the mitochondria and mitochondrial redox and metabolic changes (Subbaiah et al., 1998; Rhoads et al., 2006; Schwarzlander and Finkemeier, 2013). In a typical signal transduction pathway, receptor proteins perceive the signal and activate a regulatory cascade that transmits the information to the nucleus. At the end of the pathway, one or more transcription factors are activated and cause altered expression of stress-responsive genes. These genes can be involved in further amplifying the stress response (e.g. secondary transcription factors) or in executing defense functions. So far, only one transcription factor has been reported that is involved in stress-related mitochondrial retrograde regulation (MRR) (Giraud et al., 2009). This illustrates our lack of knowledge about the underlying regulatory processes, and the need for further original research.

Transcription factors are key regulators of spatiotemporal and stimulus-induced gene expression by binding to specific *cis*-regulatory elements in their target gene promoters and thereby activate or repress transcription. To understand how differential gene expression is regulated, it is important to identify transcription factor-DNA interactions and to model these interactions into transcriptional regulatory networks. Different methods have been developed to study protein-DNA interactions (PDIs), such as DNAse footprinting and chromatin immunoprecipitation (ChIP) (Walhout, 2006). However, these methods require the prior knowledge of the TF identity. The yeast one-hybrid (Y1H) system is a powerful assay that allows

the identification of transcriptional regulators binding to a DNA sequence of interest in a cell-based assay (Li and Herskowitz, 1993). In this approach, the DNA bait, representing a full-length or a shorter promoter fragment (e.g. containing a particular *cis*-regulatory element) is cloned upstream of a reporter gene and integrated into the yeast genomic DNA. The second component of the Y1H system is the library that expresses prey proteins fused to a constitutive transcriptional activation domain. If the prey protein interacts with the DNA bait, the reporter gene is activated. A high-throughput version of the Y1H system compatible with the Gateway cloning technique was developed by Deplancke et al., 2004. In this system, two reporter genes are used: the auxotrophic reporter *Histidin3* (*HIS3*) allowing for nutritional selection on medium lacking histidin and the color producing marker *LacZ* encoding the β-galactosidase enzyme. Using two reporter systems allows the minimization of the uncertainty of the results of one particular assay and serves to decrease false positive results.

To identify transcriptional regulators of plant mitochondrial retrograde regulation (MRR), we assembled a set of *Arabidopsis* genes whose expression significantly changed in multiple mitochondrial perturbation studies, referred to as the mitochondrial dysfunction regulon (MDR; Chapter 2). In a previous approach (Chapter 2), the co-expression of the *MDR* genes was exploited to identify a common *cis*-regulatory element, and led to the identification of five related NAC transcription factors involved in MRR. However, we hypothesized that other transcriptional regulators might be involved in the *MDR* regulation as 1) the *MDR* genes are regulated under a multitude of stress conditions (Chapter 2; Supplemental Figure 3.3; Zimmermann et al., 2003) and are therefore likely to be regulated by multiple transcription factors (Walther et al., 2007); 2) genes with a strong co-expression pattern are mostly commonly regulated by multiple TFs (Yu et al., 2003); and 3) gene regulation in eukaryotes is complex and cooperatively regulated by multiple transcription factors simultaneously (Wray et al., 2003). Here, we aimed at identifying unknown transcriptional regulators of the *MDR*, without any prior knowledge on *cis*-regulatory elements, by Y1H screening of their full promoter sequences. This approach revealed several candidate (MRR) transcriptional regulators of the *MDR*. However, further experiments will be needed to assess the biological relevance of these novel regulatory interactions *in planta*.

RESULTS

Identification of transcriptional regulators of the *MDR* by Y1H screening

Promoter sequences (2-kb intergenic regions upstream of the translational start codon) were successfully cloned for 12 *MDR* genes (*AOX1a*, *At2g04070*, *At2g41730*, *At5g09570*, *HSP23.5*, *NDB4*, *ST*, *UGT74E2* and *UPOX*; Chapter 2) and six additional genes (*At2g04040*, *At5g62480* and *UGT73C6*) that are also targets of MRR (Van Aken et al., 2007), but did not meet the stringent selection criteria for general MRR responsiveness in Chapter 2. Promoter:*HIS3* and promoter:*LacZ* reporter constructs were integrated into the genome of the YM4271 yeast strain. For each promoter, yeast clones with low self-activation of the *HIS3* and *LacZ* reporter genes were searched and the concentration of 3-aminotriazole (3-AT) necessary to abolish background *HIS3* expression was determined. Except for the *UGT74E2* and *AOX1a* promoters, yeast clones with low self-activation of the reporter genes could be selected. However, as *AOX1a* is a model gene for MRR studies and *UGT74E2* has an important role in drought tolerance in Arabidopsis, they were included for Y1H screening analyses.

First, Y1H screening was performed with a cDNA library obtained from Arabidopsis cell suspension cultures. However, screening of several promoters did not yield any proteins annotated as transcriptional regulators, except for four LATERAL ORGAN BOUNDARIES transcription factors (LBD4, LBD17, LBD18 and LBD19), of which two (LBD4 and LBD19) had been previously marked as possibly unspecific interactors in the Y1H system (Dr. B. Berckmans, personal communication) (Berckmans, 2011). Next, the promoters were screened against the REGIA transcription factor (TF) library containing prey clones of 1394 Arabidopsis TFs (Paz-Ares, 2002). An overview of the identified potential interactors obtained by screening a pool of all the REGIA TF plasmids is depicted in Supplemental Table 3.1. The predominant TFs that were isolated were the HOMEOBOX PROTEIN 22 (ATHB22) and the NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) domain-containing protein AT3G12910. As they were identified with all promoters tested, we marked them as possibly unspecific interactors (see later). Other potential interactors were isolated with low frequency (mostly once) and, except for the ETHYLENE REPONSE FACTOR protein AT5G43410, we did not retrieve any common interactors of the

different *MDR* promoters. To overcome the technical difficulties presented by the abundance of ATHB22 and AT3G12910 and to address the specificity of the interactions, high-throughput pairwise interaction tests were performed with the individual REGIA TF clones, further referred to as matrix assays. The *UGT74E2*, *UPOX* and *ST* promoters were tested for interactions with the 1394 TFs individually on 15 individual plates in the 96-well format (see Methods). After confirmation by retransformation of the reporter yeast strain, all identified interactors were further tested against eight other *MDR* promoters (Table 3.1; Figure 3.1). In addition, to test the specificity of TFs that interacted with many or all *MDR* promoters tested, the promoters of three cell cycle genes (*E2Fa*, *DEL1* and *CYC3;2*) were included as a negative control (Berckmans et al., 2011a; Berckmans et al., 2011b) (data not shown). The matrix screens yielded 50 TFs, including a total of 172 potential PDIs (Table 3.1; Figure 3.1). Although several PDIs obtained from the pooled screens could be confirmed in these matrix assays, many were not confirmed. Moreover, many new interactors were found with the individual matrix screening assays, indicating a higher coverage of this system compared to the pooled screens. The interactors represented different TF families, with the strongest enrichment for the APETALE2-ETHYLENE-RESPONSE ELEMENT BINDING PROTEIN (AP2-EREBP) family (Nakano et al., 2006), mainly DEHYDRATION RESPONSIVE ELEMENT BINDING (DREB) subfamily members (Sakuma et al., 2002) (Supplemental Figure 3.1), and the NAC family (Ooka et al., 2003) (Supplemental Figure 3.2). Moreover, several TFs were isolated that bound multiple *MDR* promoters, indicating potentially common *MDR* regulators. Several interactors, including ATHB22 and AT5G43410 were found to bind to the three cell cycle promoters (data not shown), suggesting they might unspecifically interact with the reporter vectors (e.g. the minimal promoter) in the Y1H system.

Table 3.1. Identification of transcription factors that interact with *MDR* promoters by means of the Y1H matrix screening assay.
The promoters of *UGT74E2*, *ST* and *UPOX* were screened for interactions with each of the 1394 REGIA TF-clones individually. The identified interactors were further tested against other *MDR* promoters (*AOX1a*, *At5g09570*, *At2g41730*, *HSP23.5*, *NDB4*, *At2g04040*, *UGT73C6* and *At5g62480*). Positive for *HIS3* is indicated with a grey bar, and positive for *LacZ* is indicated with a blue bar. The plus sign indicates the strength of the reporter gene activation, (+), very weak; +++, very strong. Asterisk indicates possible sticky proteins, as determined by binding negative control promoter:reporter constructs.

Transcription factor (prey)			Promoter (bait)									
Gene ID	Other name	Family[a]	UGT74E2	ST	UPOX	UGT73C6	AOX1a	At2g41730	NDB4	HSP23.5	At2g04040	At5g62480	At5g09570
AT1G53910	RAP2.12	AP2/EREBP (ERF)	+										
AT2G33710		AP2/EREBP (ERF)	+		++								
AT5G53950	CUC2	NAC	NA	+		+++	+++				++		++
				+++		+++	+++	+++	+++	++	+++	+++	+++
AT5G63790	ANAC102	NAC	++	+	+++	++	++	(+)	(+)		++		
			+++								++		
AT1G01720	ATAF1	NAC						(+)					
			+										
AT5G04410	ANAC078	NAC	++										
			+			+				+			+
AT3G15510	NARS1	NAC	NA	++		+	++	(+)					+
AT3G29035	ORS1	NAC	NA		(+)			(+)					
AT2G24430	ANAC038/39	NAC				+++	++						++
			+++	+++	+++	+++	+++	+++	+++	++	++	+++	+++
AT3G10490	ANAC051/52	NAC	(+)		(+)	+							
			(+)			++							
AT5G39660	CDF2	C2C2-Dof	NA				++						
							+++						
AT2G01150	RHA2B	-	NA	(+)			++						
AT2G17180*	DAZ1	C2H2	++	+	++	NA	NA	NA	NA	NA	NA	NA	NA
			+++										
AT4G35280*	DAZ2	C2H2	+	+	+	NA	NA	NA	NA	NA	NA	NA	NA
			+										
AT2G46870	NGA1	ABI3-VP1	NA	+	++	+	++				+		
							++						
AT2G38340	DREB19	AP2/EREBP (DREB)	(+)	(+)	+		+	+	(+)	+	(+)		
			++	+	(+)				+++	+++	+	+	
AT2G44940		AP2/EREBP (DREB)		(+)		+			(+)		+		
AT5G05410	DREB2A	AP2/EREBP (DREB)		+									
				++									

Table 3.1. Identification of transcription factors that interact with *MDR* promoters by means of the Y1H matrix screening assay. (*Continued*).

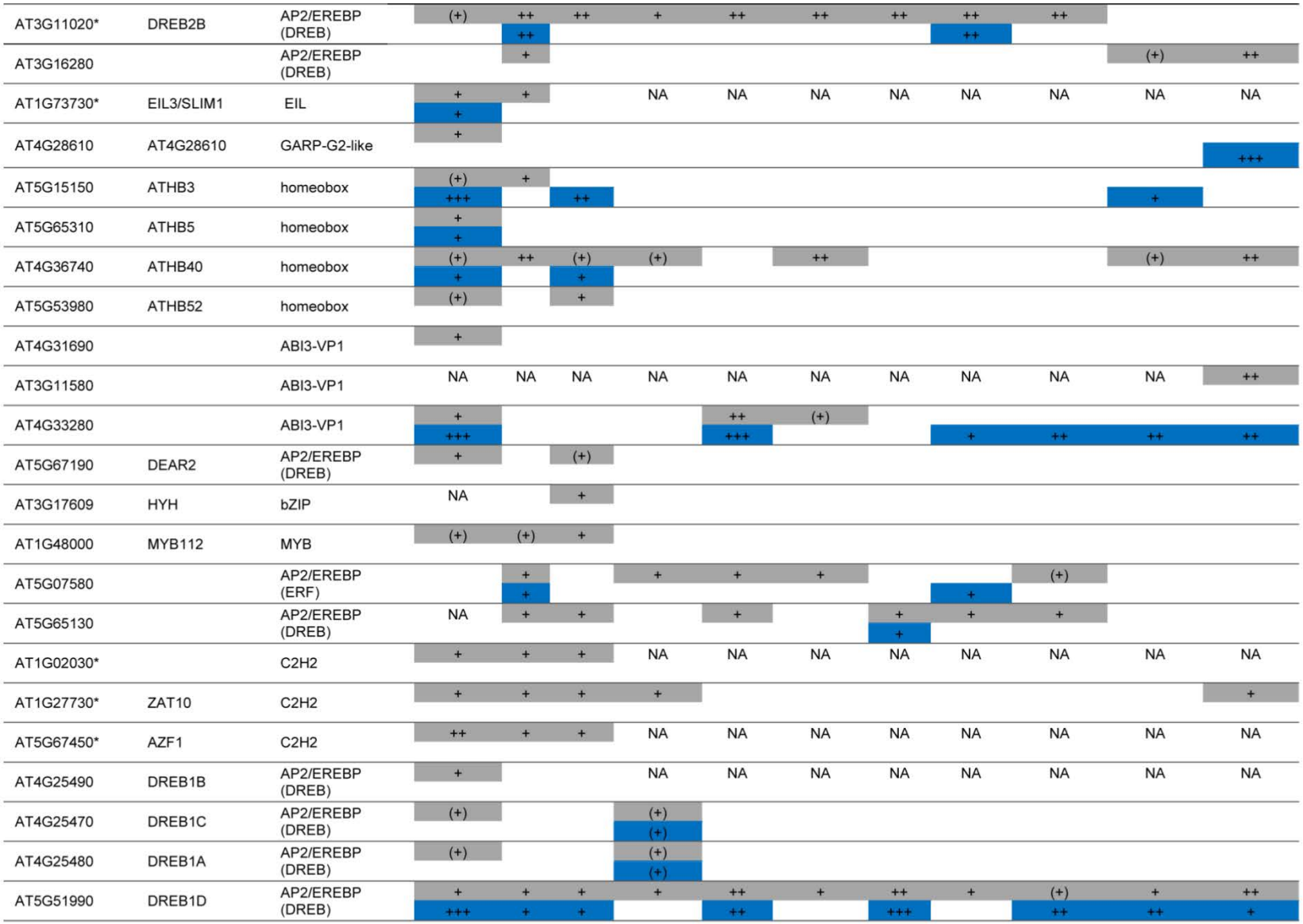


Table 3.1. Identification of transcription factors that interact with *MDR* promoters by means of the Y1H matrix screening assay. (*Continued*).

AT3G60490		AP2/EREBP (DREB)	+	++	++						+	+	
			(+)										
AT1G34190	ANAC017	NAC	+++	++	++		++		+				
			+++	++	++	+++	+++	+++	+	+++			+++
AT1G54060	ASIL1	Trihelix	++		+								
			++	+			++				+	++	+
AT3G14180	ASIL2	Trihelix	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+++
													+++
AT1G43160	RAP2.6	AP2/EREBP (ERF)	(+)		+								
			+	+			++		+				
AT3G15210	ATERF4/RAP2.5	AP2/EREBP (ERF)	(+)										
			++				++		+				
AT2G21230	bZIP30	bZIP	+	(+)	(+)	+	+	+		+	+		
			+++	+	+		+++				+++	+++	
AT2G31370	bZIP59	bZIP	(+)	+		+	+				+		(+)
AT4G38900	bZIP29	bZIP	(+)	++	(+)	(+)	(+)	+	+		++	+	
			+++	++							+	++	

[a]According to the TF family classification of the DATF database (Guo et al., 2005).

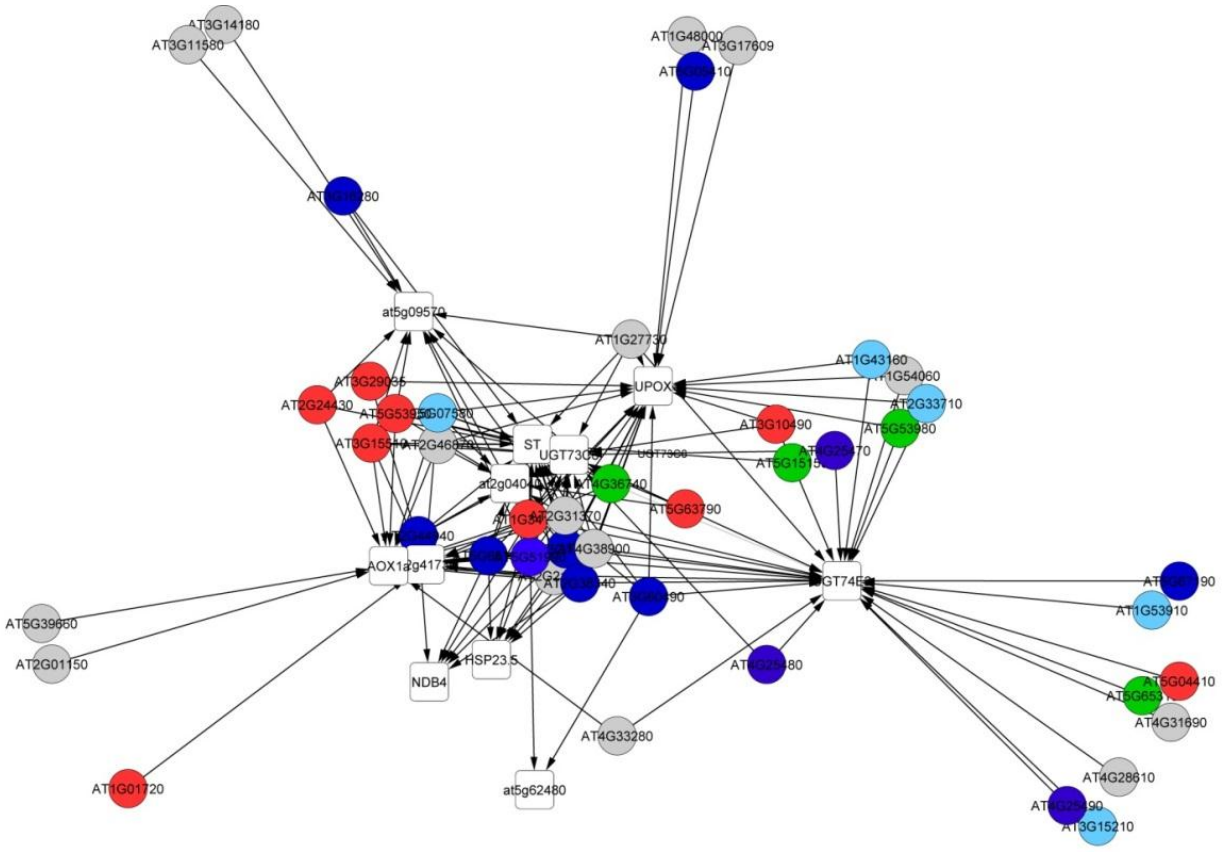


Figure 3.1. Network representation of the identified TF-promoter interactions. The network was constructed using the Cytoscape spring-embedded layout algorithm (Shannon et al., 2003). Promoters are indicated by rectangles and TFs by circles. The color code of the TFs represents the TF family: NAC (red), homeobox (green), AP2-EREBP (blue), and other (grey). Within the AP2-EREBP family, the ERF subfamily (pale blue) and the DREB (dark blue) are distinguished.

DISCUSSION

To identify transcriptional regulators of plant mitochondrial retrograde regulation, a Y1H screening approach was employed using MRR target genes (*MDR* genes) as a bait. As the Y1H system might yield false positive interactions, the biological significance of the identified PDIs needs to be further assessed *in planta*. Gene Ontology (GO) enrichment analysis of the 50 isolated TFs showed overrepresentation of the "response to stress" GO term (GO:0006950; 3-fold, $P < 0.05$). In addition, inspection of the literature revealed that 16 of the 50 identified TFs have a role in the stress response. DREB proteins are major TFs required for cold-inducible and osmotic stress-inducible gene expression (Nakashima et al., 2009). The DREB1A, DREB1B and

DREB1C transcription factors, which only bind to one or two out of the 11 *MDR* promoters tested, are known to mediate cold responsive gene expression (Liu et al., 1998). On the other hand, DREB1D, that appears to bind all *MDR* promoter tested, mediates osmotic stress responsive genes expression (Haake et al., 2002), corresponding with the expression pattern of the *MDR* genes that are induced by osmotic, but not by cold stress. *DREB2A* and *DREB2B* are also induced by osmotic stress and not by cold stress, and overexpression of the constitutively active *DREB2A* induces drought tolerance in Arabidopsis (Liu et al., 1998; Sakuma et al., 2006). Similarly to the previously identified *MDR* regulator, ANAC013 (Chapter 2), DREB2A and DREB2B proteins interact with RADICAL-INDUCED CELL DEATH1 (RCD1), that is suggested to play a role in the regulation of TF activities (Belles-Boix et al., 2000; Jaspers et al., 2009; Vainonen et al., 2012). Likewise the *MDR* and *ANAC013* expression patterns, *DREB2B*, together with another isolated TF, the ERF gene *At2g33710*, is specifically induced in proliferating leaves by stress (Skirycz et al., 2010) (Chapter 2, Addendum). Another DREB2-related protein identified in the Y1H screens, DREB19 has been shown recently to mediate increased drought and salt stress tolerance in Arabidopsis (Krishnaswamy et al., 2011). Among the isolated ERF family proteins, RELATED TO AP2.5 (RAP2.5) and RAP2.6 have been demonstrated to act as a transcriptional repressor and transactivator, respectively, in the abscisic acid (ABA)-dependent salt stress response (Yang et al., 2005; Zhu et al., 2010; Krishnaswamy et al., 2011). RAP2.12 mediates hypoxia-responsive gene expression, consistent with the *MDR* co-expression under low oxygen conditions. Interestingly, RAP2.12 belongs to the ERF family subgroup containing the HYPOXIA RESPONSIVE ERF2 (HRE2) of the MDR, that has also been implicated in the hypoxia response (Licausi et al., 2011a; Licausi et al., 2011b; Bailey-Serres et al., 2012). ANAC102, that binds multiple *MDR* promoters in yeast, is important for seed germination during low oxygen stress and regulates the expression of five *MDR* genes among which *AOX1a*, *At2g04040*, and *At2g41730* (Christianson et al., 2009). The NAC transcription factors ATAF1 and ORS1 have been implicated in multiple stress responses. Moreover, consistent with the binding results in yeast, the *At2g41730* gene is up-regulated by *ORS1* overexpression in Arabidopsis (Wu et al., 2009; Balazadeh et al., 2011). Furthermore, the ZAT10 and AZF2 Cys2/His2-type zinc finger proteins containing an associated amphiphilic repression (EAR) domain and the BASIC-LEUCINE ZIPPER bZIP30 are also involved in stress responsive

gene expression (Mittler et al., 2006; Rossel et al., 2007; Kodaira et al., 2011; Kouno and Ezaki, 2012).

For several TF families, members with closely related DNA binding domains were isolated in the Y1H screens. For example, the DREB1A, DREB1B, DREB1C and DREB1D DNA binding domains cluster in the phylogenetic analysis (Nakano et al., 2006). Moreover, the *DREB1A*, *DREB1B* and *DREB1C* genes lie in tandem in the genome and thus might have originated from recent gene duplications (Medina et al., 1999). Similarly to our results, DREB1A, DREB2B and DREB1D were commonly isolated in an Y1H screen of the stress responsive *RD29A* promoter, indicating they have similar DNA sequence preference (Mitsuda et al., 2010). Furthermore, we isolated three other closely related DREB proteins (DREB2A, DREB2B and DREB19) and three closely related BASIC LEUCINE ZIPPER DOMEIN (bZIP) proteins (bZIP29, bZIP30 and bZIP59). Although the similarity in DNA binding domain explains their similar binding capacities in the Y1H system, this does not necessarily indicate they all bind genuinely *in planta*. For example, if the TF is not expressed where and/or when the promoter is active, it is likely not a true regulator *in planta*. To prioritize candidate *MDR* regulators, the expression characteristics of the TFs were compared to that of the *MDR* genes (Supplemental Figure 3.3). As the *MDR* is mainly active under stress conditions, we clustered the identified TF and *MDR* genes under a number of perturbation conditions using the GENEVESTIGATOR hierarchical clustering tool (Zimmermann et al., 2004). *At2g33710* and *DREB19* as well as the previously identified *ANAC013* and *ANAC078* (Chapter 2) clustered together with the *MDR* genes, indicating they are potential *MDR* regulators. Moreover, *DREB2A*, *ATAF1*, *ANAC102*, *ZAT10*, *RAP2.5*, *RAP2.6* and *MYB112* also showed an expression pattern similar to that of the *MDR* genes, but under a smaller number of perturbation conditions compared to *At2g33710* and *DREB19*. Moreover, *At2g33710*, *DREB19*, *ATAF1* and *ZAT10* are induced upon multiple mitochondrial perturbations, such as antimycin A (AA), rotenone, and oligomycin treatment as well as external H_2O_2 application (Ng et al., 2012, unpublished results; Clifton et al., 2012; our unpublished results). In addition, *DREB2A* is induced by AA, H_2O_2 and in the mitochondrial complex I deficient *rug3* mutant (Kühn et al., 2011). RAP2.5 is induced by AA and H_2O_2, but down-regulated upon rotenone and oligomycin treatment, and RAP2.6 is down-regulated in the prohibitin deficient mutant *phb3* (Van Aken et al., 2007). However, other isolated TFs that do not appear to be co-expressed with the *MDR* should not be excluded as candidate regulators, as TFs

often show low expression variation and are regulated at the post-transcriptional level. To conclude, we identified several candidate *MDR* regulators, but further experiments, such as ChIP will be indispensable to confirm these novel regulatory interactions *in planta*.

METHODS

Y1H screening

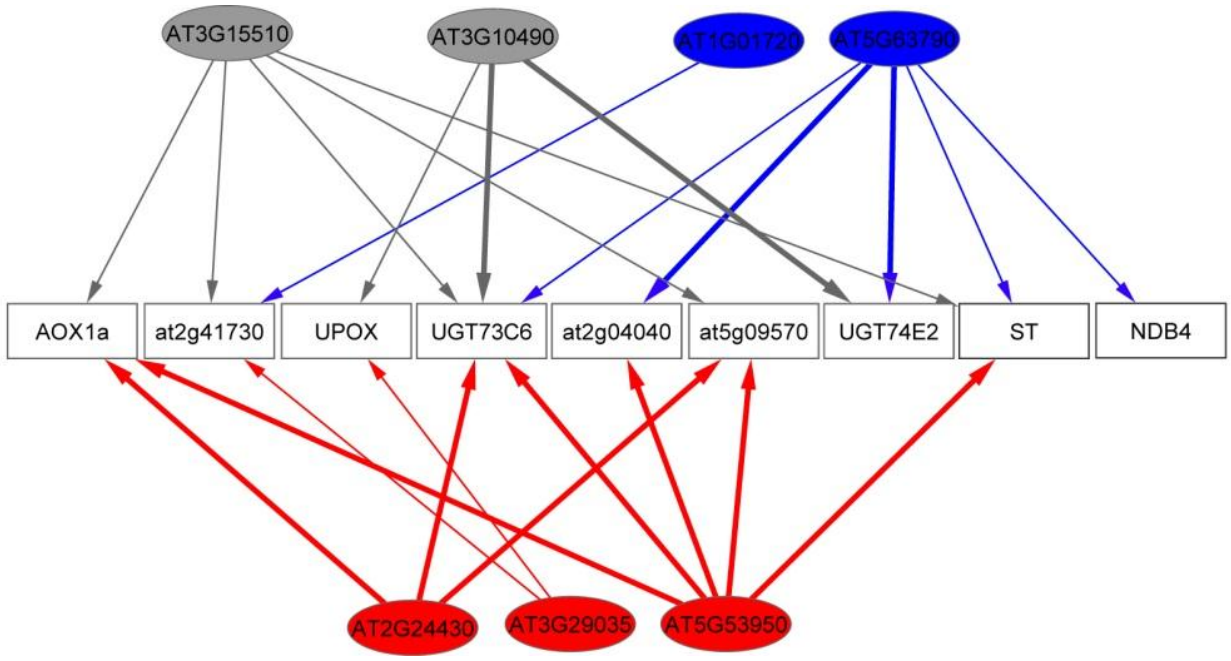
Yeast strain YM4271 and destination vectors pMW#2 and pMW#3 were obtained from Dr. M. Walhout (University of Massachusetts Medical School, Worcester, MA, USA). The REGIA collection was provided by Dr. Franziska Turck (Max Planck Institute for Plant Breeding Research, Köln, Germany). Y1H library screening was performed as described in detail in Deplancke et al. (2006). For the matrix assays (individual prey transformations), the same approach was scaled down to the 96-well plate format (Vermeirssen et al., 2007). Prey plasmid transformations were performed by combining 20 µL of competent yeast cell suspension, 100 ng plasmid, and 100 µL Tris-EDTA(TE)/lithium acetate/polyethyleneglycol per well. After heat shock (20 min at 30°C), plates were centrifuged (for 10 s) and supernatant was removed. Yeast cells were resuspended in 20 µL TE of which 5 µL was spotted on selective medium (SD-His-Ura-Trp; Clontech) and grown for 2-3 days at 30°C. Yeast transformants were freshly regrown on selective medium for 1-2 days at 30°C and subsequently transferred to selective medium containing 3-AT (at a concentration at which self-activation is minimal) and onto a nitrocellulose filter (Hybond-N; Amersham Biosciences) that has been placed on top of a non-selective YPD (Clontech) plate by means of the replicate plating technique (Deplancke et al., 2006). The 3-AT plates were replica-cleaned to remove excess yeast cells and yeast growth was monitored during 3-10 days after transfer. The YPD plate was incubated overnight at 30°C and subsequently used for the β-galactosidase assay (Deplancke et al., 2006).

Hierarchical clustering of gene expression profiles

The expression of the 50 identified TFs and the 28 *MDR* genes (Chapter 2 and Van Aken et al. (2007)) was analyzed using the GENEVESTIGATOR meta-analysis tool (Zimmermann et al., 2004) under a selection of conditions related to stress, biotic stress, temperature, hormone,

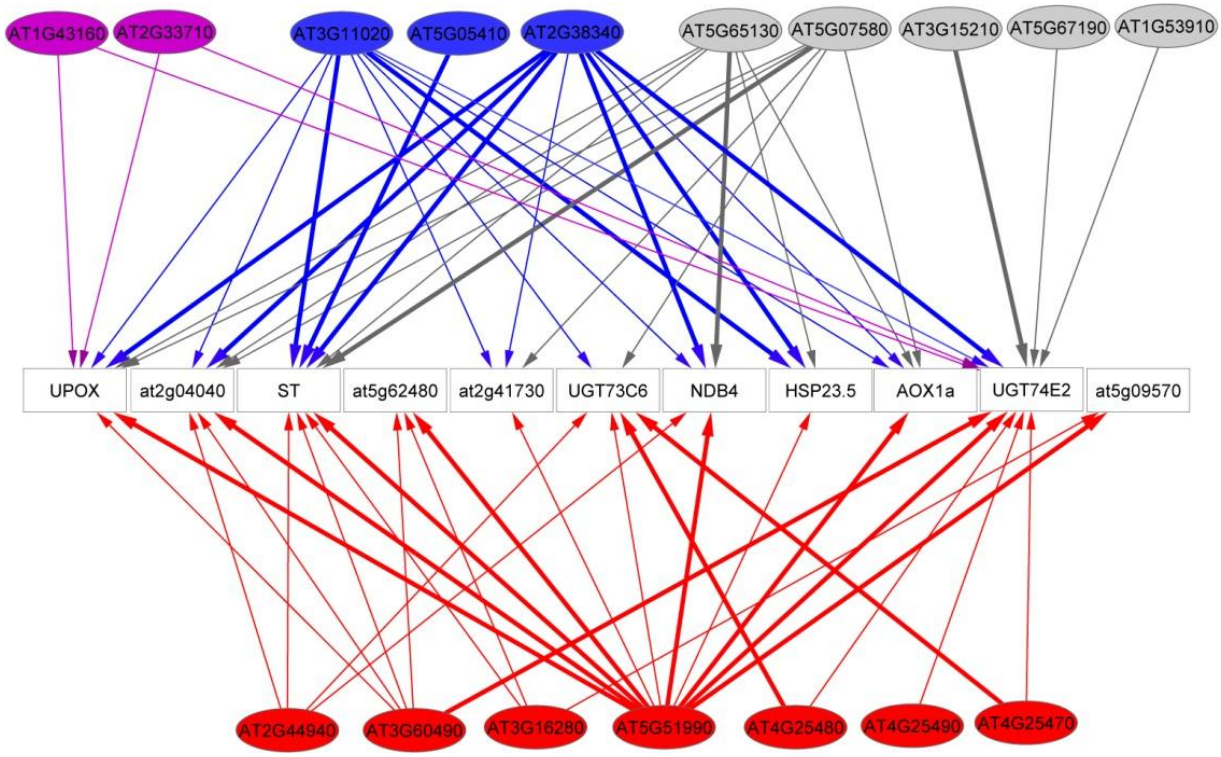
elicitor, chemical, nutrient, light intensity and light quality (1091 conditions and 3360 samples in total). Pearson correlation coefficient was used to hierarchically cluster together genes and conditions with similar behavior.

SUPPLEMENTAL DATA



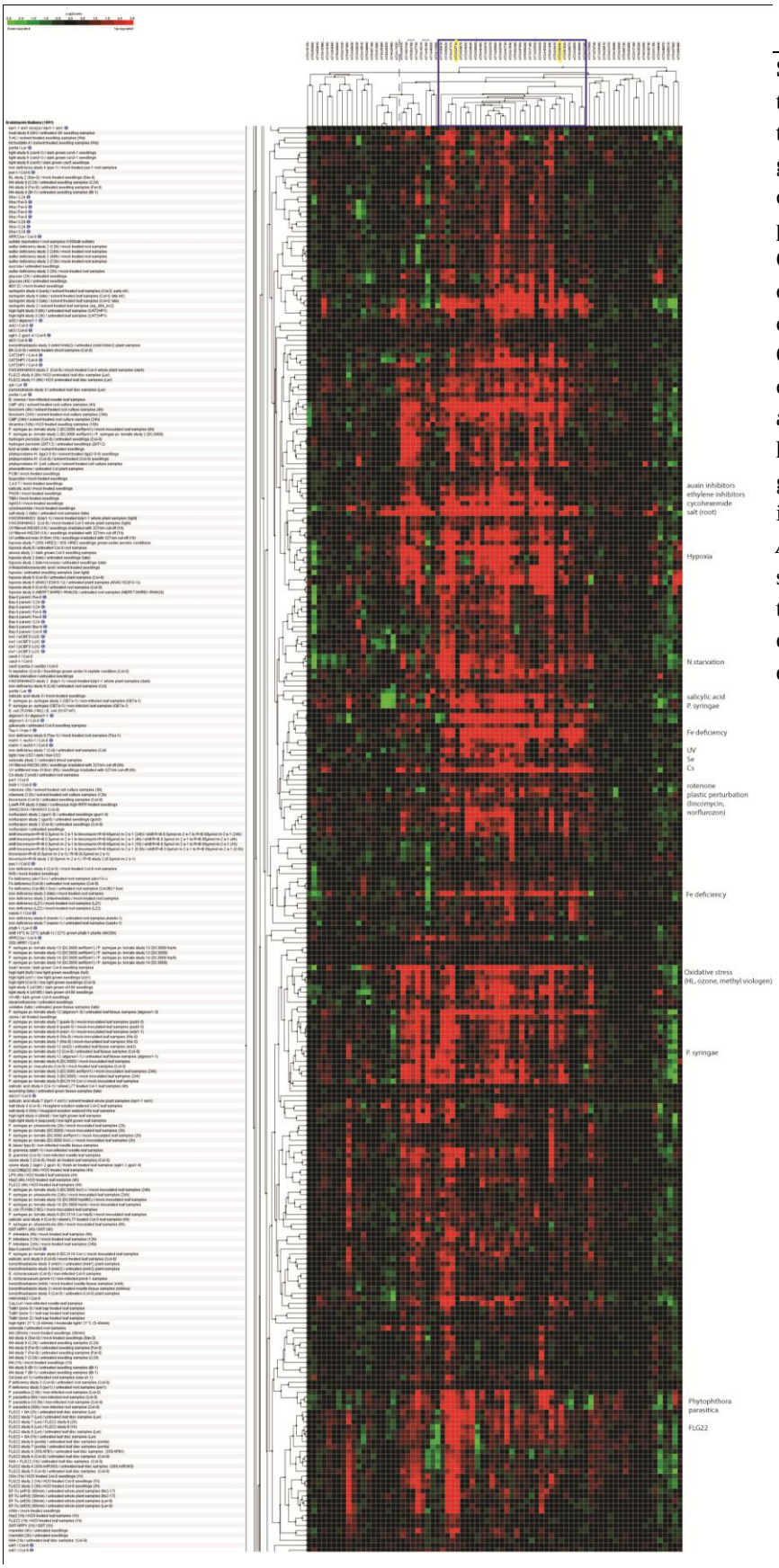
Supplemental Figure 3.1. Schematic overview of the identified regulatory interactions with NAC family transcription factors.

Promoters are indicated by white rectangles and transcription factors by ovals. The color of the transcription factor node represents the NAC subgroup based on phylogenetic classification of NAC domains according to Ooka et al. (2003): ATAF subfamily (blue), NAM subfamily (red), and other subfamilies (grey). The line width of the edges represents the relative strength of the interaction in the Y1H system: positive for both *HIS3* and *LacZ* (thick line); only positive for *HIS3* (thin line). Interactions based on the activation of the *LacZ* reporter gene only were not reported here.



Supplemental Figure 3.2. Schematic overview of the identified regulatory interactions with AP2/EREBP domain transcription factors.

Promoters are indicated by white rectangles and transcription factors by ovals. The color of the transcription factor node represents the AP2/EREBP subgroup based on phylogenetic classification of AP2/EREBP domains according to Nakano et al. (2006): group III (red), group IV (blue), group X (purple) and other (grey). The line width of the edges represents the relative strength of the interaction in the Y1H system: positive for both *HIS3* and *LacZ* (thick line); only positive for *HIS3* (thin line). Interactions based on the activation of the *LacZ* reporter gene only were not reported here.



Supplemental Figure 3.3. Comparison of the expression profiles of the identified transcription factors with that of the *MDR* genes under different stress-related conditions. The meta-analysis was performed using the GENEVESTIGATOR hierarchical clustering tool under 1091 perturbation conditions (Zimmermann et al., 2004). Only a part of the heat map, containing the conditions under which the *MDR* genes are commonly up-regulated is displayed here. The cluster containing the *MDR* genes is indicated with a purple box and includes two isolated TFs (*DREB19* and *At2g33710*) that are indicated in yellow. A second gene cluster that is closely related to the *MDR* gene cluster and includes eight identified TFs is marked with a dashed box.

Supplemental Table 3.1. Overview of the promoters screened and the identified transcription factors by means of Y1H screening with a pool of the REGIA TF clones.
Interactions that were confirmed (*HIS3* positive or positive for both *HIS3* and *LacZ*) in the Y1H matrix assays are indicated in bold underline. Interactions that were only confirmed by weak *HIS3* activation are indicated in bold and not underline. Possible sticky interactors, as evidences by binding all promoters tested, including negative control promoter:reporter constructs, are indicated with an asterisk and were excluded for sequencing analysis in further screens after PCR detection with specific primers.

Gene ID	Other name	Family	*HIS3* activation	*LacZ* activation	Hits
ST (At2g03760)					
AT2G36610*	ATHB22	homeobox	++		9
AT3G12910*		NAC	+		8
AT3G62340	WRKY68	WRKY	+		1
AT4G01580		ABI3-VP1	+		1
AT3G18960		ABI3-VP1	+		1
AT4G36740	ATHB40	homeobox	+++		1
AT4G31610	REM1	ABI3-VP1	+		1
AT5G43410		AP2-EREBP (ERF)	++		1
AT1G69780	ATHB13	homeobox	+		1
AT4G31690		ABI3-VP1	+	+	1
AT2G38340	DREB19	AP2-EREBP (DREB)	+	+	1
AT5G07580		AP2-EREBP (ERF)	+		1
AT1G43160	RAP2.6	AP2-EREBP (DREB)	+++	+	1
UGT73C6 (At2g36790)					
AT2G36610*	ATHB22	homeobox	+		4
AT3G12910*		NAC	++		4
AT4G16430		bHLH	+++		1
AT2G31370		bZIP	++	+	1
AT1G04370	ATERF14	AP2-EREBP (ERF)	++		1
AT4G28140		AP2-EREBP (DREB)	+++		1
AT2G24430	ANAC038/39	NAC	+	+++	1
UPOX (At2g21640)					
AT3G12910*		NAC	++	+	3
AT2G36610*	ATHB22	homeobox	+		1
AT3G54340	AP3	MADS	+++		1
UGT74E2 (At1g05680)					
AT2G36610*	ATHB22	homeobox	+++	++	2
AT2G16770	BZIP23	bZIP	++		2
AT4G25480	DREB1A	AP2-EREBP (DREB)	++		2
AT5G02840	LCL1	MYB-related	+++	+	2
AT3G12910*		NAC	+++		1
AT2G43220		-	++		1
AT3G47640	PYE	bHLH	++		1
AT4G11070	WRKY41	WRKY	+		1
AT4G30935	WRKY32	WRKY	++	++	1
AT3G10580		homeobox	+	+	1
AT3G15210	ATERF4	AP2-EREBP (ERF)	+++	++	1
AT4G31610	REM1	ABI3-VP1	+	+	1
AT3G05200	ATL6	-	+		1
AT5G65310	ATHB5	homeobox	+++	++	1
AT3G60490		AP2-EREBP (DREB)	++		1
AT1G12630		AP2-EREBP (DREB)	++	++	1
AT1G50640	ATERF3	AP2-EREBP (ERF)	++		1

Supplemental Table 3.1. Overview of the promoters screened and the identified transcription factors by means of Y1H screening with a pool of the REGIA TF clones. (*Continued*).

AT5G43410		AP2-EREBP (ERF)	+++	++	1
AT3G21880		C2C2-CO-like	++		1
AT1G53910	RAP2.12	AP2-EREBP (ERF)	+++	++	1
AT4G39070		C2C2-CO-like	++		1
AT2G33480	ANAC041	NAC	+++		1
AT2G47890		C2C2-CO-like	+++		1
AT3G04730	IAA16	AUX/IAA	+++	++	1
AT5G49448	CPUORF4	-	+++	+	1
AT5G49450	BZIP1	bZIP	+		1
AT3G01220	ATHB20	homeobox	+++		1
AT2G33710		AP2-EREBP (ERF)	+++	+	1
AT1G01520	ASG4	MYB-related	+++	+	1
AT5G60910	AGL8	MADS	+++	+	1
AT1G72210		bHLH	+++	+	1
AT5G58900		MYB	++	+	1
AT3G49760	BZIP5	bZIP	+++	+	1
AT1G69490	ANAC029	NAC	+++	+	1
AT5G51990	DREB1D	AP2-EREBP (DREB)	++	+	1
AT1G26260	CIB5	bHLH	++		1
AT3G46070		C2H2	(+)	+++	1
AT2G24430	ANAC038/39	NAC	(+)	+++	1
At2g04040					
AT3G12910*		NAC	++		10
AT2G36610*	ATHB22	homeobox	++		5
AT5G43410		AP2-EREBP (ERF)	++		2
AT2G38300		GARP-G2-like	++		1
AT4G37730	BZIP7	bZIP	+++		1
AT1G49130		C2C2-CO-like	+++		1
AT4G33280		ABI3-VP1	+++	+	1
At5g62480					
AT4G14540	NF-YB3	CCAAT	+++		1
AT1G75510		-	+++		1
					1
At2g04070					
at1g16490	ATMYB58	MYB	+		1
at3g61910	ANAC066	NAC	+		1
At5g09570					
at5g60200	TMO6	C2C2--Dof	+		3
at3g11580		ABI3-VP1	+		2
at3g11020	DREB2B	AP2-EREBP (DREB)	+		1
at3g14180	ASIL2	Trihelix	+		1
HSP23.5 (At5g51440)					
at2g18160	BZIP2	bZIP	+		1
AOX1a (At3g22370)					
at4g39250	ATRL1	MYB-related	+		1
at2g13570	NF-YB7	CCAAT	+		1
at3g06740	GATA15	C2C2-GATA	+		1

Supplemental Table 3.1. Overview of the promoters screened and the identified transcription factors by means of Y1H screening with a pool of the REGIA TF clones. (*Continued*).

at5g53980	ATHB52	homeobox	+	1
at2g01060		GARP-G2-like	+	1

Supplemental Table 3.2. Primers used for promoter cloning.

Primer	Sequence
AttB4F_P-UPOX	ATAGAAAAGTTGACAACATTGATCATACGAGATCAAAAAGG
AttB1r_P-UPOX	TGTACAAACTTGACGCTGAAAACAGAAAGAAATCTCATGAA
AttB4F_P-UGT73C6	ATAGAAAAGTTGTGATAATTAGTGTAACTTTCACATACTCGA
AttB1r_P-UGT73C6	TGTACAAACTTGGCGGACGATGCAACTTTAGTAAGAACC
AttB4F_P-At2g04040	ATAGAAAAGTTGGAAGACAAGACTCGCGTATTTGTA
AttB1r_P-At2g04040	TGTACAAACTTGCCGTGTTTAACGTTGAAGGCCTTTCCCTTT
AttB4F_P-ST	ATAGAAAAGTTGAATGAATCTGCTTTACCAACTTAGTTC
AttB1r_P-ST	TGTACAAACTTGCGTTGTTGAGACTTGAGAGATCG
AttB4F_P-At2g04070	ATAGAAAAGTTGGTCATAAAGGTTGTTCCTAAGAGCTATCTCAAAGT
AttB1r_P-At2g04070	TGTACAAACTTGCGTTGTTTTAGGTTGTTAATTTTTTTTTTGTCC
AttB4F_P-NDB4	ATAGAAAAGTTGCTATTTCATAAATTGAACCTTAATTAAAGACATC
AttB1r_P-NDB4	TGTACAAACTTGGTGGTTGATGATTTCTCAAACCTCAGAT
AttB4F_P-UGT74E2	GGGGACAACTTTGTATAGAAAAGTTGGATTTCACCCATGATATACTG
AttB1r_P-UGT74E2	GGGGACTGCTTTTTTGTACAAACTTGTTTCTCCTTCTTTTTAATCTTGT
AttB4F_P-AOX1a	ATAGAAAAGTTGATCTGAAGAGCTTCTAGC
AttB1r_P-AOX1a	TGTACAAACTTGTGTTTCAAATCGGAAAAAGTG
AttB4F_P-At2g41730	ATAGAAAAGTTGATCAACCGATTGATCAATTGG
AttB1r_P-At2g41730	TGTACAAACTTGGTTTGCTTATTTTGATTTGAG
AttB4F_P-HSP23.5	ATAGAAAAGTTGGTAAAATCGATCTAAACCGACTTTCAAAT
AttB1r_P-HSP23.5	TGTACAAACTTGGATTTTTGGAAAGAGAAGAAGCTTAGAAT
AttB4F_P-At5g09570	ATAGAAAAGTTGGTCTGGAAGTGGAGGATTATTTTCTAC
AttB1r_P-At5g09570	TGTACAAACTTGGTGTTTGAATTTCAGATGTTGAAGTGTTAAG
AttB4F_P-At5g62480	ATAGAAAAGTTGTATTAGCTTTCTCTGTTTGTGTTTTGG
AttB1r_P-At5g62480	TGTACAAACTTGCGATTTTTTTATTCTTCTTTAGACTTGAGAGTATT

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Chapter 4

Oxidative stress-induced retrograde relocalization of a chloroplastic NAC transcription factor

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AUTHOR CONTRIBUTIONS

I.D.C. and A.I. wrote the chapter with help of F.V.B. and S.V. I.D.C. designed, performed and analyzed the yeast one-hybrid, chromatin immunoprecipitation, qRT-PCR experiments, promoter:GUS and stress assays (Figure 4.2, 4.7, 4.8, and 4.9; Supplemental Figure 4.1). A.I. produced the transgenic GFP fusion lines, designed, performed and analyzed the subcellular (re)localization and processing experiments (Figure 4.3, 4.4, 4.5, and 4.6). S.V. produced the *ANAC102*^{OE}, *ANAC102*^{KO} and prom:GUS lines. B.v.d.C. performed the chloroplast fractionation experiments.

ABSTRACT

Due to their sessile lifestyle, plants can not evade fluctuating environmental conditions. To cope with these stresses, plants have evolved robust adaptation methods driven by extensive transcriptional reprogramming. As stress responses are energetically costly, they are tightly regulated through complex signal transduction pathways. It is becoming increasingly evident that subcellular organelles such as mitochondria and chloroplasts are involved in stress signal transduction. During adverse environmental conditions, the functioning of these organelles is rapidly perturbed. Therefore, they are considered as sensors of stress conditions that communicate to the nucleus to initiate an appropriate defense response, in a process termed retrograde regulation. However, retrograde regulation mechanisms and components of plants are still poorly understood. Here, we present the involvement of the *Arabidopsis thaliana* NAC transcription factor ANAC102 in the stress response during chloroplast-derived oxidative stress conditions. During normal conditions, ANAC102 is localized to the chloroplasts. However, upon increasing ROS production in the chloroplasts, ANAC102 relocalizes to the nucleus to alter oxidative stress-responsive gene expression. Moreover, altered *ANAC102* levels affect plant oxidative stress tolerance. Our results indicate that ANAC102 could play an important role in chloroplast retrograde regulation of the stress response.

INTRODUCTION

Plant cells contain three genetic compartments (nucleus, chloroplast and mitochondrion) with the nucleus carrying the largest part of genomic information. This genetic compartmentalization requires communication to coordinate their activities during the organelles' development and function. This communication includes both anterograde (nucleus to organelle) and retrograde (organelle to nucleus) signals. Anterograde mechanisms mediate gene expression in organelles in response to endogenous and environmental stimuli that are perceived in the nucleus. Retrograde regulation transmits signals that originate in the mitochondria or the chloroplasts to regulate nuclear gene expression, and is further referred to as mitochondrial retrograde regulation (MRR; Chapter 1) and chloroplast retrograde regulation (CRR), respectively.

One important function of chloroplast retrograde regulation is to coordinate the biosynthesis of photosynthetic and metabolic protein complexes manufactured in the chloroplast with those subunits encoded in the nucleus, for instance during chloroplast biogenesis (Pogson et al., 2008). Furthermore, CRR has been shown to be important in modulating chloroplast functioning at optimal levels in accordance to fluxes in metabolites and changes in environmental conditions. For example, during fluctuating light conditions, the expression of photosynthesis-related genes is modulated to maximize photosynthesis and decrease ROS production due to photo-inhibition (Anderson et al., 1995). In addition to the vital role in regulating chloroplastic processes, it is also becoming clear that CRR plays a significant role in the plants' adaptive response to stresses (Fernandez and Strand, 2008). Metabolic processes in the chloroplast, especially the photosynthetic reactions, are extremely sensitive to stress. The chloroplasts could therefore act as sensors of changes in the environment and coordinate the nuclear-encoded adaptive stress response. The involvement of chloroplasts in the stress response has been mainly studied under conditions of excess light. When light energy exceeds the limit that plants can use for photosynthesis, excitation pressure and consequently ROS production increases in the chloroplast, and leads to induction of antioxidant gene expression and adjustment of photosynthetic machinery to reduce light harvesting (Klenell et al., 2005; Rossel et al., 2007; Woodson and Chory, 2008). Cold stress also targets chloroplast function by damaging the thylakoid membrane and slowing down photosynthesis (Crosatti et al., 2012), resulting in increased excitation pressure and ROS production and CRR has been shown to mediate cold

acclimation responses (Heidarvand and Maali Amiri, 2010). Moreover, it is becoming increasingly evident that CRR is also important during defense responses to pathogens (Padmanabhan and Dinesh-Kumar, 2010; Nomura et al., 2012). Chloroplast-derived singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), and oleic acids have been shown to regulate defense-related and salicylic acid responsive genes (Danon et al., 2005; Jiang et al., 2009; Maruta et al., 2012).

Although the CRR pathways are mechanistically not well understood, several signals have been reported that trigger retrograde signaling from chloroplasts: photosynthetic redox signals, reactive oxygen species (ROS), intermediates of chlorophyll biosynthesis and plastid gene expression-dependent signals (Figure 4.1) (Fernandez and Strand, 2008). In addition, several chloroplastic protein components have been described that can generate the retrograde signals, such as the GENOMES UNCOUPLED1 (GUN1), EXECUTER1 and EXECUTER2 (EX1 and EX2), and STATE TRANSITION7 (STN7). Furthermore, the nuclear transcription factor ABSCISIC ACID INSENSITIVE4 (ABI4) was identified as a nuclear component of CRR, repressing light induction of photosynthetic genes when the chloroplast function is impaired (Figure 4.1) (Koussevitzky et al., 2007). However, the signaling cascades that relay these chloroplast signals to the nucleus remain unclear.

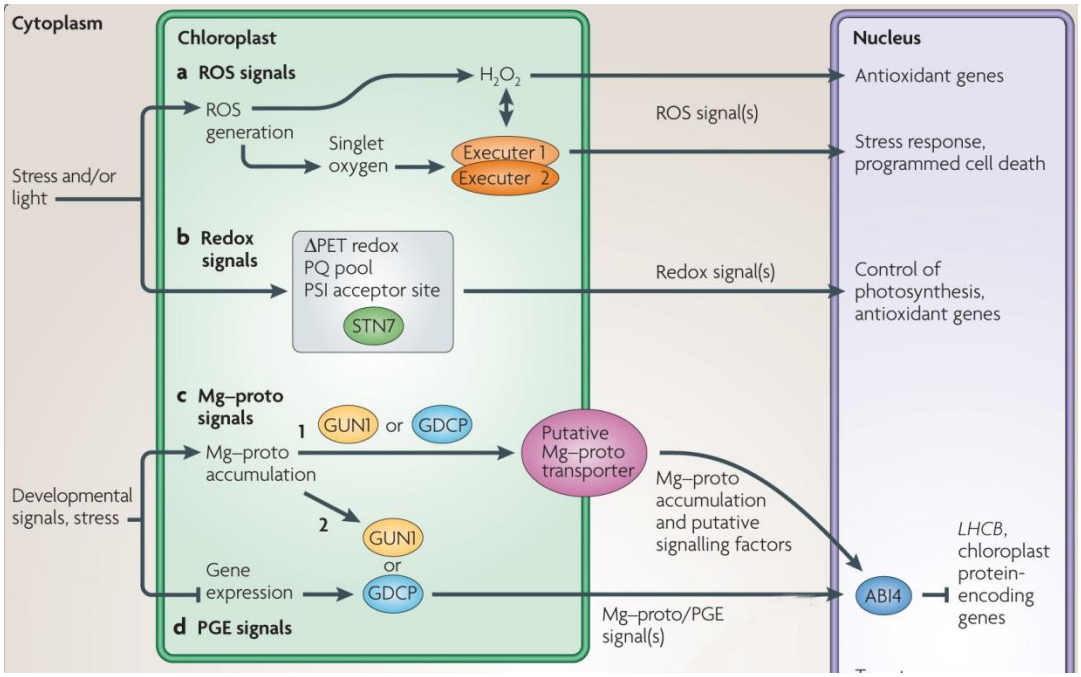

Figure 4.1. Chloroplast retrograde signaling pathways in plants.

Figure 4.1. Chloroplast retrograde signaling pathways in plants. (*Continued*).
Four different pathways are displayed (a-d). **a)** Reactive oxygen species (ROS) accumulate during exposure to stress or excess light. Singlet oxygen ($^1O_2$) accumulation in the chloroplast is mediated to the nucleus via the chloroplastic proteins EXECUTER1 and EXECUTER2 and activates genes involved in programmed cell death. Hydrogen peroxide (H_2O_2) is more stable, can migrate from the chloroplasts and induce an acclimatory response. The crosstalk between $^1O_2$ and H_2O_2-mediated signaling might contribute to the fine-tuning of the stress response and set the threshold for cell death or resistance. **b)** Redox signals are mediated through the reduction state of plastoquinone (PQ) or via elements on the reducing site of photosystem I (PSI) and are involved in modulating metabolism in response to fluctuating light conditions. The chloroplastic protein kinase STATE TRANSITION7 (STN7) participates in the transfer of the redox signal to the nucleus. **c and d)** Signals from the accumulation of chlorophyll biosynthesis intermediates (Mg-protoporphyrin IX) and inhibition of plastid gene expression (PGE) are mainly involved in genome coordination. These two pathways converge in the chloroplast at the chloroplastic GENOMES UNCOUPLED1 (GUN1) protein. In response to the GUN1-derived signal, the transcription factor ABSCISIC ACID INSENSITIVE4 (ABI4) prevents binding of factors required for light-induced expression of nuclear-encoded photosynthesis genes.
LHCB, gene encoding photosystem II chlorophyll a/b-binding protein.
From Woodson and Chory (2008).

Important candidates for mediating CRR are proteins that can move from the chloroplast to the nucleus (Krause et al., 2012). Proteins can be sequestered in the chloroplast before they act as transcriptional regulators in the nucleus. Their storage and release from chloroplasts and their subsequent translocation to the nucleus could allow a fast response to plastid perturbations upon certain triggers (Krause et al., 2012). Evidence for this was recently provided by the identification of the chloroplast envelope-bound plant homeodomain (PHD) transcription factor PHD TYPE TRANSCRIPTION FACTOR WITH TRANSMEMBRANE DOMAINS (PTM) that is, upon perturbation of chloroplastic function, proteolytically cleaved and released to the nucleus (Sun et al., 2011). PTM mediates CRR-mediated down-regulation of nuclear photosynthetic genes by activating *ABI4* transcription in a manner associated with histone modifications. Another protein candidate for mediating chloroplast to nucleus communication is Whirly1 that has been shown to move from the chloroplast to the nucleus to effect the expression of pathogenesis-related genes (Isemer et al., 2012).

In this study, we aimed at characterizing the Arabidopsis NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) family transcription factor ANAC102 that was previously identified as a putative *MDR* (mitochondrial dysfunction regulon; Chapter 2) regulator by means of Y1H screening (Chapter 3). *ANAC102* is induced upon various stress conditions, thereby showing substantial co-expression with the *MDR*. Interestingly, ANAC102 was shown to localize to the

chloroplasts (Marshall et al., 2012), indicating it could be a candidate regulator of organelle (chloroplast)-mediated stress signal transduction. We showed that upon increasing chloroplastic ROS production, ANAC102 relocalizes to the nucleus to repress *MDR* gene expression. Moreover, altered *ANAC102* levels affect plant tolerance towards oxidative stress conditions. Thus, we characterized ANAC102 as a candidate regulator of chloroplast retrograde regulation of the stress response.

RESULTS

ANAC102 interacts with *MDR* promoters in the yeast one-hybrid system

In Chapter 3, we aimed at identifying regulators of target genes of mitochondrial retrograde regulation, referred to as the mitochondrial dysfunction regulon genes (*MDR*). Therefore, we screened the *MDR* promoters for interacting transcription factors using the Y1H system. In a high-throughput Y1H matrix screening assay against the REGIA transcription factor (TF) library (Paz-Ares, 2002), the NAC family transcription factor ANAC102 was identified (Chapter 3). ANAC102 interacts with the *UPREGULATED BY OXIDATIVE STRESS* (*UPOX*), *UDP-GLYCOSYL TRANSFERASE 74E2* (*UGT74E2*), *ALTERNATIVE OXIDASE 1a* (*AOX1a*), *UGT73E2*, DETOXIFICATION1 (*AtDTX1*), SULFOTRANSFERASE (*ST*), ATP-BINDING CASSETTE B4 (*ABCB4*), NAD(P)H DEHYDROGENASE B4 (*NDB4*), and *At2g41730* promoters, as evidenced by induction of the *Histidin3* (*HIS3*) reporter construct. Induction of the *β-galactosidase* (*LacZ*) reporter gene was only observed with the *UGT74E2* and *At2g04040* promoters, but not with the other *MDR* promoters tested. The Y1H interaction data for three of the *MDR* promoters (*AOX1a*, *UPOX* and *UGT74E2*) are displayed in Figure 4.2. To confirm the interactions *in planta*, chromatin immunoprecipitation (ChIP) experiments were performed on transgenic Arabidopsis seedlings overexpressing a green fluorescent protein (GFP)-tagged version of *ANAC102* (*ANAC102-GFP*^{OE}). Preliminary ChIP data show enrichment of *UGT74E2* and *At2g04040* promoter fragments after precipitation with the anti-GFP antibody, relative to *ACTIN2* (data not shown). Although repeat experiments are necessary to confirm these ChIP results and to assess the interaction with other *MDR* promoters, these data indicate that ANAC102 is a potential regulator of the *MDR* in Arabidopsis.

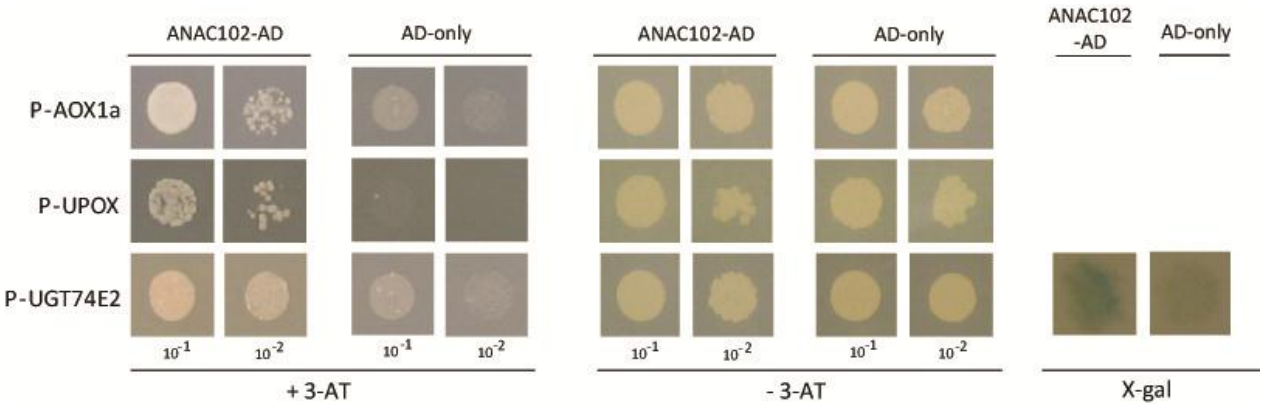

Figure 4.2. ANAC102 binds the *MDR* promoters in the yeast one-hybrid system.
ANAC102 interacts with the *AOX1a*, *UPOX*, and *UGT74E2* promoters as shown by the Y1H assay. Yeast reporter strains containing ANAC102 fused to the GAL4 activation domain (ANAC102-AD) induce the *HIS3* reporter constructs compared to yeast cells containing only the AD (AD-only), as revealed by growth on 3-aminotriazole (3-AT), a competitive inhibitor of HIS3. Induction of the *LacZ* reporter gene was only observed with the *UGT74E2* promoter, as revealed by the β-galactosidase assay (X-gal), but not with the *AOX1a* and *UPOX* promoters (data not shown).

ANAC102 localizes to the chloroplasts

It was previously demonstrated that ANAC102 potentially localizes to the chloroplasts (Marshall et al., 2012). In this study, the open reading frame (ORF) of *ANAC102* was fused in frame with *GFP* both at the N- or C-terminus and placed under the control of the constitutive cauliflower mosaic virus 35S (CaMV35S) promoter (Figure 4.3a). While transient expression of the N-terminal GFP fusion of *ANAC102* (*GFP-ANAC102*) in the leaf epidermis of *Nicotiana benthamiana* led to both a nucleocytosolic and a putative cytoskeletal localization, the C-terminal ANAC102-GFP fusion colocalized with the autofluorescence of the chloroplasts and was also present in the nucleus (Marshall et al., 2012). Next, the subcellular localization was assessed in leaf epidermis cells of at least two independent transgenic Arabidopsis plants. While no GFP-positive primary transformants overexpressing *GFP-ANAC102* could be obtained, the nuclear and chloroplastic localization of ANAC102-GFP could be confirmed in several independent *ANAC102-GFP*^{OE} plants (Figure 4.3b). Within the chloroplasts, GFP fluorescence was not evenly distributed but appeared in speckles.

Because overexpression of a GFP-tagged protein could lead to protein mistargeting, transgenic plants expressing the N- and C-terminal GFP fusion construct under the transcriptional control of the native *ANAC102* promoter were constructed (prom$_{ANAC102}$:*GFP-ANAC102* and prom$_{ANAC102}$:*ANAC102-GFP*) (Figure 4.3a). Unfortunately, no GFP fluorescence was observed for both constructs and this was probably due to the low expression of *ANAC102* in leaves during normal growth conditions. Because *ANAC102* transcript levels are constitutively increased in leaves of catalase2-deficient mutants (*cat2*) that have decreased hydrogen peroxide (H_2O_2) scavenging capacity (Vandenabeele et al., 2004; Vanderauwera et al., 2005; Vanderauwera et al., 2011), the prom$_{ANAC102}$:*ANAC102-GFP* constructs were transformed in the *cat2* mutant background, resulting in a strong GFP fluorescence (Figure 4.3b). While overexpression of *ANAC102-GFP* resulted in both a nuclear and a chloroplastic localization, native expression of *ANAC102-GFP* led to an exclusive chloroplastic localization in the *cat2* mutant (Figure 4.3b). Moreover, native expression of *GFP-ANAC102* showed no GFP fluorescence in *cat2*. In accordance with the putative chloroplastic localization, ANAC102 contains a predicted chloroplast transit peptide (cTP) at its N-terminus (Figure 4.3c) (Emanuelsson et al., 2007). Positioning of GFP at the N-terminus of ANAC102 presumably shields the cTP and, therefore, the observed cytoskeletal localization in *GFP-ANAC102*^{OE} plants probably resulted from blockage of the cTP, leading to mistargeting of ANAC102.

To further consolidate the chloroplastic localization and determine the suborganellar location of ANAC102, intact chloroplasts were isolated from leaves of both *ANAC102-GFP*^{OE} and prom$_{ANAC102}$:*ANAC102-GFP* lines. Subsequently, chloroplasts were lysed in a hypotonic medium and chloroplast thylakoids were separated from the stroma fraction by centrifugation. GFP-tagged proteins were detected by protein gel blot analysis using a monoclonal anti-GFP antibody. Preliminary results indicated that ANAC102-GFP is present in intact chloroplasts, consolidating the GFP localization data (Figure 4.3d). Within the chloroplast, ANAC102-GFP was mainly found in the thylakoid fraction.

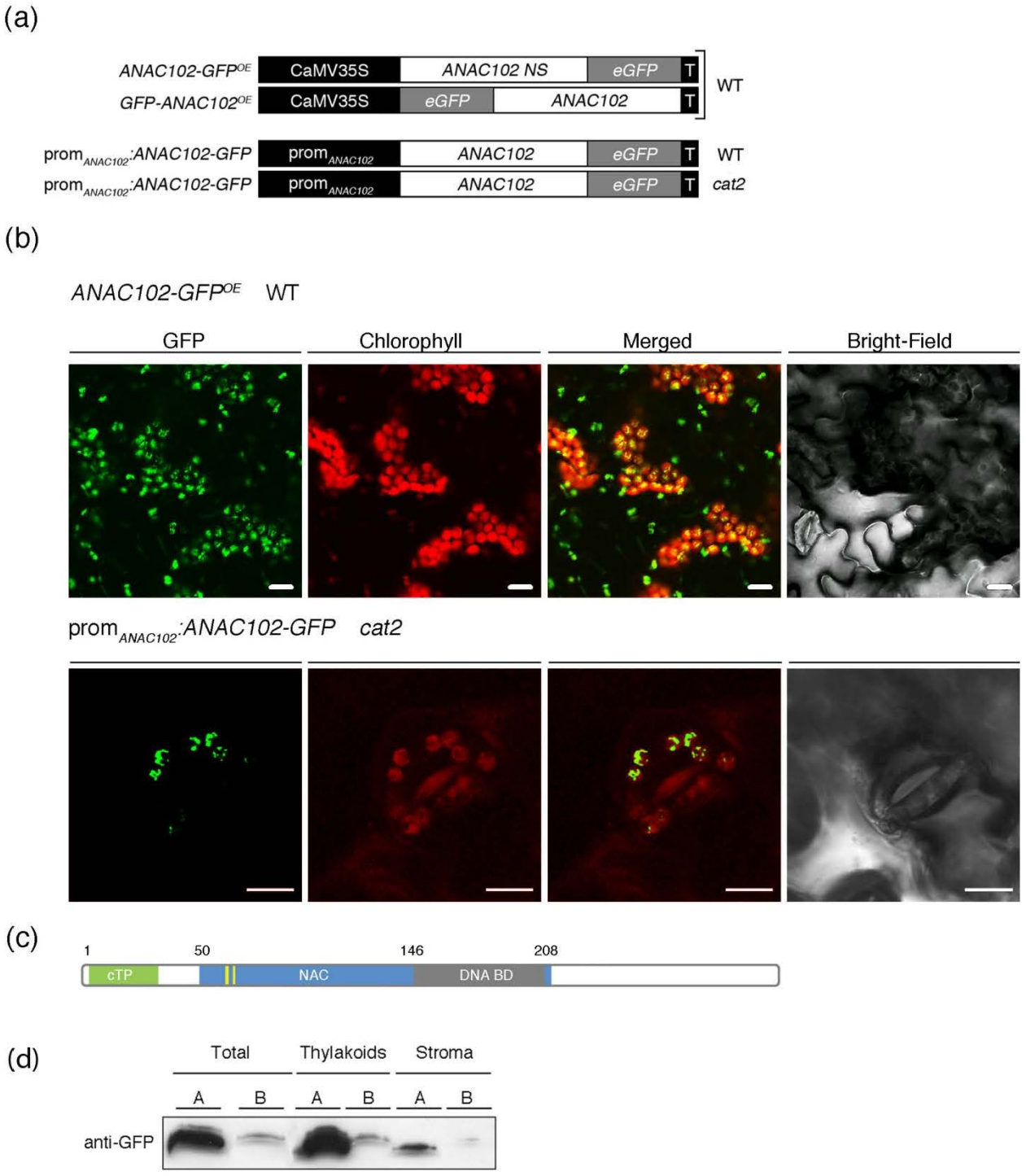

Figure 4.3. ANAC102 is targeted to the chloroplasts.
(a) Constructs used to determine the subcellular localization of ANAC102.
(b) ANAC102-GFP localizes to the chloroplasts. Subcellular localization of ANAC102-GFP in stable transgenic Arabidopsis wild-type and *cat2* lines. Bars, 20 µm. GFP, green fluorescent protein; Merged, overlay of GFP and chlorophyll (red) fluorescence images.

Figure 4.3. ANAC102 is targeted to the chloroplasts.(*Continued*).
(c) Schematic representation of the different domains present in ANAC102. *ANAC102* encodes a 312 amino acid protein with a conserved NAC domain (50-208 amino acids). The NAC domain contains the DNA binding domain (BD) and a predicted bipartite nuclear localization signal (indicated in yellow) (Kikuchi et al., 2000). The N-terminal region contains a predicted chloroplast transit peptide (cTP) (Emanuelsson et al., 2007).
(d) Suborganellar localization of ANAC102-GFP. Intact chloroplasts were isolated from leaves of *ANAC102-GFP*^{OE} and prom$_{ANAC102}$*:ANAC102-GFP* plants and fractionated into stromal and thylakoid fractions. Fifteen micrograms of each fraction was separated by SDS-PAGE, and protein gel blot analysis was performed using a monoclonal anti-GFP antibody.

Next, the targeting properties of the different domains of ANAC102 were assessed by making GFP-tagged truncated versions of ANAC102 (Figure 4.4a). First, to functionally validate the targeting properties of the predicted cTP, a construct was made in which the N-terminal domain of ANAC102 (1-43 amino acids), that includes the predicted cTP sequence, was fused in frame with GFP (*cTP$_{ANAC102}$-GFP*) and placed under the constitutive promoter CaMV35S (Figure 4.4a). While transient expression of full length *ANAC102-GFP* in *N. benthamiana* resulted in a nuclear and chloroplastic localization, cTP$_{ANAC102}$-GFP displayed a nucleocytosolic localization (Figure 4.4b). On the other hand, ANAC102-GFP proteins that lack this N-terminal domain (Δ cTP ANAC102- GFP) displayed an exclusive nuclear localization (Figure 4.4b).

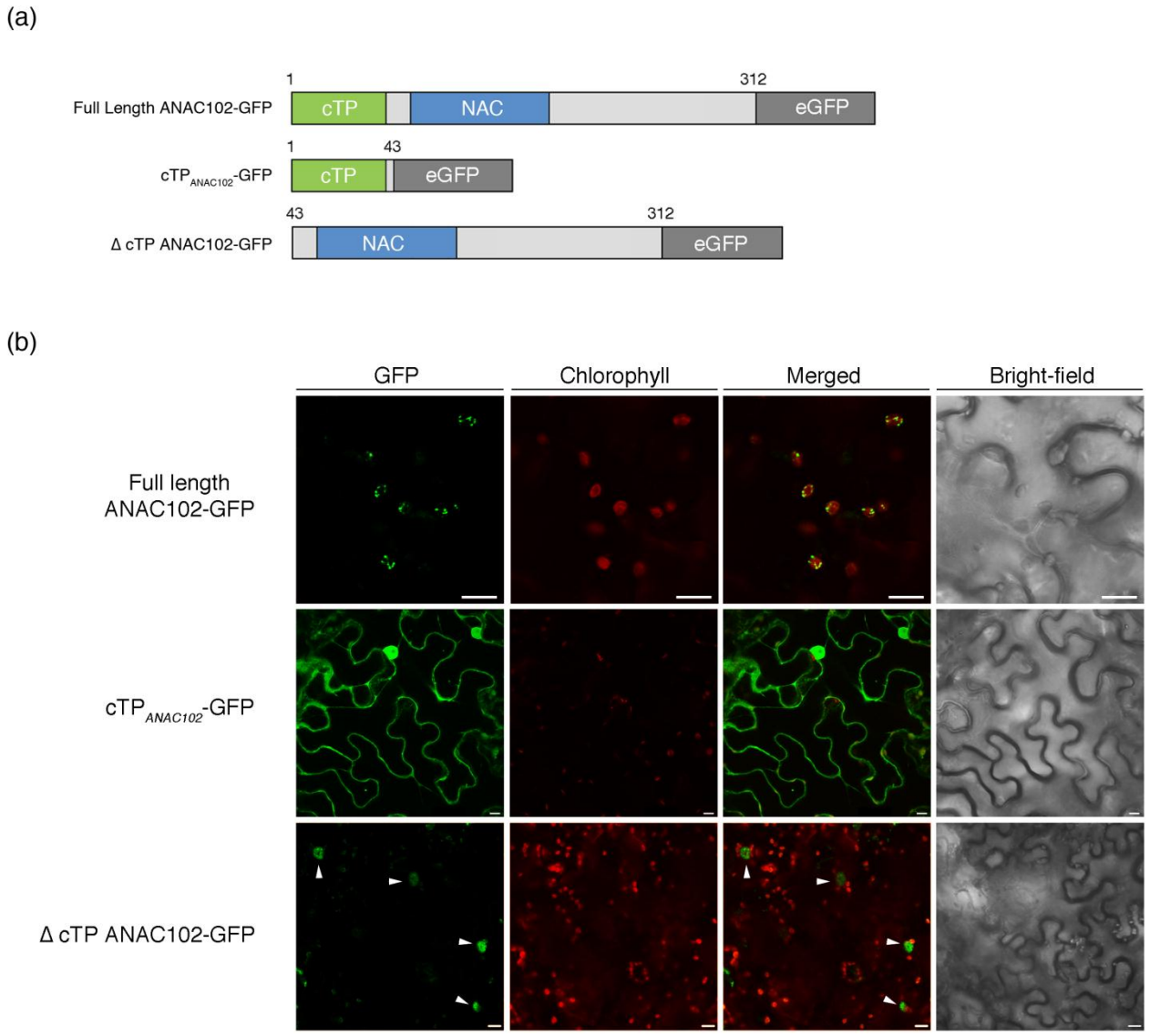

Figure 4.4. Deletion constructs of ANAC102-GFP.
(a) Schematic representation of the full-length and deletion constructs.
(b) Transient expression of the deletion constructs in leaf epidermal cells of *N. benthamiana*. Similar results were obtained with independent experiments. cTP, chloroplast transit peptide; merged, overlay of GFP and chlorophyll (red) fluorescence images. Bars, 10 µm.

Relocalization of ANAC102 during oxidative stress conditions

The chloroplastic localization of ANAC102 is at first sight contradictory with its role to regulate nuclear gene expression. Therefore, we hypothesized that ANAC102 proteins might redistribute to the nucleus under a certain stimulus. Oxidative stress-induced subcellular relocalization has been reported for a number of proteins (Ahlfors et al., 2004; Kaminaka et al., 2006). Therefore,

we assessed whether the subcellular localization of ANAC102 changes during oxidative stress conditions. Together with the mitochondria and peroxisomes, chloroplasts are the major ROS production sites. To increase ROS levels in the chloroplasts, we used the herbicide methyl viologen (MV, 1,1'-dimethyl-4,4'-bipyridinium dichloride), also known as paraquat. MV is a redox-active compound that in light accelerates the generation of superoxide radicals ($O_2^{\bullet-}$) and H_2O_2 in chloroplasts (Mehler, 1951) (Figure 4.5a). Prom$_{ANAC102}$:*ANAC102-GFP* lines (*cat2*) were grown *in vitro* for two weeks on half-strength (½) MS on nylon mesh and were subsequently transferred to ½MS containing 50 µM MV. Whereas ANAC102-GFP colocalized exclusively with chloroplasts prior to MV treatment, GFP fluorescence was also observed in nuclei after 6 h of MV treatment (Figure 4.5b).

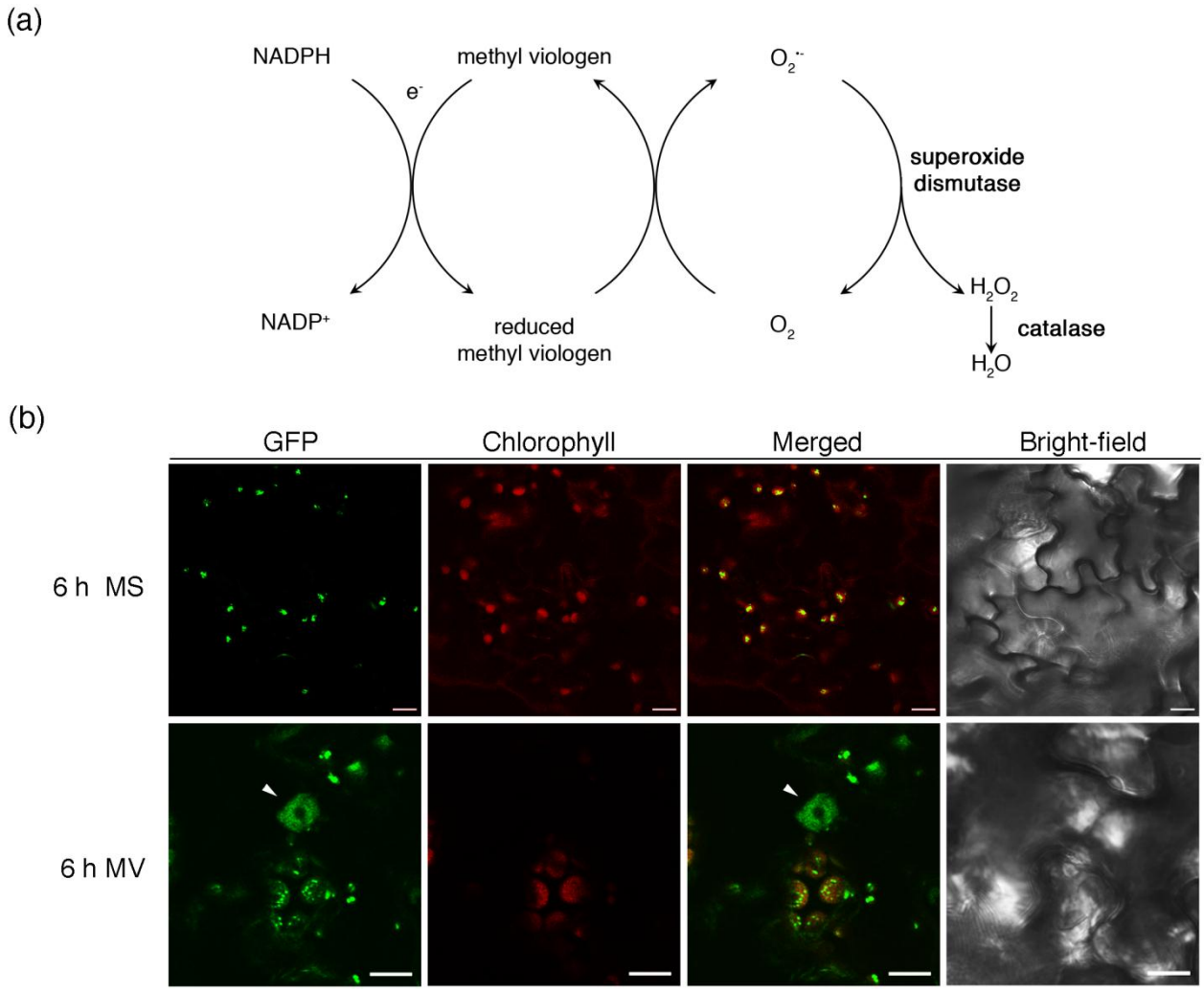

Figure 4.5. ANAC102 relocalization from the chloroplasts to the nucleus by MV.

Figure 4.5. ANAC102 relocalization from the chloroplasts to the nucleus by MV. (*Continued*)
(a) Schematic representation of the mode of action of MV in the light. MV accepts electrons (e^-) from photosystem I and transfers them to molecular oxygen (O_2). This leads to the formation of superoxide radicals ($O_2^{\bullet-}$) and regenerates oxidized MV, again available to accept electrons from photosystem I and start the cycle again. $O_2^{\bullet-}$ is converted to H_2O_2 by superoxide dismutase. H_2O_2 can freely diffuse between subcellular compartments and is scavenged by peroxisomal catalases. Enzymes are indicated in bold.
(b) ANAC102 relocalizes to the nucleus upon MV treatment in *cat2* mutants. Under non-stressed conditions, ANAC102-GFP was exclusively localized to the chloroplasts. After 6 h of MV treatment, ANAC102-GFP was localized both to the chloroplasts and the nuclei (indicated with an arrow). Similar results were obtained in independent experiments. Merged, merged images of GFP and chlorophyll fluorescence images.

Next, we assessed whether ANAC102 is proteolytically processed upon MV treatment. Therefore, we treated *ANAC102-GFP*^{OE} and prom$_{ANAC102}$:*ANAC102-GFP* (*cat2*) plants with 50 µM MV as described above and visualized GFP-tagged proteins on a protein gel blot with anti-GFP antibody. Both in the *ANAC102-GFP*^{OE} and the prom$_{ANAC102}$:*ANAC102-GFP* lines, the full length ANAC102-GFP (~63 kDa) and a band that corresponds in size with ANAC102-GFP without the N-terminal cTP (Δ cTP ANAC102-GFP; ~58 kDa) were detected (Figure 4.6). Moreover in *ANAC102-GFP*^{OE} lines, additional cleavage products of ANAC102-GFP were detected (~45 and ~30 kDa) irrespective of the MV treatment (Figure 4.6a). Because overexpression leads to a nuclear localization, the cleavage of a C-terminal region of approximately 18 kDa could be necessary for release from the chloroplasts. Unfortunately, we could not detect the presence of these cleavage products in the prom$_{ANAC102}$:*ANAC102-GFP* lines upon MV treatment (Figure 4.6b) and, thus, could not directly link the relocalization with the MV-induced processing of ANAC102.

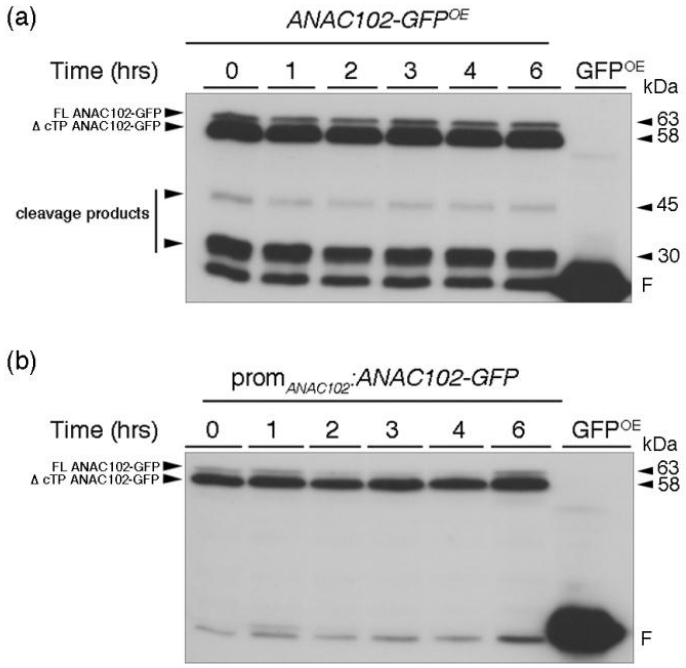

Figure 4.6. Processing of ANAC102. *ANAC102-GFP*^{OE} (a) and *prom*$_{ANAC102}$*:ANAC102-GFP* (*cat2*; b) lines were grown for 2 weeks on ½MS agar plates and subsequently treated with 50 µM MV. Fifteen micrograms of protein were separated by SDS-PAGE, and protein gel blot analyses were performed with a monoclonal anti-GFP antibody. The full-length (FL) ANAC102-GFP (63 kDa), the ANAC102-GFP without cTP (Δ cTP ANAC102-GFP; 58 kDa) are indicated by an arrow. *GFP*^{OE} plants were used as a positive control for GFP signal. F, free GFP.

Altered *ANAC102* levels affect oxidative stress tolerance

As ANAC102 likely relocalizes from the chloroplast to the nucleus during oxidative stress, we assayed the effect of altered *ANAC102* levels on plant stress tolerance under those conditions. Therefore, we generated transgenic Arabidopsis lines overexpressing *ANAC102* under the control of the CAMV35S promoter. Two independent homozygous overexpression lines (*ANAC102*^{OE1} and *ANAC102*^{OE2}) with elevated *ANAC102* transcript levels (35-fold and 143-fold, respectively; assayed with qRT-PCR analyses) were selected. The T-DNA insertion mutant SALK_030702, in which a T-DNA was inserted into the second exon of the *ANAC102* gene, was obtained from the SALK collection (Alonso and Stepanova, 2003). Homozygous mutants of this line had 6.5% residual *ANAC102* transcript levels. Two-week-old control grown wild-type and *ANAC102* mutant plants were transferred to ½MS agar plates supplemented with 1 or 2 µM MV and rosette growth was visually monitored throughout the treatment. After one week of MV treatment, the rosette area of the strongest overexpression line (*ANAC102*^{OE2}) was larger and seemed to have lower anthocyanin pigmentation (Figure 4.7). However, when the MV treatment was prolonged to five weeks, plants of both overexpression lines (*ANAC102*^{OE1} and *ANAC102*^{OE2}) had completely died, while the wild-type plants remained green with visibly high anthocyanin pigmentation (Figure 4.7).

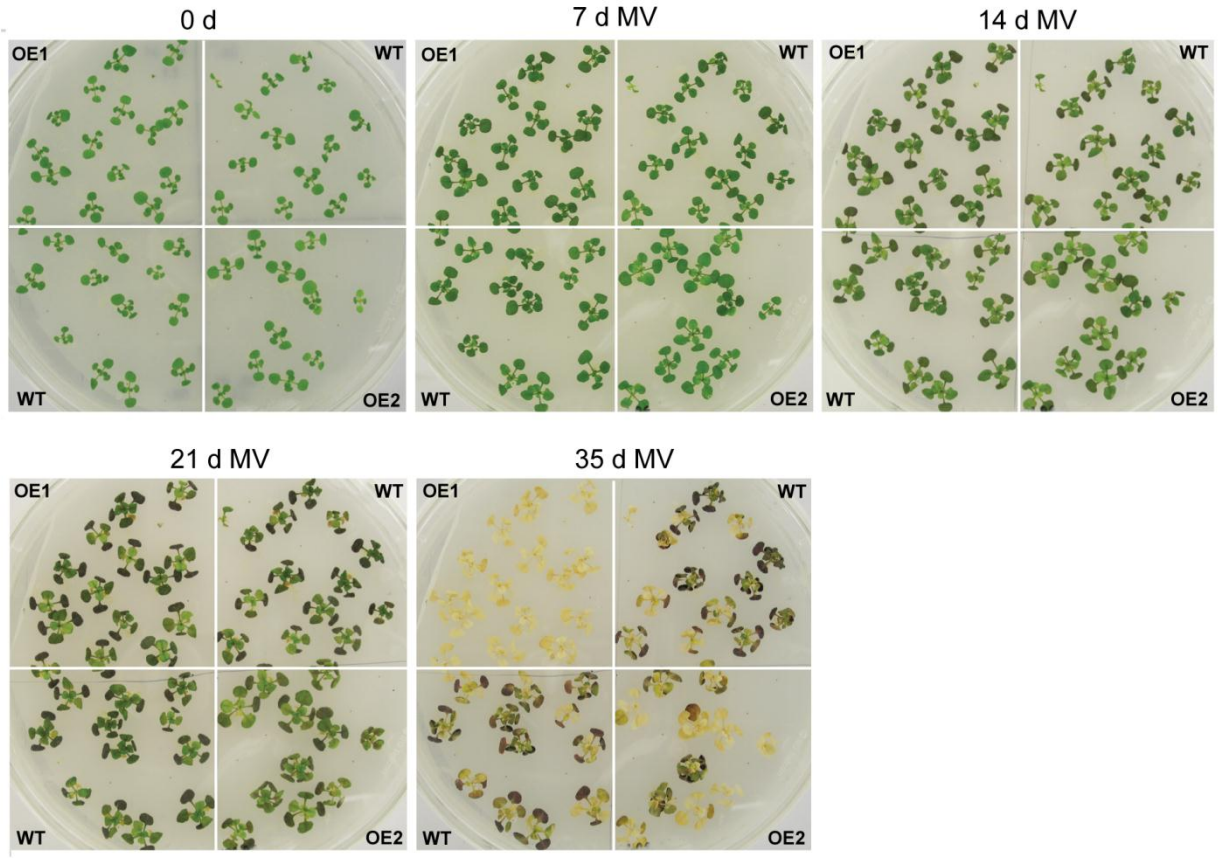

Figure 4.7. *ANAC102* overexpression lines are more sensitive to MV-mediated oxidative stress.
Phenotype of the *ANAC102*^{OE} lines during 2 µM MV treatment. Two-week-old wild-type (WT) and *ANAC102*^{OE} lines (OE1 and OE2) grown under control conditions (0 d) were transferred to 2 µM MV-containing ½MS medium and rosette growth was monitored until 5 weeks after transfer.

Next, we assessed whether alterations in *ANAC102* expression levels also affect tolerance to other oxidative stress-related conditions. First, the performance of *ANAC102*^{OE} and *ANAC102*^{KO} plants was assayed during conditions of increased photorespiration, that promotes the intracellular generation of H_2O_2. Therefore, a bioassay was used in which photorespiration in the peroxisomes is increased by restricting gas exchange within Petri plates (Mühlenbock et al., unpublished results; Vanderauwera et al., 2012; see Methods). Whereas the strongest *ANAC102* overexpression line (*ANAC102*^{OE2}) was visibly more susceptible to photorespiratory H_2O_2, the *ANAC102*^{KO} plants showed an increased performance (Figure 4.8). These observations were confirmed by measurements of the photosynthetic performance (Figure 4.8). In a second assay, postgermination growth and early development of *ANAC102* mutant lines under oxidative stress

was examined by germinating and growing the plants on ½MS medium containing the herbicide 3-amino-1,2,4-triazole (3-AT), an inhibitor of the peroxisomal H_2O_2 scavenger catalase (Gechev et al., 2005). Under low (1 µM) 3-AT concentration, *ANAC102* overexpression seedlings showed an increased rosette area, indicating they have a better growth performance under these mild oxidative stress conditions (Supplemental Figure 4.1ai). However, under severe (2 – 8 µM) 3-AT stress, *ANAC102*^{KO} had an increased performance as evidenced by a larger rosette area (under 2 µM 3-AT) and less chlorophyll loss (under 2 and 8 µM 3-AT), as assessed by visual observation (Supplemental Figure 4.1aii). In a third assay, tolerance to exogenously applied H_2O_2 was assessed by germinating and growing plants on medium supplemented with H_2O_2. Whereas no visible phenotype was observed for the overexpression lines, *ANAC102*^{KO} seedlings had a better performance on mild (1 – 2 mM) H_2O_2 concentrations (Supplemental Figure 4.1b). When grown on 1 mM H_2O_2, the rosette area of *ANAC102*^{KO} seedlings was visibly larger than that of wild-type plants. When grown on 2 mM H_2O_2, wild-type plants showed a retarded development and chlorophyll loss, whereas knockout seedlings developed with milder symptoms. To conclude, these data indicate that *ANAC102* overexpression increases sensitivity to oxidative stress, possibly by inhibiting the stress response and the concomitant growth reduction, whereas reduced *ANAC102* levels lead to higher tolerance. Morover, the impaired stress-induced growth reduction of *ANAC102* overexpressing plants appears to increase yield and performance of plants under very mild stress conditions.

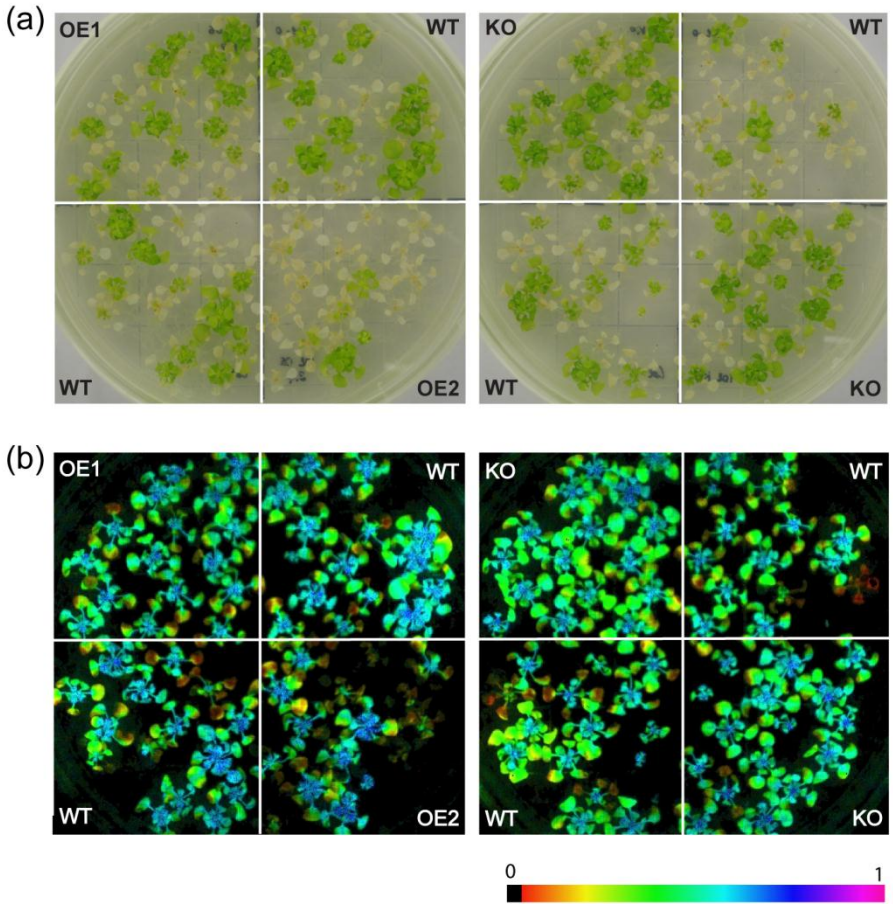

Figure 4.8. Altered *ANAC102* levels affect tolerance to photorespiratory H_2O_2 stress.
(a) Wild-type (WT), *ANAC102*^{OE} (OE1 and OE2), and *ANAC102*^{KO} (KO) plants were grown for 2 weeks under control conditions and subsequently subjected to photorespiratory H_2O_2 stress conditions for 5 weeks.
(b) Photosystem II maximum efficiency (F_v'/F_m') of WT, *ANAC102*^{OE} (OE1 and OE2), and *ANAC102*^{KO} (KO) plants subjected to photorespiratory H_2O_2 stress conditions for 3 weeks.

ANAC102 represses oxidative stress-mediated induction of the *MDR* genes

We previously showed that ANAC102 binds the promoters of several *MDR* genes in the Y1H system, indicating it could regulate the *MDR in planta*. Gene expression analysis using GENEVESTIGATOR (Zimmermann et al., 2004) revealed that the *MDR* genes as well as *ANAC102* are strongly up-regulated by MV-induced oxidative stress (Figure 4.9a). To further assess the spatiotemporal expression pattern of *ANAC102* upon MV treatment, transgenic Arabidopsis plants were generated in which the *ANAC102* promoter was transcriptionally fused to *GUS*. Under nonstressed conditions, GUS staining was observed in parts of the root system,

cotyledons and faintly in older leaves of two-week-old seedlings (Figure 4.9b), similar to results obtained in Christianson et al. (2009). However, *ANAC102* promoter activity was increased in the aerial tissues following MV exposure, with the strongest induction after 6 h and 9 h of treatment (Figure 4.9b).

To examine the role of ANAC102 in regulating *MDR* gene expression, we assessed the effect of altered *ANAC102* levels on *MDR* transcript levels during normal and oxidative stress conditions. Wild-type and mutant *ANAC102* transgenic lines were germinated and grown for 2 weeks on ½MS and subsequently transferred to ½MS supplemented with 50 µM MV, as described above. We followed the expression of five *MDR* genes (*AOX1a*, *AtDTX1*, *ST*, *UGT73E2*, and *UGT74E2*) in time in mock- and MV-treated wild-type and *ANAC102* mutant plants. These five genes were strongly induced upon MV treatment in wild-type plants (Figure 4.9c). However, the induction was partially abolished in *ANAC102*^{OE} lines compared to wild-type plants, indicating that ANAC102 could function as a transcriptional repressor of these genes under oxidative stress conditions (Figure 4.9c). In *ANAC102*^{KO} plants, the *MDR* transcript levels were not altered when compared to the wild-type situation under both nonstressed and oxidative stress conditions (data not shown).

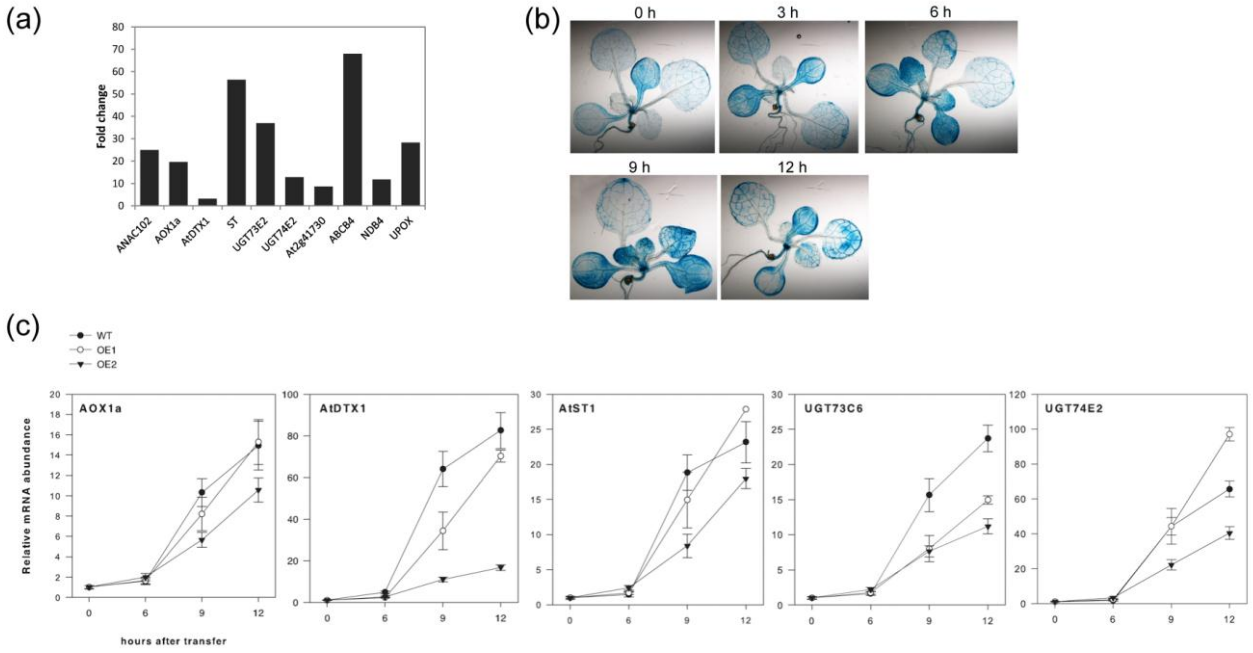

Figure 4.9. *ANAC102* overexpression partially represses oxidative stress-induced *MDR* gene expression.

Figure 4.9. *ANAC102* overexpression partially represses oxidative stress-induced *MDR* gene expression. (*Continued*).
(a) *ANAC102* and its target *MDR* genes are induced after MV treatment. Expression ratios of MV treatment relative to mock treatment in green tissue were obtained from the AtGenExpress abiotic time series (Kilian et al., 2007) with the GENEVESTIGATOR meta-analysis tool (Zimmermann et al., 2004).
(b) Spatiotemporal expression pattern of *ANAC102* during MV treatment visualized by histochemical GUS staining.
(c) Expression profile of *AOX1a*, *AtDTX1*, *ST*, *UGT73E2*, and *UGT74E2* during MV treatment in wild-type (WT) plants and two independent $ANAC102^{OE}$ lines (OE1 and OE2). Values are expressed relative to the 6 h-mock-treated wild-type (indicated by time point 0). Data represent average ± SE (n = 3 qRT-PCR technical repeats). Similar results were obtained in a biological repeat experiment.

DISCUSSION

Adverse environmental conditions such as drought, heat stress and pathogen assaults negatively affect plant growth and development. Upon exposure to stress, plants regulate a variety of genes with the aim to enable them to adapt to these stresses. Complex transcriptional regulatory networks are involved in regulating stress-mediated gene expression, involving both transcriptional activation and repression to ensure the tight regulation of the stress response (Yamaguchi-Shinozaki and Shinozaki, 2006). Several transcription factor families, such as AP2/EREBP, NAC, WRKY, bZIP, MYB, and bHLH have been found to control downstream gene expression in various stress signal transduction pathways.

The NAC family is one of the largest plant-specific transcription factor families with more than 100 members in Arabidopsis (Ooka et al., 2003) and has been implicated in a wide range of developmental processes, and pathogen defense and abiotic stress responses (Olsen et al., 2005a). The NAC proteins are characterized by the presence of a conserved N-terminal NAC domain that contains five subdomains involved in DNA binding, nuclear localization and the formation of homo- or heterodimers with other NAC proteins (Xie et al., 2000; Duval et al., 2002; Ernst et al., 2004). Based on similarities in the NAC domain amino acid sequences, NAC proteins are classified into 18 subfamilies (Ooka et al., 2003). In contrast to the N-terminal NAC domain, the C-terminal regions of NAC proteins are highly divergent (Ooka et al., 2003) and mediate the transcriptional activity (Xie et al., 2000; Jensen et al., 2010).

ANAC102 belongs to the ATAF subfamily of the NAC transcription factor family that includes the Arabidopsis ATAF1 (ANAC002), ATAF2 (ANAC081) and ANAC032 (Ooka et al.,

2003). Genes encoding these ATAF subfamily members show some degree of co-expression as they are commonly expressed in root tissue, in old leaves and during salt stress (Jensen et al., 2010). Besides the induction by salt stress, *ANAC102* transcript levels are increased by osmotic, cold, UV-B and oxidative stress and pathogen infection, but decreased by heat stress (Zimmermann et al., 2004). Most NAC genes shown to be induced by cold are repressed by heat and vice versa (Jensen et al., 2010). Moreover, *ANAC102* and *ATAF2* are induced by a combination of cold and light treatment that affects the photosynthetic machinery and chloroplast redox status (Soitamo et al., 2008). ATAF1 negatively regulates drought and biotic stress responsive gene expression (Lu et al., 2007; Wang et al., 2009b). ATAF2 functions as a repressor or activator of pathogenesis related genes, depending on the growth conditions (Delessert et al., 2005; Wang et al., 2009a). ANAC102 was recently characterized to mediate hypoxia resistance of seeds (Christianson et al., 2009).

We previously identified five closely related transmembrane domain-containing (NAC WITH TRANSMEMBRANE MOTIF 1-LIKE [NTL]) NAC transcription factors as positive regulators of the mitochondrial dysfunction regulon (*MDR*) genes during mitochondrial retrograde regulation (Chapter 2). By means of Y1H screening (Chapter 3), several other candidate TFs that bind to the *MDR* promoters were identified. Among them, ANAC102 shows substantial co-expression with the *MDR*. Interestingly, ANAC102 is localized to the chloroplasts under nonstressed conditions, indicating it could mediate chloroplast signals to regulate *MDR* gene expression. We showed that upon chloroplast-initiated oxidative stress, ANAC102 relocalizes to the nucleus and represses *MDR* gene expression. Moreover, altered *ANAC102* levels affect plant tolerance towards oxidative stress conditions. Thus, we characterized ANAC102 as a potential signaling component of chloroplast retrograde regulation of the stress response.

ANAC102 is located to the chloroplasts and relocalizes to the nucleus during oxidative stress

ANAC102 translationally fused to GFP (ANAC102-GFP) was previously demonstrated to localize to the chloroplasts (Marshall et al., 2012). Strong overexpression of *ANAC102-GFP* resulted in a dual targeting to the chloroplasts and the nucleus, indicating that ANAC102 harbors

both a functional chloroplast transit peptide (cTP) and nuclear localization signal (NLS). Interestingly, expression of *ANAC102-GFP* under the control of its native promoter resulted in an exclusive chloroplastic localization, indicating hierarchical dominance of the cTP over the NLS. Furthermore, deletion of the N-terminal end of ANAC102 containing the predicted cTP resulted in an exclusive nuclear localization, indicating that this region is important for chloroplast import. However, this N-terminal region alone did not localize to the chloroplast, indicating that other regions of ANAC102 might also be important for chloroplast import.

The existence of nuclear regulators of gene expression that are also targeted to an organelle (mitochondria or chloroplast) is becoming increasingly evident in plants (Krause and Krupinska, 2009; Krause et al., 2012). Storage or sequestration of nuclear regulators in organelles is a way to control gene expression. Upon specific environmental and developmental stimuli that are perceived in the organelle, the release of the prefabricated inactive transcription factor and subsequent translocation to the nucleus allows rapid transcriptional responses. However, for most organelle-targeted nuclear regulators, their release from the organelle has not been studied so far. We could trigger relocalization of ANAC102-GFP from the chloroplasts to the nucleus in *cat2* mutant lines expressing native levels of *ANAC102-GFP* by using MV, a redox active compound that leads to the formation of $O_2^{\bullet-}$ and H_2O_2 in the chloroplasts. Because of its relative stability, H_2O_2 can easily migrate from the chloroplasts to adjacent compartments (Bienert et al., 2006; Mubarakshina et al., 2010). The lack of the H_2O_2-scavenging enzyme catalase, that plays a crucial role in maintaining low levels of photorespiratory H_2O_2 in the peroxisomes (Mhamdi et al., 2010), but also acts as a sink for intracellular H_2O_2 (Willekens et al., 1997), might further perturb ROS homeostasis and enhance ROS-induced changes in the cellular redox state. Several cleavage products of ANAC102-GFP were observed by protein gel blot analysis of *ANAC102-GFP*^{OE} lines that contain ANAC102-GFP in both the chloroplasts and the nucleus. This could indicate that proteolytic processing might play a role in the release of ANAC102 from the chloroplasts. Unfortunately, we could not detect these cleavage products in the native GFP expression lines (under both control and MV conditions) and can therefore not rule out the possibility that the observed cleavage products are the result of *ANAC102-GFP* overexpression. On the other hand, it is also possible that the nuclear isoform has the same size as the chloroplast protein, as was observed for the dual targeted Whirly1 protein (Grabowski et al., 2008). Upon import into the chloroplasts, the N-terminal target peptide is cleaved off, and the resulting mature

protein is released from chloroplasts and accumulates in the nucleus (Krause et al., 2005; Isemer et al., 2012). Additional experiments are necessary to clarify these issues. It will be necessary to confirm that, after MV treatment, the nuclear-localized ANAC102-GFP originates from the chloroplast and is not the result of increased MV-mediated *ANAC102-GFP* expression or alternative processing. Photoactivation of chloroplast-localized ANAC102 fusion proteins to a photoactivatable 4.(PA) variant of GFP (ANAC102-PA-GFP) (Patterson and Lippincott-Schwartz, 2002) prior to MV treatment will allow tracking of their intracellular dynamics. Moreover, the development of an ANAC102-specific antibody is crucial. Antibodies raised against the full-length ANAC102 were produced, but lacked specificity presumably by the presence of the conserved NAC domain. Therefore, the more divergent C-terminal domain could be used to produce more specific antibodies.

Although we provide the first evidence for the relocalization of a chloroplastic NAC transcription factor upon chloroplast-initiated oxidative stress, the mechanism of relocalization is still unclear. The perturbed chloroplastic redox state could modify ANAC102 directly or indirectly by changing an unidentified NAC-binding protein that, in turn, binds or alters ANAC102. ANAC102 has two closely positioned cysteine residues that could serve as a redox sensor (Heine et al., 2004; Liu et al., 2005). Once modified, ANAC102 has to cross the chloroplast membrane(s). Several translocation mechanisms are plausible, such as transport by vesicles, trafficking by envelope transporters, stromule tip shedding and close intercompartimental contacts (Krause and Krupinska, 2009; Krause et al., 2012). Moreover, oxidative stress can change the permeability of membranes (Arpagaus et al., 2002; Krause et al., 2012) and/or disrupt the chloroplast structure. However, preliminary electron microscopy experiments showed that the chloroplast ultrastructure was not affected at the same times and conditions of relocalization (data not shown).

ANAC102 is a regulator of nuclear gene expression and affects oxidative stress tolerance

It was previously shown that altered *ANAC102* levels affect nuclear gene expression in Arabidopsis, more specifically, during the low oxygen stress response (Christianson et al., 2009). A NAC domain consensus binding site was overrepresented in the promoters of the differentially expressed genes, indicating that ANAC102 directly regulates nuclear gene expression by binding

the promoters of its target genes. Here, we demonstrated that ANAC102 binds the promoters of the *MDR* genes in the Y1H system and preliminary ChIP experiments confirmed these observations *in planta*. The *MDR* genes were previously identified as general targets of mitochondrial retrograde regulation (Chapter 2). However, gene expression analysis using GENEVESTIGATOR revealed that besides mitochondria-dependent stimuli, the *MDR* is also induced under specific chloroplast dysfunction conditions, such as treatment with inhibitors of photosystem II or carotenoid biosynthesis, but not by inhibitors of chloroplast protein synthesis (Supplemental Figure 4.2) (Zimmermann et al., 2004). Moreover, several *MDR* genes are amongst the most strongly induced genes in the SAL1-PAP chloroplast retrograde pathway, that is involved in high light and drought stress signaling (Estavillo et al., 2011). In addition, the *MDR* is strongly up-regulated by MV treatment that initiates ROS production in the chloroplast (Zimmermann et al., 2004; Kilian et al., 2007; Figure 4.9). These observations indicate that the *MDR* is regulated by retrograde signals from both the mitochondria and the chloroplasts. Moreover, *ANAC102* itself is upregulated by both chloroplast and mitochondrial perturbations (Van Aken and Whelan, 2012). In this regard, a recent study indicated that retrograde signals from both organelles might interact to affect nuclear gene expression, as ABI4, a well known chloroplast retrograde regulator, was shown to be involved in mitochondrial retrograde regulation as well (Giraud et al., 2009). On the other hand, it has been suggested that crosstalk between the mitochondria and the chloroplasts (likely through redox active molecules and/or ROS) or indirect effects of perturbation of one organelle on the other could affect retrograde signaling from either organelle (Woodson and Chory, 2008; Schwarzlander et al., 2012). Taken together, these observations indicate that mitochondrial and chloroplast retrograde regulation are connected processes.

MV-mediated induction of the *MDR* is dampened by *ANAC102* overexpression, indicating that ANAC102 acts as a transcriptional repressor during these conditions. Moreover, genes encoding the light-harvesting chlorophyll a/b binding proteins LIGHT-HARVESTING CHLOROPHYLL B-BINDING 2 (LHCB2:4) and EARLY LIGHT-INDUCIBLE PROTEIN2 (ELIP2) that are down- and up-regulated, respectively, upon chloroplast perturbation, are changed in the same direction in $ANAC102^{OE}$ (Nott et al., 2006; Christianson et al., 2009). In addition, expression of *ABI4* is reduced in $ANAC102^{KO}$ under low oxygen stress (Christianson et al., 2009). These data strongly indicate that ANAC102 has a role in mediating chloroplast signals

to regulate nuclear gene expression. However, further experiments integrating relocalization experiments and gene expression analysis upon various chemical treatments that inhibit chloroplast function are required to clarify the role of ANAC102 in chloroplast retrograde regulation. Moreover, it would be interesting to test the effect of altered *ANAC102* levels on the expression of CRR target genes, such as *LHCB2:4* and *ELIP2*, during these conditions.

As ANAC102 is synergistically and antagonistically involved in the low oxygen response, and the consensus NAC binding site is enriched in both the *ANAC102*^{OE} induced and repressed genes, it was suggested that ANAC102 could function both as an activator and a repressor (Olsen et al., 2005b; Christianson et al., 2009). Most NAC proteins characterized so far are transcriptional activators (Tran et al., 2004). However, increasing evidence emerges that NAC transcription factors can also function as transcriptional repressors. For instance, the calmodulin (CaM)-binding NAC protein CBNAC functions as a CaM-mediated repressor (Kim et al., 2007). Moreover, the VASCULAR-RELATED NAC-DOMAIN (VND)-INTERACTING 2 (VNI2) contains both an activator and a repressor domain, and as a result, acts either as an activator or repressor depending on the cellular conditions and the *cis*-acting elements (Yang et al., 2011). Interestingly, ATAF2, the closest homolog of ANAC102 has also been shown to function as a repressor or activator, depening on the growht conditions (Delessert et al., 2005; Wang et al., 2009b). As the *MDR* gene expression is positively regulated by NTL NAC transcription factors through the MDM NAC binding site (Chapter 2), it is possible that ANAC102 could occupy the MDM under certain conditions, thereby preventing binding and transcriptional activation by the NTLs. However, we did not observe an interaction of ANAC102 with the MDM *cis*-regulatory element in the Y1H assay (data not shown). Further detailed ChIP experiments will be needed to reveal the ANAC102 binding site in the *MDR* promoters.

Altered *ANAC102* levels affect tolerance to oxidative stress conditions. As the MDR, including AOX1a, has previously been implicated in oxidative stress tolerance (Chapter 2), failure of *MDR* induction in *ANAC102*^{OE} plants could be responsible for the observed phenotype. *ANAC102*^{OE} plants showed reduced growth retardation and anthocyanin accumulation compared to wild-type plants during early stages of MV treatment. Plants frequently reduce growth as part of the adaptive stress response to save energy and resources (Skirycz and Inze, 2010), and induction of the non-energy conserving alternative respiration has been implicated in this response (Sieger et al., 2005). In addition, a protective role for anthocyanins has been suggested

as scavengers of ROS (Zhang et al., 2011) and they commonly accumulate during various stress responses (Chalker-Scott, 1999). Moreover, prolongation of the severe MV stress resulted in completely bleached *ANAC102*^{OE} seedlings, indicating accelerated cell death, whereas the wild-type plants seemed to cope with the stress and remained partially green. In this regard, AOX has been implicated as a "survival protein" by reducing ROS production and thereby dampening programmed cell death (PCD) induction during stress (Robson and Vanlerberghe, 2002; Van Aken et al., 2009). Thus, *ANAC102*^{OE} appears to have an impaired stress response. To reveal whether altered AOX1a levels are responsible for the observed phenotype (impaired growth retardation and accelerated cell death), *AOX1a* OE and KO plants will be analyzed under MV stress conditions.

A constitutive activation of the stress response would be energetically costly, and the reallocation of resources towards defense decreases plant overall fitness (Heil and Baldwin, 2002). Therefore, plants need a tight control of the defense response involving both transcriptional activation and repression mechanisms (Kazan, 2006). In this regard, RAP2.1 was characterized as a repressor that is induced upon stress and negatively regulates the DREB-mediated cold and drought stress response by binding the DRE *cis*-regulatory element (Dong and Liu, 2010). We suggest that ANAC102 might be important for fine-tuning the stress response. Whereas under severe stress conditions, the reduced growth retardation of *ANAC102* overexpression plants decreases survival rate, this results in increased yield under very mild stress conditions. Thus, ANAC102 might be involved in the switch between growth and the stress response under adverse conditions, as was previously suggested for the AOX1a protein function (Sieger et al., 2005). Genomewide identification of ANAC102 target genes using inducible overexpression constructs as well as chromatin immunoprecipitation and determining ROS levels in *ANAC102* overexpression lines will provide further insight into the function of ANAC102 in the stress response.

METHODS

Plant growth conditions and stress treatments

A. thaliana (L.) Heynh ecotype Columbia (Col-0) plants (wild type) were grown until stage 1.04 (unless stated otherwise; 4th true leaf 1 mm in size) (Boyes et al., 2001) on half-strength (½) Murashige and Skoog (MS) medium (Duchefa Biochemie; http://www.duchefa.com/), 1% (w/v) sucrose, 0.7% (w/v) agar, pH 5.7 at 21°C and under a 16-h light/8-h dark photoperiod, 150 µmol $m^{-2}s^{-1}$ light intensity and 50% relative humidity. For the ANAC102 relocalization and processing experiments, wild-type and mutant seeds were sown and germinated on nylon mesh on ½MS plates. At stage 1.04, plants were transferred to ½MS plates containing 50 µM MV. For the ANAC102 processing experiments, a pool of 32 plants was harvested at each time point. Two independent experiments were carried out (independent sets of plants sown and treated on different dates). For the qRT-PCR analyses of the *MDR* genes, wild-type, *ANAC102* overexpression and knock-out lines were treated under the same conditions as the relocalization experiment. At each time point, three biological samples of six plants were harvested. For the MV stress assay, 2-week-old mesh-grown plants were transferred to ½MS plates supplemented with 2 µM MV. For the photorespiration-promoting conditions, plants were grown under control conditions for two weeks. Then, the petri plates were sealed with parafilm to restrict gas exchange. For the H_2O_2 stress assays, plants were germinated and grown on ½MS medium supplemented with 1, 2, 4 or 8 mM H_2O_2 (Merck) or supplemented with 1, 2, 4, or 8 µM 3-AT (Acros Organics). The stress assays were performed with at least two biological repeats. The PSII maximum efficiency (F_v'/F_m') was determined with a PAM-2000 chlorophyll fluorometer and ImagingWin software application (Walz; Effeltrich, Germany) on light-adapted plants.

Cloning of open reading frames and promoters

The full-length open reading frame (with and without stop codon), the sequence encoding the putative chloroplast targeting peptide (cTP), the ORF without the cTP, the ORF without the C-terminal region and the promoter of *ANAC102* were amplified by polymerase chain reaction (PCR) from first-strand cDNA and genomic DNA of *A. thaliana* (L.) Heynh. ecotype Columbia

(Col-0), respectively, with gene/promoter-specific primers extended with the attB sites for Gateway cloning (Invitrogen Carlsbad, CA, USA; Supplemental Table 4.1). PCR reactions were run with high-fidelity Phusion DNA polymerase (Finnzymes OY, Espoo, Finland) and fragments were cloned into the Gateway entry vectors (Invitrogen) according to the manufacturer's instructions.

Generation of transgenic Arabidopsis plants

GFP fusion plants - Constitutive promoter-driven expression clones were generated with the binary destination vectors pK7FWG2 and pK7WGF2 (Karimi et al., 2007), resulting in C- and Nterminal GFP protein fusions, respectively, under the control of the cauliflower mosaic virus 35S (CaMV35S) promoter. Endogenous promoter-driven translational fusions were created with the MultiSite Gateway technology (Invitrogen) that combined the *ANAC102* ORF and GFP fragments downstream of the endogenous promoter in the pK7m34GW destination vector (Karimi et al., 2007).

Overexpression plants – A constitutive promoter-driven expression construct was generated in the binary destination vector pK7WG2D (Karimi et al., 2007).

All constructs were transferred into the *Agrobacterium tumefaciens* strain C58C1 harboring the virulence plasmid MP90.

Loss-of-function plants – Homozygous plants were selected from the SALK_030702C T-DNA insertion line by genomic PCR with gene-specific and T-DNA-specific primers. The expression level of *ANAC102* was determined by qRT-PCR.

Transient expression and stable genetic transformation

All GFP-protein fusion constructs were transiently expressed in leaf epidermal cells of 5-week-old wild-type tobacco (*Nicotiana benthamiana*) by *A. tumefaciens*–mediated leaf infiltration (Sparkes et al., 2006). For stable expression, the constructs were transformed into wild-type or catalase-deficient plants (CAT2HP2 (*cat2*); 7% residual catalase activity) (Vandenabeele et al., 2004) by *Agrobacterium*-mediated floral dip (Clough and Bent, 1998). Kanamycin-resistant plants were selected on ½MS medium (Duchefa Biochemie, Haarlem, The Netherlands), 1%

(w/v) sucrose, 0.7% (w/v) agar, pH 5.7 and 35 mg.L-1 kanamycin (Sigma-Aldrich, St. Louis, MO, USA) at 21°C and 16-h light/8-h dark photoperiod. Homozygous lines with a single T-DNA locus were selected by segregation analysis.

Chloroplast fractionation experiments

Chloroplasts were isolated and fractionated into stroma and thylakoids from 3.4 g leaves of 4-week-old *A. thaliana* (wild type, *ANAC102-GFPOE* and prom$_{ANAC102}$:*ANAC102-GFP*) plants according to the method described by (Lamkemeyer et al., 2006).

Y1H screening

Yeast strain YM4271 and destination vectors pMW#2 and pMW#3 were obtained from Dr. M. Walhout (University of Massachusetts Medical School, Worcester, MA, USA). The REGIA collection was provided by Dr. Franziska Turck (Max Planck Institute for Plant Breeding Research, Köln, Germany). Design of the yeast reporter strains was done as described in detail (Deplancke et al., 2006). Primers used for cloning of the promoters are displayed in Supplemental Table 4.1. For the screening of the REGIA collection, the 1394 prey plasmids were individually transformed in the reporter yeast strains by means of a high-throughput transformation system in the 96-well format (Deplancke et al., 2006; Vermeirssen et al., 2007). Twenty microliter of competent yeast cell suspension, 100 ng plasmid, and 100 µL Tris-EDTA(TE)/lithium acetate/polyethyleneglycol were combined per well. After heat shock (20 min at 30°C), plates were centrifuged (for 10 s) and supernatant was removed. Yeast cells were resuspended in 20 µL TE of which 5 µL was spotted on selective (SD-His-Ura-Trp; Clontech) medium, on SD-His-Ura-Trp containing the appropriate concentration of 3-AT (Acros Organics) to minimize self-activation, and on a nitrocellulose filter (Hybond-N; Amersham Biosciences) that has been placed on top of a non-selective YPD (Clontech) plate. Growth on 3-AT was monitored during 3-10 days after transfer. The YPD plate was incubated overnight at 30°C and subsequently used for the β-galactosidase assay (Deplancke et al., 2006).

Chromatin immunoprecipitation

The ChIP experiments were done as described (Bowler et al., 2004; Berckmans et al., 2011) (Chapter 2). For the quantitative PCR analysis, specific primers were designed for the *UGT74E2* and *AtDTX1* promoter regions by means of primer-BLAST at the NCBI website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Rozen and Skaletsky, 2000) and tested for amplification specificity by melt-curve analysis before use.

qRT-PCR experiments

Total RNA and first-strand cDNA were prepared with TRIzol Reagent (Invitrogen) and iScript cDNA Synthesis Kit (Bio-Rad), respectively according to the manufacturer's instructions. As a template in the subsequent PCR, 5 μL of a 1:8 diluted first-strand cDNA was run on the iCycler iQ (Bio-Rad) with the SYBR Green I Master kit (Roche Diagnostics) according to the manufacturer's instructions. All individual reactions were done in triplicate. Primers were designed with the Universal ProbeLibrary Assay Design center ProbeFinder software (Roche; http://www.roche-applied-science.com/; Supplemental Table 4.1). For the expression analysis, values were normalized against *ACTIN-RELATED PROTEIN 7* (*ARP7*). The Δ cycle threshold method (Livak and Schmittgen, 2001) was applied for relative quantification of transcripts.

Promoter-GUS analysis

The 1.5-kb upstream region of the translational start site of *ANAC0102* was amplified by PCR from Arabidopsis Col-0 genomic DNA with primers (Supplemental Table 4.1) and cloned into pKGWFS7 (Karimi et al., 2002), generating an in-frame GFP-GUS fusion. The construct was transformed into Arabidopsis wild-type (Col-0). GUS assays were performed as described (Beeckman and Engler, 1994).

Protein extraction and western blot analysis

Total protein extracts were prepared by grinding leaf material (100 mg) in 200 µL extraction buffer (100 mM HEPES (pH 7.5), 1 mM EDTA, 10 mM β-mercaptoethanol and 1 mM phenylmethanesulfonylfluoride) and a protease inhibitor cocktail (COMPLETE; Roche). Insoluble debris was removed by centrifugation at 20800 x g for 15 min at 4°C. Protein concentrations were determined with the Bradford method (Zor and Selinger, 1996). Proteins (15 µg) were separated on a 12.5% SDS PAGE gel, transferred to a P membrane (Millipore; http://www.millipore.com/) and immunodetected with the Living Colors A.v. Monoclonal antibody (JL-8; Clontech Laboratories; http://www.clontech.com/) for the detection of GFP-tagged proteins by means of the Western Lightning kit (GE-Healthcare; http://www.gehealthcare.com/).

Fluorescence microscopy

For fluorescence microscopy, a confocal microscope 100M with software package LSM 510 version 3.2 was used (Zeiss; http://www.zeiss.com/), equipped with a 63x water-corrected objective (numerical aperture 1.2) to scan the leaf epidermis and underlying cell layers. GFP fluorescence was imaged in a single channel setting with 488 nm for GFP excitation.

Accession numbers

Sequence information of the genes mentioned in this article can be found at The Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org) under the following accession numbers: ANAC102 (At5g63790), ABCB4 (At2g47000), AOX1a (At3g22370), AtTDX1 (At2g04040), NDB4 (At2g20800), ST (At2g03760), UGT74E2 (At1g05680), UGT73C6 (At2g36790), UPOX (At2g21640), and ARP7 (At3g60830).

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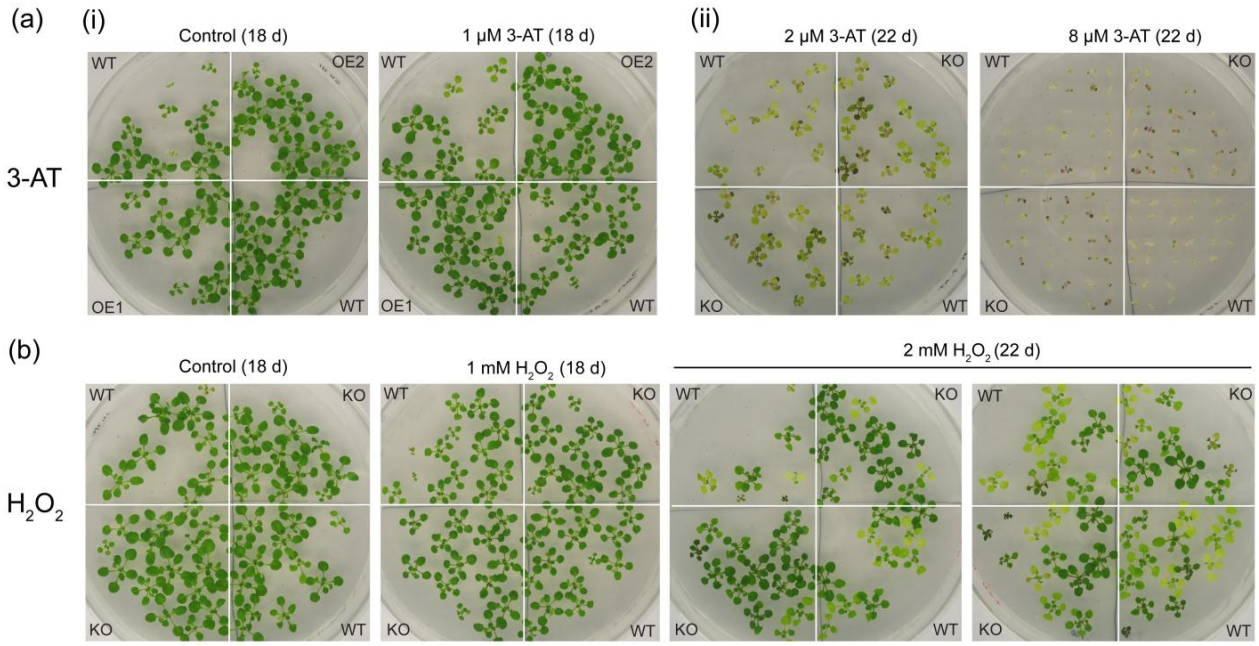
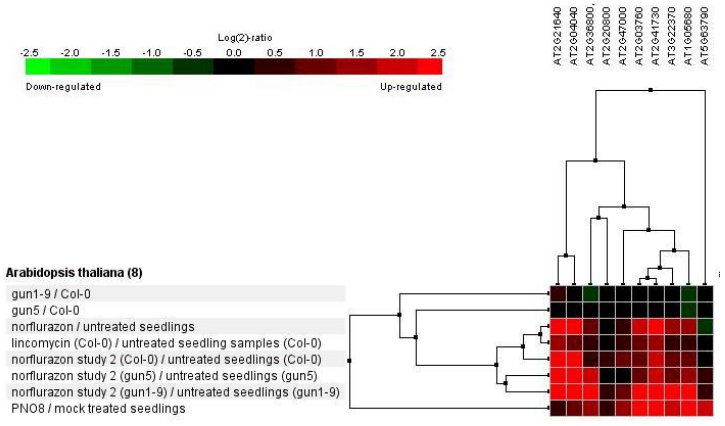
SUPPLEMENTAL DATA



Supplemental Figure 4.1. Altered *ANAC102* levels affect tolerance to 3-AT and H_2O_2-mediated oxidative stress.
(a) Phenotype of *ANAC102*^{OE} and *ANAC102*^{KO} under 3-AT-mediated oxidative stress. Wild-type (WT), *ANAC102*^{OE} (OE), and *ANAC102*^{KO} (KO) plants were germinated and grown on ½MS medium supplemented with different concentration of 3-AT. *ANAC102*^{OE} have an increased rosette area under mild (1 µM) 3-AT stress compared to that of wild-type plants (i). However, under severe (2 and 8 µM) 3-AT stress conditions, *ANAC102*^{KO} have a better performance as evidenced by an increased rosette area and less chlorophyll loss (ii).
(b) *ANAC102*^{KO} increases tolerance to externally applied H_2O_2.



Supplemental Figure 4.2. Expression profile of *ANAC102* and its target *MDR* genes during chloroplast perturbation. Color codes represent log_2-fold changes in transgenic or treated plants compared to wild-type or untreated plants, respectively. Norflurazone blocks carotenoid biosynthesis, lincomycin is an inhibitor of chloroplast protein synthesis, and N-octyl-3-nitro-2,4,6-trihydroxybenzamide (PNO8) inhibits photosystem II. The cluster was made using the GENEVESTIGATOR hierarchical clustering tool (Zimmermann et al., 2004).

Supplemental Table 4.1. Primers used.

Primers	Sequence
Promoter cloning	
AttB4F-PAt5g63790	GGGGACAACTTTGTATAGAAAAGTTGGAGATTCTACAACTGAATTACCAAAATGCCCC
AttB1rR-PAt5g63790	GGGGACTGCTTTTTTGTACAAACTTGTGGAGCTTTTATAAACGAAGGGGAGG
AttB2rF-At5g63790	GGGGACAGCTTTCTTGTACAAAGTGGGAATGGACTTTGCTCTCTTCTCCTCG
AttB3R-At5g63790	GGGGACAACTTTGTATAATAAAGTTGTTTACCCTTGAGGAGCAAAATTCCAATTC
AttB4F-PAt5g63790	GGGGACAACTTTGTATAGAAAAGTTGGAAAGACTTGGACCCAACACAGC
AttB1rR-PAt5g63790	GGGGACTGCTTTTTTGTACAAACTTGTGGAGCTTTTATAAACGAAGGG
AttB4F_P-UPOX	ATAGAAAAGTTGACAACATTGATCATACGAGATCAAAAAGG
AttB1r_P-UPOX	TGTACAAACTTGACGCTGAAAACAGAAAGAAATCTCATGAA
AttB4F_P-UGT73C6	ATAGAAAAGTTGTGATAATTAGTGTAACTTTCACATACTCGA
AttB1r_P-UGT73C6	TGTACAAACTTGGCGGACGATGCAACTTTAGTAAGAACC
AttB4F_P-AtDTX1	ATAGAAAAGTTGGAAGACAAGACTCGCGTATTTGTA
AttB1r_P-AtDTX1	TGTACAAACTTGCCGTGTTTAACGTTGAAGGCCTTTCCCTTT
AttB4F_P-ST	ATAGAAAAGTTGAATGAATCTGCTTTACCAACTTAGTTC
AttB1r_P-ST	TGTACAAACTTGCGTTGTTGAGACTTGAGAGATCG
AttB4F_P-ABCB4	ATAGAAAAGTTGGAGTTTAAATTAATAGAAATTAAATATTTATGTG
AttB1r_P-ABCB4	TGTACAAACTTGCGGTAGAGTTTCACAGATACC
AttB4F_P-NDB4	ATAGAAAAGTTGCTATTTCATAAATTGAACCTTAATTAAAGACATC
AttB1r_P-NDB4	TGTACAAACTTGGTGGTTGATGATTTCTCAAACCTCAGAT
AttB4F_P-UGT74E2	GGGGACAACTTTGTATAGAAAAGTTGGATTTCACCCATGATATACTG
AttB1r_P-UGT74E2	GGGGACTGCTTTTTTGTACAAACTTGTTTCTCCTTCTTTTTAATCTTGT
AttB4F_P-AOX1a	ATAGAAAAGTTGATCTGAAGAGCTTCTAGC
AttB1r_P-AOX1a	TGTACAAACTTGTGTTTCAAATCGGAAAAAGTG
AttB4F_P-At2g41730	ATAGAAAAGTTGATCAACCGATTGATCAATTGG
AttB1r_P-At2g41730	TGTACAAACTTGGTTTGCTTATTTTGATTTGAG
qRT-PCR analysis	
AOX1a_RT_FWD	TGGTTGTTCGTGCTGACG
AOX1a_RT_REV	CACGACCTTGGTAGTGAATATCAG
ST_RT_FWD	GGTCACCAATCCACACCTTC
ST_RT_REV	CGAAATCTGGGGACTCGTAG
UGT74E2_RT_FWD	TAACTTCTTCCACACTTCTCATAATCT
UGT74E2_RT_REV	ACAACAAAAACTAGAGTCAGTAACAAC
AtDTX1_RT_FWD	CATCAGCTGCAATGATTTGTCT
AtDTX1_RT_REV	GAACAGAGGTCTCGAGTTTCG
UGT73C6_RT_FWD	TGCCGAGGTTAAAGAGGTCA
UGT73C6_RT_REV	TCCACCAACACTCCTATCTTCTC
ARP7_RT_FWD	ACTCTTCCTGATGGACAGGTG
ARP7_RT_REV	CTCAACGATTCCATGCTCCT

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Chapter 5

Transcriptional regulatory networks of the abiotic stress response

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AUTHOR CONTRIBUTIONS

I.D.C. wrote the chapter with help of F.V.B. V.V. performed the LeMoNe and CLR runs, provided GO, Aracyc, *cis*-regulatory element and protein-protein interaction enrichment data (Suppl. Table 5.2 and 5.3). I.D.C. compiled the microarray compendium (Suppl. Table 5.1), gathered abiotic stress-related genetic perturbation microarray data, compared the different LeMoNe outputs (data not shown), analyzed and interpreted the data (Figure 5.2, Table 5.1, 5.2, and 5.4, Suppl. Table 5.3, see text). I.D.C. and S.V. produced the transgenic overexpression and knockout lines. I.D.C. selected regulators and genes, optimized the stress conditions and performed the qRT-PCR and nCounter experiments, with help of B.v.d.C., and interpreted the results (Figure 5.3, 5.4; Table 5.3). V.V. performed the statistical analysis of the nCounter data (Figure 5.4, Table 5.3). S.V. performed the stress assays on *ANAC032*-OE and KO lines (Figure 5.5).

ABSTRACT

Plants respond to changing environments by fine-tuned extensive reprogramming of their transcriptome. In order to elucidate the complex molecular mechanisms of these adaptation responses, we reverse-engineered a model of the abiotic stress transcriptional regulatory networks in *Arabidopsis thaliana*. We used the LeMoNe algorithm to extract gene co-expression modules and their predicted regulators from abiotic stress-related gene expression data. First, based on biological evidence from external datasets (GO, Aracyc metabolic pathway, protein-protein interactions), we found that LeMoNe inferred functionally coherent modules. Together, the modules spanned a wide variety of functions including various abiotic stress and pathogen responses, as well as, more general cellular functions (such as translation, photosynthesis, and cell cycle). Secondly, many predicted regulators are related to similar biological processes as their target modules. To further evaluate the predictive value of LeMoNe for regulator-target gene interactions, we compared the identified network model to regulator-target relationships inferred from a set of publicly available microarray experiments on mutant genotypes not included in the original dataset. However, the predictive power was overall low. Next, we evaluated the performance of LeMoNe in predicting novel regulatory interactions for seven transcription factors that were predicted to target one or multiple stress response modules. By analyzing gain- and loss-of-function mutants of these predicted regulators, we found indeed significant changes in expression for several of the predicted target genes, indicating LeMoNe prioritizes true regulatory interactions. Topological analysis of the experimentally obtained abiotic stress response subnetwork revealed a potentially highly connected and hierarchical structure. To conclude, we believe that the obtained results can be utilized to predict the functions of uncharacterized genes and to understand the transcriptional regulatory mechanisms of the abiotic stress response.

INTRODUCTION

When plants are exposed to adverse environmental conditions, they respond a.o. by transcriptional reprogramming to adapt metabolism and physiology to the new stress-related context. Differential gene expression during stress is a tightly controlled process, regulated through multiple interconnecting signaling pathways (Nakashima et al., 2009). To date, transcriptome studies of plants exposed to a variety of stress conditions are abundantly available, but the underlying regulatory mechanisms are largely unknown. One of the current challenges in plant biology is to shed light on the regulatory networks involved in conferring stress responses.

Most gene expression regulation occurs at the level of transcription by binding of transcription factors to *cis*-regulatory elements in the upstream regulatory regions of their target genes. Therefore, transcriptional regulatory networks that map interactions between regulatory proteins and target genes are used to study differential gene expression at a systems level (Carrera et al., 2009). Genes that are under the control of one or more shared transcription factors (co-regulated) tend to have similar patterns in expression profiles (co-expressed). Consequently, co-expressed genes incline to share functional relationships and their expression is more likely to be controlled by the same regulators (Yu et al., 2003). Moreover, it is generally assumed that regulatory proteins are themselves often regulated at the transcriptional level, so that their expression patterns provide information about their activity level. Therefore, transcription factor expression activities are often well correlated to the expression behavior of their coordinated target genes (e.g. Carrere et al., 2009). Based on these assumptions, networks of co-expressed genes and their regulators are constructed through reverse-engineering of gene expression profiles. These networks are consequently used to generate hypotheses on the functionalities of genes of unknown function and to construct comprehensive blueprints of the regulatory mechanisms of gene expression within a cell or organism (Carrera et al., 2009; Lee et al., 2010).

Different methods have been developed over the past years for the reconstruction of transcriptional regulatory networks (reviewed in Gardner and Faith, 2005; Bansal et al., 2007). Basically, two classes of methods exist to infer transcriptional regulatory networks from gene expression data. First, direct methods construct a network of individual regulator-target interactions based on the pairwise correlation between the expression profile of a transcription factor and its putative target genes. The context likelihood of relatedness (CLR) algorithm is such a direct method that scores these interactions based on the mutual information of their expression

profiles as compared to an interaction specific background distribution (Faith et al., 2007). Secondly, module-based methods assume a modular structure of the transcriptional regulatory network, with genes regulated by the same regulators (regulons) organized in co-expression modules (Segal et al., 2003). Module networks have the advantage over pairwise networks as they additionally provide functional information about the regulatory interaction through the module context. A variety of clustering methods have been developed, such as hierarchical clustering (Eisen et al., 1998), *k*-means clustering (Tavazoie et al., 1999) and self-organizing maps (Tamayo et al., 1999).

The module-based algorithm, LeMoNe (learning module networks) uses probabilistic optimization techniques for gene clustering (Michoel et al., 2007; Joshi et al., 2009). The algorithm uses a two-step procedure in which the genes are first partitioned into co-expression modules using a Gibbs sampling method. In this approach, the assignment of each gene to the module is iteratively updated and, at the same time, within each module, the conditions are clustered until a stationary state is reached (Figure 5.1). Secondly, regulators are assigned to the modules based on how well they explain the condition-dependent expression behavior of the module using a fuzzy decision tree (Figure 5.1). To obtain a statistically robust solution of co-expressed genes and their candidate regulators, the gene clustering and regulatory program are run multiple times, followed by extracting an ensemble solution from these local optima (Joshi et al., 2008). The algorithm ranks the predicted regulators by providing weights to the assigned regulators, taking into account the differential expression of the regulator across the different condition clusters, but not their absolute value. As a regulator can be assigned to more than one module, the entire module set can be assembled in a so-called module network. It has been shown previously that LeMoNe successfully predicts functional relationships and transcription regulatory interactions for *Escherichia coli*, *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Michoel et al., 2009; Vermeirssen et al., 2009).

In this study, we used LeMoNe to infer transcriptional regulatory networks for *Arabidopsis thaliana* based on abiotic stress-related transcriptome data. We verified the co-expression modules for functional coherence using external datasets. In addition, many modules were enriched for *cis*-regulatory elements, indicating co-regulation of the genes. Next, we compared the predicted regulatory interactions with known regulatory interactions obtained from publicly available microarray studies of genetic perturbations. Furthermore, we experimentally

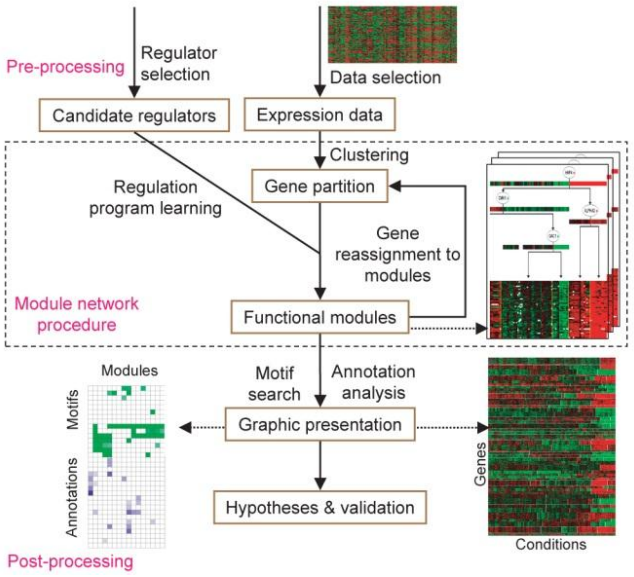

Figure 5.1. Overview of the module network algorithm and evaluation procedure. The procedure takes as input a data set of gene expression profiles and a set of regulator genes. The method itself (dotted box) is an iterative approach that determines the assignment of genes into modules and the regulation program for each module. For the regulation program, the experiment conditions are partitioned and linked in a hierarchical decision tree. For each split in this tree, a candidate regulator is found whose expression is significantly different on both sides of the split. Thus the assigned regulators explain the experiment partitions. In a post-processing phase, modules are tested for enrichment of gene annotations and *cis*-regulatory elements. From Segal et al. (2003).

demonstrated the value of LeMoNe to predict novel target genes for seven abiotic stress-related transcription factors. Overall, we demonstrate that LeMoNe predicts functional modules of co-regulated genes and prioritizes true regulatory interactions. However, despite these valuable predictions, LeMoNe still yields false negative and positive regulatory interactions, pleading for future integration of other, non-transcriptomic data types and combination of predictions from different algorithms to create a more robust regulatory network.

RESULTS

Construction of an Arabidopsis transcriptional regulatory network from abiotic stress-related transcriptome data

We assembled a compendium of Arabidopsis gene expression microarray data that includes 283 different control or perturbation conditions from 48 studies of abiotic stress treatments, hormone treatments, and genetic perturbations of abiotic stress regulators (Supplemental Table 5.1). The LeMoNe algorithm was used to construct co-expression modules and their regulation program. First, we determined the most valid co-expression network by running LeMoNe both on the absolute expression values and on the ratio expression values derived by dividing experiment

over control, corresponding to, respectively, 283 and 199 different conditions used for clustering. In addition, we applied two different probability cutoffs for the partitioning of the genes in modules: at least cluster together in 25% or 50% of the local optima module cluster solutions, further referred to as qopt25 and qopt50 solutions, respectively (see Methods) (Joshi et al., 2008). The qopt50 threshold has a higher stringency and results in more but in general smaller-sized modules compared to qopt25. Hence, we obtained a total of four output solutions: absolute-qopt25, absolute-qopt50, ratio-qopt25, and ratio-qopt50, containing a total number of 244, 638, 380, and 998 modules with an average number of 26, 12, 18, and 8 genes per module, respectively. To select the most optimal solution, we estimated the biological relevance of the various clustering options by implementing Gene Ontology (GO), Aracyc metabolic pathway and *cis*-regulatory element enrichment analysis, literature research, as well as the presence of physical protein-protein interactions within the modules (Zhang et al., 2005; De Bodt et al., 2012). We found that the ratio-qopt25 solution outperforms the other solutions in terms of functional coherence (data not shown). In this output, a total of 6710 genes is clustered in 380 modules. The modules contain an average of 18 genes, ranging from three up to 246 genes. Hence, the ratio-qopt25 solution was further used for *in silico* and experimental validation and hypothesis generation.

To compare the LeMoNe-predicted regulatory interactions with that from another reverse-engineering method, the CLR algorithm was run on the same microarray dataset. In contrast to the module-based LeMoNe algorithm, CLR infers pairwise regulator-target gene interactions (Faith et al., 2007). CLR deduced a total of 114,183 regulator-target gene interactions from our abiotic stress microarray compendium of which 7400 overlap with LeMoNe predictions.

Functional coherence

Since genes gathered in one module are co-expressed across varied conditions, a certain degree of functional coherence amongst them is expected. We assessed the functional coherence through GO enrichment analysis (see Methods). We found that 183 out of the 380 modules were significantly enriched for at least one GO biological process (GO-BP) term (Supplemental Table 5.2). Together, the modules spanned a wide variety of functions, including translation, photosynthesis, various metabolic pathways, cell cycle-related processes, and various stress responses including pathogen response. Thirty-six modules were enriched for the “response to

stress" GO-BP annotation (GO:0006950). To obtain an overview of the different stress functions, we categorized these 36 modules according to the descendant GO-BP categories of the "response to stress" GO-BP (Figure 5.2). Several modules with a role in the stress response will be discussed in the next subsections. In addition, 49 modules are enriched for a specific Aracyc metabolic pathway (Zhang et al., 2005) and 52 modules contain at least one gene pair that physically interacts at the protein level (De Bodt et al., 2012) (see Methods). Thus, based on *in silico* validation using external datasets, we conclude that LeMoNe infers functionally coherent modules.

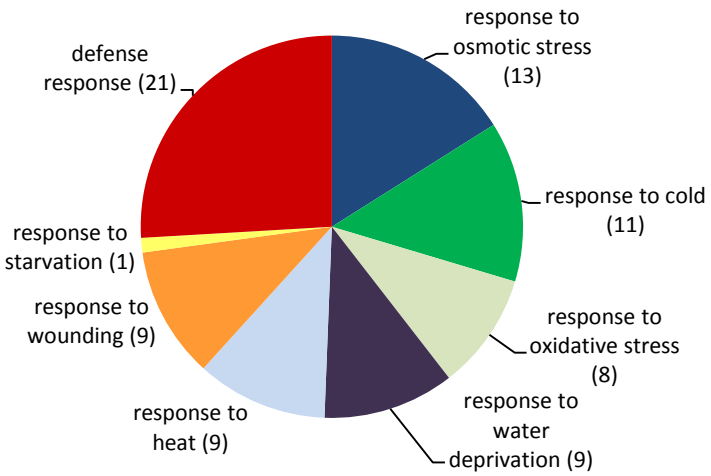

Figure 5.2. Overview of GO biological processes (GO-BP) related to the stress response in which modules were predicted to be involved. Only GO-BP terms that are direct descendants of the GO-BP "response to stress" are displayed. The numbers between brackets indicate the number of modules annotated with the GO-BP. Modules with multiple GO-BP annotations can be present in different GO-BP categories.

Regulatory coherence

Transcription factors coordinately steer gene expression through the binding with shared *cis*-regulatory elements in their target gene promoters and, as a consequence, genes targeted by the same transcription factor(s) tend to be co-expressed (Yu et al., 2003). Co-expressed genes are hence more likely to share transcriptional regulators (co-regulated) and contain common *cis*-regulatory motifs in their promoters. We analyzed the modules for enrichment of known *cis*-

regulatory elements and compared the overrepresented motifs with the module function and the function and putative binding site(s) of the predicted regulators.

The promoters (1-kb sequences upstream of the translation initiation codon) were searched for known *cis*-regulatory motifs listed in the PLACE and AGRIS databases (Higo et al., 1999; Davuluri et al., 2003). The occurrence of these motifs in each module were compared with the frequencies among all promoters in the genome and the hypergeometric *P* value was calculated and corrected for multiple testing using the false discovery rate (FDR) (Benjamini and Hochberg, 1995). We considered motifs with *P* values lower than 0.05 that are present in at least 50% of the genes within a module and more than 1.5 times enriched in the module compared to the genome to be significantly overrepresented (Supplemental Table 5.3). Eighty-six modules (or 22.6%) contain at least one enriched motif. We found that 30 modules are enriched for more than one motif. These modules are mostly involved in "general" cellular functions like translational activity, photosynthesis, and circadian rhythm, indicating that these processes are coordinately regulated at the transcriptional level. Interestingly, several motifs such as the UP1/2ATMSDN, SITEIIATCYC, I BOX, and ABRE are present in multiple modules, similar to results obtained in a previous study that predicted functional co-expression modules and *cis*-regulatory elements from Arabidopsis gene expression data (Ruan et al., 2011), indicating these motifs might be involved in regulating multiple processes. For 67 out of the 86 modules containing an overrepresented *cis*-regulatory element, this could be associated with the function of the modules and/or predicted regulators using GO enrichment data, literature information, and expression data from the GENEVESTIGATOR meta-analysis tool (Zimmermann et al., 2004) and these results are summarized in Supplemental Table 5.3. Among these 67 modules, 36 had a (potential) role related to the abiotic or biotic stress response (Supplemental Table 5.3), of which some representative examples are displayed in Table 5.1 and discussed in the next subsections.

Three modules (44, 730, and 827; Table 5.1) are enriched for the drought-responsive element (DRE) that mediates transcriptional regulation in response to cold, drought and high salinity stress in an abscisic acid (ABA)-independent manner (Yamaguchi-Shinozaki and Shinozaki, 1994). These modules are significantly co-expressed during cold (module 44 and 730), and salt and drought stress (module 730 and 827). Interestingly, LeMoNe predicted DREB family transcription factors for the three modules, indicating these could be direct regulators of

the module genes. These DREB transcription factors were previously not functionally associated with the predicted module genes.

The ABA-responsive element (ABRE) was identified in 23 modules (Supplemental Table 5.3). Among them, we found the modules 62, 77, 94, 100, 120, 133, 150, 155, 884, and 1081 up-regulated by ABA and drought stress to variable degrees, according to the function of the ABRE in ABA-dependent drought stress responsive gene expression (Table 5.1). Interestingly, these modules share regulators like RESPONSIVE TO DESICCATION 26 (RD26) and ARABIDOPSIS THALIANA HOMEOBOX 7 (ATHB-7) and ATHB-12 which are involved in the ABA-dependent drought stress response (Soderman et al., 1996; Fujita et al., 2004; Valdes et al., 2012), indicating these modules might be co-regulated. However, LeMoNe did not predict the ABRE-BINDING FACTORS (AREB/ABF) or other BASIC LEUCINE ZIPPER (bZIP) family members for any of these ABRE containing modules (Choi et al., 2000; Uno et al., 2000). This could be explained by the fact that ABFs are, in addition to transcriptional regulation by ABA, also posttranslationally activated by ABA (Furihata et al., 2006). However, the CLR algorithm that infers pairwise regulator-target gene interactions predicted ABF3 and the G-BOX BINDING FACTOR 3 (GBF3) as regulators for most of the genes of modules 62, 94, 100, 133, 150, 884, and 1081 in addition to RD26, ATHB-7 and ATHB-12 (Lu et al., 1996). This illustrates the partial complementarity of LeMoNe and CLR, as well as, the better performance of CLR in making true predictions for a higher number of regulators compared to LeMoNe recovering a higher number of known targets for fewer regulators (Michoel et al., 2009). Moreover, several genes of modules 77, 100, 133, 150, and 1081 are down-regulated in a triple homolog *abf3* mutant (Yoshida et al., 2010), indicating ABF3 is potentially a direct regulator of and co-regulates these modules by interacting with the ABRE.

Module 56 is enriched for genes involved in the unfolded protein response (UPR) and is enriched for the UPR *cis*-regulatory element (see below). Module 108 and 53 are potentially involved in the nutrient deprivation response. Module 108 is enriched for the binding site of the PHOSPHATE STARVATION RESPONSE 1, a master transcriptional activator of the phosphate (Pi) starvation response (Rubio et al., 2001). Expression analysis using GENEVESTIGATOR (Zimmermann et al., 2004; Misson et al., 2005) revealed that the genes in this module are indeed induced upon Pi starvation, a condition that was not present in our microarray compendium, and included several enzymes involved in phosphate metabolism, such as purple acid-phosphatases

which have been implicated in the Pi starvation response (Tran et al., 2010). Module 53 contains the sugar-repressive element (SRE) and its genes are induced by sugar deprivation and repressed upon high sugar concentrations (Zimmermann et al., 2004; Usadel et al., 2008), indicating that its genes could be involved in the sugar starvation response. The evening element (EE), that is necessary for circadian control of gene expression is enriched in modules 30 and 168 that are involved in circadian rhythm and the cold stress response (see below). Interestingly, it has been shown that the circadian clock interacts with cold response signaling (see below) (Espinoza et al., 2008).

Three modules enriched for the MYB transcription factor binding site are involved in secondary metabolism: synthesis of the defense-related glucosinolates (module 111 en 122) and flavonoid biosynthesis (module 76; see below), processes that have been shown before to be regulated by several MYB family members. For module 111, LeMoNe and CLR predicted, respectively, the MYB28 and MYB34 transcription factors, that were previously shown to regulate glucosinolate biosynthesis genes (Celenza et al., 2005; Gigolashvili et al., 2007). However, for module 122, no MYB family member was predicted as regulator. In addition, eight modules are enriched for the W box, the DNA binding site of WRKY transcription factors (TF). The genes of these modules are involved in the defense response and/or are responsive to various pathogen infections (Zimmermann et al., 2004), in agreement with the function of several WRKY TFs in the biotic stress response (Pandey and Somssich, 2009). For three of these modules, LeMoNe predicted WRKY6 or WRKY75 that are known to regulate defense-related genes (Robatzek and Somssich, 2002; Encinas-Villarejo et al., 2009). In addition, CLR predicted two other as yet uncharacterized WRKY TFs (WRKY15 and WRKY28) (see Table 5.1), that could be novel candidate regulators of the defense response. The ETHYLENE REPONSE FACTOR (ERF) binding site, named the GCC box, is enriched in two modules (192 and 197) of which module 197 contains several genes involved in the defense response (mainly chitinases). Several ERF family members have been shown to control the expression of defense genes (McGrath et al., 2005) and an as yet unstudied ERF gene is predicted as regulator for module 192. Furthermore, four modules (35, 41, 58, and 137) are enriched for the CGCG box, a binding site for calmodulin-binding/DNA-binding proteins involved in multiple signal transduction pathways in plants (Yang and Poovaiah, 2002). Although the specific function of this DNA motif is not

clear at this stage, the modules appear to be involved in stress signaling and/or defense response, based on GO enrichment analysis and published gene functional data.

To conclude, we found several modules enriched for a *cis*-regulatory element and, in most cases, the function of the element could be associated with the function of the module and/or with the potential binding site of the LeMoNe and/or CLR predicted regulators. The presence of common *cis*-regulatory elements within the modules does not only indicate co-regulation of the module genes but also provides an additional means to predict the corresponding direct regulators.

Table 5.1. Representative modules with a significantly overrepresented *cis*-regulatory element that are (potentially) involved in the stress response.
The function of the module and/or regulator(s) is displayed if it corresponds to the function of the motif (column 6 and 7, respectively). In column 8 is depicted whether the putative binding site of the predicted regulators corresponds to the motif. %, percentage of genes in the module that contain the motif.
DRE, drought-responsive element; ABRE, ABA-responsive element; UPR, unfolded protein response; PHR1, phosphate starvation response 1; SRE, sugar-repressive element; EE, evening element. [1], Dong and Liu (2010); [2], Tsutsui et al. (2009); [3], Valdes et al. (2012); [4], Li et al. (2008); [5], Grossniklaus et al. (1998); [6], Mauch-Mani and Flors (2009); [7], Fujita et al. (2004); [8], Kodaira et al. (2011); [9], Rawat et al. (2011); [10], Para et al. (2007); [11], Gigolashvili et al. (2007); [12], Encinas-Villarejo et al. (2009); [13], Robatzek and Somssich (2002); [14], Gigolashvili et al. (2007); [15], Ren et al. (2000).

Motif		M	*P* value	%	Module function	Regulator(s) involved in the same function as the motif	Regulator(s) with binding site corresponding to the motif	Ref.
DRE	RCCGAC	44	8.1E-07	64	responsive to cold	RAP2.1	RAP2.1 (DREB)	[1]
	TGGCCGAC	730	8.1E-04	50	responsive to cold, drought, salt	CEJ1	CEJ1 (DREB)	[2]
	AGCCGACCA	827	2.1E-03	68	responsive to cold, drought, salt		AT4G28140, AT2G20880 (DREB)	
ABRE	ACGTGKC	62	1.5E-04	50	ABA and drought response	ATHB-12, RD26, ATHB-7		[3]
	CACGTGG	77	2.2E-05	50	seed and embryo development, hyperosmotic response	ATHB-7, NF-YA5; MEA		[3] [4] [5]
	CACGTG	94	5.2E-04	56	responsive to ABA, drought, salt	ATAF1, RD26, AZF2		[6] [7] [8]
	ACGTGTC	100	1.0E-03	53	ABA and drought response	RD26, ATHB-7		[7] [3]

Table 5.1. Representative modules with a significantly overrepresented *cis*-regulatory element that are (potentially) involved in the stress response. (*Continued*).

	CACGTG	133	9.9E-06	70	responsive to ABA and drought	RD26		[7]
	ACGTGTC	150	1.1E-04	73	ABA and drought response	ATHB-7, ATHB-12		[3]
	MACGYGB	155	8.5E-03	100	responsive to ABA, drought, salt			
	ATACGTGT	884	2.5E-02	60	responsive to ABA, drought, salt	ATHB-7		[3]
	ACGTGTC	1081	2.0E-02	71	ABA and drought response	RD26, ATHB-12		[7] [3]
UPR element	$CCN_{12}CCACG$	56	2.4E-07	50	unfolded protein response			
PHR1-binding site	GNATATNC	108	9.7E-05	73	responsive to phosphate starvation			
SRE	TTATCC	53	7.9E-04	73	carbohydrate deprivation response; sugar metabolism			
EE	AAAATATCT	168	1.6E-07	69	circadian regulation, light and cold response	APPR5		[9]
	AAAATATCT	30	6.8E-15	57	starch catabolism, response to cold, circadian rhythm	APRR3		[10]
MYB	CACCAACC	111	3.7E-03	50	glucosinolate biosynthesis	MYB28	MYB28	[11]
	ACCWWCC	122	7.1E-05	92	tryptophan and glucosinolate biosynthesis, response to wounding			
	ACCTACC	76	9.7E-06	50	flavonoid biosynthesis			
W box	TTGACT	15	1.5E-04	78	defense response		WRKY15	
	TTGACT	28	4.3E-03	79	defense response			
	TTGACT	33	9.6E-03	72	defense response			
	TTTGACY	50	5.9E-03	78	responsive to pathogen infection	WRKY75	WRKY75	[12]
	TTGACT	70	1.8E-04	86	defense response		WRKY15	
	TTGACT	80	1.2E-03	85	defense response	WRKY6	WRKY6, WRKY28	[13]
	TTTGACY	99	1.9E-06	72	responsive to pathogen infection	WRKY6	WRKY6	[13]
	TTTGACY	128	4.3E-02	81	defense response		WRKY15, WRKY30	
GCC box	GCCGCC	192	4.2E-02	100			ERF15	
	GCCGCC	197	2.9E-03	100	defense response			
CGCG box	VCGCGB	35	1.E-08	69	stress signaling and transcriptional regulation	AZF2, MYB28		[8] [14]
	VCGCGB	41	7.E-11	76	defense response	TIP		[15]

Table 5.1. Representative modules with a significantly overrepresented *cis*-regulatory element that are (potentially) involved in the stress response. (*Continued*).

	VCGCGB	58	9.E-09	75	response to biotic stress, defense response	
	VCGCGB	137	3.E-03	60	-	TIP [15]

LeMoNe recovers a small part of the true biological regulatory network

To further evaluate the predictive power of LeMoNe for regulatory interactions in plants, we searched the literature for microarray expression data resulting from genetic perturbation (overexpression or knockout) of stress regulators in Arabidopsis and could retrieve a total of 48 studies. We considered a gene to be a true target of a TF (direct or indirect) if that gene is significantly mis-regulated by genetic perturbation of that TF. The lists of differentially expressed genes were directly obtained from the statistical analyses that were performed in the respective publications, and resulted in a total of 12,040 true regulatory interactions between 56 regulators and 6678 genes. For the predicted regulator-target gene interactions, we considered three types of relations: direct regulatory interactions between a regulator and its target module (regulator → module), indirect regulatory interactions encompassing an intermediary regulator that regulates a second module (regulator → module → module), and co-expression interactions for which regulator and target are present in the same module (module → module). For 42 out of the 56 regulators, no overlap was found between the predicted and true regulatory interactions, indicating a low coverage of LeMoNe in the prediction of differentially expressed genes from genetic perturbations. For the remaining 14 regulators, we found at least one differentially expressed gene predicted as a target (Table 5.2). Again, we observed an overall low coverage in predicting genes that are mis-regulated, as for most datasets less than 1% and for only two datasets more than 10% of the differential genes were predicted. However, many of the differentially expressed genes might be the result of secondary effects in stable mutants and are therefore no regulatory targets of the regulator. This might partially account for the high number of false negative predictions. In contrast, the number of false positive predictions was relatively lower, indicating that LeMoNe predicts regulatory interactions with a relatively high quality but low sensitivity.

As gene expression profiles often measure indirect regulator-gene interactions, we assessed the LeMoNe predictions with a set of direct TF-DNA interactions obtained from large-scale yeast one-hybrid (Y1H) analyses, containing a total of 172 interactions between 50 TFs and 11 target genes (Chapter 3). No overlap was found between the two datasets, indicating that LeMoNe does not prioritize direct regulatory interactions, in agreement with previous studies (Vermeirssen et al., 2009). Nevertheless, these Y1H data have not been validated *in planta*, thus they could include false positive regulatory interactions.

Table 5.2. Comparison of LeMoNe predicted regulatory interactions with experimentally-derived regulatory interactions resulting from genetic perturbation of stress regulators in Arabidopsis. Three types of predicted regulatory interactions were considered: direct regulatory interactions (regulator → module); indirect regulatory interactions (regulator → module → module); and co-expression (module → module). The percentage of predicted interactions (only direct and co-expression) that were experimentally validated (column 10) and the percentage of differential genes resulting from genetic perturbation that were predicted by LeMoNe (only direct and co-expression) (column 11) were calculated per regulator. Dir, direct regulatory interactions; Ind, indirect regulatory interactions; Co, co-expression. Asterisk indicates a multiple knockout mutant. [1], Tran et al. (2007); [2], Tran et al. (2004); [3], Fujita et al. (2004); [4], Jensen et al. (2008); [5], Sakuma et al. (2006); [6], Suzuki et al. (2011); [7], Brodersen et al. (2006); [8], Sonderby et al. (2010); [9], Osakabe et al. (2010); [10], Vanderauwera et al. (2012); [11], Ogawa et al. (2007); [12], Rashotte et al. (2006).

Total # differential genes					Total # predictions		Validated predictions					
Regulator	Gen. pert.	Treatment	# differential genes		Dir	Co	Dir	Co	Ind	% of pred-ictions	% of diff. genes	Reference
AHK1	KO	drought	down	190		16		1		6.3	0.5	[1]
ANAC019	OE		up	145	56	0	1		8	1.8	0.7	[2]
ANAC055	OE		up	160	112	27	1		1	0.7	0.6	[2]
ANAC072	OE		up	20	38		2		2	13.2	0.6	[3]
	OE		down	38					3			[2]
	OE		up	262			3		9			[2]
ATAF1	KO	pathogen	up	341	25		1			4.0	0.3	[4]
DREB2A	OE		up	483	19	9	1	3	32	21.4	0.6	[5]
	KO	heat	down	49				2	3			
MBF1c	KO	heat	down	36	66		4		2	6.1	11.1	[6]
MPK4	KO		up	111	5			4	12	80.0	3.6	[7]
MYB28	KO		down	210	47		8			31.9	2.0	[8]
	OE		up	191			7					
RPK1	KO		down	39		27		1		3.7	2.6	[9]
WRKY15	OE		down	750	56	59		16	42	29.6	0.1	[10]
	OE	salt	down	363				5	14			
	OE	salt	up	134			2		1			
	OE		up	598			9	2	1			
ZFHD1				981		56		3	9	5.4	0.3	[1]
HSFA2	OE		up	37	62		13			21.0	35.1	[11]
CRF6	KO*		down	180	29				1	0.0	0.0	[12]

LeMoNe predicts novel regulatory interactions

Next, we evaluated the performance of LeMoNe in predicting new TF-target gene interactions by analyzing gain- and loss-of-function mutants of predicted regulators for altered expression of their module genes. Based on their expression landscape, the overrepresentation of *cis*-regulatory elements, GO information and published data, we selected modules with a function related to the abiotic stress response (data not shown). The NAM, ATAF AND CUC 13 (ANAC013), ANAC053, ANAC032, ETHYLENE-RESPONSE FACTOR6 (ERF6), RELATED TO AP2 1 (RAP2.1), RAP2.6L, and WRKY6 transcription factors were a.o. assigned as highly-ranked regulators to one or more of these selected abiotic stress-related modules. Interestingly, ANAC013, ANAC053, and WRKY6 and RAP2.1 and ANAC032 share one or more target modules, indicating interwoven transcriptional regulation of these modules by these TFs. We generated and retrieved gain- and loss-of-function lines for these seven TFs (with the exception of a RAP2.1 overexpression line) (see Methods). Within the target modules of these seven TFs, we selected a total of 73 genes for expression analysis of the TF mutants, together with ten non-predicted, stress-regulated target genes as a negative control, the seven TFs themselves to reveal potential cross-regulation and ten housekeeping genes for normalization. For all the TF target modules, a significant expression correlation was observed under salt stress conditions. Therefore, we chose to carry out the downstream perturbational studies under salt stress condition. We first performed a molecular phenotypic analysis using different salt concentrations and a time-course analysis of the response to salt stress to select specific parameters for the expression analysis (see Methods). qRT-PCR analysis revealed that a 12 h treatment with 150 mM NaCl induced the TF genes (Figure 5.3) and several target genes (data not shown).

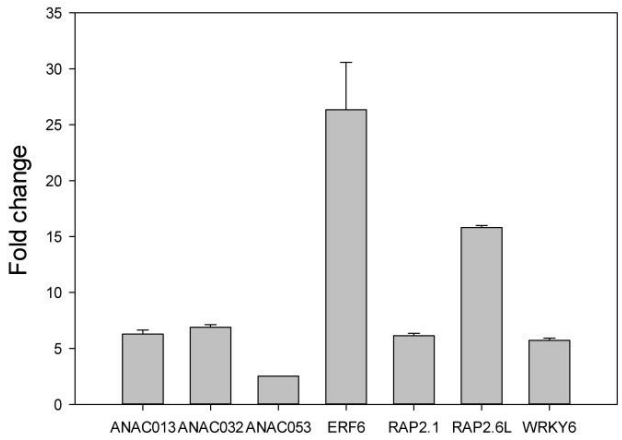

Figure 5.3. Expression pattern of the seven selected transcription factors under salt stress conditions.
Wild-type Arabidopsis plants were grown for 2 weeks under standard conditions and subsequently transferred to medium supplemented with 150 mM NaCl for 12 hours. Expression was analyzed by qRT-PCR. Bars represent average fold changes of salt treated versus untreated from two biological replicates (± SE).

The seven TFs were predicted by LeMoNe as positive regulators of their target modules. Therefore, we analyzed the knockout/down lines under salt stress conditions to reveal whether stress-mediated gene induction is impaired by reduced or absent levels of the TFs. On the other hand, the transcriptome of overexpression lines was examined under nonstressed conditions to see whether the TFs can constitutively activate the target genes in the absence of stress. As WRKY6 has been reported to act both as an activator and a repressor (Robatzek and Somssich, 2002; Chen et al., 2009; Kasajima et al., 2010), its overexpression line was additionally examined under stress conditions. The nCounter Analysis System (NanoString Technologies) (Geiss et al., 2008) was used to measure the expression levels of the predicted target and control genes in the TF mutants. In total, we obtained 114 differential genes by overexpression and only 14 upon knockout/down of the transcription factors, indicating potential redundancy. Moreover, there was no overlap between the overexpression and loss-of-function network, indicating that overexpression and knockout reveal different parts of the biological regulatory network. Except for the RAP2.1 targets, all experimentally identified target genes appeared to be positively regulated by the TFs (induced and repressed upon TF overexpression and knockout, respectively), consistent with the reported transcriptional activator function for ANAC013, ANAC053, RAP2.6L, and WRKY6 (Chapter 2; Lee et al., 2012; Krishnaswamy et al., 2011; Robatzek et al., 2002). RAP2.1 acts as a negative regulator of the stress response (Dong and Liu,

2010) and, consequently, we found for the *RAP2.1* knockdown lines a stronger stress-mediated induction of its target genes compared to the wild-type situation. The positive expression correlation in LeMoNe between *RAP2.1* and its target genes could be explained by the fact that RAP2.1 acts as a negative subregulon of the DREB activators: DREB TFs mediate stress induction of their target genes and, at the same time, induce *RAP2.1* to dampen the induction of the DREB target genes as well as its own expression by a negative feedback loop (Dong and Liu, 2010). Thus, *RAP2.1* and its target genes are subject to the same transcriptional control, which likely explains their similar expression behavior.

To evaluate the predictive power of LeMoNe, we calculated precision, recall and *F*-measure values for each of the seven TFs. Precision is defined as the fraction of predicted targets that are correct whereas recall is defined as the fraction of true targets that were predicted by the model. As precision and recall are necessarily negatively correlated, the *F* value provides the absolute efficiency by measuring the balance between these two parameters. Depending on the TF, we observed different predictive performance measures of LeMoNe with respect to recall and precision (Table 5.3). The best predictions were obtained for ANAC013 (*F*-measure 0.51), with a relatively low number of false positive and false negative target gene predictions. The second-best predictions were observed for ERF6 (*F*-measure 0.32), for which all ten predicted targets are confirmed (precision 1), but with a low coverage of the true targets (recall 0.21). Thus, this illustrates the ability of LeMoNe to reveal regulatory interactions that have not yet been validated. However, for several of the TFs tested, LeMoNe still predicts a relatively high number of false positive and/or false negative regulatory interactions.

Table 5.3. Performance of LeMoNe in predicting novel regulatory interactions. For seven transcription factors, the predicted target genes were compared with the true target genes obtained from expression analysis of the TF overexpression (OE) and knockout (KO) lines. The predictive performance was evaluated by calculating the precision, recall, and *F*-measure.

	Predicted targets	True targets (OE)	True targets (KO)	Predicted true targets	Precision	Recall	*F*-measure
ANAC013	34	48	0	21	0.62	0.44	0.51
ANAC032	19	1	0	0	0	0	0
ANAC053	19	1	0	1	0.05	1	0.10
ERF6	10	48	0	10	1	0.21	0.34
RAP2.1	17	NA	6	2	0.12	0.33	0.17
RAP2.6L	23	2	1	3	0.13	1	0.23
WRKY6	21	14	7	3	0.14	0.14	0.14

To analyze the structure of the obtained transcriptional regulatory network, we visualized the experimentally derived TF-target gene interactions in a network using Cytoscape (Shannon et al., 2003) (Figure 5.4). The constructed model includes a total of 128 regulatory interactions between the seven TFs and 73 unique target genes. The network is highly connected as three TFs target 21-48% of the 100 genes measured and 31 target genes are regulated by at least two TFs. This could indicate the coordinate direct regulation of the target genes by multiple TFs at the promoter level. On the other hand, indirect effects may account for the large number of regulators we observe per target. ANAC013 and ERF6 regulate, respectively, five and three of the other studied TFs (*ANAC053*, *ERF6*, *WRKY6*, *RAP2.1*, and *ANAC032* by ANAC013; and *ANAC053*, *RAP2.6L*, and *ANAC013* by ERF6) and also modulate the expression of several of these TFs target genes, pointing to a hierarchical structure of the transcriptional regulatory network in which ANAC013 and ERF6 are master regulators. Judged by the number of target genes, including TF genes, and the magnitude of the response (data not shown), the most influential TF in the network appears to be ANAC013 (Figure 5.4). The consensus ANAC013 binding site is present in 13 (of which 10 predicted) out of the 48 true target gene promoters, among which eight were experimentally confirmed to be bound by the ANAC013 protein in chromatin immunoprecipitation (ChIP) experiments (Chapter 2). This indicates that LeMoNe predicted both direct and indirect ANAC013 targets and the latter could be indirectly regulated through the TFs that are up-regulated by ANAC013. Among the 48 ERF6 true target genes, only five (of which 2 predicted) contain the GCC box (GCCGCC), indicating that most identified targets are indirectly regulated by ERF6. RAP2.6L, RAP2.1, ANAC053, and ANAC032 appear to be more specific regulators, affecting the expression of only 1-6% of the tested genes. The W box (TTTGAC[C/T]) is present, although not significantly enriched, in 10 (among which the three predicted true targets) of the WRKY6 true targets. The DRE element (RCCGAC) is present in four out of the six RAP2.1 true target genes, consistent with the enrichment of the DRE motif in the predicted RAP2.1 target modules. Thus, we conclude that LeMoNe correctly predicts target genes of which several are (potentially) direct targets and others are possibly indirectly regulated. Analysis of the structure of the experimentally obtained regulatory network reveals a potential hierarchical organization including several layers of transcriptional control. However, care has to be taken in the interpretation of the obtained regulatory model as only 93 target genes were studied and transcriptional analysis of TF overexpression and knockout might disclose false positive (e.g. due

to secondary effects in stable mutants) and false negative (e.g. due redundancy of among TFs) regulatory interactions. Many ANAC053 target genes might have been missed in our analysis because of the weak transgene expression in its overexpression line. Moreover, regulatory interactions inferred from expression data are often indirect, involving several hidden, intermediate regulators (Joshi et al., 2010). Therefore, testing whether the TF directly targets its differentially expressed genes using the yeast one-hybrid or ChIP technology will provide more insight into the underlying biological network.

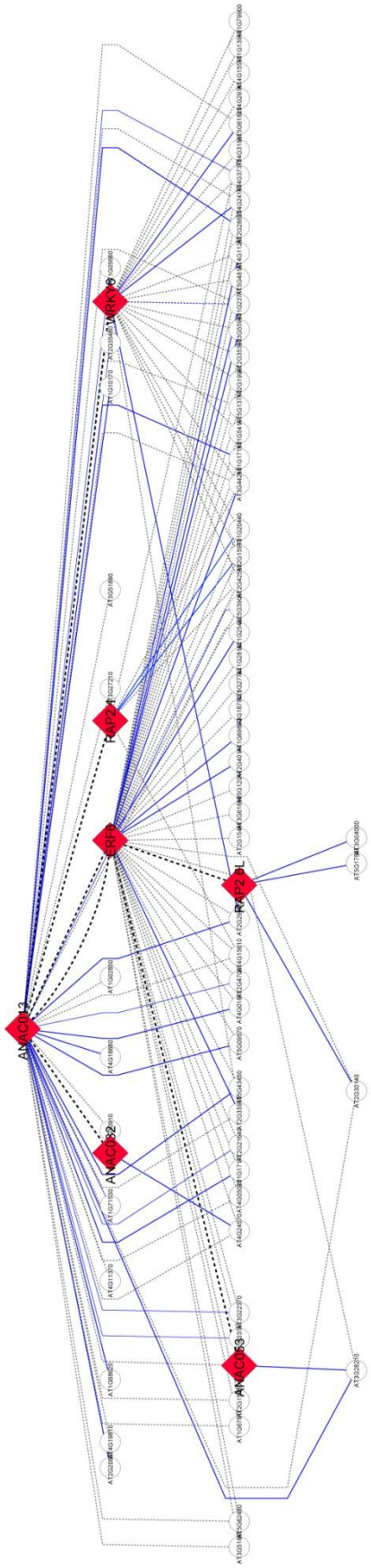

Figure 5.4. Transcriptional regulatory network consisting of experimentally-derived regulatory interactions. Each transcription factor is connected to the genes that are differentially expressed after overexpression or deletion of the transcription factor. The network was constructed using Cytoscape 2.2 (Shannon et al., 2003).
Red diamonds, transcription factors; white circles, target genes. Regulatory interactions are depicted as grey dashed lines; blue solid lines, regulatory interactions that were also predicted by LeMoNe.

LeMoNe prioritizes biologically relevant regulators

As the co-expression modules and their regulatory program were constructed from abiotic stress-related expression data, we first assessed whether LeMoNe prioritized regulators involved in the abiotic stress response. Therefore, we examined regulators that were assigned to multiple modules (Table 5.4). Fifteen regulators were predicted to regulate five or more modules. Among these 15 predicted master regulators, ten are known to have a role the abiotic and/or biotic stress response, based on published experimental evidence. In addition, four transcription factors were identified in yeast one-hybrid screens for transcription factors binding to stress-responsive promoters in Chapter 2 and 3. Hence, LeMoNe prioritized abiotic stress regulators as master regulators.

Table 5.4. Transcription factors with the most regulatory effects (highest outgoing connectivity).
Fifteen transcription factors regulate at least five modules. TFs indicated in bold have a function in the stress response. TFs that were identified in yeast one-hybrid (Y1H) screens for transcription factors binding to stress-responsive promoters (see Chapter 2 and 3) are indicated in column 5.

TF	Outgoing connectivity	Name	Function	Y1H	Reference
AT2G26150	**7**	**ATHSFA2**	**heat stress**		Ogawa et al. (2007)
AT2G46680	**7**	**ATHB-7**	**ABA and drought stress**		Valdes et al. (2012)
AT4G36540	7	BEE2	brassinosteroid signaling		Friedrichsen et al. (2002)
AT1G32870	**6**	**ANAC013**	**oxidative stress**	**+**	Chapter 2
AT3G11020	**6**	**DREB2B**	**homolog of DREB2A, drought-responsive gene expression**	**+**	Nakashima et al. (2000)
AT3G22780	6	TSO1	meristem organization		Song et al. (2000)
AT3G30260	6	AGL79	-		
AT3G49530	**6**	**ANAC062/NTM1**	**biotic and abiotic stress**		Li et al. (2010)
AT5G22290	**6**	**ANAC089**	**antioxidant defense**		Klein et al. (2012)
AT1G48000	5	MYB112	-	+	
AT1G62300	**5**	**WRKY6**	**nutrient starvation; defense response**		Robatzek and Somssich (2002); Chen et al. (2009); Kasajima et al. (2010)
AT3G10500	**5**	**ANAC053**	**drought stress and senescence**	**+**	Lee et al. (2012)
AT3G19580	**5**	**AZF2**	**osmotic stress response**		Kodaira et al. (2011)
AT3G24500	**5**	**MBF1c**	**biotic, heat and osmotic stress**		Suzuki et al. (2005)
AT4G01460	5	bHLH	-		

In the previous section, we selected seven transcription factors that were predicted to target one or multiple abiotic stress-related modules and hence might have an important role during stress. Indeed, for five of them, a function in the abiotic stress response was recently demonstrated: RAP2.6L increases salt and drought stress tolerance (Krishnaswamy et al., 2011); WRKY6 affects plant performance under nutrient deprivation conditions (Chen et al., 2009; Kasajima et

al., 2010); RAP2.1 is important for the tight control of the cold and drought stress response by repressing DRE-mediated gene expression (Dong and Liu, 2010); ANAC053 negatively affects drought tolerance (Lee et al., 2012); and *ANAC013* overexpression plants show increased oxidative stress tolerance but are more sensitive to salt stress (Chapter 2). In addition, ERF6 was recently shown to be involved in the pathogen defense response (Moffat et al., 2012).

As ANAC032 had not yet been characterized, we analyzed whether altered *ANAC032* levels affect tolerance to osmotic and salt stress, conditions under which its target module genes are most prominently up-regulated. Therefore, transgenic Arabidopsis plants were generated that constitutively expressed *ANAC032* under the control of the CAMV35S promoter. Two independent *ANAC032* overexpression (*ANAC032-OE*) lines with high transgene expression were selected using qRT-PCR for further analysis. An *ANAC032* knockout line (KO) was obtained from the ABRC collection (see methods). Interestingly, *ANAC032-OE* displayed increased plant biomass under osmotic stress conditions (Figure 5.5). However, no significant phenotype was observed under salt stress conditions (data not shown). Hence, we conclude that LeMoNe prioritizes biologically relevant regulators in its regulation program.

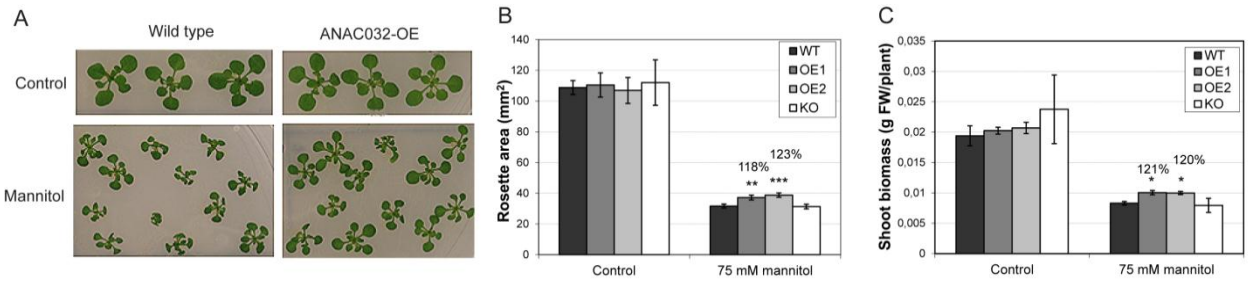

Figure 5.5. Osmotic stress resistance phenotype of *ANAC032* overexpression plants.
(A) Wild-type and *ANAC032* overexpression (*ANAC032-OE*) plants grown on normal MS medium (Control) and on MS medium supplemented with 75 mM mannitol.
(B) and **(C)** Rosette area and shoot fresh biomass of wild-type, *ANAC032-OE* and *ANAC032* knockout (KO) plants under control and mannitol stress conditions. Data represent average (n = 25 plants for control conditions, n = 75 plants for mannitol stress conditions) ± SE (Student's *t* test; * $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

Examples of functionally coherent modules related to the stress response

Iron deficiency response

In plants, iron is essential for many important metabolic processes and excessive concentrations lead to the formation of reactive oxygen species (ROS) (Ravet and Pilon, 2012). Therefore, iron homeostasis is tightly regulated and involves both the control of uptake from the rhizosphere and the internal mobilization from intracellular stores. Despite the absence of a significant GO enrichment, expression analysis using GENEVESTIGATOR revealed that the genes of module 129 (eight genes) are specifically and strongly up-regulated during iron deficiency (Zimmermann et al., 2004), a condition that was not present in our microarray compendium. Two genes in the module have been implicated in Fe homeostasis: *FERRIC REDUCTASE-OXIDASE* (*FRO3*) encodes an iron reductase; and OLIGO PEPTIDE TRANSPORTER (OPT3) is involved in long-distance iron transport (Stacey et al., 2008) (Figure 5.6).

The top regulator of the module, BASIC HELIX-LOOP-HELIX 39 (bHLH039) is a binding partner of the IRON DEFICIENCY-INDUCED TRANSCRIPTION FACTOR (FIT) bHLH transcription factor that is known as a master regulator of the iron deficiency response (Colangelo and Guerinot, 2004; Yuan et al., 2008). Overexpression of either of the two bHLH proteins did not result in mis-regulation of two tested Fe deficiency responsive genes (Jakoby et al., 2004; Yuan et al., 2008). However, overexpression of both *bHLH039* and *FIT* was sufficient to constitutively activate these genes, indicating they function together in the transcriptional regulation of the Fe deficiency response possibly by forming a functional heterodimer. The second-best predicted regulator, POPEYE (PYE) is a bHLH family member that is important for Fe mobilization within the plant and, consequently, *pye* mutants are more sensitive to low Fe availability (Long et al., 2010). PYE regulates the expression of the eight module genes, of which *FRO3* is a direct target, as evidenced by ChIP analysis. Although PYE was predicted by LeMoNe as a positive regulator of module 129, the module genes are more strongly up-regulated in *pye* mutants compared to wild-type plants under Fe deficiency conditions, indicating that PYE functions as a repressor. In agreement with the regulation by bHLH TFs, the E-box (CACGTG) is enriched among the module gene promoters (5-fold; P value < 0.05) and present in six out of the eight genes (Meshi and Iwabuchi, 1995; Chaudhary and Skinner, 1999). Moreover, the module gene *BRUTUS* (*BTS*), a putative E3 ligase protein with metal ion binding and DNA binding

domains, is known to negatively regulate the iron deficiency response (Long et al., 2010). Due to the fact that PYE and BTS commonly interact with two members of the bHLH family but do not interact with each other and due to the opposite phenotypes of the *pye* and *bts* mutants under Fe deprivation, it was suggested that their simultaneous presence in the cell ensures the balanced expression of iron homeostasis genes (Long et al., 2010; Ivanov et al., 2012). Thus, BTS that is present as a gene in the module could actually regulate the module. To conclude, the genes in module 129 are likely regulated through a complex network of bHLH and other regulatory proteins, of which two (bHLH039 and PYE) were predicted as regulators and one (BTS) is present in the module itself.

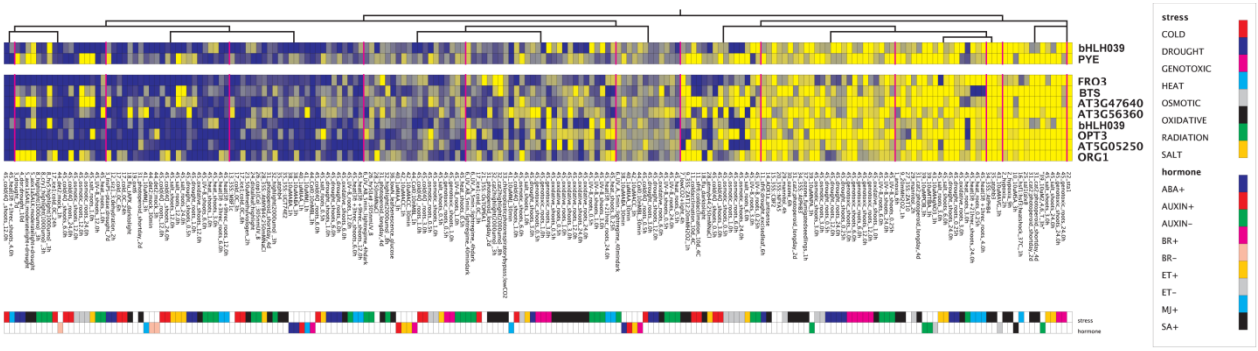

Figure 5.6. LeMoNe module 129 with module genes (bottom) and predicted regulators (top) involved in the iron deficiency response. Yellow, up-regulated genes; blue, down-regulated genes; red vertical lines partition condition clusters.

Cold acclimation

Module 30 (42 genes) is GO enriched for starch metabolism, response to temperature stimulus, and circadian rhythm. The genes in the module are up-regulated during cold and high light stress and, to a lesser extent, by high salt and drought conditions. Among the nine genes annotated to be involved in starch metabolism, seven mediate starch degradation. Starch is stored during the day inside chloroplasts and broken down to soluble sugars (maltose and glucose) when energy is needed e.g. during the night. In addition, levels of soluble sugars also increase during cold stress, and this was suggested to be due to increased expression of starch degradation enzymes (Kaplan and Guy, 2004; Lu and Sharkey, 2006). Soluble sugars such as maltose can protect proteins and the photosynthetic electron transport chain under freezing stress most likely by acting as compatible solutes (Kaplan and Guy, 2004). In accordance with the importance of starch

degradation in cold/freezing tolerance, a mutant in the starch degradation enzyme *STARCH EXCESS1* (*SEX1*), that is a member of module 30, showed impaired freezing tolerance (Yano et al., 2005). Moreover, the protein GLYCINE-RICH RNA-BINDING PROTEIN 7 (GRP7) in module 30 functions as an RNA chaperone in the post-transcriptional regulation of the cold stress response (Kim et al., 2007; Kim et al., 2008).

According to the circadian rhythm GO enrichment, the genes in the module are modulated by the circadian clock (Zimmermann et al., 2004; Dalchau et al., 2010) and 24 of the module genes contain the Evening Element (EE), a DNA regulatory element that has a role in circadian-regulation of gene expression and induces peak expression in the evening (Harmer et al., 2000). Interestingly, the EE has been shown previously to be required for cold-regulated gene expression and was suggested to integrate cold- and clock-regulated transcription (Mikkelsen and Thomashow, 2009). The circadian clock and cold acclimation are intimately linked in plants: cold affects the expression of clock genes and the clock regulates the cold response pathway (Bieniawska et al., 2008; Espinoza et al., 2008). Moreover, genes encoding starch metabolizing enzymes show diurnal changes in expression, peaking at the end of the day (Smith et al., 2004) and maltose shows strong night specific accumulation during cold acclimation (Espinoza et al., 2010). Altogether, this indicates that module 30 is potentially involved in a diurnal-regulated cold stress response, possibly by activating cold acclimation when temperatures decrease during the night.

Several MYB transcription factors have been identified to mediate clock-regulated gene expression by binding the EE element (Harmer and Kay, 2005; Gong et al., 2008), but none of them was predicted by LeMoNe as a regulator of the module. However, the second-best predicted regulator, PSEUDO-RESPONSE REGULATOR3 (PRR3) binds and modulates the stability of another PRR protein TIMING OF CAB EXPRESSION1 (TOC1) (Para et al., 2007), that directly or indirectly (by inhibiting a repressor) regulates expression of the *CIRCADIAN CLOCK ASSOCIATED1* (*CCA1*) and *LATE ELONGATED HYPOCOTYL* (*LHY*) MYB transcription factors and other MYB(-related) TFs (Alabadi et al., 2001; Pruneda-Paz et al., 2009; Huang et al., 2012). CCA1 and LHY are known to bind the EE element in the promoters of clock-regulated genes (Gong et al., 2008) and thus could be direct regulators of the genes in the module, although experimental evidence is lacking. Thus, although LeMoNe did not predict direct regulator(s) of the module genes, we could infer a potential regulatory path consisting of physical interactions

(protein-protein and protein-DNA) that might connect the predicted regulator to the module genes. Interestingly, LHY and TOC1 were, in addition to PPR3, predicted by CLR as regulators of respectively 23 and 12 genes of the module. This again demonstrates that CLR is a "regulator-centric" approach, predicting a higher number of true regulators compared to LeMoNe (Michoel et al., 2009).

Unfolded protein response

Module 56 (18 genes) is GO enriched for response to endoplasmic reticulum (ER) stress. Stresses that disrupt the ER function lead to accumulation of unfolded or misfolded proteins in the ER, a condition known as ER stress. Cells adapt to this stress by activating a signal transduction pathway, called the unfolded protein response (UPR) to synthesize more ER-associated chaperones and degradation machinery components (ERAD) in an attempt to restore ER homeostasis and function. Accordingly, module 56 contains 12 genes involved in protein folding, such as protein disulfide isomerases (PDIL), calnexins, calreticulins, and heat shock proteins; two UDP-glucose/UDP-galactose transporters involved in the re-glucosylation of unfolded glycoproteins in the ER (Reyes et al., 2006; Reyes et al., 2010); and a translocation-related protein that might translocate unfolded proteins to the cytosol for degradation (Pilon et al., 1997) (Figure 5.7). In addition, 13 module genes were previously reported in a transcriptomic study to be induced upon various treatments with chemicals that cause protein misfolding in the ER (Martinez and Chrispeels, 2003), conditions that were not present in our microarray compendium. However, the expression profile of the genes in module 56 shows a strong up-regulation by heat, UV, and oxidative stress and upon salicylic acid application. The UPR has been implicated in various abiotic and biotic stress responses as mutants in ER stress signaling components have altered resistance to these adverse conditions (Moreno and Orellana, 2011). Moreover, the UPR plays an essential role in the salicylic acid-mediated production of pathogenesis-related (PR) proteins in the ER during plant immune responses (Moreno et al., 2012). The predicted regulators of the module have not yet been characterized. However, ten of the module genes are regulated by bZIP28 under ER stress conditions (Liu and Howell, 2010) and the bZIP28 binding site (ER stress-responsive element I [ERSE-I]) is enriched among the module promoters (present in 9 genes), indicating that bZIP28 directly regulates several of the module genes. Although bZIP28

was not predicted as a regulator, these data indicate that LeMoNe correctly clusters co-regulated genes.

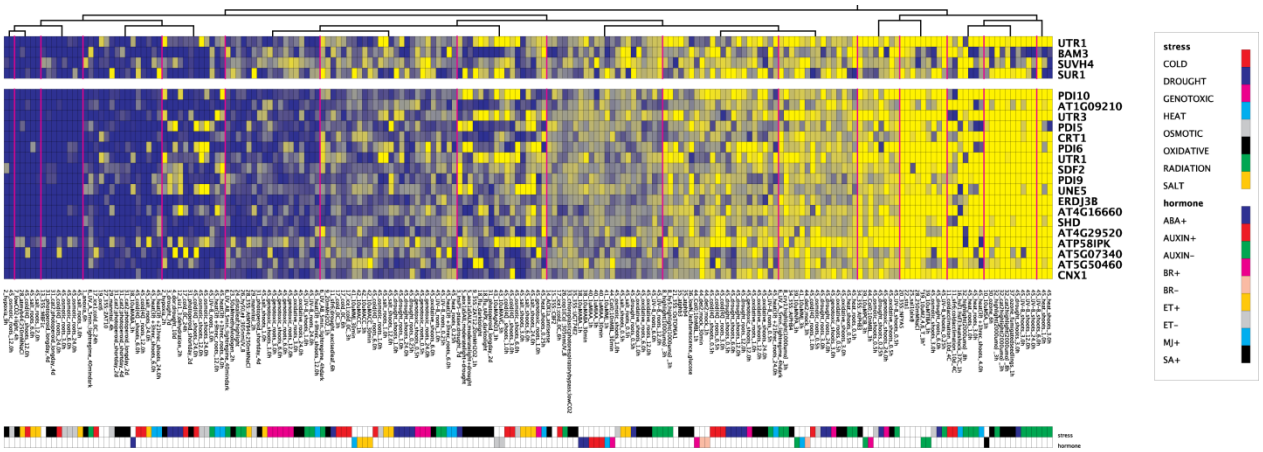

Figure 5.7. LeMoNe module 56 with module genes (bottom) and predicted regulators (top) involved in the unfolded protein response. Yellow, up-regulated genes; blue, down-regulated genes; red vertical lines partition condition clusters.

Flavonoid biosynthesis

Flavonoids are secondary metabolites that serve as flower pigments, phytoalexins, signaling molecules, and UV-B protectants (Iwashina, 2003). Flavonoid biosynthesis is part of a larger phenylpropanoid pathway, which produces a range of secondary metabolites from the aromatic amino acid phenylalanine. The flavonoid pathway starts with the general phenylpropanoid pathway and subsequently consists of 1) the early biosynthetic genes, which are common to different flavonoid subpathways and are induced prior to 2) the late biosynthesis genes (Figure 5.8) (Shirley et al., 1995; Pelletier and Shirley, 1996; Pelletier et al., 1997; Winkel-Shirley, 2001). The best characterized subpathways are those leading to flavonol and anthocyanin synthesis. LeMoNe inferred three modules (177, 76 and 134) that are each related to a specific part of the flavonoid biosynthesis pathway (Figure 5.8). First, module 177 (six genes) contains two enzymes of the general phenylpropanoid biosynthesis pathway: PHE AMMONIA LYASE 1 (PAL1) and PAL2. Secondly, the proteins CHALCONE ISOMERASE (CHS), CHALCONE ISOMERASE (CHI), FLAVANONE 3-HYDROXYLASE (F3H), FLAVONOID 3'-

HYDROXYLASE (F3'H), and FLAVONOL SYNTHASE (FLS) in module 76 (16 genes) represent the complete early flavonoid pathway that results in basic flavonols. In addition, module 76 contains two glycosyltransferases that generate functional flavonol glycosides having antioxidant activity and strong UV absorbance properties. However, other module genes have not been characterized yet or are involved in other parts of the pathway, such as an anthocyanidin glycosyltransferase (UGT78D2) and the 4-COUMARATE-COA LIGASE 3 (4CL3) that mediates the last step of the general phenylpropanoid pathway. Thirdly, module 134 contains five genes that are involved in the anthocyanin branch from the flavonoid pathway (Tohge et al., 2005) (Figure 5.9). The expression profile of the genes in modules 177, 76, and 134 shows an overall up-regulation during high light, UV radiation, and oxidative stress consistent with the function of flavonols and anthocyanins as antioxidants and to prevent photoinhibition and photodamage through the absorption of excess light (Chalker-Scott, 1999).

Enzymes of the flavonoid pathway are largely regulated at the transcriptional level and different transcription factor regulons correspond to distinct subpathways (Hichri et al., 2011). First, the general phenylpropanoid pathway is regulated by the MYB4 transcription factor (Jin et al., 2000). Despite MYB4 was not predicted as a regulator of module 177, another uncharacterized MYB family member was proposed, indicating this could be a novel regulator of phenylpropanoid biosynthesis. Secondly, the early flavonoid biosynthesis enzymes are regulated by three MYB transcription factors (MYB11, MYB12, and MYB111) (Stracke et al., 2007). Correspondingly, a MYB binding site is enriched in module 76 and nine genes are down-regulated in the triple mutant *myb11 myb12 myb111*, of which one gene was indicated to be a direct target by protoplast co-transfection assays (Stracke et al., 2007). However, these MYB TFs were not predicted as regulators by LeMoNe. The predicted top regulator is the bZIP transcription factor ELONGATED HYPOCOTYL5-HOMOLOG (HYH), a homolog of HY5. HY5 has been shown to regulate the *MYB12* gene in response to light and UV-B radiation and *hy5* mutants have altered expression of 10 genes in module 76, indicating HY5 indirectly regulates these genes through MYB12 (Stracke et al., 2010). As HYH is partially or completely redundant with its homolog HY5 with respect to the UV-B-mediated transcriptional response (Brown and Jenkins, 2008), it is possible that HYH is also an indirect regulator of the module genes by regulating *MYB12*. In addition to HYH, CLR predicted MYB12 and HY5 as regulators for respectively six and five out of the 16 genes in this module. Thirdly, MYB/bHLH/WD-repeat

transcription factor complexes specifically regulate the anthocyanin branch (Gonzalez et al., 2008) and one of these MYB TFs, PRODUCTION OF ANTHOCYANIN PIGMENT1 (PAP1) was predicted as a regulator of module 134. All five genes of module 134 are strongly up-regulated by *PAP1* overexpression (Tohge et al., 2005). In addition, three genes (*FDR*, *GST12*, and *UGT75C1*) are down-regulated in a multiple knock-down mutant of *PAP1* and related MYB TFs and are likely direct PAP1 targets, as revealed by protoplast co-transfection assays (Gonzalez et al., 2008). In addition to PAP1, CLR predicted PAP2 and TRANSPARENT TEST8 (TT8), that are also involved in anthocyanin biosynthesis, for all five genes of module 134 (Gonzalez et al., 2008).

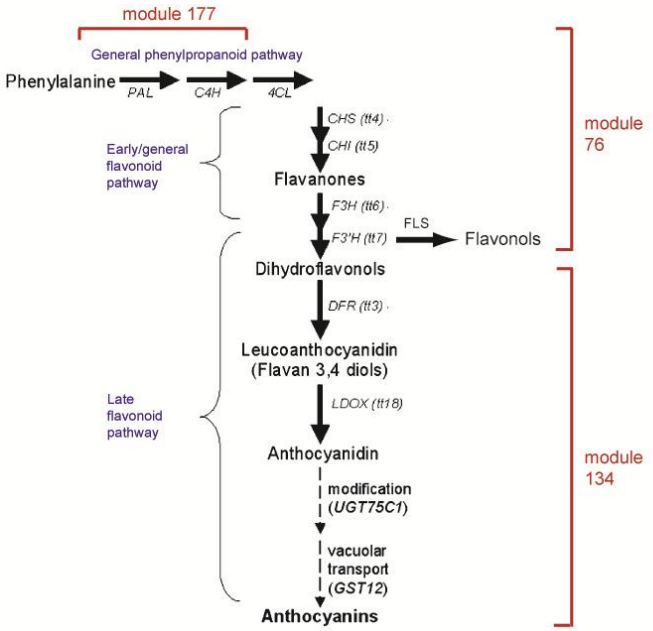

Figure 5.8. Schematic overview of the flavonoid biosynthesis pathway yielding flavonols and anthocyanins.
The flavonoid biosynthesis pathway starts with the general phenylpropanoid pathway and subsequently consists of early and late divisions, leading to the production of specific flavonoids (flavonol and anthocyanin are shown here). LeMoNe modules 177, 76, and 134 that represent three different subpathways are indicated. Adapted from Gonzales et al., 2008.

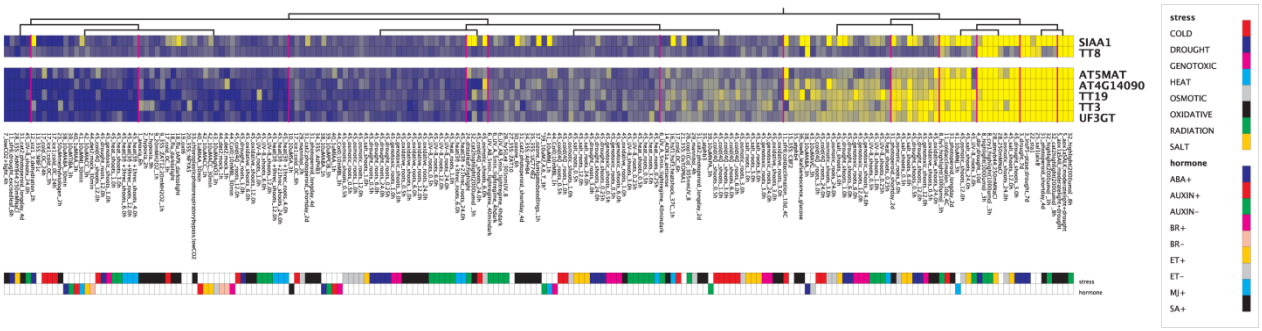

Figure 5.9. LeMoNe module 134 with module genes (bottom) and predicted regulators (top) involved anthocyanin biosynthesis. Yellow, up-regulated genes; blue, down-regulated genes; red vertical lines partition condition clusters.

DISCUSSION

In this study, we have used the LeMoNe algorithm for identifying gene co-expression modules and their regulators from abiotic stress-related gene expression profiles. Previous studies have demonstrated that co-expression data can be used to predict functional and co-regulation relationships between genes (Eisen et al., 1998; Yu et al., 2003). First, using GO and Aracyc metabolic pathway enrichment and/or the presence of physical protein-protein interactions as a measure of the functional coherence, we found biological evidence for 53% of the modules. Together, the modules spanned a wide variety of functions including various abiotic stress and pathogen responses, as well as, more general cellular functions related to translation, photosynthesis, specific metabolic pathways, and cell cycle processes. Secondly, for about a quarter of the co-expression modules, we observed potential co-regulation of the genes, as evidenced by the overrepresentation of one or more *cis*-regulatory motifs in their promoters. The actual number of modules with overrepresented motifs might in fact be higher, as many *cis*-elements have not yet been identified or might not be significantly enriched in the modules due to their short and degenerate nature. In addition, based on literature evidence, we found that several modules contain genes that share a regulator, whether or not this regulator was predicted by LeMoNe. To conclude, we demonstrated that LeMoNe infers functionally and regulatory coherent modules.

Although it has been widely shown that co-expression can be used to infer functional and/or co-regulation relationships between genes, the prediction of regulator-target gene relations from expression information is still challenging. Many studies have inferred transcriptional

regulatory networks from expression data, but so far they have largely not been validated (Amit et al., 2009; Nero et al., 2009). We found that many predicted regulators are related to similar biological processes as their target modules. To further evaluate the predictive power of LeMoNe for regulatory interactions, we compared the identified network model to regulator-target relationships inferred from a set of publicly available microarray experiments on mutant genotypes not included in the original dataset. We found an overall low coverage in predicting regulatory interactions, probably due to the fact that many of these regulators are not significantly regulated at the transcript level. The regulation of many transcription factors is post-transcriptionally through dimerization, co-factor binding, post-translational modifications, and subcellular localization and/or sequestration and this cannot be recovered from gene expression data (Lee et al., 2006). In addition, expression profiling of regulator mutants reveals many non-regulatory target genes, that change due to secondary effects (Joshi et al., 2010), which could also account for the high number of false negative predictions.

As a proof of concept, we have used the identified transcriptional regulatory network to predict novel regulatory interactions. For seven TFs that were predicted to regulate one or more stress-related modules, we experimentally assessed the predictions by analyzing TF mutants and showed that, depending on the TF tested, the expression of several predicted target genes indeed changed. The overall higher coverage compared to that of the previous regulatory network derived from public microarray data, could be attributed to the fact that these seven selected transcription factors are significantly regulated at the transcriptional level, as they were predicted by LeMoNe as regulators of abiotic stress-regulated modules. The predictive performance was the best for ANAC013 targets, with a relatively low number of false positive and negative predictions. This could be explained by the fact that ANAC013 is strongly regulated at the transcript level and, in addition, autoregulates its own transcription (Chapter 2), resulting in a high expression correlation with its target genes. Similarly, in *S. cerevisiae* and *E. coli* regulatory networks, a large fraction of the regulators for which true targets could be predicted by LeMoNe are autoregulators (Michoel et al., 2009). The seven studied TFs were predicted by LeMoNe as positive regulators of their target modules. Accordingly, for all of them except RAP2.1, their target genes appear to be positively regulated, being induced or repressed by TF overexpression or knockout, respectively. RAP2.1, that has been reported previously as a repressor of the stress response (Dong and Liu, 2010) also negatively regulates the stress-induction of its target genes in

our experiments. The positive expression correlation between RAP2.1 and its target genes could be explained by the fact that they are under the same or similar regulatory control: both *RAP2.1* and its target genes are up-regulated by DREB transcription factors and under the negative regulation of RAP2.1 itself (Dong and Liu, 2010). Furthermore, from the analysis of TF binding sites in the true predicted target genes, we assume that LeMoNe predicted both direct and indirect regulatory interactions for these seven TFs. The prediction of indirect regulatory interactions is not surprising as these are often measured from expression data (Balaji et al., 2008; Joshi et al., 2010). We conclude that LeMoNe prioritizes true regulatory interactions and this is mainly true for transcription factors that are largely regulated at the transcriptional level.

Analysis of the topology of the experimentally obtained regulatory network suggests a high connectivity and potential hierarchical structure, in agreement with results from a previous study that inferred an Arabidopsis transcriptional regulatory network of the stress response (Carrera et al., 2009). This high control could be indicative of the robust transcriptional regulation of these genes during stress and could explain why single knockout of the TFs hardly affected target gene expression. ANAC013 was revealed as a potential master regulator of the obtained subnetwork, with a major role in the regulation of stress responsive genes, potentially through activating a cascade of intermediary transcription factors. However, as transcriptome analyses of TF mutants, especially overexpression lines, reveals many indirect target genes as well as genes that change due to secondary effects (e.g. altered physiology of the cell), care should be taken in the interpretation of the proposed network (Balaji et al., 2008; Joshi et al., 2010). To gain a better understanding of the underlying true biological network, evaluation of the identified regulatory interactions using the ChIP or Y1H technology will be necessary.

We have used a reverse engineered model of the Arabidopsis abiotic stress response transcriptional regulatory networks to predict unknown regulatory interactions with some promising results that reveal insight into abiotic stress-mediated transcriptional regulation. We conclude that predicted gene regulatory networks can be a powerful tool in understanding transcriptional regulatory mechanisms, especially when integrated with experimental validation. In the future, integration of other data types such as posttranscriptional and posttranslational regulation and physical (protein-DNA and protein-protein) interactions will enable the creation of a more robust network that more accurately reflects the underlying biological network. In addition, as different predictive methods, such as LeMoNe and CLR, infer complementary

information, it will be useful to combine their predictions. To conclude, we believe that the obtained results can be utilized to predict the functions of uncharacterized genes and to understand the transcriptional regulatory mechanisms of the abiotic stress response.

METHODS

Microarray compendium

Affymetrix ATH1 expression profile data were assembled from three sources: the Gene Expression Omnibus (GEO), ArrayExpress from the European Bioinformatics Institute database and the Nottingham Arabidopsis Stock Centre microarray (NASC) (Edgar et al., 2002; Craigon et al., 2004; Parkinson et al., 2009). We gathered expression data (CEL files) for 45 experimental series, representing in total 642 individual arrays and 283 different experimental conditions, including controls (Supplemental Table 5.1). The data were preprocessed in Bioconductor, R (http://www.bioconductor.org/). Through the robust multi-array average (RMA) method, a background-adjusted, quantile normalized and summarized log-transformed expression value was obtained for each probe set. Next, replicate conditions were summarized and both absolute expression values and ratio expression values derived by dividing experiment over control were obtained. In order to limit data inclusion from off-target hybridization, we used a custom *A. thaliana* cdf file, that consists of 19 937 probe sets of at least eight probes, each targeting to its transcript with perfect sequence identity and not aligning to any other gene's transcript with zero or one mismatches (Casneuf et al., 2007). For LeMoNe and CLR analysis, we removed 6087 genes for which the ratio hardly (standard deviation lower than 0.25) changed in any of the conditions, in order to identify regulation programs for different types of abiotic stresses and not the general abiotic stress response. Hence, we obtained an Arabidopsis transcript abundances profile compendium consisting of average expression values for 13850 genes in 283 (absolute expression values) and 199 (ratio expression values) conditions, respectively.

LeMoNe analysis

We ran 20 independent Gibbs sampler LeMoNe (http://bioinformatics.psb.ugent.be/software/details/LeMoNe) runs, generating 20 local optima

module clusters solutions, from which an ensemble-averaged solution of coexpression modules was created. We varied the ensemble clustering parameter qopt (i.e. at least cluster together in 5 out of the 20 runs _ qopt25, at least cluster together in 10 out of the 20 runs _ qopt5, with a higher stringency leading to smaller size modules) to create the modules. For the regulator list, We selected 1340 transcription factors based on the presence on the ATH1 array and the presence in the database Plant TFDB v2.0, Peking University, China (Zhang et al., 2011). Next, LeMoNe predicted a ranked list of weighted regulators for each module, based on an ensemble of 10 regulatory program trees with maximum three levels per module built using significant experiment sets found from 10 different experiment partitions and significant regulators sampled from 100 candidate regulator-split value pairs for each split between significant experiment clusters (Joshi et al., 2009). The weight of a regulator is the sum of split scores over the different regulatory programs (10), for each regulator sampled (100) and for each level in the tree (3) taking into account the proportion of conditions covered. The split score (0-1) of a regulator indicates how well the expression-split value of the regulator explains the partition in conditions in the module. We only considered regulators having a weight of 10 or higher (the top 2% of all regulators assigned).

CLR analysis

CLR was applied to the same microarray compendium (http://gardnerlab.bu.edu/clr.html) providing mutual information z-scores for target gene interactions. We used a cut-off for the z-score at 5 (p-value < 3.10^{-7}) and only retained at most the 10 best regulators for each gene.

Functional analysis on gene modules

Each module was analyzed for GO Biological Process enrichment with BiNGO using a gene-based custom annotation file for *Arabidopsis* (created from annotation and ontology files downloaded from www.geneontology.org on 18/10/2011), the whole annotation as a reference set (24453 *Arabidopsis* genes have a Biological Process annotation) and Benjamini and Hochberg False Discovery Rate multiple hypothesis testing correction with a confidence level of 95% (ref. Bingo). In a similar manner, each module was assessed for AraCyc 8.0 metabolic pathway enrichment (2095 *Arabidopsis* genes are annotated with a specific metabolic pathway). We kept

the significant GO or AraCyc enrichment only if more than one gene in the module had the GO Biological Process or Aracyc metabolic pathway annotation. Through 32955 experimental protein-protein interactions present in the CORNET database (De Bodt et al., 2012), we also calculated for each module the percentage of genes in the module that shared protein-protein interactions.

Cis-regulatory motifs

Known *cis*-regulatory motifs from the PLACE and AGRIS databases (Higo et al., 1999; Davuluri et al., 2003) present in the promoters, defined as 1-kb upstream of translation start sites or in the intergenic region if the adjacent upstream gene is located within a smaller distance, of all *Arabidopsis* genes were obtained from the ATCOECIS resource (http://bioinformatics.psb.ugent.be/ATCOECIS/) (Vandepoele et al., 2009). *Cis*-regulatory motif enrichment was calculated for each module using hypergeometric enrichment and Bonferroni multiple hypothesis testing correction with a confidence level of 95%. In addition, cis-regulatory motif enrichment in a module was only considered 1) when the motif is present in at least 50% of the genes and is more than 1.5 times enriched in the module compared to the genome, or 2) is more than 100 times enriched and is present in at least 20% of the genes in the module, and in both cases the motif should be present in more than one gene.

Cloning of open reading frames

The full-length open reading frames were amplified by polymerase chain reaction (PCR) from first-strand cDNA of *A. thaliana* (L.) Heynh. ecotype Columbia (Col-0) with gene-specific primers extended with the attB sites for Gateway cloning (Invitrogen Carlsbad, CA, USA; Supplemental Table 5.4). PCR reactions were run with high-fidelity Phusion DNA polymerase (Finnzymes OY, Espoo, Finland) and fragments were cloned into the Gateway entry vectors (Invitrogen) according to the manufacturer's instructions.

Generation of transgenic Arabidopsis plants

Overexpression plants – A constitutive promoter-driven expression construct was generated in the binary destination vector pK7WG2D (Karimi et al., 2007). Transgene expression levels were determined by qRT-PCR.

MicroRNA lines - *ANAC013*-specific sequences were identified with the Web MicroRNA Designer (WMD) (www.weigelworld.org). The miR precursors were constructed according to Schwab et al. (2006) (Supplemental Table 5.4) and cloned into pK7WG2D.

Constructs were transformed into Arabidopsis Columbia-0 (Col-0) by Agrobacterium-mediated floral dipping (Clough and Bent, 1998). Homozygous lines with a single T-DNA locus were selected via segregation analysis and transgene expression was monitored via qRT-PCR analysis.

Loss-of-function plants – We obtained T-DNA insertion mutants (Supplemental Table 5.5) from the ABRC at Ohio State University. Homozygous plants were selected by genomic PCR with gene-specific and T-DNA-specific primers. The expression level of the genes was determined by qRT-PCR.

Plant growth conditions and stress treatments

A. thaliana (L.) Heynh ecotype Columbia (Col-0) plants (wild type) were grown until stage 1.04 (Boyes et al., 2001) on half-strength (½) Murashige and Skoog (MS) medium (Duchefa Biochemie; http://www.duchefa.com/), 1% (w/v) sucrose, 0.75% (w/v) agar, pH 5.7 at 21°C and under a 16-h light/8-h dark photoperiod, 150 µmol $m^{-2}s^{-1}$ light intensity and 50% relative humidity. For the optimization of the salt stress conditions, seeds were either directly sown and germinated on ½MS medium supplemented with 25, 50, and 75 mM NaCl or grown on a nylon mesh on ½MS for 2 weeks and subsequently transferred to ½MS supplemented with 100 or 150 mM NaCl for different periods (6, 12, 24h). For the Nanostring nCounter experiments, a combination of wild-type and three transgenic lines were grown for 2 weeks on a nylon mesh on a ½MS plate and subsequently transferred to ½MS supplemented with 150 mM NaCl. Twelve hours after transfer, three biological samples of 5 – 10 plants were harvested. For the mannitol stress assays, wild-type and transgenic plants were sown and germinated on ½MS supplemented with 75 mM mannitol.

RNA extraction and qRT-PCR

Total RNA and first-strand cDNA were prepared with TRIzol Reagent (Invitrogen) and iScript cDNA Synthesis Kit (Bio-Rad), respectively according to the manufacturer's instructions. As a template in the subsequent PCR, 5 µL of a 1:8 diluted first-strand cDNA was run on the iCycler

iQ (Bio-Rad) with the SYBR Green I Master kit (Roche Diagnostics) according to the manufacturer's instructions. All individual reactions were done in triplicate. Primers were designed with the Universal ProbeLibrary Assay Design center ProbeFinder software (Roche; http://www.roche-applied-science.com/; Supplemental Table 5.4). For the expression analysis, values were normalized against ACTIN-RELATED PROTEIN 7 (ARP7). The Δ cycle threshold method (Livak and Schmittgen, 2001) was applied for relative quantification of transcripts.

Nanostring nCounter data analysis

The raw nCounter data were rescaled by dividing per experiment by two factors: 1) the sum of the positive controls per experiment divided by the median of the sums of the positive controls over all experiments, in order to correct for technical errors, 2) the geometric mean of the 3 most stable household genes, selected by geNorm (Vandesompele et al., 2002), per experiment divided by the total geometric mean of these 3 household genes over all experiments, in order to correct for differences in mRNA content of the samples. We identified the differential expression analysis between wild-type and perturbed transcription factor plants using the DESeq package for count data in Bioconductor, R using an adjusted *P* value cut-off of 0.1 (Anders and Huber, 2010). The raw nCounter data were fed into the package and the above rescaling factors were written in the sizeFactors slot of the CountDataSet object for the package DESeq. We compared the replicate perturbed transcription factor plants against the wild-type plants that were together with the former on the same plates. We applied a local fit for the estimation of the dispersions using the method "per-condition".

Accession numbers

ANAC013, At1g32870; ANAC053, At3g10500; ANAC032, At1g77450; ERF6, At4g17490; RAP2.1, At1g46768; RAP2.6L; At5g13330; WRKY6, At1g62300; RD26, At4g27410; ATHB-7, At2g46680; ATHB-12, At3g61890; ABF3, At4g34000; GBF3, At2g46270; MYB28, At5g61420; MYB34, At5g60890; FRO3, At1g23020; WRKY75, At5g13080; WRKY15, At2g23320; WRKY28, At4g18170; OPT3, At4g16370; bHLH039, At3g56980; PYE, At3g47640; BTS, At3g18290; SEX1, At1g10760; GRP7, At2g21660; PPR3 At5g60100; PAL1, At2g37040; PAL2, At3g53260; CHS, At5g13930; CHI, At5g05270; F3H, At3g51240; F3'H, At5g07990; FLS,

At5g08640; UGT78D2, At5g17050; 4CL3, At1g65060; HYH, At3g17609; PAP1, At1g56650; FDR, At5g42800; GST12, At5g17220; UGT75C1, At4g14090.

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SUPPLEMENTAL DATA

Supplemental Table 5.1. The compendium of 45 series of stress-related microarray experiments containing a total of 642 arrays in 283 conditions.
For each experiment is given a brief description; the database in which the experiment has been published with the corresponding database ID; the respective publication, if available. Series 45 actually contains different experiments which were carried out by different groups, as indicated.

Study	Description	Database	ID	Reference
1	Effects of anoxia and sucrose on seedling growth	GEO	GSE2133	Loreti et al., 2005
2	Dynamics of mRNA abundance and translation in response to short and prolonged hypoxia and reoxygenation	GEO	GSE9719	Branco-Price et al.
3	Global expression profiling of wild type and transgenic Arabidopsis plants in response to water stress	GEO	GSE10670	Perera et al., 2008
4	Transcription profiling of Arabidopsis dor mutant and wild-type plants in response to drought stress.	GEO	GSE10643	Zhang et al., 2008
5	Transcription profiling of SALK_084897 or SAIL_303_D08 Arabidopsis plants grown under normal conditions or with moderate light and drought treatment applied	ArrayExpress	E-ATMX-32	Giraud et al., 2008
6	AtGenExpress: Light treatments	TAIR	ME00345	
7	Carbon fixation (endogenous sugar) and light-dependent gene expression	GEO	GSE3423	Bläsing et al., 2005
8	Genome-wide gene expression analysis reveals a critical role for CRY1 in the Response of Arabidopsis to High Irradiance	GEO	GSE7743	Kleine et al., 2007
9	Hydrogen peroxide stress and Zat12 over-expression in Arabidopsis.	GEO	GSE5530	
10	AtGenExpress: Effect of ibuprofen, salicylic acid and daminozide on seedlings	TAIR	ME00364	
11	The effects of the sfr2, sfr3 and sfr6 mutations on lyotropic stress responses	GEO	GSE6177	
12	Genome-wide transcriptome analysis of Arabidopsis and siz1-3 response to drought stress	GEO	GSE6583	Catala et al., 2007
13	Over-expression of MBF1c enhances stress tolerance	GEO	GSE5539	
14	WTv.AOXantisense	GEO	GSE2406	Umbach et al., 2005
15	Response to CBF2 expression	GEO	GSE5536	
16	Transcription profiling of heat stress response in Arabidopsis wild type and hsf1x3 double knockout mutant lines	ArrayExpress	E-MEXP-98	Busch et al., 2005
17	ICE1 regulation of the Arabidopsis Cold-Responsive Transcriptome	GEO	GSE3326	Lee et al., 2005
18	Expression data from thylakoidal ascorbate peroxidase overexpressor Arabidopsis thaliana (Col) rosette leaves	GEO	GSE10812	Laloi et al., 2007
19	A polyadenylation factor subunit implicated in regulating oxidative stress responses in Arabidopsis thaliana	GEO	GSE7211	
20	NFYA5, a CCAAT binding transcription factor important for drought resistance in Arabidopsis	GEO	GSE12029	Li et al., 2008
21	Gene expression in wild-type and transgenic plants overexpressing rice OsTOP6A1 gene	GEO	GSE6812	Jain et al., 2008
22	STA1, a stress-upregulated nuclear protein, is required for pre-mRNA splicing, mRNA turnover and stress tolerance	GEO	GSE4662	Lee et al., 2006
23	Transcription profiling of Arabidopsis seedlings treated with the herbicide methyl viologen	ArrayExpress	E-ATMX-28	Scarpeci et al., 2008
24	Transcription profiling of Arabidopsis distal leaves directly exposed to and shaded from high light levels	ArrayExpress	E-ATMX-19	Rossel et al., 2007
25	Transcription profiling of Arabidopsis wild type and SAL1 mutant plants grown under normal conditions	ArrayExpress	E-MEXP-1495	Wilson et al., 2009
26	Transcription profiling of Arabidopsis wild type, cop1-4, hy5-1 mutant seedlings exposed to polychromatic radiation	ArrayExpress	E-MEXP-557	Oravecz et al., 2006
27	The effect of overexpression of Zat10 in Arabidopsis leaf tissue	ArrayExpress	E-ATMX-20	Rossel et al., 2007
28	Transcription profiling of Arabidopsis wild type, AtMYB44 knock-out and AtMYB44 over-expressing plants treated with NaCl	ArrayExpress	E-ATMX-30	Jung et al., 2008
29	Transcription profiling of Arabidopsis treated with sugars (glucose, mannose) and abcissic acid	ArrayExpress	E-MEXP-475	Li et al., 2006

Supplemental Table 5.1. The compendium of 45 series of stress-related microarray experiments containing a total of 642 arrays in 283 conditions. (*Continued*).

30	Transcription profiling of Arabidopsis thaliana response to ozone	ArrayExpress	E-MEXP-342	
31	catalase2 knockout			
32	CATALASE2 knockdown + high light stress			
33	low CO2			
34	Mitochondrial type-I prohibitins of Arabidopsis thaliana are required for supporting proficient meristem development.	-	-	Van Aken et al., 2007
35	Perturbation of indole-3-butyric acid homeostasis by the UDP-glucosyltransferase UGT74E2 modulates Arabidopsis architecture and water stress tolerance.	-	-	Tognetti et al., 2010
36	Expression patterns of genes induced by sugar accumulation during early leaf senescence	NASC/NascArrays	NASCARRAYS-136	Pourtau et al., 2006
37	Functional Genomics of Ozone Stress in Arabidopsis.	GEO	GSE5722	
38	AtGenExpress: ABA time course in wildtype seedlings	TAIR	ME00333	
39	AtGenExpress: Effect of auxin inhibitors on seedlings	TAIR	ME00358	
40	AtGenExpress: IAA time course in wildtype seedlings	TAIR	ME00336	
41	AtGenExpress: Methyl Jasmonate time course in wildtype seedlings	TAIR	ME00337	
42	Experiment: AtGenExpress: ACC time course in wildtype seedlings	TAIR	ME00334	
43	AtGenExpress: Effect of ethylene inhibitors on seedlings	TAIR	ME00360	
44	AtGenExpress: Brassinolide time course in wildtype and det2-1 mutant seedlings	TAIR	ME00335	
45	AtGenExpress: tissue comparison, abiotic treatment	TAIR		
	AtGenExpress: Cold stress time course	TAIR	ME00325	
	AtGenExpress: Osmotic stress time course	TAIR	ME00327	
	AtGenExpress: Salt stress time course	TAIR	ME00328	
	AtGenExpress: Drought stress time course	TAIR	ME00338	
	AtGenExpress: Genotoxic stress time course	TAIR	ME00326	
	AtGenExpress: Oxidative stress time course	TAIR	ME00340	
	AtGenExpress: UV-B stress time course	TAIR	ME00329	
	AtGenExpress: Heat stress time course	TAIR	ME00339	

Supplemental Table 5.2. Overview of the modules with a significant GO enrichment.
Only the most significant GO biological process term is displayed.

Module	# genes	# genes with GO	*P* value	GO-ID	GO Description
1	62	58	2.80E-87	6412	translation
2	70	20	1.81E-09	10467	gene expression
3	71	30	3.79E-27	6412	translation
4	74	4	4.52E-04	16116	carotenoid metabolic process
5	56	5	1.81E-07	42775	mitochondrial ATP synthesis coupled electron transport
6	69	11	2.52E-15	7018	microtubule-based movement
7	111	2	4.16E-02	19348	dolichol metabolic process
8	63	12	3.60E-06	16070	RNA metabolic process
9	181	7	1.06E-08	9773	photosynthetic electron transport in photosystem I
10	134	14	5.35E-08	6511	ubiquitin-dependent protein catabolic process
11	71	63	2.03E-91	6412	translation
12	68	5	3.05E-02	48580	regulation of post-embryonic development
13	51	6	5.88E-04	10016	shoot morphogenesis
14	45	15	2.65E-11	6412	translation
15	55	13	3.51E-05	6468	protein amino acid phosphorylation
16	46	2	3.91E-02	7155	cell adhesion
17	36	23	1.11E-39	15979	photosynthesis
18	67	10	1.50E-06	16051	carbohydrate biosynthetic process
19	54	4	1.15E-04	9062	fatty acid catabolic process
20	52	2	2.21E-02	10304	PSII associated light-harvesting complex II catabolic process
22	42	9	1.51E-04	6412	translation
27	84	2	1.23E-03	45337	farnesyl diphosphate biosynthetic process
28	37	7	3.80E-05	45087	innate immune response
29	32	11	4.25E-08	44248	cellular catabolic process
30	37	9	1.80E-15	5982	starch metabolic process
32	218	65	3.36E-33	10467	gene expression
33	55	13	3.59E-15	10200	response to chitin
34	23	4	2.12E-02	6979	response to oxidative stress
35	36	2	2.76E-02	43193	positive regulation of gene-specific transcription
36	22	3	1.15E-02	6073	cellular glucan metabolic process
37	28	5	1.56E-04	9698	phenylpropanoid metabolic process
38	21	12	1.22E-21	6260	DNA replication
39	33	4	1.63E-03	9642	response to light intensity
40	22	11	3.80E-17	15979	photosynthesis
41	38	7	4.94E-02	6468	protein amino acid phosphorylation
43	38	4	3.67E-02	9755	hormone-mediated signaling pathway
45	24	2	2.80E-02	32318	regulation of Ras GTPase activity
46	35	7	2.04E-07	7167	enzyme linked receptor protein signaling pathway
47	101	7	3.62E-02	16192	vesicle-mediated transport
48	17	5	1.58E-04	6979	response to oxidative stress
49	30	7	7.46E-09	6260	DNA replication
50	19	3	8.57E-04	9404	toxin metabolic process
51	34	17	1.40E-03	65007	biological regulation
52	27	5	9.27E-04	6457	protein folding
55	17	2	1.64E-04	10623	developmental programmed cell death
56	15	6	1.16E-14	34976	response to endoplasmic reticulum stress
57	21	3	4.82E-08	80003	thalianol metabolic process
58	32	13	4.85E-19	10200	response to chitin
59	81	4	1.63E-03	43085	positive regulation of catalytic activity
60	17	15	2.26E-36	6334	nucleosome assembly
61	22	7	8.81E-08	6631	fatty acid metabolic process
62	33	5	1.59E-03	9414	response to water deprivation

Supplemental Table 5.2. Overview of the modules with a significant GO enrichment. (*Continued*).

64	31	8	1.75E-02	23052	signaling
65	25	11	7.18E-16	15979	photosynthesis
68	77	9	7.55E-07	15979	photosynthesis
69	13	4	5.49E-09	10584	pollen exine formation
70	34	14	1.36E-08	6468	protein amino acid phosphorylation
71	19	10	3.79E-15	9611	response to wounding
72	24	7	4.16E-06	6259	DNA metabolic process
73	23	7	3.09E-03	5975	carbohydrate metabolic process
76	15	8	3.30E-16	9813	flavonoid biosynthetic process
77	21	5	3.02E-03	48316	seed development
80	32	4	7.27E-03	10200	response to chitin
81	21	8	7.24E-11	9408	response to heat
82	13	3	1.13E-03	6073	cellular glucan metabolic process
84	45	11	7.93E-09	46394	carboxylic acid biosynthetic process
85	14	3	3.89E-02	9617	response to bacterium
86	11	6	4.35E-14	16144	S-glycoside biosynthetic process
88	31	3	2.05E-03	18193	peptidyl-amino acid modification
89	22	3	2.33E-03	9738	abscisic acid mediated signaling pathway
90	20	3	3.34E-04	9404	toxin metabolic process
94	21	7	7.06E-04	9725	response to hormone stimulus
95	50	5	1.96E-02	30001	metal ion transport
98	14	13	1.67E-26	9408	response to heat
100	16	9	4.40E-13	9414	response to water deprivation
104	13	3	9.02E-06	6949	syncytium formation
105	157	3	3.73E-02	10020	chloroplast fission
106	9	3	2.16E-06	10212	response to ionizing radiation
107	13	2	4.01E-02	6869	lipid transport
108	7	2	2.60E-03	16036	cellular response to phosphate starvation
110	35	4	1.74E-05	15995	chlorophyll biosynthetic process
111	7	6	2.97E-10	6790	sulfur metabolic process
112	7	3	1.33E-05	45333	cellular respiration
113	26	5	1.14E-04	10200	response to chitin
115	10	3	1.52E-06	103	sulfate assimilation
116	18	3	9.48E-05	16072	rRNA metabolic process
119	10	8	4.97E-13	6457	protein folding
120	9	6	2.50E-10	10876	lipid localization
121	29	5	1.12E-02	9793	embryonic development ending in seed dormancy
122	10	5	2.98E-10	9073	aromatic amino acid family biosynthetic process
123	6	6	5.69E-08	34645	cellular macromolecule biosynthetic process
125	26	2	4.00E-03	6743	ubiquinone metabolic process
130	37	2	1.44E-03	6552	leucine catabolic process
134	4	2	3.73E-05	9718	anthocyanin biosynthetic process
135	12	3	5.32E-05	9853	photorespiration
139	23	3	2.86E-02	9698	phenylpropanoid metabolic process
140	7	2	1.46E-03	9404	toxin metabolic process
142	31	6	3.09E-06	9751	response to salicylic acid stimulus
145	4	4	3.32E-09	45454	cell redox homeostasis
146	5	3	3.88E-05	9408	response to heat
147	6	2	7.41E-04	9834	secondary cell wall biogenesis
149	5	4	3.82E-06	6091	generation of precursor metabolites and energy
150	11	4	9.34E-05	9414	response to water deprivation
152	6	2	3.02E-05	48571	long-day photoperiodism
153	7	2	3.21E-02	6631	fatty acid metabolic process
154	15	4	3.75E-04	9611	response to wounding
155	8	4	1.91E-05	9611	response to wounding
156	5	2	2.73E-02	9408	response to heat

Supplemental Table 5.2. Overview of the modules with a significant GO enrichment. (*Continued*).

159	8	4	1.02E-05	9408	response to heat
160	3	3	1.39E-07	71368	cellular response to cytokinin stimulus
161	63	16	6.12E-10	6412	translation
163	5	2	9.84E-05	10311	lateral root formation
166	5	3	1.24E-04	10876	lipid localization
168	15	2	4.56E-04	48586	regulation of long-day photoperiodism, flowering
169	6	3	1.92E-02	6952	defense response
172	11	5	4.87E-03	6796	phosphate metabolic process
173	5	4	5.40E-03	6950	response to stress
177	6	3	7.19E-04	9698	phenylpropanoid metabolic process
181	10	3	5.02E-03	9611	response to wounding
185	3	2	3.42E-03	9733	response to auxin stimulus
186	4	3	9.91E-03	9725	response to hormone stimulus
188	5	4	1.41E-10	6568	tryptophan metabolic process
189	3	3	2.23E-06	6869	lipid transport
190	4	2	2.58E-03	6073	cellular glucan metabolic process
196	2	2	3.45E-03	46686	response to cadmium ion
197	4	2	8.71E-03	9814	defense response, incompatible interaction
199	15	2	8.17E-03	9738	abscisic acid mediated signaling pathway
200	3	3	1.78E-02	50896	response to stimulus
203	5	3	1.20E-02	9725	response to hormone stimulus
208	8	4	1.83E-04	9733	response to auxin stimulus
210	17	2	2.08E-02	9404	toxin metabolic process
214	3	2	2.24E-03	9408	response to heat
217	3	2	3.73E-04	10193	response to ozone
224	4	3	2.99E-02	9628	response to abiotic stimulus
226	3	2	5.48E-06	10039	response to iron ion
234	4	2	3.45E-02	9639	response to red or far red light
235	3	2	1.37E-02	9737	response to abscisic acid stimulus
246	2	2	1.29E-05	19758	glycosinolate biosynthetic process
247	5	2	4.47E-02	9908	flower development
254	3	2	4.06E-02	31323	regulation of cellular metabolic process
255	3	3	3.29E-02	50896	response to stimulus
266	2	2	1.38E-03	9611	response to wounding
268	3	2	3.42E-03	9733	response to auxin stimulus
274	3	2	1.26E-03	6260	DNA replication
277	7	2	4.37E-02	9414	response to water deprivation
289	3	2	4.42E-02	9628	response to abiotic stimulus
290	3	2	1.59E-02	9056	catabolic process
311	3	3	1.14E-03	5975	carbohydrate metabolic process
320	6	2	3.10E-02	46686	response to cadmium ion
327	3	3	1.43E-02	6950	response to stress
330	9	2	4.20E-02	50832	defense response to fungus
340	4	3	9.78E-06	6915	apoptosis
670	3	2	9.73E-05	6949	syncytium formation
719	3	2	3.17E-02	45449	regulation of transcription
827	2	2	1.18E-02	6355	regulation of transcription, DNA-dependent
833	3	3	5.60E-03	6464	protein modification process
862	2	2	1.99E-03	51707	response to other organism
876	3	2	3.51E-02	51707	response to other organism
939	3	2	4.15E-03	43632	modification-dependent macromolecule catabolic process
1081	7	2	7.20E-04	9788	negative regulation of abscisic acid mediated signaling pathway
1217	4	2	8.45E-03	10200	response to chitin
1223	4	2	3.80E-02	6396	RNA processing
1267	5	5	1.42E-03	44267	cellular protein metabolic process

Supplemental Table 5.2. Overview of the modules with a significant GO enrichment. (*Continued*).

1408	3	2	3.21E-03	9611	response to wounding
1429	3	2	2.39E-02	9628	response to abiotic stimulus
1491	4	2	1.85E-02	32787	monocarboxylic acid metabolic process
1526	4	2	1.94E-02	42398	cellular amino acid derivative biosynthetic process
1563	3	2	3.95E-02	45449	regulation of transcription
1583	6	3	1.82E-02	6952	defense response
1616	3	2	2.61E-02	45449	regulation of transcription
1656	3	2	6.39E-04	15698	inorganic anion transport
1733	2	2	5.47E-05	38	very-long-chain fatty acid metabolic process
2030	5	2	1.73E-02	6281	DNA repair
2053	3	2	3.06E-02	6810	transport
2196	3	3	7.92E-04	6468	protein amino acid phosphorylation
2341	3	2	4.77E-02	44281	small molecule metabolic process
2596	3	2	2.04E-02	16070	RNA metabolic process

Supplemental Table 5.3. LeMoNe modules containing a significantly overrepresented *cis*-regulatory element.
The function of the module and/or regulator(s) is displayed if it corresponds to the function of the motif (column 7 and 8, respectively). Modules indicated in bold have a function related to the stress response. Members of transcription factor families with (potential) binding sites corresponding to the motif are displayed in column 9. %, percentage of genes in the module that contain the motif; E, fold enrichment; *P* value, FDR corrected hypergeometric *P* value.

M	Motif	Name of motif	%	E	*P* value	Module function	Regulator(s) with similar function	Regulator(s) with corresponding binding site
1	AAACCCTA	AAACCCTAUP2ATMSD	87	4.46	6.6E-28	translation		
	AAACCCTAA	TELOBOXATEEF1AA1;AAACCCTAA	58	4.67	4.6E-15			
	TGGGCY	SITEIIATCYTC	87	2.37	7.7E-14			
	GGGCC	SORLIP2AT;GGGCC	81	2.66	1.0E-13			
	GGCCCAWWW	UP1ATMSD	53	3.61	4.6E-10			
2	AAACCCTA	AAACCCTAUP2ATMSD	69	3.56	6.1E-21	gene expression; ribosome biogenesis		
	AAACCCTAA	TELOBOXATEEF1AA1;AAACCCTAA	53	4.26	6.7E-17			
	GGGCC	SORLIP2AT;GGGCC	56	1.86	1.5E-04			
	TGGGCY	SITEIIATCYTC	62	1.70	4.0E-04			
3	GATAAGR	IBOXCORENT	54	2.27	5.7E-06	translation; photosynthesis		
	GATAAG	IBOX;GATAAG	66	1.84	3.4E-05			
	TGGGCY	SITEIIATCYTC	64	1.73	7.5E-04			
	GGGCC	SORLIP2AT;GGGCC	55	1.83	1.9E-03			
	TTATCC	SREATMSD	57	1.62	3.2E-02			
5	TGGGCY	SITEIIATCYTC	67	1.82	3.6E-04	mitochondrial respiration		
	GGGCC	SORLIP2AT;GGGCC	54	1.78	2.2E-02			
6	AACGG	MYBCOREATCYCB1	93	1.86	1.7E-13	cell cycle, microtubuli-based movement		
	YAACKG	MYB2CONSENSUSAT	89	1.60	1.1E-07			
	AWTTCAAA	ERELEE4	64	1.92	1.7E-05			
11	AAACCCTA	AAACCCTAUP2ATMSD	83	4.26	2.9E-28	translation		
	GGCCCAWWW	UP1ATMSD	63	4.30	2.9E-18			
	GGGCC	SORLIP2AT;GGGCC	83	2.74	1.3E-17			
	TGGGCY	SITEIIATCYTC	87	2.38	3.5E-16			
14	AAACCCTA	AAACCCTAUP2ATMSD	52	2.65	1.9E-05	translation		
	GGGCC	SORLIP2AT;GGGCC	59	1.94	2.4E-03			
15	TTGACT	TTGACT	78	1.70	1.5E-04	defense response		WRKY15
	TTTGACY	WBBOXPCWRKY1	63	1.80	3.3E-03			
17	GATAAGR	IBOXCORENT	71	2.97	7.5E-08	photosynthesis	GLK2	
	ACGTGKC	ACGTABREMOTIFA2OSEM	54	3.72	1.2E-06			
	GATAAG	IBOX;GATAAG	78	2.17	1.2E-05			
	GCCAC	SORLIP1AT	80	2.00	4.5E-05			
18	CCGAC	LTRECOREATCOR15	60	1.76	7.1E-04			
20	ACGTG	ABRELATERD1	77	1.54	9.6E-03	chloroplast localized proteins	COP1	
22	AAACCCTA	AAACCCTAUP2ATMSD	77	3.94	8.2E-18	translation		
	AAACCCTAA	TELOBOXATEEF1AA1;AAACCCTAA	61	4.89	4.6E-15			
	TGGGCY	SITEIIATCYTC	63	1.70	2.0E-02			

Supplemental Table 5.3. LeMoNe modules containing a significantly overrepresented *cis*-regulatory element. (*Continued*).

23	GATAAG	IBOX;GATAAG	62	1.73	2.5E-02			
24	TTATCC	SREATMSD	72	2.07	6.0E-05	carbohydrate metabolism		
	GATAAG	IBOX;GATAAG	70	1.95	5.5E-04			
	GATAAGR	IBOXCORENT	53	2.24	3.8E-03			
28	TTGACT	TTGACT	79	1.71	4.3E-03	defense response		
30	AAAATATCT	EVENINGAT;AAAATATCT	57	8.04	6.8E-15	starch catabolism, response to cold, circadian rhythm	APRR3	
33	MACGYGB	ABRERATCAL	62	1.91	5.5E-04			
	TTGACT	TTGACT	72	1.57	9.6E-03	defense response		
35	VCGCGB	CGCGBOXAT	69	3.95	1.0E-11	stress signalling and transcriptional regulation	AZF2, MYB28	
	MACGYGB	ABRERATCAL	62	1.91	1.2E-02			
	RYCGAC	CBFHV	67	1.79	1.6E-02			
38	WTTSSCSS	E2FCONSENSUS	87	3.41	2.3E-07	DNA replication		
	GGGCC	SORLIP2AT;GGGCC	74	2.44	4.7E-03			
	TGGGCY	SITEIIATCYTC	78	2.13	1.3E-02			
39	ACGTGKC	ACGTABREMOTIFA2OSEM	54	3.75	5.6E-06	response to light intensitiy; chlorophyll biosynthesis	GLK2	
41	VCGCGB	CGCGBOXAT	76	4.37	3.2E-14	defense response	TIP	
	MACGYGB	ABRERATCAL	62	1.90	2.5E-02			
44	RCCGAC	DRECRTCOREAT	64	3.19	8.1E-07	responsive to cold	RAP2.1	RAP2.1 (DREB)
	RYCGAC	CBFHV	79	2.14	2.4E-05			
	CCGAC	LTRECOREATCOR15	74	2.18	1.0E-04			
49	WTTSSCSS	E2FCONSENSUS	77	3.01	1.6E-06	cell cycle; DNA replication		
50	TTTGACY	WBBOXPCWRKY1	78	2.25	5.9E-03	responsive to pathogen infection	WRKY75	WRKY75
	KCACGW	RHERPATEXPA7	87	1.89	1.4E-02			
52	GGGCC	SORLIP2AT;GGGCC	66	2.16	2.6E-02			
53	TATCCA	TATCCAOSAMY	81	2.16	2.0E-05	carbohydrate depriviation response; carbohydrate metabolism		
	TATCCAY	TATCCAYMOTIFOSRAMY3D	59	2.87	8.9E-05			
	TTATCC	SREATMSD	73	2.09	7.9E-04			
	GATAAG	IBOX;GATAAG	73	2.03	1.4E-03			
56	CCNNNNNNNNNNNNCCACG	UPRMOTIFIIAT	50	16.09	2.4E-07	unfolded protein response		
	CACGTG	CACGTGMOTIF;CACGTG	67	4.53	2.0E-04			
	CACGTGG	IRO2OS	50	6.94	3.4E-04			
	ACGTGKC	ACGTABREMOTIFA2OSEM	61	4.24	1.6E-03			
	TGACGT	TGACGTVMAMY	67	3.38	4.7E-03			
	ACGTG	ABRELATERD1	94	1.90	1.7E-02			
	MACGYGB	ABRERATCAL	78	2.39	2.7E-02			
57	ACGTG	ABRELATERD1	96	1.93	3.4E-04			
	CATGCA	RYREPEATBNNAPA	71	2.73	1.3E-03			
58	VCGCGB	CGCGBOXAT	75	4.30	1.5E-11	defense response		
	MACGYGB	ABRERATCAL	78	2.39	8.6E-06			
	TTGACC	ELRECOREPCRP1;TTGACC	61	1.98	4.6E-02			
60	VCGCGB	CGCGBOXAT	65	3.71	4.5E-03			
	TGACGT	TGACGTVMAMY	65	3.28	1.5E-02			

Supplemental Table 5.3. LeMoNe modules containing a significantly overrepresented *cis*-regulatory element. (*Continued*).

62	ACGTGKC	ACGTABREMOTIFA2OSEM	50	3.47	1.5E-04	ABA and drought response	ATHB12, RD26, ATHB7	
	MACGYGB	ABRERATCAL	67	2.05	9.0E-03			
66	YAACKG	MYB2CONSENSUSAT	100	1.79	3.6E-03			
	AACGG	MYBCOREATCYCB1	95	1.89	8.9E-03			
67	AAACCCTA	AAACCCTAUP2ATMSD	65	3.38	1.3E-09			
68	GATAAGR	IBOXCORENT	50	2.13	8.4E-06	photosynthesis		
	TTATCC	SREATMSD	61	1.76	6.7E-05			
	GATAAG	IBOX;GATAAG	59	1.65	1.4E-03			
	TATCCA	TATCCAOSAMY	57	1.52	3.3E-02			
70	TTGACT	TTGACT	86	1.87	1.8E-04	defense response		WRKY15
71	CACGTG	CACGTGMOTIF;CACGTG	62	4.20	2.4E-04			
	MACGYGB	ABRERATCAL	81	2.49	1.8E-03			
	ACGTG	ABRELATERD1	90	1.82	2.5E-02			
	ACACNNG	DPBFCOREDCDC3	95	1.68	2.8E-02			
76	ACCTACC	ACCTACC	50	13.28	9.7E-06	flavonoid biosynthesis		
	ACCWWCC	BOXLCOREDCPAL	75	3.59	1.4E-03			
	CCWACC	MYBPZM	75	2.68	3.3E-02			
77	ACGTGTC	GADOWNAT	73	8.28	1.3E-10	seed and embryo development, hyperosmotic response	ATHB-7, NF-YA5; MEA	
	ACGTGKC	ACGTABREMOTIFA2OSEM	77	5.36	1.6E-08			
	CACGTGG	IRO2OS	50	6.94	2.2E-05			
	ACGTG	ABRELATERD1	100	2.01	5.4E-05			
	ACACGTG	ACACGTG	50	5.12	4.8E-04			
	CACGTG	CACGTGMOTIF;CACGTG	59	4.01	5.0E-04			
	MACGYGB	ABRERATCAL	82	2.51	7.0E-04			
	ACACNNG	DPBFCOREDCDC3	95	1.68	1.7E-02			
80	TTGACT	TTGACT	85	1.85	1.2E-03	defense response	WRKY6	WRKY6, WRKY28
	TTGACC	ELRECOREPCRP1;TTGACC	70	2.26	1.4E-03			
	TTTGACY	WBBOXPCWRKY1	70	2.00	1.2E-02			
86	MACGYGB	ABRERATCAL	91	2.79	2.2E-02			
90	TGACGT	TGACGTVMAMY	70	3.55	3.5E-04	carbohydrate metabolism		
94	CACGTG	CACGTGMOTIF;CACGTG	56	3.80	5.2E-04	responsive to ABA, drought, salt	ATAF1, RD26, AZF2	
	ACGTGKC	ACGTABREMOTIFA2OSEM	52	3.61	2.9E-03			
	TATCCA	TATCCAOSAMY	76	2.03	2.7E-02			
95	TTGACT	TTGACT	72	1.56	3.8E-02			
99	TTTGACY	WBBOXPCWRKY1	72	2.06	1.9E-06	responsive to pathogen infection	WRKY6	WRKY6
	TTGACT	TTGACT	75	1.63	1.4E-03			
100	ACGTGKC	ACGTABREMOTIFA2OSEM	65	4.49	7.6E-04	ABA and drought response	RD26, ATHB-7	
	ACGTGTC	GADOWNAT	53	6.03	1.0E-03			
	ACACGTG	ACACGTG	53	5.42	2.4E-03			
	MACGYGB	ABRERATCAL	82	2.53	8.9E-03			
	RYCGAC	CBFHV	82	2.21	4.7E-02			
108	GNATATNC	P1BS	100	6.02	9.7E-05	responsive to phosphate starvation		

Supplemental Table 5.3. LeMoNe modules containing a significantly overrepresented *cis*-regulatory element. (*Continued*).

110	GATAAGR	IBOXCORENT	67	2.84	4.5E-07	chlorophyll biosynthesis		AT1G19000 (MYB)
	GATAAG	IBOX;GATAAG	70	1.94	1.9E-03			
111	CACCAACC	CACCAACC	50	21.23	3.7E-03	glucosinolate biosynthesis	MYB28	MYB28
	AACGTG	T/GBOXATPIN2	88	4.24	2.0E-02			
112	AGCGGG	BS1EGCCR	57	13.60	1.7E-02			
	VCGCGB	CGCGBOXAT	86	4.91	2.9E-02			
116	AAACCCTA	AAACCCTAUP2ATMSD	94	4.84	2.8E-09	RNA processing; ribosome biogenesis		
	AAACCCTAA	TELOBOXATEEF1AA1;AAACCCTAA	83	6.71	3.2E-09			
119	AAACCCTA	AAACCCTAUP2ATMSD	82	4.19	3.1E-03			
120	ACACGTG	ACACGTG	78	7.96	4.9E-04	lipid localization; seed germination		
	CATGCAY	RYREPEATLEGUMINBOX	89	5.59	6.1E-04			
	CATGCA	RYREPEATBNNAPA	100	3.86	1.0E-03			
	CACGTG	CACGTGMOTIF;CACGTG	78	5.28	7.9E-03			
	CATGCAT	RYREPEATGMGY2	67	5.67	3.1E-02			
121	MACGYGB	ABRERATCAL	67	2.05	1.7E-02	seed development	MEA	
122	ACCWWCC	BOXLCOREDCPAL	92	4.38	7.1E-05	tryptophan and glucosinolate biosynthesis, response to wounding		
	ACCAACC	ACCAACC	58	6.42	5.9E-03			
123	GTCGAC	CRTDREHVCBF2	67	11.26	2.3E-02			
125	GCCAC	SORLIP1AT	82	2.04	1.8E-03	response to light	SPA1 (SUPPRESSOR OF PHYA-1051)	
	AGCCAC	AGCCAC	54	3.26	1.9E-03			
128	TTTGACY	WBBOXPCWRKY1	81	2.34	4.3E-02	defense response	MYB15	WRKY15, WRKY30
129	ACGTGKC	ACGTABREMOTIFA2OSEM	88	6.07	2.1E-03			
	GACGTGGC	GACGTGGC	50	25.34	2.2E-03			
	TGACGTGG	HEXAT;TGACGTGG	50	17.29	1.0E-02			
	ACGTGGC	BOXIIPCCHS	63	8.62	2.1E-02			
	MACGYGB	ABRERATCAL	100	3.07	2.9E-02			
	CACGTG	CACGTGMOTIF;CACGTG	75	5.09	5.0E-02			
133	ACGTGTC	GADOWNAT	60	6.83	3.7E-06	responsive to ABA and drought	RD26	
	CACGTG	CACGTGMOTIF;CACGTG	70	4.75	9.9E-06			
	ACACGTG	ACACGTG	60	6.14	1.2E-05			
	ACGTGKC	ACGTABREMOTIFA2OSEM	65	4.51	9.2E-05			
135	ACGTGTC	GADOWNAT	62	7.00	6.6E-04	photosynthesis; photorespiration		GBF6 (bZIP)
	ACGTGKC	ACGTABREMOTIFA2OSEM	69	4.80	2.4E-03			
	ACACGTG	ACACGTG	54	5.51	1.9E-02			
137	VCGCGB	CGCGBOXAT	60	3.44	6.2E-05		TIP	
145	TACGTGGC	TACGTGGC;TACGTGGC	75	48.50	2.1E-03	redox homeostasis		bZIP34

Supplemental Table 5.3. LeMoNe modules containing a significantly overrepresented *cis*-regulatory element. (*Continued*).

150	ACGTGTC	GADOWNAT	73	8.28	1.1E-04	ABA and drought response	ATHB-7, ATHB-12	
	ACGTGKC	ACGTABREMOTIFA2OSEM	82	5.67	2.6E-04			
	ACACGTG	ACACGTG	64	6.52	4.6E-03			
152	ACGTGKC	ACGTABREMOTIFA2OSEM	100	6.93	1.7E-03	circadian regulation	CCA1, PIL6	
	AGCCAC	AGCCAC	100	6.09	3.8E-03			
	CACGTGTC	CACGTGTC	67	14.54	1.2E-02			
153	MACCWAMC	MYBPLANT	100	3.61	2.4E-02			
154	ACGTG	ABRELATERD1	90	1.82	2.5E-02			
155	MACGYGB	ABRERATCAL	100	3.07	8.5E-03	responsive to ABA, drought, salt		
161	AAACCCTA	AAACCCTAUP2ATMSD	50	2.56	7.8E-06	translation; gene expression		
	TGGGCY	SITEIIATCYTC	62	1.69	7.0E-03			
168	AAAATATCT	EVENINGAT;AAAATATCT	69	9.68	1.6E-07	circadian regulation, light and cold response	APPR5	
192	GCCGCC	GCCCORE;GCCGCC	100	15.18	4.2E-02	responsive to pathogen infection		ORA59 (ERF), ERF15
197	GCCGCC	GCCCORE;GCCGCC	100	15.18	2.9E-03	defense response		
	AGCCGCC	AGCBOXNPGLB	75	34.98	5.9E-03			
234	TACGTGGA	TACGTGGA	75	44.73	3.0E-03	light response; regulation of photosynthesis genes	LZF1 (Light regulated Zinc Finger 1)	
241	GCGTNNNNNNNACGC	VOZATVPP	100	214.17	1.3E-05			
274	TYTCCCGCC	E2FAT	67	59.81	4.5E-02	DNA replication		
730	TGGCCGAC	TGGCCGAC	50	84.40	8.1E-04	responsive to cold, drought, salt	CEJ1	CEJ1 (DREB)
827	AGCCGACCA	AGCCGACCA	67	316.16	2.1E-03	responsive to cold, drought, salt		AT4G28140,AT2G20880 (DREB)
	TGGACGG	CMSRE1IBSPOA	100	38.68	2.8E-03			
	CCATACATT	S2FSORPL21	67	81.46	3.2E-02			
884	ATACGTGT	ZDNAFORMINGATCAB1;ATACGTGT	60	24.44	2.5E-02	responsive to ABA, drought, salt	ATHB-7	
1081	ACGTGTC	GADOWNAT	71	8.13	2.0E-02	ABA and drought response	RD26, ATHB12	
1621	CTAACCA	MYBATRD22;CTAACCA	71	6.70	4.7E-02			
1917	TAGTGGAT	NRRBNEXTA	100	33.70	3.7E-03			
2196	GCGGCAAA	E2FBNTRNR	67	73.77	3.9E-02			

Supplemental Table 5.4. Primers used.

Primer	Sequence
ANAC013-ORF_FWD	AAAAAGCAGGCTCCACCATGGACTTGTCGGTTGAGAA
ANAC013-ORF_REV	AGAAAGCTGGGTCTCACCATAACAAAGGCCTCC
ANAC053-ORF_FWD	AAAAAGCAGGCTCCACCATGGGTCGTGGCTCAGTAAC
ANAC053-ORF_REV	AGAAAGCTGGGTCTCACCTGGAAGAGACCAAAATG
ANAC032-ORF_FWD	AAAAAAGCAGGC TCCACCATGATGAAATCTGGGGCTGATTTGC
ANAC032-ORF_REV	AGAAAGCTGGGTCTCAGAAAGTTCCCTGCCTAACC
ERF6-ORF_FWD	GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCTACACCAAACGAAG
ERF6-ORF_REV	GGGGACCACTTTGTACAAGAAAGCTGGGTATCAAACAACGGTCAATTGTG
ANAC013-I miR-s	gaTGTAACGGTAAGTCCGTCGTTtctctcttttgtattcc
ANAC013-II miR-a	gaAACGACGGACTTACCGTTACAtcaaagagaatcaatga
ANAC013-III miR*s	gaAAAGACGGACTTAGCGTTACTtcacaggtcgtgatatg
ANAC013-IV miR*a	gaAGTAACGCTAAGTCCGTCTTTtctacatatatattcct

Supplemental Table 5.5. T-DNA insertion lines.

Gene	KO line
ANAC053	SALK_009578C
ANAC032	SALK_012253
ERF6	SALK_030723
RAP2.1	SALK_092889C
RAP2.6L	SALK_051006C

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Chapter 6

General conclusion and future perspectives

GENERAL CONCLUSION AND FUTURE PERSPECTIVES

Plants are not able to evade fluctuating changes in the environment and have therefore evolved robust methods to cope with environmental stresses. Differential gene expression is an important driving force in the stress response and is a highly tuned process regulated through multiple highly interconnected signaling pathways. We are currently facing the challenge to unwire the complex regulatory networks of the plant stress response.

Retrograde regulation of the stress response in plants

During adverse environmental conditions, the functioning of subcellular organelles including mitochondria and chloroplasts is rapidly perturbed. Mitochondria and chloroplasts are therefore considered as stress sensors that communicate to the nucleus to activate an appropriate defense response, in a process termed retrograde regulation. In contrast to other eukaryotes, such as yeast and animals, plant retrograde mechanisms and components are only beginning to be understood. Although several signaling components of plant chloroplast retrograde regulation (CRR) have been discovered, almost nothing was known about the regulatory mechanisms of plant mitochondrial retrograde regulation (MRR) at the beginning of this Ph.D. project. The main aim of this research work was to identify protein signaling components, more specifically transcription factors involved in MRR.

We applied a bottom-up approach in which MRR target genes were used as a bait to identify transcriptional regulators of MRR by means of the yeast one-hybrid method. Therefore, we first compiled a set of genes that are robust targets of mitochondrial dysfunction, named the mitochondrial dysfunction regulon (MDR). Besides their general responsiveness to mitochondrial perturbation, the *MDR* genes showed a similar expression behavior during various exogenous stress cues that could indirectly target mitochondrial function, hinting at their co-regulation. Within the *MDR* promoters, a common novel *cis*-regulatory element was discovered that is necessary and sufficient for MRR-mediated gene expression, named the mitochondrial dysfunction motif (MDM). Yeast one-hybrid screening revealed five closely related NAC family transcription factors (ANAC013, ANAC016, ANAC017, ANAC053 and ANAC078) binding the MDM, which was confirmed independently by electrophoretic mobility shift assays and, for

ANAC013, by chromatin immunoprecipitation. Our results showed that ANAC013 is necessary for full MRR-mediated *MDR* gene induction. Interestingly, parallel research identified ANAC017 in genetic screen for mutants with impaired MRR responsiveness (Ng et al., 2012, unpublished results). As individual overexpression of the NAC transcription factors commonly induces the *MDR* genes *in planta*, it is likely that, in addition to ANAC013 and ANAC017, the other three isolated NAC transcription factors also steer MRR gene expression. However, further experiments will be needed to confirm this. Despite the seemingly redundancy in target, *MDR* gene expression, it is possible that, based on their distinct spatiotemporal and stimulus-specific expression patterns, these factors act in an additive manner, mediating different aspects of MRR.

The five MDM-binding NAC transcription factors were previously designated as putative membrane-associated due to the presence of a transmembrane motif (Kim et al., 2007). Stimulus-induced release of prefabricated inactive transcription factors, including membrane sequestered transcription factors provides a rapid means to initiate an appropriate transcriptional response upon abrupt environmental changes (Seo et al., 2008). Thus, it is likely that mitochondrial retrograde regulation involves the release and activation of these putative transmembrane NAC transcription factors upon mitochondrial triggers. Surprisingly, ANAC013 as well as ANAC017 appear to be associated with endoplasmic reticulum (ER) membranes (Ng et al., 2012, unpublished results). However, these data need confirmation with independent techniques, such as immunodetection with a specific antibody against the NAC proteins and cell fractionation experiments. It has been shown in mammalian cells that the ER can physically associate at some points with the mitochondria, which is important for local communication between the two organelles, likely through calcium signals (Pizzo and Pozzan, 2007; Kornmann and Walter, 2010). In that regard, it is interesting to note that ANAC017 also colocalizes with F-actin of the cytoskeleton (Ng et al., 2012; unpublished results), which mediates the motility of both the mitochondria and the ER and has been implicated in tethering the two organelles (de Brito and Scorrano, 2008; Kornmann et al., 2011). In addition, mitochondrial calcium fluxes have been associated with the *MDR* induction and this appears to involve calcium crosstalk with the ER (Vanderauwera et al., 2012). Although these observations shed some light on plant MRR signal transduction mechanisms, including the cytosolic storage and release of transcription factors, further research is necessary to unravel the pathway in detail. Therefore, the proteolytic activation and concomitant nuclear relocalization of the cytosolic sequestered NAC transcription factors

upon mitochondrial perturbation first needs to be confirmed through processing analysis using protein gel blots and GFP fusion or immunogold labeling localization experiments. Furthermore, integration of mitochondrial perturbation treatments with ROS scavengers, (mitochondrial) calcium channel blockers or protease inhibitors in these relocalization and processing experiments together with the identification of protein interaction partners will help to understand the mechanisms underlying activation of these NAC transcription factors.

The general process of mitochondria-to-nucleus communication is conserved among eukaryotes, but the signaling mechanisms and components as well as the specific functions appear not to be conserved across eukaryotic species (Liu and Butow, 2006; Rhoads, 2011). This might reflect the different metabolic complexity as well as the different kind of "stresses" species encounter, such as nutrient deprivation in yeast and mitochondrial diseases in animals. Due to their sessile lifestyle, plants have evolved a battery of complex adaptation responses to environmental stresses. This is reflected by their higher number of genes encoding transcription factors, compared to other eukaryotes, including plant specific transcription factor families, which together may provide a tight control of stress responsive gene expression (Riechmann et al., 2000; Chen et al., 2002). We identified transcription factors of the plant specific NAC family as regulators of mitochondrial retrograde regulation of the plant stress response. Although the signaling mechanisms are divergent, this NAC-mediated retrograde regulation of the *MDR* appears to be functionally equivalent to the yeast and animal MRR-mediated oxidative stress and detoxification response, indicating some basic/general features of MRR are conserved. Differences of plant MRR compared to that of other (heterotrophic) eukaryotes might indeed be expected, due to the altered metabolic flexibility of plant mitochondria, containing the alternative respiratory pathway, and their metabolic interactions with chloroplasts. For example, the alternative respiratory pathway can alleviate photo-oxidative damage in the chloroplasts by efficiently dissipating excess reducing equivalents (Yoshida et al., 2006, 2007). Similarly, ANAC013 increases tolerance to chloroplast-initiated oxidative stress (by methyl viologen; MV), likely through MRR regulation of the MDR, including the alternative oxidase (AOX). However, as the strongest phenotypic effects of altered ANAC013 levels were observed after MV treatment (compared to mitochondrial stress treatments) and the MDR genes are also responsive to various chloroplast perturbations (Van Aken and Whelan, 2012), we can not exclude that ANAC013 and the MDM motif also mediate chloroplast signals to the nucleus. As mitochondria and chloroplast

functions are tightly connected, it is reasonable to assume that overlapping/interacting mechanisms of retrograde regulation exists, as was shown for the ABI4 transcription factor that mediates both MRR and CRR (Giraud et al., 2009; Rhoads, 2011).

Besides the five MDM element-binding NAC transcription factors, we provide a list of other, candidate MRR regulators, that bind the *MDR* promoters in the yeast one-hybrid system via other, yet unknown *cis*-regulatory elements. However, further experiments will be needed to confirm these novel regulatory interactions *in planta* (e.g. by chromatin immunoprecipitation) and to assess the potential role of these factors in MRR or other intracellular stress signaling pathways. Particularly interesting amongst these identified transcription factors is ANAC102 that is localized to the chloroplasts, suggesting it could mediate chloroplast signals to regulate *MDR* gene expression. Several transcription factors that regulate nuclear gene expression are found in the chloroplasts and they are suggested to allow a fast transcriptional response to changes in chloroplast-localized processes upon certain environmental triggers (Krause et al., 2012). However, except for the recently reported WHIRLY1 and PTM protein relocalization (Sun et al., 2011; Isemer et al., 2012), the release of these transcription factors from chloroplasts and concomitant translocation to the nucleus has not been studied so far. In this work, we provide preliminary evidence for the translocation of ANAC102 from the chloroplasts to the nucleus to regulate *MDR* gene expression upon chloroplast-initiated oxidative stress induced by MV. This indicates that ANAC102 mediates chloroplast retrograde regulation of the *MDR*. However, this relocalization needs confirmation by independent techniques, for example by protein gel blot analysis of isolated nuclei and chloroplasts before and after MV treatment. Moreover, it will be necessary to confirm that, after MV treatment, the nuclear-localized ANAC102 originates from the chloroplast and is not the result of increased MV-mediated *ANAC102* expression or alternative processing. In contrast to ANAC013, ANAC102 appears to repress *MDR* gene expression by a yet unknown mechanism (active or passive repression) and renders plants more sensitive to MV-mediated oxidative stress. This is apparent from the altered growth response and accelerated cell death phenotype of *ANAC102* overexpression lines during oxidative stress, indicative of an impaired stress response. The contrasting stress phenotypes resulting from *ANAC013* and *ANAC102* overexpression (resistance versus accelerated cell death), respectively, could also be explained by the elevated and impaired induction of the *AOX* gene in these lines. AOX has been implicated as a “survival protein” by dampening programmed cell death (PCD)

induction during stress, likely through determining cellular ROS levels and antioxidant defense responses (Robson and Vanlerberghe, 2002; Amirsadeghi et al., 2006; Giraud et al., 2008; Van Aken et al., 2009). AOX might thus significantly impact the stress response including the decision to initiate PCD or attempt to recover. It is therefore not surprising that *AOX* and the other *MDR* genes are under several levels of control, potentially regulated by various organelle signaling pathways and involving both transcriptional activation and repression. Moreover, the complexity of organelle-to-nucleus regulation has been strengthened by the observation that retrograde signals from both mitochondria and chloroplast interact through the ABI4 transcription factor (Giraud et al., 2009). Another possibility is that retrograde regulation from the chloroplast and/or mitochondrion is influenced by chloroplast-mitochondrial crosstalk (Woodson and Chory, 2008). Therefore, the exact signals that trigger ANAC102 as well as ANAC013 relocalization and concomitant altered *MDR* gene expression remain to be determined using mutants or treatments that specifically impair chloroplast or mitochondrial function. Moreover, genomewide identification of ANAC102 and ANAC013 target genes using inducible overexpression constructs as well as chromatin immunoprecipitation combined with genome-tiling microarrays (ChIP-chip) or by next-generation sequencing (ChIP-seq) will reveal the contribution of these organelle signaling pathways to the response of plants to environmental stresses.

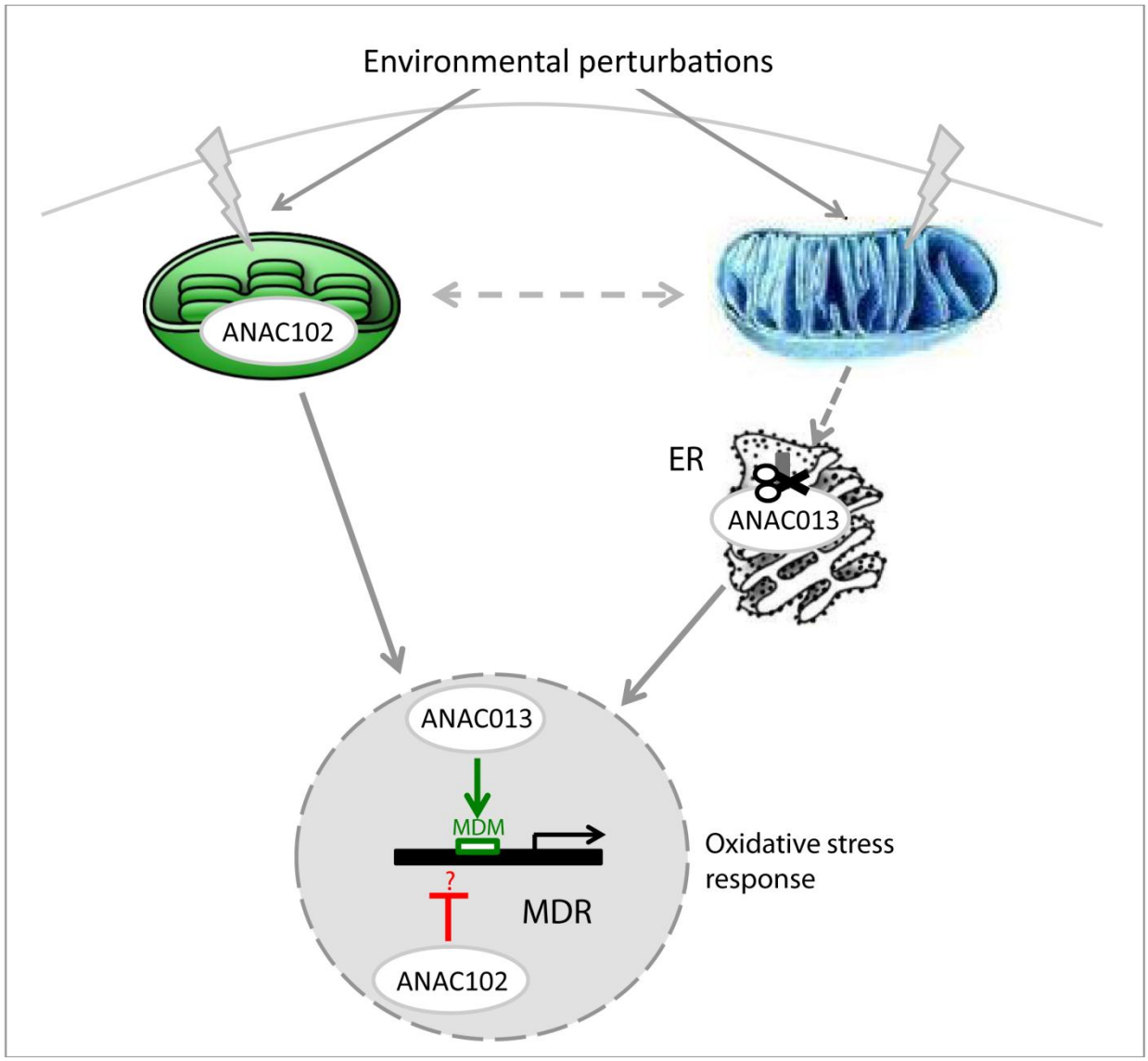

Figure 6.1. Scheme summarizing the results obtained in this thesis concerning organelle-to-nucleus signaling by NAC transcription factors.

Upon mitochondrial perturbation, ANAC013 positively regulates *MDR* (for mitochondrial dysfunction regulon) gene expression by binding the MDM (for mitochondrial dysfunction motif) *cis*-regulatory element, thereby increasing organelle-initiated oxidative stress tolerance. ANAC013 contains a transmembrane domain and is anchored to the endoplasmic reticulum (ER) under normal conditions. Therefore, we hypothesize that upon mitochondrial triggers, ANAC013 is proteolytically released from the ER and translocated to the nucleus to mediate MRR-induced gene expression. ANAC102, on the other hand, is a negative regulator of the *MDR* during chloroplast perturbation conditions. Under normal conditions, ANAC102 is targeted to the chloroplasts. Preliminary data revealed the potential relocalization of ANAC102 from the chloroplasts to the nucleus upon chloroplast perturbation, indicating it is a candidate regulator of chloroplast-to-nucleus signaling to fine-tune the oxidative stress response.

Reverse engineering of regulatory networks of the abiotic stress response

Plants continually adapt to changing conditions by altering gene expression patterns that are fine-tuned through complex networks of signal transduction pathways (Nakashima et al., 2009). In the recent years, many experimental data have been generated to elucidate the regulatory mechanisms of the stress response. Large-scale mutant analyses/screens have identified many players in the stress response and genome-wide expression profiling studies revealed transcriptional responses to various stress conditions. Therefore, the organization of these molecular data to reconstruct the gene regulatory networks is one of the central tasks in systems biology. To date, most gene regulatory networks are reverse-engineered by meta-analysis of microarray data, as they provide important information about the cells transcriptional response and they are one of the most abundant sources of data.

We used the LeMoNe algorithm to infer transcriptional regulatory networks from abiotic stress-related gene expression profiles (Michoel et al., 2007). LeMoNe groups genes in modules and predicts their regulators, based on similarity in gene expression profiles (co-expression). We obtained functionally coherent modules of co-regulated genes and predicted regulators related to similar biological functions as their target modules. In agreement with previous studies (Yu et al., 2003), we found that gene expression data provide much better support for functional and co-regulation relationships than for regulator-target gene relations. The predictive coverage for known regulatory interactions inferred from publicly available microarray studies of genetic perturbations was overall low for our identified network. Thus, regulatory predictions from expression data appear to reveal only a small part of the underlying true biological network. This could be explained by the fact that these predictions rely on the assumption that regulators are themselves transcriptionally regulated, so that their expression profiles provide information about their activity level. However, for many transcription factors, the major mode of regulation is probably post-transcriptional (Lee et al., 2006). Next, we experimentally assessed the performance of LeMoNe in predicting novel regulations. Overexpression or inhibition of transcription factors that were predicted to regulate abiotic stress-related modules revealed that, depending on the perturbed transcription factor, expression of several genes in the target modules indeed changed significantly. Overall, the true/correct regulatory predictions are likely to correspond to the fraction of transcription factors that are mostly transcriptionally regulated and

are subject to only minor post-transcriptional regulation. In agreement, we observed the best predictive performance for the ANAC013 transcription factor, that is strongly regulated at the transcriptional level and, in addition, autoregulates its own transcription. We conclude that the prediction of regulatory networks from gene expression data can provide valuable information for at least part of the regulatory network.

Transcription factor activity is regulated at multiple levels, such as post-translational modification (e.g. (de)phosphorylation), dimerization and co-factor binding, subcellular localization and/or sequestration, which can not be recovered from expression data. In the future, co-expression data should be simultaneously combined with other data types, such as physical interactions (protein-protein, protein-DNA), post-transcriptional and post-translational modifications to generate an integrated network (Tan et al., 2008). Indeed, it has been shown in yeast, plants and mammalian cells that combining diverse data types enhances the predictive power of the gene network compared to using expression data alone (Hartemink et al., 2002; Tan et al., 2008; Zhu et al., 2008; Huttenhower et al., 2009; Kourmpetis et al., 2011; Heyndrickx and Vandepoele, 2012). Moreover, different algorithms recover largely distinct parts of the underlying regulatory network and optimal results will be obtained by integrating the predictions of various methods (Michoel et al., 2009).

LeMoNe prioritizes biologically relevant regulators, as most predicted master regulators (regulators that were assigned to multiple modules) have been shown previously to have a function in the stress response, among which ANAC013. In agreement, ANAC013 was revealed as a top regulator of our experimentally-derived abiotic stress subnetwork (see above), likely through the regulation of several downstream transcription factors. These observations further corroborate the importance of mitochondrial retrograde regulation in the stress response. Hence, our predicted regulatory network can be a valuable source for the identification of gene candidates for the development of stress-tolerant crops. To conclude, our predicted model of the abiotic stress response provides a powerful starting point as both a hypothesis generator and as a descriptive tool to explain stress-mediated gene expression changes.

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SUMMARY

Plants have evolved robust adaptation mechanisms to cope with continually changing environmental conditions. Differential gene expression is an important driving force in the stress response and is a highly tuned through multiple highly interconnected signaling pathways. Unraveling the molecular mechanisms of how plants sense, transduce and adapt to stresses are of primary importance for the development of stress tolerant crops.

A recently emerging concept concerns the role of mitochondria and chloroplasts as sensors of adverse conditions that communicate to the nucleus to activate an appropriate defense response in a process termed retrograde regulation. Despite their recognized importance in stress signal transduction, plant retrograde mechanisms are still poorly understood. Although several signaling components of plant chloroplast retrograde regulation (CRR) have been discovered, almost nothing was known about the mechanisms of plant mitochondrial retrograde regulation (MRR) at the beginning of this Ph.D. project **(Chapter 1)**.

In this work, we aimed at identifying regulators involved in plant MRR. Therefore, we applied a bottom-up approach using MRR target genes as a bait to identify transcription factors involved in MRR by means of the yeast one-hybrid method. We first compiled a set of robust MRR target genes in *Arabidopsis thaliana* through a meta-analysis of transcriptome data of mitochondrial perturbations. In the upstream regions of these MRR target genes, a *cis*-regulatory element was discovered that is necessary and sufficient for gene expression upon various mitochondrial perturbations, named the mitochondrial dysfunction motif (MDM) **(Chapter 2)**. Five transmembrane domain-containing NAC transcription factors (ANAC013, ANAC016, ANAC017, ANAC053 and ANAC078) bind the MDM *cis*-regulatory element. We further demonstrated that ANAC013 mediates MRR-induced gene expression by binding the MDM and alters plant stress tolerance. ANAC013 appears to be associated with endoplasmic reticulum (ER) membranes under nonstressed conditions. Although experimental evidence is currently lacking, we hypothesize that upon certain mitochondrial triggers, ANAC013 is proteolytically released from the ER and translocated to the nucleus to mediate MRR-responsive gene expression.

In addition to the MDM-binding NAC transcription factors, we provide a set of other, candidate MRR regulators that bind the MRR target gene promoters in the yeast one-hybrid system **(Chapter 3)**. However, further experiments *in planta* are necessary to assess these novel regulatory interactions and evaluate the potential role of these transcription factors in MRR or

other intracellular stress signalling pathways. Moreover, this approach also led to the identification of a transcription factor (ANAC102) that is potentially involved in chloroplast retrograde regulation of the stress response **(Chapter 4)**. ANAC102 is localized in the chloroplasts under nonstressed conditions. Upon chloroplast-initiated oxidative stress conditions, preliminary results show the translocation of ANAC102 to the nucleus where it alters gene expression. However, ANAC102 appears to repress stress responsive gene expression and negatively affects plant stress tolerance, indicating it might be involved in the fine-tuning of the stress response. To conclude, we identified one of the first transcription factors involved in MRR as well as a potential CRR regulator that is activated through retrograde relocalization from chloroplast to the nucleus.

In a second part of the Ph.D. project, we reverse-engineered a model of the Arabidopsis abiotic stress transcriptional regulatory networks **(Chapter 5)**. Therefore, we used the LeMoNe algorithm to extract gene co-expression modules and their predicted regulators from abiotic stress-related gene expression data. We showed that LeMoNe infers functionally coherent modules of co-regulated genes and predicts regulators involved in the same biological functions as their target gene modules. Comparison of the predicted regulator-target gene interactions with known regulatory interactions obtained from publicly-available genetic perturbation studies revealed that the predictive coverage of the "complete" biological regulatory network is rather low. This might be attributed to the fact that many transcription factors are regulated at the post-transcriptional level and, thus, their activity is not apparent from gene expression data alone. In addition, we experimentally tested the regulatory predictions for seven abiotic stress-related transcription factors and found that several of their predicted target genes indeed changed significantly in the transcription factor gain- or loss-of-function lines. Overall, the correctly predicted regulatory interactions are likely to correspond to the fraction of transcription factors that are significantly regulated at the transcriptional level. We conclude that inferring regulatory networks from gene expression data provides valuable information for at least part of the underlying true biological network. However, integration of other, non-transcriptomic data types and combining predictions from different algorithms will be needed to create a more robust regulatory network.

SAMENVATTING

Planten hebben doorheen de evolutie robuuste adaptatiemechanismen ontwikkeld om te kunnen omgaan met de voortdurend schommelende omgevingsomstandigheden. Het begrijpen van de moleculaire mechanismen waarmee planten de omgeving waarnemen en deze signalen omzetten in adaptatiereacties is belangrijk voor de ontwikkeling van transgene gewassen die beter bestand zijn tegen dergelijke ongunstige omstandigheden.

Recente bevindingen tonen aan dat subcellulaire organellen zoals de mitochondriën en chloroplasten een belangrijke rol spelen in het waarnemen van de stress en daaropvolgend communiceren met de nucleus om een verdedigingsreactie te activeren. De communicatie van organellen naar de nucleus om genexpressie aan te sturen wordt retrograde regulatie genoemd. Desondanks het geweten is dat retrograde regulatie een belangrijke rol speelt in stresssignaaltransductie in planten, zijn de onderliggende mechanismen slechts beperkt ontrafeld. Hoewel er reeds een aantal signaaltransductiecomponenten van de chloroplast-retrograde-regulatie (CRR) bekend zijn, was er bij de aanvang van dit doctoraatsproject nauwelijks iets geweten over de mitochondriale retrograde-regulatie (MRR) mechanismen in planten **(Hoofdstuk 1)**.

Dit werk had als doelstelling om regulatoren betrokken in plant MRR te identificeren. Om dit te bewerkstelligen werd een “bottum-up” benadering gehanteerd, waarin MRR-doelgenen gebruikt werden om transcriptiefactoren betrokken in MRR te identificeren door gebruik te maken van de yeast one-hybrid methode. Hiervoor werd eerst een lijst van genen samengesteld die robuust gereguleerd worden door verstoring van de mitochondriale functie in *Arabidopsis thaliana*. In deze groep van genen werd een nieuw *cis*-regulatorisch element gevonden dat noodzakelijk en voldoende is voor MRR-geïnduceerde genexpressie en daarom het mitochondriaal dysfunctie motief (MDM) genoemd werd **(Hoofdstuk 2)**. Vijf NAC transcriptiefactoren met een transmembraandomein (ANAC013, ANAC016, ANAC017, ANAC053 en ANAC078) binden op dit MDM *cis*-regulatorisch element. We tonen verder aan dat ANAC013 MRR-geïnduceerde genexpressie reguleert *in planta* door het binden op het MDM en tevens stresstolerantie van de plant beïnvloedt. ANAC013 is geassocieerd met het endoplasmatisch reticulum (ER) onder controleomstandigheden. Hoewel experimenteel bewijs op dit moment ontbreekt, veronderstellen we dat volgende op signalen afkomstig van de

mitochondriën, ANAC013 vrijgegeven wordt van de ER membranen en zich verplaatst naar de nucleus om genexpressie aan te sturen.

Naast deze MDM-bindende NAC transcriptiefactoren, identificeerden we een reeks andere mogelijke MRR regulatoren, die de MRR-doelgenpromoteren binden in het yeast one-hybrid systeem **(Hoofdstuk 3)**. Verdere experimenten *in planta* zijn echter noodzakelijk om deze potentiële regulatorische interacties te valideren en om de mogelijke rol van deze transcriptiefactoren na te gaan in MRR of andere subcellulaire stresssignaaltransductie pathways. Deze benadering leidde bovendien tot de identificatie van een transcriptiefactor (ANAC102) die mogelijk betrokken is in chloroplast retrograde regulatie van de stress respons **(Hoofdstuk 4)**. ANAC102 is aanwezig in de chloroplasten onder controleomstandigheden. Preliminaire resultaten tonen de verplaatsing van ANAC102 van de chloroplast naar de nucleus aan tijdens oxidatieve stressomstandigheden geïnitieerd in de chloroplasten. ANAC102 blijkt stress-geïnduceerde genexpressie te inhiberen en heeft een negatief effect op de stresstolerantie van de plant. Dit duidt erop dat ANAC102 betrokken is de nauwgezette controle van de stressrespons. Dus, in dit deel van het doctoraatsproject werden één van de eerste transcriptiefactoren betrokken in MRR geïdentificeerd alsook een mogelijke CRR regulator die geactiveerd wordt door retrograde-geïnduceerde verplaatsing van de chloroplasten naar de nucleus.

In een tweede deel van dit doctoraatswerk, werd regulatorisch netwerken van de abiotische stress respons in Arabidopsis gemodelleerd **(Hoofdstuk 5)**. Het LeMoNe algoritme werd gebruikt om modules met co-geëxpresseerde genen en hun regulatoren te voorspellen op basis van abiotische stress-gerelateerde genexpressiedata. We tonen aan dat LeMoNe functioneel coherente modules afleidt, bestaande uit co-gereguleerde genen, alsook regulatoren voorspelt met functies gelijkaardig aan die van hun doelmodules. Het vergelijken van de voorspelde regulator-doelgen interacties met gekende regulatorische interacties afgeleid van beschikbare genetische perturbatiestudies, toonde aan dat LeMoNe slechts een klein deel van het “volledige” biologische netwerk voorspelt. Dit is mogelijk te wijten aan het feit dat veel regulatoren gereguleerd worden op post-transcriptioneel niveau, en dus hun activiteit niet blijkt uit hun expressieprofiel. Daarnaast hebben we voor zeven abiotische stress-gerelateerde transcriptiefactoren de voorspelde regulaties experimenteel getest. De expressie van verschillende voorspelde doelgenen veranderde inderdaad significant door overexpressie of het uitschakelen van de transcriptiefactor. De correct voorspelde regulaties komen waarschijnlijk overeen met de fractie van de transcriptiefactoren die

significant gereguleerd worden op transcriptioneel niveau. We besluiten dat het modelleren van netwerken op basis van genexpressiedata waardevolle informatie kan geven over tenminste een deel van het onderliggende biologische netwerk. Om meer robuuste netwerken te maken zal het belangrijk zijn om in de toekomst andere datatypes te integreren en de voorspellingen van verschillende algoritmen te combineren.