Comparative Transcriptomics to Analyse the Stress Responses in Monocotyledons and Dicotyledons and the Contribution of Alternative Oxidase Isoforms to Stress Tolerance in Arabidopsis

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LIST OF ABBREVIATIONS

$^1O_2$	singlet oxygen
2-OG	2-oxoglutarate
3AT	3-Amino-1,2,4-triazole
AA	antimycin A
ABA	abscisic acid
AOX	alternative oxidase
ATP	adenosine triphosphate
Az	sodium azide
bp	base-pair
BR	brassinosteroids
C	cysteine
Ca^{2+}	calcium ions
CaMV	cauliflower mosaic virus
Chl	chlorophyll
CK	cytokinin
CM	crista membrane
CYS	cysteine residue
DEGs	differentially expressed genes
EMS	methanesulfonate
ER	endoplasmic reticulum
ET	ethylene
GA	gibberellic acid
GWAS	genome wide association studies
H_2O_2	hydrogen peroxide
HO^-	hydroxyl radical
HPLC	high-performance liquid chromatography
HRM	high-resolution melt
IMM	inner mitochondrial membrane
JA	jasmonic acid
LIN	lincomycin
MDM	mitochondrial dysfunction motif
MDS	mitochondrial dysfunction stimulon
MFA	monofluoroacetate
MNU	methylnitrosourea
mRNA	messenger RNA
MRR	mitochondrial retrograde response
mtETC	mitochondrial electron transport chain
MV	methyl viologen
N	nitrogen
NF	norflurazon
NGS	next generation sequencing
O_2^-	superoxide radical
OAA	oxaloacetate
OGs	orthogroups
OMM	outer mitochondrial membrane

OXPHOS	oxidative phosphorylation
PCD	programmed cell death
Pyr	pyruvate
QfO	quest for orthologs
QTLs	quantitative trait loci
RAO	regulators of AOX
RNA	ribonucleic acid
RNAseq	RNA-sequencing
ROS	reactive oxygen species
rRNA	ribosomal RNA
S	serine
SA	salicylic acid
SAR	systemic acquired resistance
SD	segmental duplication
SNP	single nucleotide polymorphism
Succ	succinate
TCA	tricarboxylic acid
TD	tandem duplication
TFs	transcription factors
TILLING	targeting induced local lesions in genomes
TRX	thioredoxin
UQ	ubiquinone
UV	ultraviolet
WGD	whole-genome duplication
WGT	whole-genome triplication

ABSTRACT

Throughout their lifecycle plants are exposed to a variety of adverse environmental conditions that are not optimal for growth and productivity. Over evolutionary scales plants respond by adaptations as transgenerational mechanism, acclimate as medium-term response and/or trigger short-term stress responses. Understanding the response mechanisms that translate into improved stress tolerance can increase agricultural yields and is important for plant breeding approaches.

While information gained in the model plant *Arabidopsis thaliana* represents an excellent reference source, knowledge transfer into crop species is often hindered by limited comparative data. The first manuscript (Chapter 2) analyses the stress responses in monocots (rice and barley) and dicots (Arabidopsis) to different stimuli that target organellar function and hormonal signalling pathways. Comparative transcriptomics on the basis of orthology was used to identify common and species-specific responses to stress to provide greater insight into how research findings in Arabidopsis can be translated to crop species.

The alternative oxidase (AOX) is a key marker of the MRR and plays an important role in the stress response. As the terminal oxidase of the nonenergy-conserving alternative pathway in plant mitochondria AOX balances cellular energy and carbon metabolism under adverse condition. The lack of AOX1a in Arabidopsis, the main stress-responsive isoform, cannot be fully compensated by other AOX isoforms under stress conditions. The second manuscript (Chapter 3) analyses the transcriptional regulation of AOX isoforms in response to impaired mitochondrial function. Transgenic lines harbouring promoter-swap constructs of the different AOX isoforms and their native promoters as well as overexpression lines have been analysed for transcript and protein levels. Results show that translational regulation mediated by the *AOX1a* promotor and the amount of protein, regardless of the expressed isoform, is required to compensate the lack of AOX1a.

STATEMENT OF AUTHORSHIP

This thesis includes work by the author that has been published or accepted for publication as described in the text. Except where reference is made in the text of the thesis, this thesis contains no other material published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma.

No other person's work has been used without due acknowledgment in the main text of the thesis.

This thesis has not been submitted for the award of any degree or diploma in any other tertiary institution.

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CHAPTER 1 – INTRODUCTION

1.1 Plant stress responses and how to improve tolerance

1.1.1 Environmental stress and plant performance

As sessile organisms in a highly dynamic and ever-changing environment plants are consistently exposed to unfavourable or even adverse environmental conditions that limit growth and development. During the course of evolution, plants developed a multitude of specific sensing and response mechanisms to gradual and rapid environmental changes to survive and reproduce. These responses are very complex and alter gene expression, ultimately leading to morphological, physiological, biochemical and molecular changes. Re-establishing cellular homeostasis as well as functional and structural protection of proteins and membranes is the aim of these changes (Zhang et al., 2018, Wang et al., 2003).

In the context of agriculture and food security, biotic and abiotic stresses have a large impact on productivity by limiting average plant yields by up to 50 % (Wang et al., 2003). Abiotic stresses such as heat, cold, drought, salinity, oxidative stress and nutrient deficiency, are primarily responsible and can reduce average yields severely. Biotic stresses including pests and pathogens, like fungi, bacteria, viruses, nematodes, and herbivorous insects have a severe impact on plant performance as well and significantly increase agronomic costs through necessary pesticide treatments. Global food production is facing dramatic challenges as climate change significantly increases biotic and abiotic stresses and leads to a further decrease of arable land (Jaggard et al., 2010, Powell et al., 2012, Zandalinas et al., 2018). Extreme weather conditions like flood and drought are is already impacting people and threatening food supply and the required infrastructure (Benton, 2019). At the same time, a fast-growing global population has a much higher demand for agricultural products (FAO, 2009). This demand requires an increase of agricultural production by 50-100% to satisfy the growing demand of 9-11 billion people that are projected to inhabit this planet by 2050 (Kummu et al., 2017, Alexandratos and Bruinsma, 2012).

1.1.2 Stress sensing and responses in plants

In response to adverse environmental stress conditions plants specifically change gene expression, metabolism and physiology, which suggests that plants have specific sensing mechanisms (Zhu, 2016). Plant cells are thought to directly perceive signals when challenged with a biotic or abiotic stressor via sensors or receptors triggered by physiological or chemical changes as well as transient chemical signals on the cell surface

(Zhang et al., 2018). Responses can be triggered indirectly by rapid changes of physical parameters that translate into changes in the status of cellular components or metabolite levels within the different subcellular compartments in the cell (Zhu, 2016).

Linear pathways in response to adverse environmental conditions overlap with other branches as part of a more complex signalling network (Knight and Knight, 2001). Genes can be induced by several stimuli which lead to a high degree of complexity. Each stress condition, individual or combinatorial, requires a unique response mechanism, adapted to the cellular needs. This is facilitated by multiple signal transduction pathways that integrate the different signals and interact with each other to regulate gene expression. The response is thereby not limited to a local event but also comprises systemic signalling pathways that affect other parts of the plant rapidly within seconds to minutes (Kollist et al., 2019).

Intensive research in *Arabidopsis thaliana* (hereafter referred to as Arabidopsis) resulted in fundamental knowledge about the molecular principles, physiology, metabolism and development in plants and provides a knowledge base as well as a variety of valuable tools for improving stress tolerance in crop species (Kramer, 2015). In their natural habitats plants are subjected to various combinations of abiotic and/or biotic stress conditions (Mahalingam, 2015). In order to explore and enhance multiple stress responses, synergistic and antagonistic components of stress signalling cascades in the context of crosstalk between different stresses need to be identified and characterised (Shaik and Ramakrishna, 2013).

The multilevel cellular responses that integrate various environmental signals is orchestrated by calcium ions (Ca^{2+}), numerous plant hormones, reactive oxygen species (ROS), kinases, phosphatases and other regulatory proteins, as well as compounds and small molecules (Figure 1) with many transcription factors (TFs) orchestrating the transcriptional responses (Peck and Mittler, 2020).

Free calcium ions (Ca^{2+}) are second messengers that are released in response to environmental factors. Ca^{2+} fluxes across membranes show stimuli-specific signatures and result in temporally and spatially defined concentration changes within cell compartments (Steinhorst and Kudla, 2013). Specific Ca^{2+} binding proteins relay the information that lead to downstream responses. These responses include protein phosphorylation mediated by Ca^{2+}-regulated kinases as well as specific Ca^{2+}-regulated TFs and promotor elements that effect gene expression (Kudla et al., 2010).

ROS, i.e. singlet oxygen ($^1O_2$), superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^-), are toxic at elevated levels and are often released in cells as a harmful by-products of aerobic metabolism such as photosynthesis and respiration (Apel

and Hirt, 2004). Despite their harmful effects, ROS emerged as major regulatory molecules in plants and play an integral role as signalling molecules in controlling biological processes such as growth, development and responses to environmental stress stimuli (Baxter et al., 2014). Plants evolved various enzymatic and non-enzymatic scavenging mechanisms to maintain redox balance (Tripathy and Oelmüller, 2012). The redox balance can be perturbed by various adverse environmental conditions that lead to higher ROS levels. Oxidative stress induces proteins involved in the scavenging machinery as well as other cellular rescue responses (Desikan et al., 2003). The underlying signalling networks, however, regarding perception of ROS perception and the immediate downstream processes are almost completely unknown (Waszczak et al., 2018).

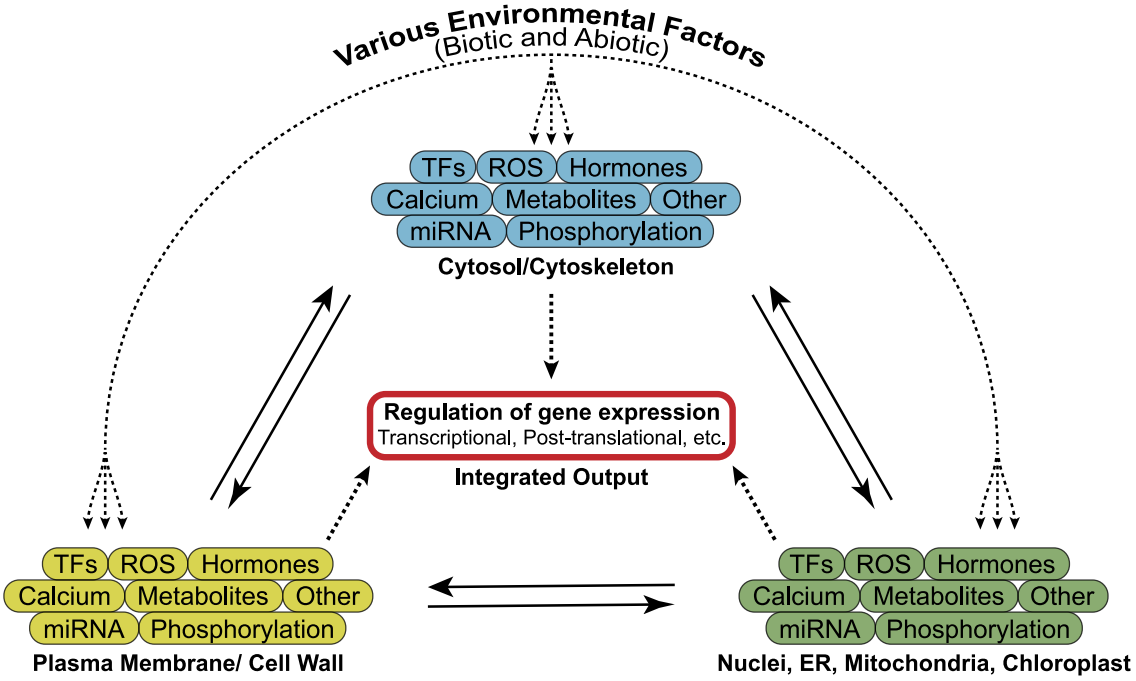

Figure 1. Perception and integration of environmental stimuli in plant cells to impact gene expression.

Multiple biotic and abiotic environmental stimuli are integrated by different subcellular compartments via different signalling complexes. These trigger specific signal transduction cascades to regulate gene expression as a response to restore cellular homeostasis. Crosstalk between signalling pathways within and between compartments leads to stress signatures that are specific for each combination of environmental stimuli. TFs, transcription factors; ROS, reactive oxygen species; miRNA, micro RNA; ER, endoplasmic reticulum. Redrawn and modified figure from Peck and Mittler (2020).

Phytohormones are chemical messengers that are produced within plants and are involved in diverse physiological processes including growth and development. They further mediate plant acclimation and adaptation responses to adverse environmental

conditions (Verma et al., 2016, Berens et al., 2017). Auxin, cytokinin (CK), gibberellic acid (GA), brassinosteroids (BR), ethylene (ET), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and strigolactones are the phytohormone families that can be found in plants. Hormonal signalling pathways are intertwined in an antagonistic or synergistic crosstalk (Berens et al., 2017). Especially ABA is the central regulator of the many abiotic stress responses. Increased levels under adverse environmental conditions initiate signal transduction and consequently specific cellular responses (Sah et al., 2016). SA, JA and ET have important functions in activating defence responses to biotic stresses. SA responds to biotrophic and hemi-biotrophic pathogens and imparts systemic acquired resistance (SAR) to the plant and JA and ET to necrotrophic pathogens and herbivorous insects (Gaffney et al., 1993, Bari and Jones, 2009).

As master regulators of gene networks, TFs are involved in most biological processes and are essential for plant development and stress responses. TFs play an important role in signal transduction processes activating and repressing the transcription of their target genes via sequence-specific DNA binding and protein–protein interactions. The extraordinary status of TFs in plant regulation is reflected in a high proportion of TF encoding genes in plant genomes. The average proportion of TFs identified in monocot and dicot species so far is around 5% of all genes in a given genome (Jin et al., 2016). 58 TF families can be found in plants of which many play important roles in sensing environmental changes including APETALA2 /ETHYLENE RESPONSIVE FACTOR (AP2/ERF), NAM (No Apical Meristem) ATAF1/2 (Arabidopsis Activating Factor) and CUC2 (Cup-shaped Cotyledon) (NAC), WRKY, basic Leucine Zipper (bZIP) and MYB (Sharma et al., 2018). Being important regulatory switches, TFs have emerged as promising candidates for molecular genetics and plant engineering strategies to increase abiotic stress tolerance and resistance to certain pathogens (Sharma et al., 2018). In this context, transgenic plants overexpressing TFs increase tolerance and resistance in response to adverse environmental conditions as shown for many different species including agronomically relevant crops (comprehensive list can be found in (Sharma et al., 2018).

The DEHYDRATION-RESPONSIVE ELEMENT-BINDING (DREB) family of TFs, a subfamily of the AP2/ERF TF family, regulates the expression of many stress-inducible genes and play a vital role in improving the abiotic stress tolerance in plants (Yamaguchi-Shinozaki and Shinozaki, 1994, Dubouzet et al., 2003, Nakashima et al., 2000, Sakuma et al., 2002). DREB2A is a very good example for a complex mechanism in plants in response to abiotic stress. This TF increases tolerance to heat and drought stress in Arabidopsis by inducing heat- and drought-responsive genes while supressing plant growth (Sakuma et al., 2006a, Sakuma et al., 2006b). This induction depends on the

stability of DREB2A which was shown to rely on phosphorylation of a specific domain under non-stress conditions that results in protein degradation. Under stress conditions, however, dephosphorylation activates the TF and enables induction of stress-responsive genes (Mizoi et al., 2019). This sophisticated mechanism allows plants to rapidly respond to fluctuating environmental conditions. The improved drought tolerance, resulting in higher yield and productivity in field conditions, was conferred to transgenic sugarcane (*Saccharum spp*. Hybrid) expressing the constitutively active AtDREB2A protein that lacks the specific domain responsible for phosphorylation and consecutively degradation (de Souza et al., 2019). The importance of the DREB2 family in the response to abiotic stress has been shown in important crop species like rice (*Oryza sativa*) (Herath, 2016), wheat (*Triticum aestivum*) (Mondini et al., 2015) and sorghum (*Sorghum bicolor*) (Akbudak et al., 2018).

1.1.3 Plant breeding strategies

Plant breeding strategies have substantially improved the yields and nutritional value of crops every year since the beginning of the green revolution, but these increases have plateaued in some regions (Li et al., 2018). This is due to a narrow genetic base, the limitation of diversity as a result of domestication and selective breeding (Louwaars, 2018), which is further challenged by extreme adverse environmental conditions and dangerous pathogens that impose much higher threats. New breeding strategies and technologies must be developed to meet the demands imposed by climate change and population growth.

As outlined by Arbona et al. (2017) there are four major strategies to improve stress tolerance in crops. The first strategy is the identification of variability in stress tolerance from natural populations and the use of genetic markers referred to as Quantitative Trait Loci (QTLs). The development of Next Generation Sequencing (NGS) technologies that enable low-cost high-throughput DNA sequencing has revolutionised the field of plant breeding. Whole-genome re-sequencing of large populations of thousands of plants further helped to identify genetic markers that are related to a beneficial trait. High-throughput phenotyping technologies have accelerated the assessment of tolerance traits. The use of Genome Wide Association Studies (GWAS) further improves the understanding of the molecular basis of complex traits and is beneficial for QTL mapping at a much higher resolution (Barabaschi et al., 2016). Exploring the genomic variation to identify Single-Nucleotide-Polymorphism (SNPs) as molecular markers is very important for molecular genetics and plant breeding.

The second strategy is based on polyploidy, which refers to the duplication of an entire genome, as a stress tolerance trait. Polyploid organisms are often associated with

increased vigor and can outperform their diploid relatives in terms of yield and product quality as well as increased tolerance to biotic and abiotic stresses (Sattler et al., 2016). Increased organ size and heterozygosity, buffering of deleterious mutations and heterosis are the important features of polyploidy in plant breeding.

The third strategy is a phenotype-driven selection of stress-tolerant varieties via in vitro mutagenesis, using chemically or physically induced variations in their DNA sequence (Arbona et al., 2017). High throughput technologies like Targeting Induced Local Lesions in Genomes (TILLING) have been developed to generate and identify variation in candidate genes or genes of interest (Sikora et al., 2011). Chemically induced single base-pair (bp) changes, single nucleotide polymorphisms (SNPs), GC to AT or AT to GC shifts via ethyl methanesulfonate (EMS), sodium azide (Az) and methylnitrosourea (MNU) introduce random mutations across the entire genome and are then screened for very large populations. Screening methods are NGS, High-Performance Liquid Chromatography (HPLC), High-Resolution Melt (HRM) and different forms of electrophoresis (Sikora et al., 2011).

The fourth strategy is the identification of functional and regulatory genes as well as specific promotors from related or other plant species that might confer tolerance to adverse environmental conditions or pathogens using genetic transformation and genome editing. In addition to identifying and utilizing genomic variation in plant breeding, the generation of new allelic variation via genome editing provides new opportunities for crop improvements (Zafar et al., 2020). Transgenic technology can introduce novel exogenous genes into the host organism or alter the expression levels of endogenous genes to improve stress tolerance (Wang et al., 2016). Advances in targeted genome-editing technologies like Clustered Regularly Interspaced Short Palindromic Repeats /CRISPR-associated Protein 9 (CRISPR/Cas9) enable efficient targeted modifications in most crops. An important advantage of CRISPR/Cas9 system is that transgenes or selection genes cannot be traced which could result in engineered plants not being considered as genetically modified organisms (Arbona et al., 2017). Due to the advances in NGS technologies, reference DNA sequences for the most important crop species are available and the vast amount of sequence data and the downstream bioinformatic analysis let to the discovery of many new genes and regulatory sequences and therefore new molecular markers.

Strategies in plant engineering via genome editing target either functional genes that encode for proteins with direct functions to protect cells from stresses or regulatory genes involved in signal transduction and signalling pathways that alter gene expression in response to different stresses (Wang et al., 2016). As outlined in the previous section,

TFs are master regulators and promising candidates for plant engineering strategies to increase abiotic stress tolerance and resistance to certain pathogens as outlined for DREB2A.

Many members of the NAC TF family have been identified as important targets for engineering crops with improved tolerance. Overexpression of *SNAC3* in rice enhanced heat, drought, and oxidative stress tolerance while repression led to the opposite effect in response to all adverse conditions (Fang et al., 2015). This TF is thought to balance cellular redox homeostasis by regulating the expression of genes encoding for ROS-scavenging enzymes. Other studies that analysed transgenic Arabidopsis plants expressing different NAC TFs from rice, wheat, maize and chickpea showed enhanced tolerance to drought, heat and high concentrations of salt (Yu et al., 2014, Huang et al., 2015, Lu et al., 2012, Hong et al., 2016, Guo et al., 2015). The plant-specific WRKY family is important in defence responses as reported for *GhWRKY39-1* from cotton (*Gossypium hirsutum*) that confers resistance against bacterial and fungal pathogens but also enhances salt- and oxidative stress tolerance when overexpressed in transgenic tobacco (*Nicotiana benthamiana*) plants (Shi et al., 2014). In rice, *OsWRKY30* increases drought tolerance via Mitogen-Activated Protein (MAP) kinases-mediated activation (Shen et al., 2012), *TaWRKY22* increases drought tolerance and grain yields when overexpressed in transgenic wheat (Gao et al., 2018) and the maize *ZmWRKY106* has been shown to improve drought and heat tolerance in transgenic Arabidopsis plants (Wang et al., 2018a).

OsRR22, most likely a member of the MYB-like TF family, represents an example of breeding strategies using CRISPR/Cas9 TF knockdowns in rice to improve tolerance to abiotic stress (Takagi et al., 2015, Zhang et al., 2019). CRISPR/Cas9 induced mutations into the coding region of this TF significantly improved salinity tolerance of rice seedlings. A comprehensive list of genome-modified plants using the CRISPR-Cas technology with enhanced tolerance to adverse growth conditions that target TFs and other regulatory or functional genes including all major crops can be found in Tofazzal (2019).

In order to engineer crop plants with increased stress-tolerance and resistance the identification of key genes especially master regulators and the corresponding pathways is fundamental and requires researchers to unravel the complex and multilayered molecular mechanisms underlying perception and responses (Wang et al., 2016). Due to the complex and unpredictable ever-changing environment it is important, to focus on multiple pathways to understand how each contributes to stress tolerance as interactions between them may have significant impact on overall plant performance as a result of stress (Jacob et al., 2017, Wang et al., 2016).

1.2 Comparative genomics to understand stress responses in crops

1.2.1 Ortholog inference

High-throughput sequencing technologies have generated a vast amount of sequence information for complete genomes of many different organisms and therefore provide an unprecedented amount of information on evolutionary scales. Comparing gene sequences within and between genomes from different species allows for the reconstruction of their evolutionary relationships and is therefore fundamental to comparative biological research summarised as ‘comparative genomics’ (Koonin, 2005). Studying the evolution of gene functions across species allows for the prediction of gene function in yet uncharacterised species. In addition, it enables the transfer of biological knowledge and experimentally confirmed functional information from model organisms to newly sequenced or less annotated genomes of high interest (Gabaldon and Koonin, 2013, Koonin, 2005). However, developing methods that accurately construct functional annotation for novel genomes is very challenging as genes belong to large multigene families that evolved through multiple duplication events (Figure 2A) (Vaattovaara et al., 2019). These events include whole genome duplication or even triplication, sub-genomic duplication events like tandem- or segmental duplication and transposable element mediated duplication (Panchy et al., 2016). Decreased selection pressure on duplicated genes and increased mutation rates lead to sub- or neo-functionalisation as well as gene loss or pseudogenization (Vaattovaara et al., 2019). These evolutionary events play a central role in plant diversification and provide the basis for the adaptive evolution (Flagel and Wendel, 2009).

In this context, the identification of homology relationships between sequences represents an important first step to identify the evolutionary processes. Homologs are evolutionary related genes that have descended from a common ancestor and can further be classified into two fundamentally distinct types, orthologs and paralogs. As proposed by Fitch (1970), orthologs are gene pairs in different species that are derived via a speciation event while paralogs are derived from duplication events. This distinction is crucial and represents the basis for gene function prediction and the knowledge transfer between species. Based on the ortholog inference, it is assumed that orthologs retain their ancestral function across distinct species, while paralogs that have been retained during evolutionary processes, can evolve different or specialized functions (Altenhoff et al., 2012).

The basic definition of orthology and paralogy is fundamental for comparative genomics as a concept that aims to dissect extremely complex evolutionary processes (Gabaldon

and Koonin, 2013). This concept remains relatively straightforward as long as it focusses on the relationship of two genes in related species. When dealing with multiple species, however, the application of this concept becomes complicated as orthology and paralogy are not transitive (Figure 2B) (Shiao et al., 2008).

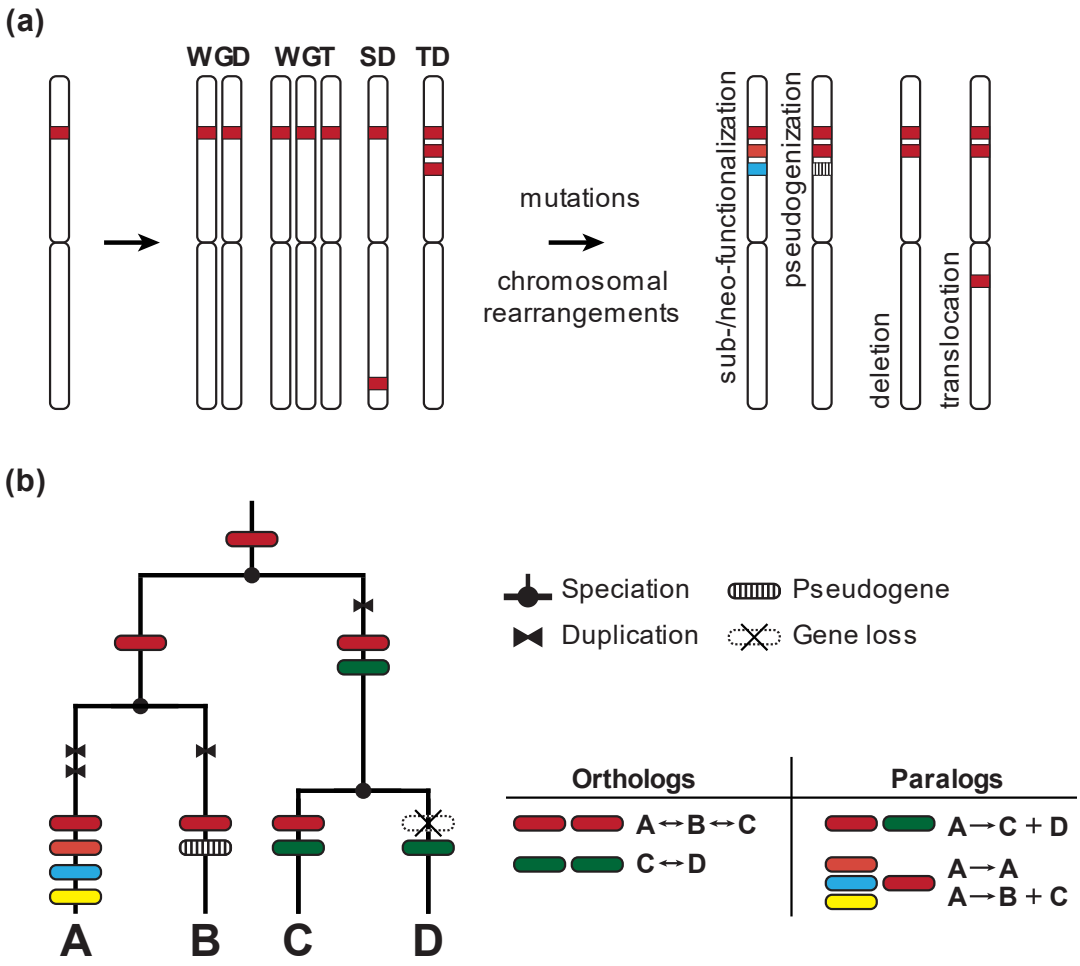

Figure 2. Simplified diagrams to illustrate the complex evolution of gene-families and phylogenetic relationships of the genes within.

(**a**) Different types of evolutionary events including whole-genome duplication (WGD), whole-genome-triplication (WGT), segmental duplication (SD) within or between chromosomes and tandem duplication (TD), leading to duplicated/multiplicated genes. Mutations lead to the distribution of ancestral functions (sub-functionalization) or even completely novel functions (neo-functionalization) between those genes and can further result in pseudogenes. Deletion and consequently gene-loss can be a result of chromosomal rearrangements as well as gene translocation. **(**b**)** Exemplary gene evolution of an ancestral gene leading to four individual populations (A-D) as a result of different speciation and duplication events. These events lead to orthologous genes (separated by speciation) and paralogous genes (separated by speciation). Specific examples of ortholog- and paralog-relationships are shown and demonstrate that homology relationships are not transitive. Pseudogenization (species B) and gene loss (species D) further visualize the complexity of gene families. Redrawn and modified figure from Vaattovaara et al. (2019).

Multiple computational approaches to infer orthology have been developed and compared in the literature with strengths and weaknesses based on their conceptual characteristics (Altenhoff et al., 2019a, Fernández et al., 2019). Further differences between the available tools are required processing power and consequently run-time, accuracy, versatility, and scalability but also the applicability for users without programming knowledge. In order to assess and compare the performance of orthology inference methods for precision and recall a publicly accessible web service has been developed, the 'Quest for Orthologs' (QfO) benchmarking server, that provides a reference proteome data set (Glover et al., 2019).

Generally, computational orthology inference methods can be classified into two major groups, tree- and gene-based methods that are based on different concepts. The tree-based method uses gene/species tree reconciliation and the annotation of all splits of a given gene tree as duplication or speciation to infer all pairs of orthologous and paralogous genes (Fernández et al., 2019). This methodology is the most appropriate method for disentangling orthologous and paralogous genes, but it is less applicable as it is computationally expensive to produce especially for a large number of organisms and genes (Kristensen et al., 2011). Tools that use this methodology are PhylomeDB (Huerta-Cepas et al., 2014), TreeFam (Schreiber et al., 2014) or ETE3 (Huerta-Cepas et al., 2016). Graph-based algorithms compare sequences in a pairwise fashion within and between species and build a graph with genes as nodes and measures for sequence similarity as proxies for their evolutionary relationship as edges (Fernández et al., 2019). Common tools using a graph-based approach are OMA (Altenhoff et al., 2019b), OrthoDB (Kriventseva et al., 2019), SonicParanoid (Cosentino and Iwasaki, 2019), OrthoVenn (Wang et al., 2015) or OrthoFinder (Emms and Kelly, 2019).

To deal with the complex ortholog evolution in multiple species some methods aim to define orthologous groups referred to as 'orthogroups' (OGs). The simplest type of these groups contains only sets of genes for which members are orthologous in a one-to-one relationship that is strictly transitive (Fernández et al., 2019). These types are rare in plants, and more often complete OGs contain sets of genes that descended from a single gene in the last common ancestor of all the species considered in the analysis, therefore containing orthologs and paralogs (Fernández et al., 2019, Emms and Kelly, 2015). The latter approach is the logical extension of orthology to multiple species and a frequently used unit of comparison (Emms and Kelly, 2015).

One of the tools that infers complete OGs and outperformed all competing methods in the QfO benchmark comparison is OrthoFinder. The first principle stage of this tool is the OG inference, using an all-vs-all BLAST that is corrected for gene length bias and normalised

for phylogenetic distance between species (Emms and Kelly, 2015). This is followed by the second stage which is the inference of unrooted and rooted gene- and species-trees (Emms and Kelly, 2019). The phylogenetic information is then used in the third stage to accurately infer orthologs. OrthoFinder has been used in many studies that utilized ortholog inference in mammals, bacteria, protists and fungi (Moreno-Santillán et al., 2019, Bradwell et al., 2018, Rodrigues et al., 2019). Studies in plants include multiple comparative analyses in the family of grasses (*Poacea*), containing important crop species maize, rice and barley, focussing on various gene families (Kong et al., 2019a, Kong et al., 2019b, Kong et al., 2019c, Cai et al., 2018). Furthermore, this tool has been used in comparative research to analyse the transcriptional control of photosynthesis in the dicot model plant Arabidopsis and the agronomically important species rice (Wang et al., 2017).

1.2.2 Transcriptomic profiling

Transcriptomics describe the study of the entirety of RNA molecules, mostly ribosomal RNA (rRNA), messenger RNA (mRNA) and a variety of non-coding RNA (ncRNA), within a single cell or tissue. The transcriptome represents a snapshot of the transcribed genes at a specific time point, developmental stage and in an environmental condition, and therefore reflects the cellular state and thus serves as a quantitative read-out of RNA-status. Investigating the transcriptome and the comparison of gene expression profiles between different conditions or tissues helps to identify important genes or even regulatory pathways and is therefore a link between genetic information and phenotype.

The advances and decreasing costs of high-throughput technologies like NGS technologies, that enable RNA-sequencing (RNAseq), has revolutionized the transcriptomic landscape in the last decade. Working at single nucleotide resolution, RNAseq has enabled the de novo assembly of transcriptomes (Montero-Pau et al., 2018, Weinberg et al., 2019, Chen et al., 2017) the identification of unknown transcripts and helped to reveal alternative splicing (Wang et al., 2019) as well as gene fusion events (Hrdlickova et al., 2017). The quantification of mRNA abundance to identify differentially expressed genes (DEGs) between different biological samples represents one of the most commonly used approaches that utilize RNAseq technology. This approach typically analyses transcript abundance as a proxy for gene expression to detect significant changes between experimental conditions or developmental stages. To quantify the transcript abundance, RNAseq reads need to be mapped to a reference transcriptome or genome, which are available for a wide range of eukaryotic and prokaryotic model organisms. De novo assemblies of transcriptomes can be used as reference for non-model organisms, which clearly highlights advantages of NGS compared to older

techniques like microarrays. Transcriptomic profiling can help to identify candidate genes and cis-regulatory elements and predict their function.

1.2.3 Cross-species comparison

Many aspects of plant development and physiology have been characterised using Arabidopsis and have fundamentally improved our understanding of similar biological processes in the crop counterparts (Meinke et al., 1998). Arabidopsis has been established as an excellent reference source for comparative and translational research in crop species.

The transfer from Arabidopsis to crops was shown for the expression of a MYB TF which is a known regulator of the flavonol biosynthesis in Arabidopsis and induces phenylpropanoids biosynthesis pathways in tomato (*Solanum lycopersicum*). Expression of AtMYB12 in tomato under the control of the fruit specific E8 promoter resulted in 10 % increase of the fruit dry weight and high levels of certain anti-oxidants in the fruit that are linked to human health-benefits (Zhang et al., 2015a, Luo et al., 2008).

The availability of high-quality reference genomes for many important cereals as well as highly sophisticated bioinformatic tools to infer orthology have significantly improved the translational research in the context of plant breeding. These reference genomes include the most important cereals rice, wheat, maize and barley (Kawahara et al., 2013, Jiao et al., 2017, Mascher et al., 2017, The International Wheat Genome Sequencing Consortium (IWGSC), 2014).

Different studies aimed to understand the regulatory networks in response to stress in the model species rice and Arabidopsis representing monocots and dicots, respectively. Based on a meta-analysis using microarray studies in responses to drought and bacterial stress, Shaik and Ramakrishna (2013) discovered biological processes, cellular pathways and TF families that are commonly and exclusively altered. A similar approach by Narsai et al. (2010) utilized orthology and transcriptomic data to investigate the level of similarity in transcriptional networks across organs in both species and further compared the responses to abiotic stress. This genome wide overview revealed a significant divergence between both species in response to abiotic stress and overall provides a rational basis for the selection of candidate genes in translational research.

Other studies have been conducted focussing on specific pathways or networks between Arabidopsis and rice or other crop species. Obertello et al. (2015) constructed a cross-species network to study nitrogen (N)-regulated gene networks in rice by using orthology and gene-interaction information from Arabidopsis. They identified two N-regulated TFs in rice that have been experimentally validated to mediate the N response in Arabidopsis.

A genome-wide comparative analysis of flowering-related genes in Arabidopsis, wheat, and barley was based on a combination of orthology and gene expression profiling (Peng et al., 2015). This study assembled a comprehensive collection of flowering-related genes in wheat and barley as resource to select candidate genes. This is important for plant breeding as flowering time is a critical agronomic trait that impacts yield and grain quality.

1.3 Organellar communication in stress signalling

1.3.1 Endosymbiont to organelle

Besides the nucleus, two additional genetic compartments can be found in plant cells, namely chloroplasts and mitochondria. Phylogenetic data suggest that these organelles derived from prokaryotic endosymbiont ancestors, i.e. free-living prokaryotes. Most likely and widely accepted, a revolutionary single ancient event of endosymbiosis occurred around 1.5 billion years ago (Dyall et al., 2004). The endosymbiosis, probably of an α-proteobacterium–like ancestor, either by being engulfed or by an event of invasion, is the origin of mitochondria within eukaryotic cells. By a similar process, chloroplasts descended from a cyanobacterium-like ancestor and its assimilation into a mitochondrion-possessing eukaryote 1.5-1.2 billion years ago (Dyall et al., 2004, Woodson and Chory, 2008). The incorporation by a eukaryotic cell led to an evolutionary milestone from prokaryotic endosymbionts to functional and highly specialized organelles. The engulfed endosymbionts represented an additional genetic and biochemical compartment that led to major changes and transformations of the whole cell. Changes in biological functions of the endosymbiont due to a new intracellular existence with beneficial features for the host cell resulted in a semiautonomous organelle.

During this process, a massive gene transfer from the endosymbiont to the nucleus occurred and metabolic pathways had to be reorganised. In addition, novel signalling networks to control and regulate development and metabolic processes within the new organelle evolved (Bräutigam et al., 2007). During the course of evolution, redundant genes were eliminated, and most genes were transferred to the nucleus (Kurland and Andersson, 2000, Burger et al., 2013, Zimorski et al., 2014). The gene transfer is still an ongoing evolutionary process and is happening at different rates between plant families. The mitochondrial genome in angiosperms, which is the largest reported mitochondrial genome, has expanded since the plants colonised the land (Kubo and Newton, 2008). This is contrary to mammalian mitochondrial genomes which have become smaller and more compact.

Besides this extensive gene transfer, both organelles retained their own core set of genes. In mitochondria, the retained genome mainly encodes genes required for the

mitochondrial electron transport chain (mtETC) and the essential ribosomal machinery for their synthesis (Allen, 2003). This enables mitochondria to control their expression, to maintain redox balance and to avoid the overproduction of ROS in a nuclear independent manner (Kleine et al., 2009).

1.3.2 Organellar signalling

Coordinated gene expression in compartments separated by partially impermeable lipid bilayers with specific molecule- and protein-specific transporters requires complex and tightly coordinated signalling networks. This communication is crucial for cellular homeostasis and avoidance of oxidative stress in plant cells. In order to ensure gene expression in response to tissue-specific, developmental, internal and external stimuli new bi-directional signalling networks between organelles and the nucleus evolved as a consequence of the endosymbiotic events (Goldschmidt-Clermont, 1997, Koussevitzky et al., 2007).

Changing the expression of nuclear genes encoding organellar proteins allows for a direct control over processes within the organelle to modify and coordinate developmental as well as metabolic activities depending on cellular needs (Ng et al., 2014). This signalling from the nucleus to mitochondria or other organelles is called anterograde signalling. This top-down control occurs at different levels, i.e. transcriptional and post-translational. It is further controlled by signals induced under varying developmental or environmental conditions (Leister, 2005, Giraud et al., 2009).

Organelles play a crucial role in incorporating environmental cues into metabolic responses acting as sensors to adjust and maintain cellular homeostasis (Kessler and Schnell, 2009, Caldana et al., 2012, Vanlerberghe, 2013). This signalling pathway, sensing the status of the organelle to communicate it back to the nucleus is called retrograde signalling. These signals then modulate the nuclear gene expression as a feedback signal to address the initial defects leading to dynamic adjustments of gene expression (Woodson and Chory, 2008, Van Aken et al., 2016b).

Retrograde signalling, which has been well established for chloroplasts, can be grouped into two different categories, biogenic and operational control. The biogenic control refers to a developmental-dependent control during biogenesis of organelles to ensure the accurate assembly by coordinating the availability of required subunits and co-factors in correct stoichiometry (Pogson et al., 2008). Operational control describes fast adjustments in energy metabolism from mature organelles to ensure optimal production and the avoidance of oxidative stress while coping with changed environmental or developmental conditions (Pogson et al., 2008).

1.3.3 Chloroplast retrograde signalling

The inhibition of chloroplast biogenesis using carotenoid synthesis inhibitor norflurazon (NF) and plastid translation inhibitor lincomycin (LIN) has been used to characterize biogenic chloroplast-to-nucleus retrograde signalling pathways (Woodson et al., 2013). The resulting down-regulation of photosynthesis related genes like LIGHT HARVESTING CHLOROPHYLL A/B BINDING PROTEIN 1.2 (LHCB1.2) was de-repressed in certain mutant lines, defined as *genomes uncoupled* (*gun*) mutants (Zhang et al., 2015b). Six *gun* mutants have been identified of which five (*gun2/3/4/5/*6) rescue the repression of gene expression by NF and encode enzymes involved in the tetrapyrrole biosynthesis pathway which demonstrates the importance of this pathway in the biogenic signalling (Koussevitzky et al., 2007). Tetrapyrroles include chlorophyll (Chl), heme, siroheme, and phytochromobilin are therefore essential for photosynthesis, respiration and the assimilation of nitrogen/sulfur (Tanaka et al., 2011).

GUN1, a pentatricopeptide repeat (PPR) protein, is not related to the synthesis of tetrapyrroles and represents the only *gun* mutant that shows the *gun* phenotype after treatment with NF and exclusively with LIN (Hernandez-Verdeja and Strand, 2018). The involvement of GUN1 in retrograde signalling pathways linked to the tetrapyrrole biosynthesis pathway, plastid gene expression, and photosynthetic electron transport has been shown but the underlying mechanisms are not well understood (Jia et al., 2019).The TF ABSCISIC ACID INSENSITIVE 4 (ABI4) was reported to be involved in the retrograde signal transduction downstream of GUN1 (Nott et al., 2006). A recent study, however, systematically assessed ABI4 in relation to the chloroplast-to-nucleus retrograde response and could not find consistent evidence that supports the proposed involvement in this signalling pathway (Kacprzak et al., 2019). This study analysed the expression of several retrograde-regulated nuclear genes in response to inhibitors of chloroplast development in various *abi4* alleles.

Accumulation of plastidial metabolites derive from disturbed plastid metabolism are proposed to be stress-specific operational retrograde signals, i.e. 3'-phosphoadenosine 5'-phosphate (PAP), MEP 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcPP) and β-cyclocitral (β -CC) (Koussevitzky et al., 2007, Leister, 2012, Xiao et al., 2013, de Souza et al., 2017). Under non-stress conditions the phosphatase SAL1 enzymatically degrades and detoxifies PAP which results in very low basal levels (Phua et al., 2018). Adverse environmental conditions, however, lead to an inactivation of redox regulated SAL1 due to oxidative stress which then leads to the accumulation of PAP that may act as retrograde signal leading to the up-regulation of stress-responsive genes (de Souza et al., 2017, Estavillo et al., 2011, Chan et al., 2016). MEcPP, a precursor of isoprenoids synthesised

by the methylerythritol phosphate (MEP) pathway in the chloroplast, shows stress-mediated induction as well and alters nuclear stress-responsive genes (Xiao et al., 2012). Its accumulation correlates with that of the chloroplast targeted HYDROXYPYRUVATE REDUCTASE 3 (HPL) protein and is linked to higher SA levels that increased resistance to infection by biotrophic pathogens. β-CC, the product of β-carotene oxidation, is participating in retrograde signalling by enhancing the tolerance to photo-oxidative stress and the induction of SA synthesis (Ramel et al., 2012). Furthermore, accumulation of ROS generated in the chloroplast and redox-signals from the photosynthetic electron transport chain have been characterised as retrograde signals (Gray et al., 2003, Nott et al., 2006, Woodson and Chory, 2008).

1.3.4 Mitochondrial retrograde signalling

Retrograde signalling in mitochondria, was firstly reported in yeast by Liao and Butow (1993) who described the mitochondrial retrograde response (MRR) and identified two pivotal TFs, RTG1 and RTG2, that control inter-organellar communication between mitochondria, peroxisomes and the nucleus. Furthermore, they showed that nuclear gene expression is sensitive to the functional state of mitochondria and characterised a pathway that controls expression of genes that alter metabolism in response to perturbed respiratory function. In comparison to chloroplast retrograde signalling, few components of the mitochondrial pathways are known in plants.

ALTERNATIVE OXIDASE 1A (AOX1a) represents the most widely used marker to study MRR. In species like Arabidopsis, tobacco, soybean and maize AOX expression is increased in response to mitochondrial dysfunction induced by either mtETC complex III inhibitor antimycin A (AA) and/or monofluoroacetate (MFA), a tricarboxylic acid (TCA) cycle inhibitor (Djajanegara et al., 2002, Zarkovic et al., 2005, Karpova et al., 2002, Vanlerberghe and McLntosh, 1996). The analysis of the promotor region of *AOX1a* revealed a 6-bp sequence whose deletion increased the promoter activity and indicated a repressor binding motif (Ho et al., 2008). This sequence overlaps with a cis-acting regulatory element and potential binding site of *ABI4*, a nuclear localized AP2/EREBP family TF participating in ABA signalling (Koussevitzky et al., 2007, Woodson and Chory, 2008). ABI4 was shown to negatively regulate *AOX1a* under normal growth conditions. while this repression is relieved after rotenone and ABA treatment (Giraud et al., 2009). ABI4 also plays an important role in other plant functions like root growth, nitrogen signalling or pathogen resistance and is also required for the redox response (León et al., 2012). As outlined above, ABI4 was also proposed to be part of plastid-to nucleus retrograde signalling and therefore represents a putative convergence point between mitochondrial and plastidial retrograde signalling pathways (Koussevitzky et al., 2007).

This involvement of ABI4 in chloroplast retrograde signalling, however, has been questioned by a recent study (Kacprzak et al., 2019).

In order to identify molecular components of MRR a forward genetic screen was conducted and revealed regulators of AOX (RAOs) (Ng et al., 2013a). *RAO1* encodes the nuclear localized CYCLIN-DEPENDENT KINASE E1 (CDKE1) which regulates diverse cellular stress signalling pathways and is required for the expression of *AOX1a* in Arabidopsis (Ng et al., 2013a). Moreover, CDKE1 regulates AOX1a post-translationally as indicated by a stronger reduction on the protein level. CDKE1 interacts with the nucleus- and cytosol-localised PROTEIN KINASE 10 (KIN10), a Snf1-related protein kinase (SnRK) with essential functions in plant protection and survival under stress. KIN10 plays an important role in vegetative and reproductive growth and developmental transition under normal growth conditions (Baena-González et al., 2007). Gene expression analyses of *kin10* RNAi lines revealed a large overlap with CDKE1 regulated genes. The transport of KIN10 to the nucleus to interact with CDKE1 as part of a protein kinase signalling cascade represents a mechanism in plants to integrate signals from multiple input sources (Ng et al., 2013a). CDKE1 is also involved in the regulation of LIGHT HARVESTING COMPLEX B2.4 (LHCB2.4) and therefore responds to signals originating in both mitochondria and chloroplasts (Blanco et al., 2014).

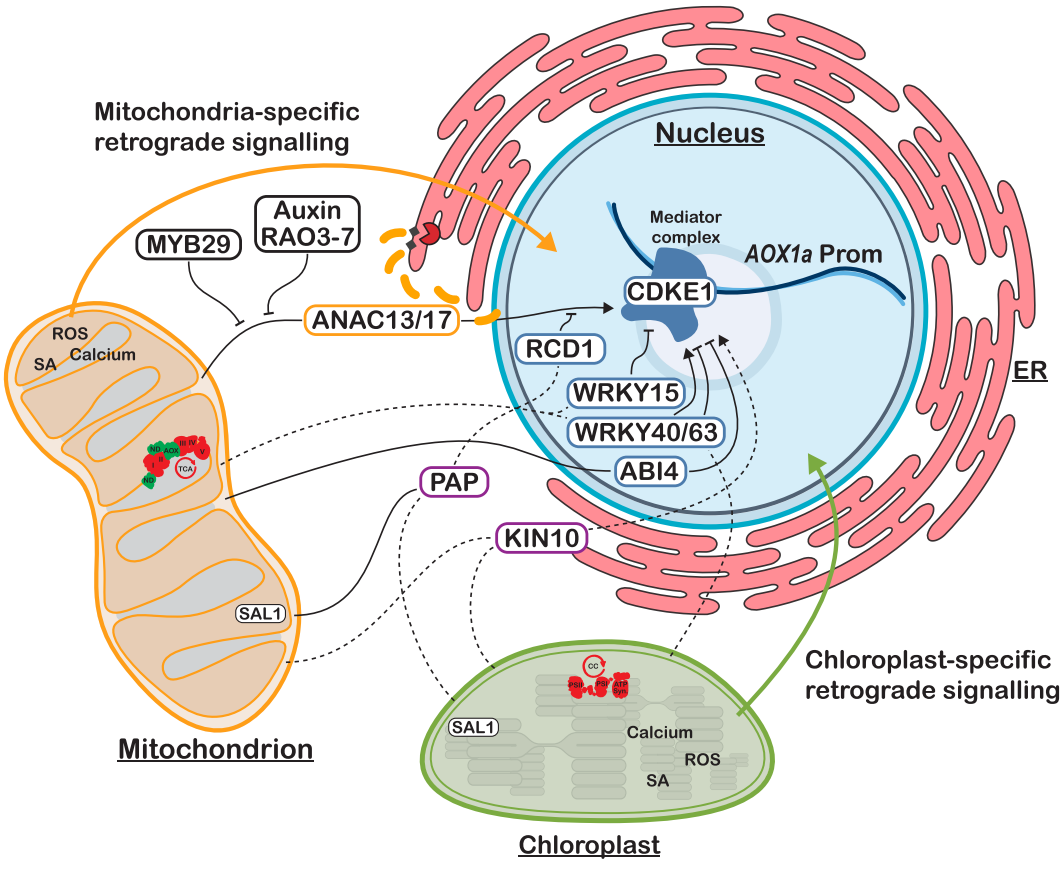

Figure 3. Mitochondrial and chloroplast retrograde signalling exemplary for AOX1a.
Several mitochondrial and chloroplast derived components of retrograde signalling pathways have been identified, that are either organelle-specific or putatively shared. AOX1a represents the key marker gene for the mitochondrial retrograde response (MRR) in Arabidopsis and a large number of components that regulate its expression. Mitochondrial dysfunction leads to the activation of a number of ER-bound NAC transcription factors (TFs) including master-regulator ANAC017 and ANAC013 which itself is a regulatory target of ANAC017. WRKY40 is another positive regulator of AOX1a. A subunit of the kinase module of the Mediator complex, CDKE1, is important for the induction of AOX1a. KIN10, a central mediator of stress and energy signalling links mitochondrial and chloroplast retrograde signalling and relays the information to CDKE1. Negative regulators of AOX1a are WRKY15 and WRKY63 as well as ABI4 which link to chloroplast is not conclusive. MYB29 and several components involved in auxin signalling (RAO 2,4,5,6 and 7) are negative regulators as well. RCD1, was shown to be a repressor of ANAC13/17 as well as other MDS genes and overlaps or converges with the SAL1-PAP dependent pathway which integrates mitochondrial and chloroplast retrograde signalling. Where a role has been experimentally shown, it is indicated with a solid line. Roles that are proposed based on changes of transcript abundance alone or questioned are indicated with dashed lines. MYB29, MYB DOMAIN PROTEIN 29; RAO, Regulator of Alternative Oxidase 1A; ANAC, NAC (NAM, ATAF, CUC2) transcription factor 13/17; WRKY15/40/63, WRKY DOMAIN PROTEIN 15/40/63; RCD1, RADICAL-INDUCED CELL DEATH PROTEIN 1; CDKE1, CYCLIN-DEPENDENT KINASE E1; ABI4, A BA INSENSITIVE 4; MDS, mitochondrial dysfunction stimulon genes; SAL1, phosphatase-like protein; PAP, 3'-phosphoadenosine 5'-phosphate; ROS, reactive oxygen species; ER, endoplasmic reticulum; SA; salicylic acid. Redrawn and modified figure from Wang et al. (2020).

A second mutant (*rao2*) was identified as an integral cellular component of the MRR in plants using forward and reverse genetic screens (De Clercq et al., 2013, Ng et al., 2013b). The *RAO2* gene encodes the ANAC017 TF that is bound to the endoplasmic reticulum (ER) via its C-terminal transmembrane domain. Activation via proteolytic cleavage, presumably by a rhomboid protease, leads to the migration of ANAC017 to the nucleus to regulate gene expression (Ng et al., 2013b). ANAC017 represents a master regulator of cellular responses and its overexpression leads to growth retardation, altered leaf development with decreased cell size and viability, and early leaf senescence. It further induces the transcript abundance of genes related to mitochondrial stress, cell death/autophagy, and leaf senescence (Meng et al., 2019, Van Aken et al., 2016b, Van Aken et al., 2016a).

MYB DOMAIN PROTEIN 29 (MYB29)/RAO7 is another TF involved in the regulation of AOX1a in Arabidopsis and is a negative regulator of mitochondrial stress responsive genes. However, MYB29 does not bind to the *AOX1a* promotor indicating an indirect involvement in the regulation of the mitochondrial retrograde gene expression (Zhang et al., 2017). Several MYB TFs, including MYB29, are transcriptional regulators of the glucosinolate biosynthesis that is linked to biotic plant defence, response to abiotic stress conditions and in hormonal signalling (Sonderby et al., 2010). This points to a

convergence of stress signals and hormonal networks with the MRR in Arabidopsis (Zhang et al., 2017). The MRR and consequently the transcript abundance of *AOX1a* seems to be reciprocally regulated via auxin signalling, with auxin repressing the MRR and preventing the induction of AOX1a. The involvement of auxin in MRR regulation is further supported by the identification of additional *rao* mutants (Ivanova et al., 2014). The underlying genes encode for proteins associated with auxin transport, namely RAO3/BIG, RAO4/PIN1, RAO5/MULTIDRUG-RESISTANCE 1 as well as RAO6/ASYMMETRIC LEAVES 1.

De Clercq et al. (2013) performed a meta-analysis of microarray-derived transcriptome data resulting from experiments on plants with impaired mitochondrial function by short-term treatments with respiratory inhibitors or by genetic mutation of mitochondrial proteins. This study identified a cis-regulatory element which was conserved in the promotor regions of a set of mitochondrial stress-responsive genes, referred to as mitochondrial dysfunction stimulon (MDS) genes. A regulatory promotor element, termed mitochondrial dysfunction motif (MDM), was required for MRR-mediated gene expression. Five putative membrane-associated NAC TFs were identified as transcriptional regulators that can specifically bind the MDM, including ANAC013 and ANAC017. *ANAC013* is an MDS gene itself and was shown to act downstream of *ANAC017* which specifically bind its promotor. As positive signal-transducing component and central regulator of the MRR ANAC013 might be activated by a similar proteolytic mechanism as shown for ANAC017 (De Clercq et al., 2013).

The nuclear protein *RADICAL-INDUCED CELL DEATH1* (*RCD1*) is a negative regulator of ANAC013/17 and directly interacts with both TFs as shown *in vivo* (Shapiguzov et al., 2019). Inactivation of RCD1 was shown to increase expression of MDS genes including AOX1a and this accumulation affects the chloroplast redox status. RCD1 is thought to integrate organellar retrograde signals from mitochondria and chloroplasts which is further supported by the involvement of RCD1 in the PAP mediated signalling pathway (Shapiguzov et al., 2019, Sipari et al., 2020, Brosche et al., 2014). Other members of the SIMILAR TO RCD-ONE (SROs) family, that includes RCD1, play an important role in plant development and the responses to multiple stresses (Zhao et al., 2019)

Many mitochondrial proteins contain a special motif (W-box) that represents the core binding site for WRKY TFs that are involved in regulating stress responses (Van Aken et al., 2013). This motif is present in the promotor regions of several genes including *AOX1a*, *OUTER MITOCHONDRIAL MEMBRANE PROTEIN OF 66 KDA* (*OM66*) and *NADH DEHYDROGENASE B2* (*NDB2*) which are part of a core set of widely used stress responsive mitochondrial genes in Arabidopsis (Van Aken et al., 2009). Two WRKY TFs,

AtWRKY40 and AtWRKY63, were identified to modulate gene expression of stress responsive genes encoding mitochondrial proteins (Van Aken et al., 2013). Both TFs significantly alter gene expression of mitochondrial stress marker genes, with a repressing function for WRKY40 and activating function of WRKY63. The regulatory target of both TFs, *OM66*, is highly responsive to the plant hormone SA and overexpressing lines show a higher SA content. Because *OM66* shows a similar expression pattern in response to SA as the *PATHOGEN-RELATED GENE 1A* (PR-1a), a SA-response marker gene, OM66 seems to be under the direct control of SA (Shah, 2003, Ho et al., 2008). WRKY15 represents another potential negative regulator of MDS genes (Vanderauwera et al., 2012).

Hormonal networks are expected to interact with mitochondrial function and participate in the MRR. Along with auxin, ABA and SA as discussed above, JA, CK and ethylene are also involved in the regulation of mitochondrial function by largely unknown mechanisms (Berkowitz et al., 2016). In addition, the role of Ca^{2+} in the MRR and the retrograde signalling pathways in general is discussed. The identification of several Ca^{2+} sensors revealed specific Ca^{2+} signalling in and between organelles and studies demonstrated Ca^{2+} signals in response to biotic and abiotic stresses derived from mitochondria and chloroplasts (Stael et al., 2011, Rocha and Vothknecht, 2012, Nomura and Shiina, 2014). Several mitochondrial proteins with calcium binding motifs indicate its regulatory function (Schwarzlander et al., 2012). However, very little is known about transporters and signalling proteins in plant mitochondria related to Ca^{2+}.

1.3.5 Function of plant mitochondria in stress responses

Plant mitochondria are complex organelles with unique features that perform fundamental functions ranging from ATP synthesis and providing metabolic intermediates to incorporating environmental cues and being involved in programmed cell death (PCD). Overall, these functions contribute significantly to stress tolerance mechanisms by providing energy and contributing to metabolic acclimation. Compared the mitochondrial electron mtETC and the presence of chloroplasts as an energy-producing organelle led to more complex interorganellar communication and regulatory networks.

1.3.6 Mitochondrial structure

Mitochondria are dynamic organelles with variations in size and shape and an uneven distribution within the cell (Sheahan et al., 2005, Millar et al., 2008). Processes of fusion and fission control these variations in size, shape and number of mitochondria. In addition, the number of mitochondria within each cell depends on the cell type and the physiological state (Logan, 2006).

Mitochondria represent an intracellular compartment, separated from the cytosol, which is defined by the inner and outer mitochondrial membrane (IMM/OMM). The OMM consists of a phospholipid bilayer that is closely associated with the ER. Integral membrane proteins, porins, within the OMM enable ions and small, uncharged molecules to diffuse freely. The most prominent porin located in the OMM is the mitochondrial VOLTAGE-DEPENDENT ANION CHANNEL (VDAC) (Carraretto et al., 2016). Many different VDACs can be found in plants and they represent promising candidates in enabling the passage of signals, originating from the inner side of the outer membrane, the inter membrane space, the crista-space or crista–membrane and the inner IMM (Schwarzländer and Finkemeier, 2013) (Figure 4). The import of larger molecules like proteins require special translocases. Most proteins are thought to be imported via the TRANSLOCASE OF THE OUTER MEMBRANE (TOM) complex as part of the general import pathway (Duncan et al., 2013).

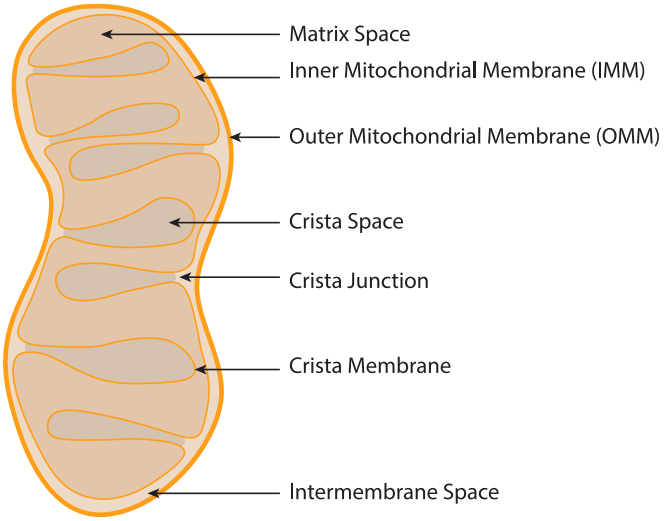

Figure 4. The compartmentalization of plant mitochondria.
Outer mitochondrial membrane (OMM) and inner mitochondrial membrane (IMM) define the intracellular compartment and build the inter membrane space. Folding of the IMM leads to the functionally distinct crista membrane (CM). Crista junction represent an important connection to the IMM. Figure redrawn and modified from Schwarzländer and Finkemeier (2013).

The IMM is non-porous and represents an impermeable barrier for all molecules allowing for further compartmentalisation. The IMM encloses the mitochondrial matrix and the cristae space, the innermost compartments of mitochondria (Figure 4). The crista membrane houses all the multimeric protein complexes of the mtETC and the ATP-synthase responsible for the oxidative phosphorylation (OXPHOS). A proton gradient between the IMS and these compartments drives the ATP synthase. The transport of

proteins across the IMM requires many different specific transport proteins like members of the translocase of the inner membrane (TIM) complex family that forms a TIM/TOM protein transport supercomplex within the intermembrane space. Other transporters mediate the passage of metabolites, cofactors, metal ions, as well as nucleic acids and polypeptides (Schwarzländer and Finkemeier, 2013). In addition, the matrix contains the mitochondrial genome, the protein synthesis machinery, and many important enzymes, especially the TCA cycle enzymes.

1.3.7 Mitochondrial function

Mitochondria are primarily responsible for energy generation within the cell and are involved in various physiological processes such as the production of biosynthetic precursors, optimisation of photosynthesis, PCD and the balancing of the cellular redox state (Mackenzie and McIntosh, 1999, Plaxton and Podestá, 2006). Two out of three main pathways of plant respiration are localized in mitochondria, namely the TCA cycle and the mtETC (Figure 5) (Fernie et al., 2004). The TCA cycle uses photosynthesis-derived carbohydrates to provide intermediates for biosynthesis. Furthermore, it couples the oxidation of carbons with the reduction of $NAD(P)^+$ to NAD(P)H, reducing equivalents for biosynthetic reactions, which can also be oxidized by the mtETC.

The mtETC is comprised of four large multiprotein complexes (complex I, II, III, IV) which are localized in the CM and includes two pathways: 1) the cytochrome c pathway and 2) the alternative pathway. In the cytochrome c pathway, complex I and II transfer electrons, derived from oxidation of NADH and succinate, respectively, to ubiquinone (UQ) which is oxidized by complex III. The electrons are then transferred by the electron carrier cytochrome c to complex IV. Electron transport by complexes I, III and IV is coupled with the translocation of protons to the IMS. This translocation creates a proton motive force, which is used by the adenosine triphosphate (ATP)-synthase to generate ATP (Figure 5).

In addition to the energy-conserving mtETC for ATP production, plant mitochondria possess five additional non-energy conserving enzymes, namely four NAD(P)H-dehydrogenases and the AOX. Two NAD(P)H-dehydrogenases are attached to the outer surface of the IMM and two NAD(P)H-dehydrogenases are present on the matrix surface. The AOX is an interfacial membrane protein, which directly couples the oxidation of ubiquinol with the reduction of molecular oxygen to water. This enzyme receives electrons from the ubiquinol pool, thereby bypassing complex III and IV without translocating protons. Consequently, the AOX is not linked to the OXPHOS system. As a result, less ATP is synthesized and most of the energy is dissipated as heat. In contrast to the cytochrome respiration, this pathway is cyanide resistant and called alternative respiration (Berthold and Stenmark, 2003, Umbach et al., 2006).

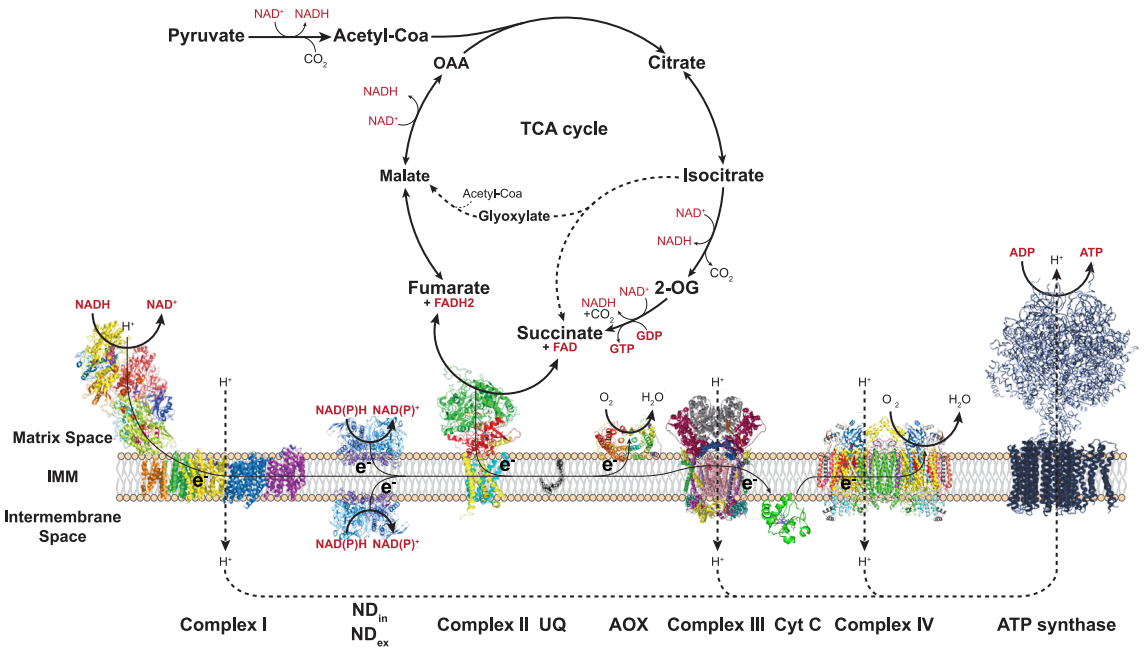

Figure 5. Schematic representation of the tricarboxylic acid (TCA) cycle and the mitochondrial electron transport chain (mETC) in plant cells.

The TCA cycle in the mitochondrial matrix space generates the reducing equivalents NADH and FADH2 via a series of enzymatic reactions starting with pyruvate. This provides electrons to the mETC which is located at the inner mitochondrial membrane. Electron transport along the membrane via different complexes in the energy-conserving cytochrome c pathway (Cyt C) generates a mitochondrial membrane potential which is then used to produce ATP via the adenosine triphosphate (ATP)-synthase. Plant mitochondria possess five additional non-energy conserving enzymes as part of the alternative pathway, namely four NAD(P)H-dehydrogenases (ND_{in}/ND_{ex}) and the alternative oxidase (AOX). The AOX is post-translationally regulated by different TCA cycle intermediates in an isoform specific manner including pyruvate, 2-oxoglutarate (2-OG), oxaloacetate (OAA) and glyoxylate. The latter is part of the glyoxylate shunt which is indicated by dashed lines. UQ, ubiquinone. Figure redrawn and modified from Selinski (2014).

Besides energy-generation, mitochondria also perform many important additional functions. Mitochondria synthesize nucleotides and are responsible for the biosynthesis of certain lipids, which are required for cell growth and cell division (Mekhedov et al., 2000, Millar et al., 2005, Li et al., 2011). Further biosynthetic pathways for amino acids, vitamins or non-vitamin coenzymes as well as organic acid intermediates for wider cellular biosynthesis can be found in mitochondria (Rébeillé et al., 2007). Mitochondria are well known to be involved in PCD by generation of ROS, the loss of electrical potential across the IMM and release of IMS proteins such as cytochrome c as inducers of PCD activation (Schwarzländer and Finkemeier, 2013, Petrov et al., 2015). Furthermore, mitochondria are crucial in many cell signalling pathways controlling mitosis, cell specification, cell death and fertilization within the embryo (Martin et al., 2014).

1.4 The Alternative oxidase

1.4.1 The importance of mitochondrial alternative oxidase in stress responses

As outlined above, plants are constantly subjected to various environmental stress conditions which have a high impact on metabolism and affect plant growth and productivity. For example, metabolic imbalances often lead to enhanced generation and accumulation of ROS causing oxidative stress. The mtETC is a major source of ROS, which can cause irreversible oxidative damage to cellular components (Juszczuk and Rychter, 2003). The alternative respiration, especially through activity of the alternative oxidase (AOX), represents an important mechanism to uncouple electron transport from ATP production by keeping the ubiquinone pool sufficiently oxidized when the electron flow through the cytochrome pathway becomes limited (Siedow and Umbach, 2000). AOX thereby has an impact on the generation of mitochondrial derived ROS which is an important signalling molecule. This links AOX to mitochondrial metabolism and is leading to changes of nuclear gene expression (Ng et al., 2014). The dynamic flexibility of the AOX is not restricted to coordinate mitochondrial function and was shown to be involved in inter-organellar energy-dissipating systems, necessary for the optimal operation of photosynthesis in the chloroplasts (Vishwakarma et al., 2015). Excess reducing equivalents produced by photochemical reactions in the chloroplasts are transported indirectly to mitochondria via the malate/oxaloacetate shuttles and malate dehydrogenases (MDHs) (Selinski and Scheibe, 2019). AOX is important for the protection of photosynthetic components against these harmful effects of excess light and concomitant overreduction leading to photoinhibition by the disruption of the photosynthetic apparatus (Liao et al., 2016, Dinakar et al., 2010, Vishwakarma et al., 2015).

1.4.2 Structure and characteristics of AOX

The cyanide-resistant respiration in plants was discovered at the beginning of the 20th century in the thermogenic plant *Sauromatum guttatum*. A shortcut in the mtETC facilitated by the AOX heats up the reproductive tissues to attract pollinators. AOX proteins are ubiquitously present in all plant species and can also be found in many fungi, protists as well as α-proteobacteria and cyanobacteria (McDonald and Vanlerberghe, 2006, Affourtit et al., 2002, Chaudhuri et al., 2006, Moore and Albury, 2008, Stenmark and Nordlund, 2003). The restricted distribution of AOX to proteobacteria and to the eukaryotic lineages supports the endosymbiotic origin of mitochondria (Costa et al., 2014). Apart from mammals, AOX-encoding genes have been characterised in animals such as

molluscs, nematodes and chordates (McDonald and Vanlerberghe, 2006, Vanlerberghe, 2013). However, AOX has been most widely investigated in the plant kingdom, especially in angiosperms. The AOX is a member of the non-heme-di-iron carboxylate proteins in which metal atoms are bound by glutamate and histidine residues within a 4-helix bundle (Andersson and Nordlund, 1999, Berthold et al., 2000). Although di-iron proteins show a diverse range of different functions, they share the common characteristics of reacting with oxygen and involvement in redox reactions.

The present model of the AOX characterizes a monotopic integral membrane protein. It is associated with the IMM via hydrophobic interactions with its catalytic centres oriented towards the matrix (Juszczuk and Rychter, 2003, Albury et al., 2010, Moore and Albury, 2008, Shiba et al., 2013, May et al., 2017). Besides an active oxygen-binding site for the reduction of oxygen to water, AOX harbours a binding site for the reducing substrate ubiquinol (Albury et al., 2009).

AOX genes in higher plants can be divided into two major subfamilies, AOX1 and AOX2. AOX1 is present in all angiosperms and is often induced by stress stimuli in many different tissues. AOX2 is present in most dicot species and absent in all monocots, even though both AOX families can be found in the common ancestors (Costa et al., 2017). AOX2 is differentially expressed in developmental stages and does not respond to stress (Considine et al., 2002, Juszczuk and Rychter, 2003, Clifton et al., 2006). Due to a new classification scheme, the two major subfamilies can further be divided into four phylogenetic clades: AOX1a–c/1e, AOX1d, AOX2a–c and AOX2d (Costa et al., 2014). As a nuclear encoded multigene family, the number of AOX isoenzymes is very diverse between different plant species and combinations of different subfamilies and types can be found (Costa et al., 2014, Considine et al., 2002). The presence of several isoform combinations within each plant species point to different functions and a different regulation.

1.4.3 AOX function and regulation

All AOX-isoenzymes are located in mitochondria and their expression is tissue-specific and depends on the developmental stage, changes in cellular metabolism and several biotic and abiotic stresses as shown for Arabidopsis (Juszczuk and Rychter, 2003, Van Aken et al., 2016b, Van Aken et al., 2009). *AOX1a* represents the gene encoding the main isoform that is ubiquitously expressed throughout all plant developmental stages and tissues. Furthermore, it represents the main stress responsive isoform. Flower-specific expression is postulated for *AOX1b*, especially during floral induction, early flower development and reproductive stages (Clifton et al., 2006). The expression level of *AOX1c* is constitutive throughout the whole plant development but much lower than *AOX1a*. In

addition, expression analyses revealed that *AOX1c* seems to be unresponsive to a variety of treatments, including stresses, suggesting a role as a housekeeping gene (Clifton et al., 2006). Transcriptome analyses revealed the association of *AOX1d* expression with senescence in leaves (Guo et al., 2004). The expression of *AOX2* appears to be specific to seeds, suggesting a role during seed maturation and early stages of germination (Clifton et al., 2006). In addition, AOX2 was shown to be imported into chloroplasts to replace the activity of a SUPPRESSED PLASTID TERMINAL OXIDASE (PTOX), pointing to a function during early events in chloroplast biogenesis by supplementing PTOX activity (Fu et al., 2012). As AOX1a represents the main stress-responsive isoform in Arabidopsis, it represents the most important and best-characterised marker gene for the MRR (see Chapter 1.3.4). *AOX1a* is highly responsive to dysfunctions in the mitochondrial-respiratory metabolism, especially complex III or IV, resulting in insufficient cytochrome-pathway capacity downstream of the ubiquinone pool (Clifton et al., 2006, Vanlerberghe, 2013). Further induction was reported by dysfunctions of complex I, the inhibition of ATP synthase and multitude of chemical treatments that disrupt mitochondrial functions (Zarkovic et al., 2005, Clifton et al., 2005). Furthermore, *AOX1a* transcripts increase during accumulation of TCA cycle intermediates such as citrate and thus respond to the status of upstream respiratory metabolism. As AOX activity represents an energy wasteful process under optimal growth conditions, it is consequently under a very strong repression mediated by the TF ABI4 (Giraud et al., 2009) and tight control of its inducer ANAC017 (Meng 2019). The MRR and consequently the transcript abundance of *AOX1a* is reciprocally regulated with auxin signalling, hormonal control of growth and development, as indicated by *AOX1a* induction

Besides transcriptional regulation, AOX activity is regulated by post-translational modifications and the involvement of different metabolites (Selinski et al., 2017, Wang et al., 2018b, Selinski et al., 2018a, Selinski et al., 2018b) (Figure 6). Its post-translational regulation is controlled by two inter-related mechanisms (Moore and Albury, 2008). Most of the AOX isoforms have two highly conserved cysteine residues (CysI and CysII) which are present in the N-terminal domain of the protein. CysI is responsible for the formation of an intermolecular disulfide bond with the corresponding CysI on the adjacent subunit of the AOX homodimer under oxidizing conditions. By contrast, reducing conditions lead to a non-covalently linked dimer resulting in an active protein. To what extend this mechanism affects AOX activity *in vivo* as well as the proposed role of the mitochondrial thioredoxin (TRX) system to mediate and maintain this reduction is debated in the literature (Florez-Sarasa et al., 2019, Schwarzländer and Fuchs, 2019)

The protein activity of an activatable AOX dimer can further be stimulated by 2-oxo acids, most notably pyruvate and glyoxylate but also oxaloacetate (OAA) and 2-oxoglutarate (2-

OG), the only TCA cycle intermediates belonging to the group of 2-oxo acids. (Rhoads et al., 1998, Umbach et al., 2006, Wang et al., 2018b, Selinski et al., 2018a, Selinski et al., 2017). The relative amount of oxidized or reduced protein seems to depend on the redox state of the pyridine nucleotide pool which itself depends on mitochondrial metabolism (Juszczuk and Rychter, 2003). Activity measurements with Arabidopsis AOX isoforms (AtAOX 1A/1C/1D) revealed the involvement of both regulatory cysteine residues (CysI and CysII) in effector activation by 2-oxo acids. However, CysII represents a secondary, less effective activation site (Selinski et al., 2017).

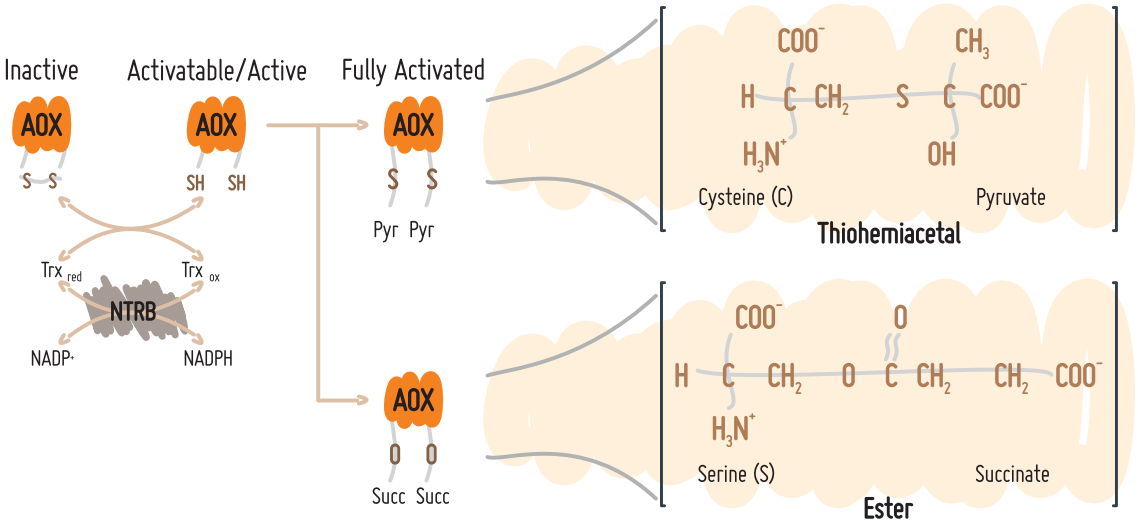

Figure 6. Post-translational activation of alternative oxidase (AOX) protein.
Inactive AOX protein due to covalent linkage of the dimeric enzyme that form an intermolecular disulfide bond under oxidizing conditions. Conversion of the inactive AOX to the activatable/active form under reducing conditions is thought to be mediated by mitochondrial thioredoxin (Trx) system. The resulting free thiol group can interact with 2-oxo acids like pyruvate (Pyr) to form a thiohemiacetal which leads to the fully active form. Some AOX isoforms possess a serine residue instead of the first regulatory cysteine residue (CysI) and are insensitive to 2-oxo acids. These isoforms can be activated by the dicarboxylic acid succinate (Succ), presumably by forming an ester bond. Figure adapted from Selinski et al. (2018b).

Several AOX isoforms contain a serine residue at the position of CysI, like AOX isoforms in tomato, maize (*Zea mays*) and lotus (*Nelumbo nucifera*) (Holtzapffel et al., 2003, Grant et al., 2009, Karpova et al., 2002). AOX1B from tomato is insensitive to 2-oxo acid activation but activated by succinate, a dicarboxylic acid intermediate of the TCA cycle (Holtzapffel et al., 2003). This succinate activation can be transferred to mutated Arabidopsis AOX1A proteins containing a serine or an alanine residue at the position of CysI, contrary to the insensitive wild-type protein (Djajanegara et al., 1999, Selinski et al., 2017). In Arabidopsis, this succinate activation is specific for AOX1A, as corresponding mutants of AOX1C and 1D remain insensitive. This specificity points to different amino

acid environment and structural differences in close vicinity to CysI, enabling an isoform-specific activation (Selinski et al., 2017, Selinski et al., 2018b). Overall, the postulated model describes differentially activated AOX1 isoforms depending on the type of effector and differences in the amino acid composition.

1.4.4 Non-redundancy of AOX isoforms in response to stress

In Arabidopsis, *AOX1a* is the isogene with the highest expression ubiquitously throughout all plant developmental stages and tissues and shows the highest transcriptional changes and abundance in response to stress (Clifton et al., 2005). As outlined previously, *AOX1a* is a key marker to study MRR due to its high sensitivity especially to the commonly used respiratory inhibitor AA. A*ox1a*-knockout mutants do not show any apparent phenotypic changes under non-stress conditions but have greatly altered transcriptional changes compared to WT plants (Giraud et al., 2008). Compensation of the lack of AOX1a could be explained by metabolic adjustments and induction of alternative pathways rather than compensation by other AOX isoforms. In *aox1a*-T-DNA lines no AOX isoforms other than AOX1D can be detected on transcript or protein level under non-stress conditions (Giraud et al., 2008, Watanabe et al., 2010, Watanabe et al., 2008, Strodtkötter et al., 2009).

Treatment of *aox1a*-knockout plants with AA, inducing mitochondrial dysfunction, leads to inhibition of photosynthetic processes, increased ROS and membrane leakage resulting in necrosis while the leaflets of wild type plants have no visible phenotypic changes (Strodtkötter et al., 2009). Moreover, the photosynthetic electron transport is strongly affected in *aox1a*-knockout plants accompanied with metabolic imbalances. Under stress-conditions *AOX1d*, the second highly stress-responsive isoform in Arabidopsis, shows highly induced expression but cannot functionally compensate the lack of AOX1a (Strodtkötter et al., 2009, Kühn et al., 2015). Despite this induction the total protein level might not be sufficient to compensate for the lack of *Aox1a*. Another explanation could be differences in the post-translational regulation of the AOX isoforms and specific fine-tuning by certain metabolites as shown by Selinski et al. (2018a). Post-translationally, AOX activity requires reducing condition which lead to a non-covalently linked dimer that were proposed to be homodimers (Umbach and Siedow, 1993). Due to the lack of distinguishability of homo- and heterodimers, the existence of a heterodimer is possible and could add another level of fine-regulation (Selinski et al., 2018a, Selinski et al., 2018b). The localisation of AOX isoforms and cell-specific expression pattern represent another level of regulation that needs to be elucidated (Selinski et al., 2018a).

1.5 Scope and Research Objectives

1.5.1 Comparative transcriptomic analysis of stress responses in Arabidopsis and the crop species rice and barley

Understanding the sensory and response mechanisms to adverse environmental conditions that ultimately translate into stress tolerance and higher yields has become a central focus in plant research (Zhu 2016). This is increasingly important due to interconnected challenges in food security that are exacerbated by the severe impact of climate change. While the direct transfer of molecular mechanisms between evolutionary distant species is challenging, Arabidopsis represents an excellent reference for comparative research in crop species due to the immense knowledge gained in this model species. Exploiting this resource of experimentally confirmed functional information to get a better understanding of the stress responses in agronomically relevant plant species is the aim of chapter 2.

Aim 1: Define the global transcriptomic responses to different stress treatments in Arabidopsis, rice and barley

RNAseq followed by transcriptomic profiling of the whole-genome responses to six different stress treatments that activate biotic and abiotic stress signalling pathways in the well-characterised dicot model Arabidopsis and two agronomically relevant monocot species rice and barley.

Aim 2: Integrate transcriptomic data and phylogenetic information to perform a comparative analysis between monocots and dicots to identify common and opposite stress responses

The ortholog relationships between genes within and between different species, which represents the basis for comparative transcriptomics, will be computationally inferred for all protein-encoding genes of the three species. Combining phylogeny with transcriptomic profiling will then be used to identify common and opposite responses to stress. This will help to understand how dynamic regulatory networks in species with different morphology and adaptations to different environments respond to adverse environmental conditions.

Aim 3: Identify regulatory pathways for both conserved and opposite responses to stress in the different species

Dissect regulatory mechanisms by focussing on specific gene families or groups of genes with important functions in the plant stress response, especially transcription factors and their corresponding cis-regulatory motifs. The focus will be on organellar signalling pathways whose importance in the plant stress response have only recently emerged in Arabidopsis, while their counterparts in monocot species is mostly unknown.

1.5.2 Analysis of the transcriptional and post-transcriptional regulation of AOX isoforms

Plants lacking AOX1a do not show any apparent phenotypic changes under non-stress conditions (chapter 1.4.1), while induction of mitochondrial dysfunction leads to severe phenotypic changes including necrotic leaves. Several explanations why other AOX isoforms cannot compensate the lack of AOX1a under stress conditions have been proposed in the literature, but the underlying regulatory mechanisms are still unknown. Therefore, the aim of chapter 3 is to analyse whether different transcriptional and/or post-translational regulation is the reason why different AOX isoforms cannot compensate for each and to better understand their specific roles.

Aim 1: Determine if insufficient protein amounts of other AOX isoforms prevent the compensation for a loss of AOX1a function.

Using transgenic *aox1a* knock-out mutants complemented with coding sequences of *AOX1a, AOX1c and AOX1d* under the control of the constitutively active Cauliflower Mosaic Virus (CaMV) 35S promoter, to how transcript abundance translates into protein level and ultimately in stress tolerance.

Aim 2: Identify differences in the transcriptional regulation of AOX isoform that explain the lack of compensation for AOX1a mutation.

In an *aox1a* mutant background transgenic lines will be generated that harbor constructs of all reciprocal AOX promoter-coding sequence combinations. This will reveal isoform-specific transcriptional regulatory mechanisms and comparative analysis of transcript and protein levels in the corresponding lines after stress treatment will explain the lack of compensation.

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CHAPTER 2

Cross-species transcriptomic analyses reveals common and opposite responses of conserved genes in Arabidopsis, rice and barley following oxidative stress and hormone treatment

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2.1 Abstract

Translation of knowledge from model to crop species requires insight into the level of conservation of response networks. Transcriptomic responses of *Arabidopsis thaliana*, *Hordeum vulgare* and *Oryza sativa* to oxidative stress and hormone treatments was undertaken to compare responses between species. The number of differentially expressed genes (DEGs) was observed to vary greatly between treatments and species. At least 70% of DEGs overlapped with at least one other treatment within a species, indicating overlapping response networks. Responses differed between species irrespective of phylogenetic distance. Orthologous DEGs were grouped as common or oppositely responsive between at least two species for each treatment. Remarkably, 15% to 34% of these showed opposite trends, indicating diversity in responses, despite orthology. Greater conservation was observed between rice and barley responses, with notable exceptions, e.g. more overlap between barley and Arabidopsis in response to ABA. DEGs with common responses to four treatments across the three species were identified and these correlated with experimental data showing the functional importance of these genes in biotic/abiotic stress responses. The mitochondrial dysfunction response was highly conserved in all three species in terms of responsive genes and regulation via the mitochondrial dysfunction element. Many opposite responses among the three species were opposite in multiple stresses, highlighting fundamental differences in the responses and regulation of these between species. Prominent among these differences were confirmed salt-stress responsive genes, suggesting a distinct, species-specific regulatory role for these components. This provides a roadmap for translation of knowledge or functions between species.

Keywords: *Arabidopsis thaliana*, *Oryza sativa*, *Hordeum vulgare*, Arabidopsis, Rice, Barley, stress, oxidative, hormone, transcriptome, orthology

2.2 Introduction

Throughout their lifecycle, plants are exposed to a variety of non-optimal growth conditions lasting from hours and days to seasons. For survival, plants must respond to these conditions using adaption as a long-term transgenerational means, acclimate as a medium-term response and/or trigger a stress response for short term response. The ability to sense and respond to changes in different time spans will ultimately decide if a species survives. Plants are challenged by abiotic and biotic stresses. For biotic stress the perception of microbial pathogens is often by a specific receptor-ligand interaction and thus the ability to recognise a pathogen is often the difference between tolerance and susceptibility (Chiang and Coaker, 2015, Li et al., 2020, Wilkinson et al., 2019). For abiotic challenges, ranging from drought, heat and flooding to nutrient or light limitation or excess, the perception and survival can be more complex. For instance, with flooding, the non-optimal condition is multi-factorial where in addition to oxygen being limited, light is also much reduced and the aqueous environment may leave a plant more sensitive to biotic infection (Voesenek et al., 2016). Various proteins that are altered by these sub-optimal conditions can trigger a signalling pathway, are essential for acclimation and have greatly increased our understanding of abiotic stress perception (Lamers et al., 2020). As plants are exposed to multiple abiotic and biotic stimuli, along with variations in the environment; from soil to beneficial microbial interactions, integration of these various signals is required throughout development to optimise growth (Peck and Mittler, 2020).

Reactive oxygen species (ROS) have emerged as key signalling molecules for a variety of adverse conditions. ROS play key roles in plant growth and development, thus their signalling role in stress responses integrates plant growth with environmental conditions (Yamada et al., 2020, Waszczak et al., 2018). The type of ROS produced, and where, defines signalling cascades and how the specificity of signalling is achieved, is clearly an important feature, determined in part by the fact that most ROS species, with the exception of hydrogen peroxide (H_2O_2), cannot travel far beyond the site of production. Therefore, their immediate interaction with proteins and metabolites is a feature of how ROS signals are transmitted (Waszczak et al., 2018, Willems et al., 2016). Classification of ROS signalling signatures using tools such as ‘ROS wheel’ or ‘Rosmeter’ allows for the comparison of changes in transcriptomic patterns to known perturbations to determine the site of stress perception and signal transduction (Willems et al., 2016, Rosenwasser et al., 2013). At a tissue level, the role of ROS in the regulation of root elongation is detailed to the extent of defining the REDOX sensitive transcription factors regulating this process, and perturbation of ROS abundance in root cells alters growth (Yamada et al., 2020, Fernandez-Marcos et al., 2017, Tsukagoshi et al., 2010). In response to non-optimal growth conditions, ROS also plays a fundamental role, with apoplastic enzymes such as

peroxidases, polyamine oxidases and the respiratory burst oxidase homolog (RBOH) responsible for producing ROS, with the latter considered responsible for the production of ROS from a variety of abiotic and biotic sources (Waszczak et al., 2018).

ROS signalling takes place intra- and inter-cellularly, as well as systematically in plants. Peroxisomes, chloroplasts and mitochondria are intracellular sources, which constantly produce different ROS species that initiate signalling pathways (Noctor et al., 2018). While the peroxisomes are a major producer of H_2O_2, efficient detoxification systems such as catalase mean that under steady state conditions there is little signal generation as a result (Foyer and Noctor, 2016). However, inactivation of catalase by salicylic acid (SA) (Rao et al., 1997), activation by Ca^{2+} (Zou et al., 2015), and interacting proteins such as NO CATALASE ACTIVITY or NUCLEREDOXIN 1 (Li et al., 2015, Kneeshaw et al., 2017), all regulate the activity of catalase. This shows the complexity of ROS signalling due to the interaction of different pathways, i.e. inactivation of catalase by SA results in an inhibition of both auxin and jasmonic acid signalling (Yuan et al., 2017). Chloroplasts produce a variety of ROS, including uniquely singlet oxygen ($^1O_2$) that is inactivated by interaction with carotenoids and other molecules (Ramel et al., 2012). The interaction with carotenoids produces β-cyclocitral that is involved in retrograde signalling (D'Alessandro et al., 2018). Both chloroplast and mitochondria produce superoxide ($O_2^{\cdot-}$) that is rapidly converted to H_2O_2 by superoxide dismutase. In both organelles ROS signalling via H_2O_2 triggers transcriptional responses with various sensors, mediators and effectors characterised (Noctor et al., 2018). In the last decade, chloroplasts and mitochondria have emerged as important hubs for sensing and responding via retrograde signalling. Five chloroplast signalling pathways depending on tetrapyrrole, redox/ROS, plastid gene expression, metabolites such as 3′-PHOSPHOADENOSINE-5′-PHOSPHATE (PAP), 2-C-METHYL-D-ERYTHRITOL-2,4-CYCLOPYROPHOSPHATE (MEcPP), and dual-located proteins have all been demonstrated to be involved in signalling to different extents (Crawford et al., 2018, Leister, 2019, Chan et al., 2016). Mitochondrial signalling pathways are required for optimal growth and development as disruption of these signalling pathway leads to severely altered growth and stress response phenotypes (Meng et al., 2019, Ng et al., 2014). Along with a variety of plant hormones that regulate growth and development (auxin, cytokinins) or play role in stress responses (abscisic acid, ABA; salicylic acid, SA; jasmonic acid, JA; ethylene) this leads to a complex network of interacting signalling pathways to control plant growth and development.

Environmental constraints are predicted to increase over the next decades due to changing climate conditions with heat waves, drought periods, water scarcity but also increased duration and frequency of precipitation causing flooding, significantly reducing agricultural productivity with great economic consequences (Zhao et al., 2017, Zandalinas

et al., 2018, Wang et al., 2003). The main focus of crop breeding programs in the past, however, has been the maximisation of crop yields, rather than efforts that aim to improve stress tolerance, especially abiotic stress tolerance (Gilliham et al., 2017). The natural adaptation to new environmental conditions is limited by the fast-changing climate (Messerer et al., 2018). Extensive research efforts are necessary to tackle these issues, hence understanding the mechanisms of plants to adjust to adverse environmental conditions is amongst the most significant domains in plant research (Loudet and Hasegawa, 2017).

In order to increase abiotic stress tolerance in crop species, research has targeted regulatory genes to alter the underlying signalling network (Peck and Mittler, 2020), and many transcription factors have been identified in crop species that confer abiotic stress tolerance (Li et al., 2014, Shen et al., 2017, Sarkar et al., 2019, Visioni et al., 2019). Transcription factors are central switches in the regulatory circuitry and represent ideal tools for engineering crop species with enhanced stress tolerance potentially against multiple stresses simultaneously (Wang et al., 2016). Advances in in functional genomics tools and NGS technology and the availability of high-quality reference genomes (Kawahara et al., 2013, Appels et al., 2018, Mascher et al., 2017) for the most important crop species have and will improve the identification of candidate genes and further enable the comparison between species (Barabaschi et al., 2016).

In this work, a comparative transcriptome analysis in response to different treatments between dicot model *Arabidopsis thaliana* and the two agronomic monocot species *Oryza sativa* (rice) and *Hordeum vulgare* (barley) was performed to identify conserved or opposite responses. A variety of treatments to generate changes in ROS and alter hormones levels were used to investigate conserved and opposite responses between species. Construction of orthogroups (OGs) in combination with comprehensive gene expression profiling was used to identify commonalities and species-specific differences in a defined biological context.

2.3 Results

2.3.1 Dynamic expression responses to stress in Arabidopsis, rice and barley

The transcriptome responses of a model species (Arabidopsis) and two crop species (rice and barley) to six treatments designed to mimic stress responses was analysed by using either hormones (ABA and SA); treatments that cause oxidative stress (3AT, MV); inhibit respiration (AA) or induce genetic damage (UV) (Table 1). To ensure efficacy of application for the six treatments (Table 1) in this study, the expression of marker genes

was examined by qRT-PCR in all three species and the induction of these were as expected (Table S1). RNAseq analysis for the response to the different treatments (Table 1) revealed that out of the 19,700, 22,609 and 24,541 detected genes annotated in the Arabidopsis (Tair10), rice (IRGSP-1.0) and barley (IBSCv2) genomes, respectively, a total of 10,462, 13,735 and 14,470 genes were responsive to at least one treatment when compared to the mock treatment (Figure 1a; Table S2a-c).

Table 1. Overview of treatments used in this study to induce stress response pathways

Treatment	Target	Stress	References
Antimycin A	Inhibition of mitochondrial electron transport, cytochrome bc1 complex.	ROS Oxidative stress	Slater, 1973
3-amino-1,2,4-triazole	Inhibition of catalases and carotenoid biosynthesis	ROS Oxidative stress	Margoliash et al., 1960 Yang et al., 2019 Su et al., 2018
Methyl viologen (Paraquat)	Competes for electrons with PSI (inhibition of photosynthesis)	ROS Oxidative stress	Hassan, 1984 Fuerst and Norman, 1991
Salicylic acid	SA Receptors	Activation of stress-signalling pathways	Ding et al., 2018 Kaltdorf and Naseem, 2013
Abscisic acid	ABA Receptors	Activation of stress-signalling pathways	Ma et al., 2009 Park et al., 2009 Santiago et al., 2009
Ultraviolet radiation (UV-C)	DNA, protein and lipids Photosynthesis	DNA damage Photoinhibition	Stapleton, 1992 Gao et al., 2008 Urban et al., 2016

For all three species, the largest number of differentially expressed genes (DEGs; FDR < 0.05 and |log2FC| >1) was observed in response to ultra-violet (UV) treatment and smallest number observed in response to antimycin A (AA) treatment (Figure 1a). The number of treatment specific DEGs for each treatment ranged from 2% to 30% in the three species, with only 2% of AA responsive DEGs in Arabidopsis showing a treatment specific response (Figure 1a). Twice the number of DEGs were responsive to catalase inhibitor 3-amino-1,2,4-triazole (3AT) in the monocots (rice: 7032 DEGs; barley: 6107 DEGs) compared to the dicot species Arabidopsis (2758 DEGs; Figure 1a). The response to 3AT is also more distinct in monocots, with 20.8% in rice and 19.9% in barley of treatment specific DEGs observed in these, compared to 8.3 % in Arabidopsis. Notably, the number of DEGs following ABA treatment was more similar between Arabidopsis (4692 DEGs) and barley (5227 DEGs) compared to almost half that number in rice (2805 DEGs).

The ABA response in Arabidopsis was also the most distinct compared to other treatments with 30.2% of all DEGs being treatment specific. Similarly, the number of DEGs following SA treatment was more similar between Arabidopsis (5329 DEGs) and rice (5223 DEGs) compared to barley (1617 DEGs), which showed around one third of that number of DEGs (Figure 1a). The response to methyl viologen (MV), which leads to oxidative stress and

the formation of ROS under illumination in the chloroplast differed between all three species with the largest number of DEGs observed for barley (6811 DEGs), followed by Arabidopsis (4538 DEGs) and rice (3122 DEGs). For each species, two-way comparisons of the number of overlapping DEGs between treatments were carried out (Figure 1b). Overall, the overlaps were greater in Arabidopsis than in rice or barley (Figure 1b). For example, more than half of all DEGs responsive to AA and 3AT treatment in Arabidopsis overlapped with the other four stresses, with the exception of ABA (Figure 1b), while the DEGs responsive to AA and 3AT were more distinct in barley and rice, having a smaller percentage of overlapping DEGs (Figure 1b). 3AT and MV responses displayed the greatest overlap in rice, whereas in barley the greatest overlap was observed between 3AT and UV (Figure 1b). Thus, the extent of responses and overlaps of the DEGs were not always more similar in rice and barley when compared to Arabidopsis, indicating species-specific responses irrespective of a closer phylogenetic relationship between the monocot species.

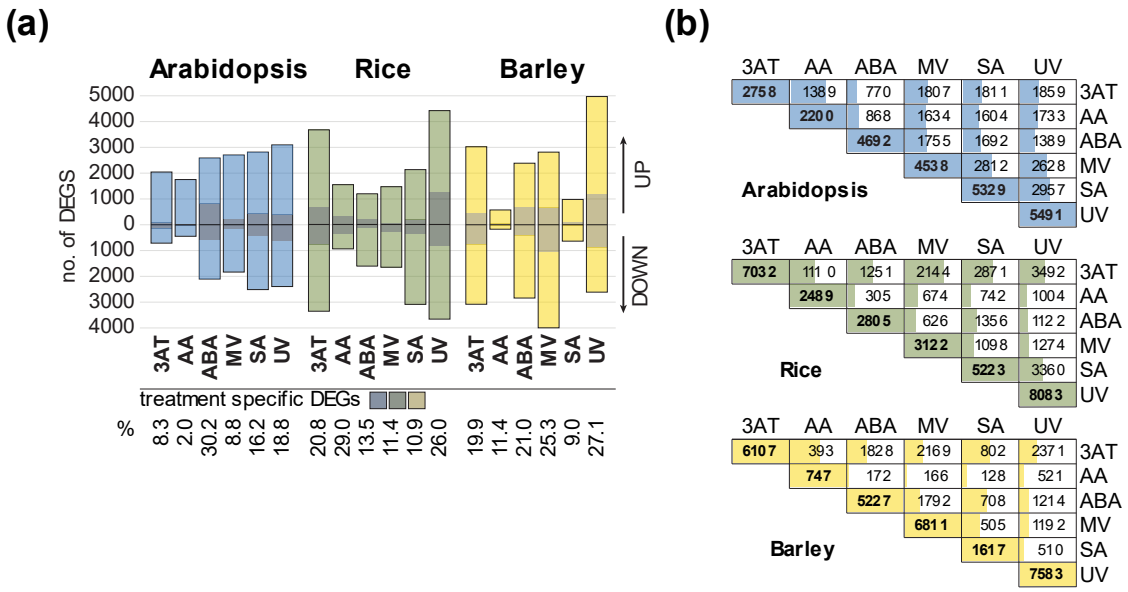


Figure 1 Summary of transcriptome responses to treatments in Arabidopsis, rice and barley.
(a) Numbers of differentially expressed genes (DEGs; |log2 (fold change)|>1, FDR<0.05) and for each treatment in Arabidopsis (At), rice (Os) and barley (Hv). The percentage of species-specific DEGs for each treatment are indicated by dark shading; 3AT = 3-amino-1,2,4-triazole, AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation. (b) Matrix showing the number of overlapping DEGs between treatments (two-way comparisons) in all species.

To gain insight into the functions of these DEGs, a PageMan over-representation analysis (Usadel et al., 2006) was carried out for all three species and the significantly over-represented categories (Fisher's test, $p<0.05$; Table S3) in the up- and down-regulated genes across at least three treatments were identified (Figure S1a, b). Many of these were

also common across the three species, revealing conserved functional responses at a transcriptome level to treatments (Figure 2a). As expected, genes encoding oxidative phosphorylation, tyrosine kinase-like (TKL) protein kinases, ABC transporters and miscellaneous oxidoreductases were significantly enriched in the up-regulated gene-sets of all three species, as well as genes encoding APETALA2/ETHYLENE responsive element binding factors (AP2/ERF), heat shock factors (HSF) and NAC and WRKY domain containing transcription factors (Figure 2a). Enrichment of photosynthesis, RNA biosynthesis and RNA processing were observed among the down-regulated genes in all three species (Figure 2b). However, there were also notable differences between species. For example, protein modification was over-represented in up-regulated Arabidopsis genes in almost all sub-categories in response to all treatments, but this was seen less so in rice and barley, particularly for phosphorylation and TKL protein kinases (Figure 2a). The pattern of enrichment for protein biosynthesis functions in the down-regulated Arabidopsis genes also differed substantially compared to rice and barley (Figure 2a). Thus, the effect of the treatments on protein modification, homeostasis and synthesis seemed to be different between dicots and monocots. Likewise, the processes of solute transport seemed to be less affected in Arabidopsis in down-regulated genes compared to the two monocots (Figure 2b). The enrichment of vesicle trafficking among up-regulated genes was more similar between Arabidopsis and rice, while these were under-represented in barley. Overall, fewer functional categories were enriched in the up-regulated rice genes compared to Arabidopsis and barley, while the down-regulated genes had similar enriched categories. By contrast, the down-regulated genes in response to ABA were more similar in Arabidopsis and barley than rice e.g. for photosynthesis (Figure 2b).

Species specific differences were also revealed in this way, for example the genes encoding oxidoreductases were enriched among the up-regulated genes following three stresses in Arabidopsis, five stresses in barley and only one stress in rice (Figure 2b). Among the down-regulated genes, significant enrichment of genes encoding ribosomal proteins were observed following 3AT, ABA and MV treatment in both rice and barley, while this was only observed in response to ABA in Arabidopsis (Figure 2b). Furthermore, the PLATZ family of TFs in Arabidopsis, MYB family in rice and C2H2-ZF family in barley were enriched in the up-regulated genes in response to three stresses in each species respectively while not showing the same enrichment pattern in the other two species (Figure S1a; Table S3). Thus, the number and enriched functional categories differed between species and treatments indicating these differ irrespective of phylogenetic distance.

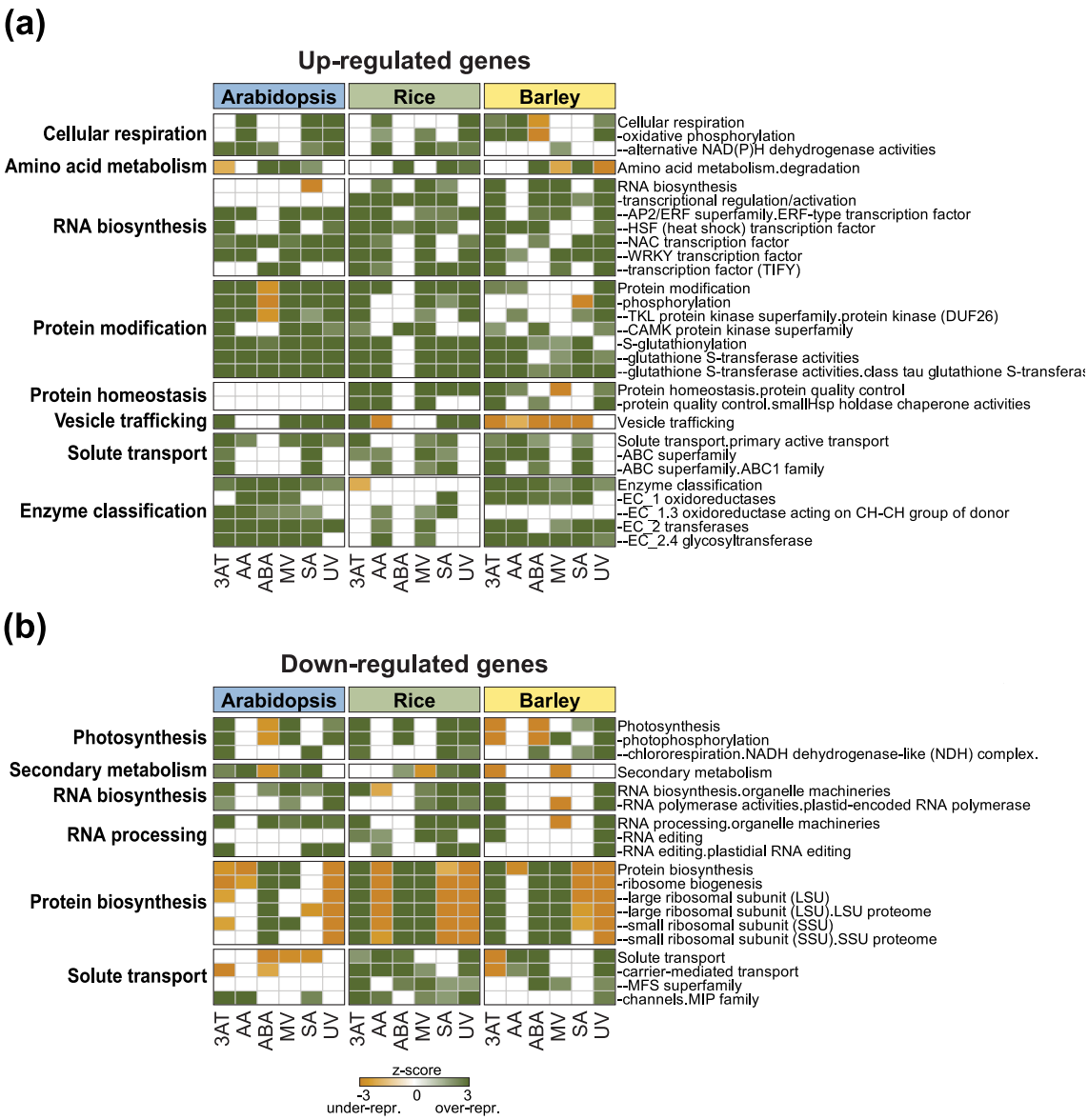


Figure 2 Conserved over-represented functional categories.

Analysis of functional categories in all species was performed using PageMan (Usadel et al., 2006) for (a) up-regulated and (b) down-regulated genes responsive to different treatments in Arabidopsis, rice and barley; 3AT = 3-amino-1,2,4-triazole, AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation. Only the functional categories that were significantly over-represented (PageMan- Fisher's test, $p<0.05$) in response to at least three stresses and two species are shown.

2.3.2 Responses of orthologous genes between species

In order to directly compare transcriptomic responses between the three species, orthologous genes were identified using OrthoFinder (Emms and Kelly, 2015) to define sets of orthologous genes, termed orthogroups (OGs), which contain orthologs and paralogs in the three species. In this way, 9371 orthogroups were identified containing DEGs responsive to at least one stress in at least one species (Table S4). Orthogroups

containing DEGs responsive in the same manner (i.e. up/down-regulated) between species were defined as common, while those containing oppositely responsive orthologous DEGs between at least two species were defined as opposite (Figure 3a). For orthogroups containing more than one gene, if the group contained some genes that were up-regulated and other genes that were down-regulated from the same species within an orthogroup, the orthogroup was excluded from the analyses. This revealed that of the 2758, 7032 and 6107 DEGs in Arabidopsis, rice and barley, respectively, responsive to 3AT (Figure 1a), 845, 1843 and 1794 genes had orthologs that showed a conserved response with at least one other species (Figure 3a). In Arabidopsis, the 845 DEGs represented 30% of all DEGs responsive to 3AT, with 331 of these genes showing a conserved response with their orthologous genes in both rice and barley, while 290 and 224 genes showed a conserved response with rice only and barley only, respectively (Figure 3a). By contrast, of all Arabidopsis DEGs responsive to 3AT (Figure 1A), 8% (228 genes) showed opposite responses to rice and/or barley. Twenty-five genes showed an opposite response to orthologous genes in both rice and barley, while 107 and 96 genes had an opposite response to orthologous genes in rice and barley only, respectively (Figure 3a). The conserved responses and opposite responses to 3AT in barley and rice contained more than twice as many genes as Arabidopsis. Most of these, i.e. 1202 and 1219 genes, showed a conserved response between rice and barley, respectively, while 402 and 409 were oppositely responsive (Figure 3a). Similar trends were observed in response to UV with 1501, 2803 and 2938 DEGs showing a conserved response with at least one other species (Figure 3a), making up 27 to 39% of all DEGs (Figure 1a).

Overall, at least twice as many genes showed conserved responses with one or more species than those that showed opposite responses (Figure 3a). In barley more genes showed a conserved response with orthologous rice genes than Arabidopsis genes for 3AT, AA, MV, SA and UV (Figure 3a). Similarly, in rice greater conservation was seen with the orthologous genes in barley in response to 3AT, ABA, MV and UV (Figure 3a). Thus for at least four stresses, rice and barley showed more conservation in their response compared to Arabidopsis, which was not surprising given their closer phylogenetic relationship. However, identifying greater conservation with Arabidopsis for rice in response to AA (227 genes) and SA (569 genes), and barley in response to ABA (575 genes) indicates that there are exceptions where greater similarity with the dicot Arabidopsis is seen (Figure 3a).

Examination of the oppositely responsive genes revealed that in response to 3AT and UV, 7 to 9% of all genes (228-581 DEGs) showed opposite responses in at least one other species (Figure 3a). Oppositely responsive genes in rice make up 10% and 11% of all DEGs responsive to ABA and MV, respectively, with 293 and 345 genes observed in these

sets, while the 238 oppositely SA-responsive DEGs in barley make up 14% of all the DEGs (Figure 3a). Thus, apart from AA, 228 to 581 DEGs were orthologous to oppositely responsive genes in at least one other species (Figure 3a), making up 6% to 14% of all DEGs in response to stress in all five stresses. Thus, when only the differentially expressed orthologous genes are considered, there is greater conservation observed based on phylogeny (Figure 3) than with the number of genes and enriched functional categories (Figure 1 & 2). However, the greater conservation between Arabidopsis and rice for AA and SA, and Arabidopsis and barley for ABA (Figure 3a) reveals diversification of responses do occur independent of phylogeny.

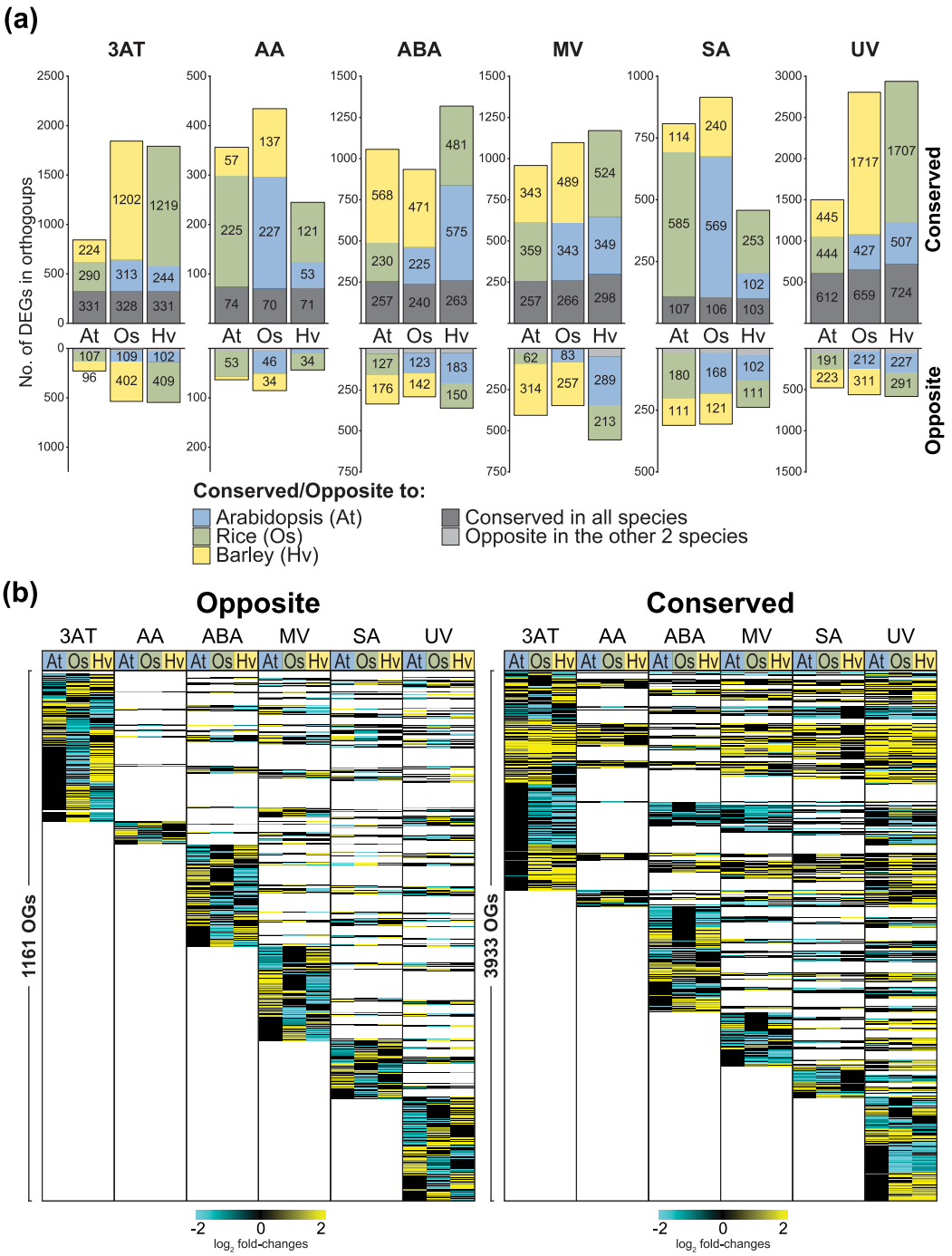


Figure 3. Transcriptome responses of orthologous genes in Arabidopsis, rice and barley in response to different treatments.

(a)The number of DEGs in Arabidopsis (At), rice (Os) and barley (Hv) that were orthologous and showed conserved or opposite responses to different stress treatments are indicated for each species. Conserved responses were defined as genes that were orthologous with at least one other species that were also up- or down-regulated in transcript abundance in response to the treatment. An opposite response was defined as genes that were orthologous in at least one species that showed the opposite response in one or both of the other species. Both conserved and opposite responses exclude any orthogroups in which genes displayed both up- and -down-regulation within the same orthogroup. Orthogroups were defined using OrthoFinder as outlined in methods. (b) Heatmap of log2-transformed fold-changes for all orthogroups (OGs) that contain differentially expressed genes (DEGs) showing opposite and conserved responses (up/down-regulated) in all three species in response to the six treatments. 3AT = 3-amino-1,2,4-triazole; AA = antimycin A; ABA = abscisic acid; MV = methyl viologen; SA = salicylic acid; UV = ultraviolet radiation.

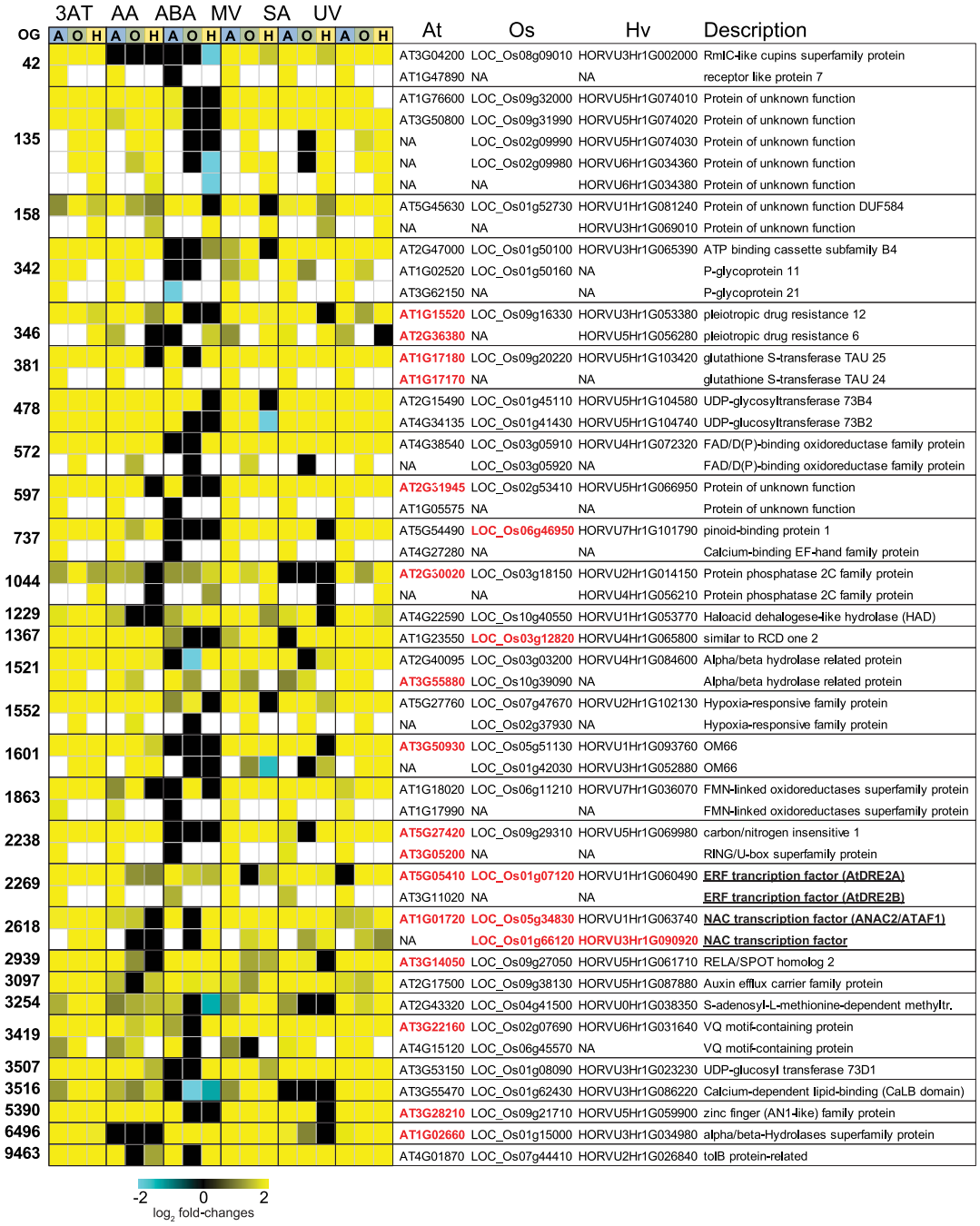
Overall, the examination of oppositely responsive DEGs revealed 1661 orthogroups containing 2275, 2365 and 2426 orthologous DEGs in Arabidopsis, rice and barley, respectively, that were oppositely responsive to at least one species and in at least one treatment (Figure 3b). Remarkably, 38% of these 1661 orthogroups also showed opposite responses in more than one treatment, with 628 orthogroups containing 1024, 1083 and 1115 DEGs in Arabidopsis, rice and barley that were oppositely responsive in more than one stress (Figure 3b). The greatest number of oppositely responsive genes within orthogroups were observed in response to UV, followed by MV, 3AT, ABA and SA with the smallest number observed for AA (Figure 3b; Figure S2). When the numbers of specific and overlapping oppositely responsive OGs were examined, 407 out of the 1161 OGs showed overlapping responses i.e. also opposite in other stresses. For example, 200 OGs out of the 445 OGs within the oppositely responsive orthogroups in response to 3AT also showed opposite responses in at least one other stress (Figure S2).

Similar examination of genes showing conserved responses (Figure 3a) revealed 3933 orthogroups containing 5186, 5560 and 5680 Arabidopsis, rice and barley orthologous DEGs, respectively, shared a conserved response with at least one other species and in at least one stress (Figure 3b). Visualisation of these revealed that many of these show conserved responses in more than one stress, making up 48%, i.e. 1874 out of the 3933 orthogroups (Figure 3b; Figure S2). When the numbers of treatment-specific and overlapping OGs were examined per stress, it is evident that apart from UV, the majority of orthogroups showed overlapping responses in other stresses (Figure S2).

2.3.3 Identification of common responsive genes across species

Identification of common responses of the DEGs in at least 3 out of 6 treatments across the three species revealed 105 OGs with 158, 146 and 157 Arabidopsis, rice and barley genes, respectively (Table S5a). GO annotations of the Arabidopsis genes in this set

revealed 93 of the 158 genes were in the response to stimulus category and over-representation analysis revealed the top two categories were; response to molecule of bacterial origin and response to chitin (Fisher's test, FDR p<0.05) (Table S6a).



OG	At	Os	Hv	Description
42	AT3G04200	LOC_Os08g09010	HORVU3Hr1G002000	RmlC-like cupins superfamily protein
	AT1G47890	NA	NA	receptor like protein 7
135	AT1G76600	LOC_Os09g32000	HORVU5Hr1G074010	Protein of unknown function
	AT3G50800	LOC_Os09g31990	HORVU5Hr1G074020	Protein of unknown function
	NA	LOC_Os02g09990	HORVU5Hr1G074030	Protein of unknown function
	NA	LOC_Os02g09980	HORVU6Hr1G034360	Protein of unknown function
	NA	NA	HORVU6Hr1G034380	Protein of unknown function
158	AT5G45630	LOC_Os01g52730	HORVU1Hr1G081240	Protein of unknown function DUF584
	NA	NA	HORVU3Hr1G069010	Protein of unknown function
342	AT2G47000	LOC_Os01g50100	HORVU3Hr1G065390	ATP binding cassette subfamily B4
	AT1G02520	LOC_Os01g50160	NA	P-glycoprotein 11
	AT3G62150	NA	NA	P-glycoprotein 21
346	AT1G15520	LOC_Os09g16330	HORVU3Hr1G053380	pleiotropic drug resistance 12
	AT2G36380	NA	HORVU5Hr1G056280	pleiotropic drug resistance 6
381	AT1G17180	LOC_Os09g20220	HORVU5Hr1G103420	glutathione S-transferase TAU 25
	AT1G17170	NA	NA	glutathione S-transferase TAU 24
478	AT2G15490	LOC_Os01g45110	HORVU5Hr1G104580	UDP-glycosyltransferase 73B4
	AT4G34135	LOC_Os01g41430	HORVU5Hr1G104740	UDP-glucosyltransferase 73B2
572	AT4G38540	LOC_Os03g05910	HORVU4Hr1G072320	FAD/D(P)-binding oxidoreductase family protein
	NA	LOC_Os03g05920	NA	FAD/D(P)-binding oxidoreductase family protein
597	AT2G31945	LOC_Os02g53410	HORVU5Hr1G066950	Protein of unknown function
	AT1G05575	NA	NA	Protein of unknown function
737	AT5G54490	LOC_Os06g46950	HORVU7Hr1G101790	pinoid-binding protein 1
	AT4G27280	NA	NA	Calcium-binding EF-hand family protein
1044	AT2G30020	LOC_Os03g18150	HORVU2Hr1G014150	Protein phosphatase 2C family protein
	NA	NA	HORVU4Hr1G056210	Protein phosphatase 2C family protein
1229	AT4G22590	LOC_Os10g40550	HORVU1Hr1G053770	Haloacid dehalogese-like hydrolase (HAD)
1367	AT1G23550	LOC_Os03g12820	HORVU4Hr1G065800	similar to RCD one 2
1521	AT2G40095	LOC_Os03g03200	HORVU4Hr1G084600	Alpha/beta hydrolase related protein
	AT3G55880	LOC_Os10g39090	NA	Alpha/beta hydrolase related protein
1552	AT5G27760	LOC_Os07g47670	HORVU2Hr1G102130	Hypoxia-responsive family protein
	NA	LOC_Os02g37930	NA	Hypoxia-responsive family protein
1601	AT3G50930	LOC_Os05g51130	HORVU1Hr1G093760	OM66
	NA	LOC_Os01g42030	HORVU3Hr1G052880	OM66
1863	AT1G18020	LOC_Os06g11210	HORVU7Hr1G036070	FMN-linked oxidoreductases superfamily protein
	AT1G17990	NA	NA	FMN-linked oxidoreductases superfamily protein
2238	AT5G27420	LOC_Os09g29310	HORVU5Hr1G069980	carbon/nitrogen insensitive 1
	AT3G05200	NA	NA	RING/U-box superfamily protein
2269	AT5G05410	LOC_Os01g07120	HORVU1Hr1G060490	ERF trancription factor (AtDRE2A)
	AT3G11020	NA	NA	ERF trancription factor (AtDRE2B)
2618	AT1G01720	LOC_Os05g34830	HORVU1Hr1G063740	NAC transcription factor (ANAC2/ATAF1)
	NA	LOC_Os01g66120	HORVU3Hr1G090920	NAC transcription factor
2939	AT3G14050	LOC_Os09g27050	HORVU5Hr1G061710	RELA/SPOT homolog 2
3097	AT2G17500	LOC_Os09g38130	HORVU5Hr1G087880	Auxin efflux carrier family protein
3254	AT2G43320	LOC_Os04g41500	HORVU0Hr1G038350	S-adenosyl-L-methionine-dependent methyltr.
3419	AT3G22160	LOC_Os02g07690	HORVU6Hr1G031640	VQ motif-containing protein
	AT4G15120	LOC_Os06g45570	NA	VQ motif-containing protein
3507	AT3G53150	LOC_Os01g08090	HORVU3Hr1G023230	UDP-glucosyl transferase 73D1
3516	AT3G55470	LOC_Os01g62430	HORVU3Hr1G086220	Calcium-dependent lipid-binding (CaLB domain)
5390	AT3G28210	LOC_Os09g21710	HORVU5Hr1G059900	zinc finger (AN1-like) family protein
6496	AT1G02660	LOC_Os01g15000	HORVU3Hr1G034980	alpha/beta-Hydrolases superfamily protein
9463	AT4G01870	LOC_Os07g44410	HORVU2Hr1G026840	tolB protein-related

Figure 4 Conserved responses in gene expression of orthologous genes to stress in Arabidopsis, rice and barley.

Log2-transformed fold-changes shown as a heatmap for the 20 orthogroups (OGs) that contain differentially expressed genes (DEGs) showing conserved responses to 4 out of 6 stress treatments (3AT = 3-amino-1,2,4-triazole, AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation) for each of the three species (At = Arabidopsis, Os =Rice and Hv = Barley). Arabidopsis genes with known functions (Supplemental Table 7a) (mutants/over-expressors) in development, defence and/or redox homeostasis are indicated in red. Underlining indicates transcription factors.

Six of the top ten significantly enriched categories involved response to oxygen, decrease in oxygen levels or hypoxia (Table S6a). Orthologous DEGs that showed an up-regulated response in at least 4 out of 6 treatments in all three species revealed a set of 29 OGs with 43, 40 and 39 genes in Arabidopsis, rice and barley respectively (Figure 4; Table S5b). This set identifies genes with both genetic and transcriptomic conservation, suggesting that the regulation of these may also be conserved.

Closer examination of the Arabidopsis genes revealed that 24 genes showed high or maximal expression during senescence in leaves compared to all other developmental tissues in Arabidopsis (Toufighi et al., 2005, Fucile et al., 2011) (Table S7a). It was also observed that ten genes out of the 43 genes (23%) produce known cell-to-cell mobile mRNAs, representing a significant enrichment (chi-square, $p<0.05$), considering only 2006 genes (approx. 6.7% of all genes in the genome) in total are cell-to-cell mobile (Thieme et al., 2015). Examination of gene functions revealed 19 out of 43 Arabidopsis genes in this common set are known to have a functional role, whereby alteration e.g. mutation/ RNAi/ over-expression of these genes resulted in plants with altered stress responses/development (Table S7a). Five of these genes resulted in altered redox-related changes in the cell (Table S7a). For example, mutation of the gene encoding GLUTATHIONE S-TRANSFERASE (CLASS TAU) 24 (GSTU24, At1g17170) caused increased overall GST activity and altered redox homeostasis (Horvath et al., 2020). A gene encoding a transmembrane protein (At2g31945) and another encoding a glycerolipid A1 lipase annotated as PLASTID LIPASE 2 (At1g02660) (Figure 4) also resulted in reduced oxidative stress tolerance in knock-out plants for these genes (Luhua et al., 2013). Similarly, the zinc finger (AN1-like) family protein STRESS-ASSOCIATED PROTEIN (SAP) 12 has been shown to be under redox-dependent regulation (Stroher et al., 2009) and elevated expression of its regulator miR408 leads to SAP12 induction as well as an increase in cellular antioxidant capacity (Ma et al., 2015) (Table S7a).

Additionally, alterations in the expression of seven genes common in the three species (Figure 4) results in altered biotic stress responses (Table S7a), including for the two ABCG genes (ABCG40 and ABCG34). Mutations of these two results in compromised *Phytophthora brassicae* and *Phytophthora capsici* as well as necrotrophic pathogen resistance, respectively (Wang et al., 2015, Khare et al., 2017). Similarly, the loss-of-function of a MAPK phosphatase (At2g30020) (Shubchynskyy et al., 2017), the over-expression of a cytochrome BC1 synthesis-like outer mitochondrial membrane protein OM66 (At3g50930) (Zhang et al., 2014) and the over-expression of ATL6 (At3g05200) and ATL31 (At5g27420) (Maekawa et al., 2012, Maekawa et al., 2014) result in increased resistance to *Pseudomonas syringae* infection, while the silencing of the VQ motif-containing gene JAV1 (At3g22160) enhances jasmonate-regulated defence against

Botrytis cinerea (Hu et al., 2013) (Table S7a). Two genes encoding auxin transporting ATP-BINDING CASSETTE proteins (At2g47000 and At3g62150) as well as a calcium binding protein encoding gene (At4g27280) were observed to result in altered auxin responses and root formation in corresponding mutant plants (Terasaka et al., 2005, Kamimoto et al., 2012, Hazak et al., 2019). Thus, this set of stress-responsive genes are conserved across Arabidopsis, rice and barley and are involved in redox and defence maintenance, and their conservation in expression implies that these roles and possibly their regulation may also be conserved in the three species. In fact, loss-of-function of the calcium-binding protein OsCCD1 (LOC_Os06g46950), an orthologue of At4g27280 (OG0000737) (Figure 4), is less tolerant to osmotic and salt stresses while overexpression significantly enhances this tolerance in rice (Jing et al., 2016) (Table S7a).

The orthogroup OG0002618 represents an example of conservation across all three species and contains NAC transcription factors that are important for stress responses (Figure 4) (Puranik et al., 2012). ATAF1 (ANAC2; AT1G01720) plays an important role in the crosstalk between abiotic and biotic stress response pathways and acts as an ABA-dependent switch between plant abiotic stress tolerance and defence (Mauch-Mani and Flors, 2009, Liu et al., 2016). Overexpressing ATAF1 in Arabidopsis increases drought tolerance (Wu et al., 2009) and overexpression of the Arabidopsis gene in transgenic rice conferred tolerance to salt stress (Liu et al., 2016). Overexpression of the closest ATAF1 homologs in transgenic rice confers cold and salt tolerance (OsSNAC2; LOC_Os01g66120) (Hu et al., 2008) as well as drought tolerance (OsNAC52; LOC_Os05g34830) (Gao et al., 2010) in an ABA-dependent matter. One of the closest homologs in barley, HvNAC6 (HORVU3Hr1G090920), mediates ABA-dependent defence responses and corresponding knock-down lines are more susceptible to powdery mildew (Chen et al., 2013) (Table S7a).

2.3.4 The mitochondrial dysfunction stimulon is a conserved organellar response

Mitochondria have an important function for stress responses by providing energy. Their endosymbiotic origin and subsequent integration into the cellular processes also made them stress sensors and signalling hubs (Wang et al., 2018). The mitochondrial dysfunction stimulon (MDS) which is part of the mitochondrial retrograde signalling pathway that signals mitochondrial dysfunction caused by genetic, pharmacological or environmental conditions to alter the expression of nuclear genes such as mitochondrial stress marker AOX (De Clercq et al., 2013). NAC transcription factors such as ANAC013 and ANAC017 bind a *cis*-regulatory motif, called mitochondrial dysfunction motif (MDM) which is present in the promoter of several genes that have altered expression in response

impaired mitochondrial function (De Clercq et al., 2013). A motif search identified these binding sites to be present in the promotor region of various *AOX* genes in rice, barley and wheat which suggests conserved MRR pathways across plant families. Conservation of these pathways in monocots is further supported by interaction of ER-membrane bound OsNAC054, involved in ABA-induced leaf senescence in rice that has been shown to specifically bind the MDM (Sakuraba et al., 2020).

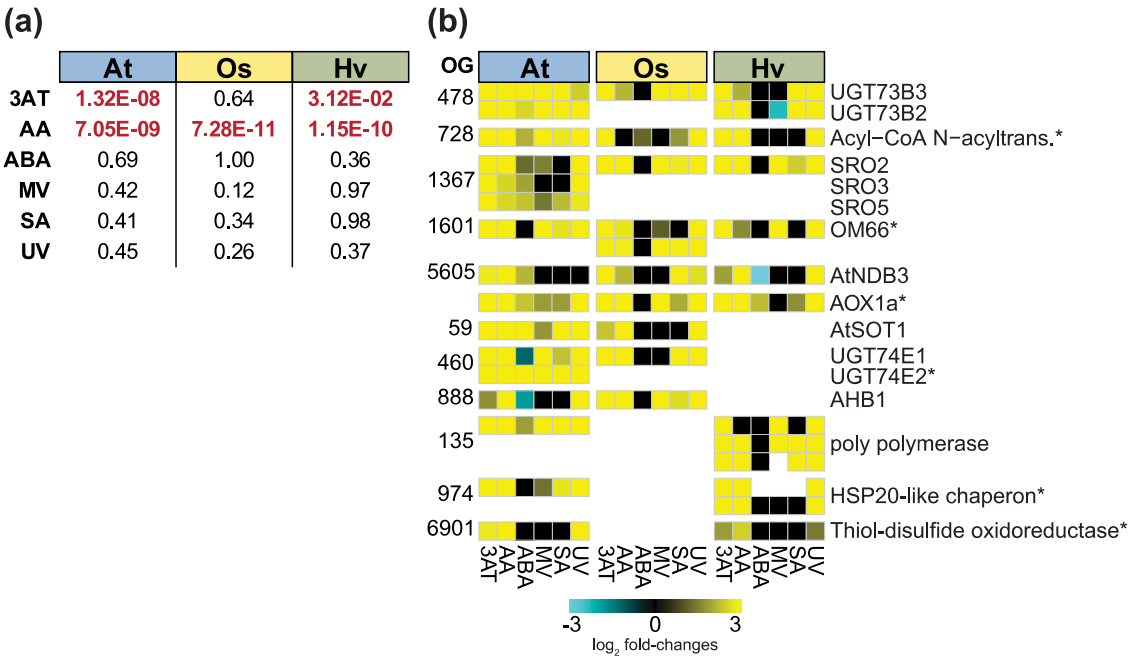


Figure 5 Mitochondrial dysfunction motif (MDM) enrichment in stress treatment specific gene sets and conservation of potential mitochondrial dysfunction stimulon (MDS) genes in Arabidopsis (At), rice (Os) and barley (Hv).

(a) Enrichment analysis of the MDM in the upstream promotor region of specific subsets containing all differentially expressed genes (DEGs; |log2 (fold change)|>1, FDR<0.05) for each stress treatment in At, Os and Hv; 3AT = 3-amino-1,2,4-triazole, AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation. The presence of the stringent MDM in all expressed genes in each species and each stress was used to analyse the significance (p-values; chi-square test) (b) Heatmap showing gene expression levels for DEGs of MDS candidate genes in At, Os and Hv. All genes contain the MDM in their 2 kb promotor region and show differential expression in response to AA and/or 3AT and at least 3 out of 6 stresses in total. The number of each orthogroup (OG) is indicated and known MDS genes are highlighted with an asterisk. Gene names/annotations refer to the genes in Arabidopsis.

Our orthology analysis showed conservation of genes encoding mitochondrial proteins, across Arabidopsis, rice and barley (Table S4) and a common up-regulated expression of *OM66* in four out of the six stress treatments (Figure 4). In addition to this, other mitochondrial components were observed to show common expression responses across species in response to at least one stress (Table S5). In order to further investigate this conservation in monocots and dicots, a pattern search of the stringent MDM

(CTTGNNNNNCAMG) was conducted in the promotor region (2 kb upstream of the translation start site) for all genes that were expressed in this study, not allowing for any permutations, using the Regulatory Sequence Analysis Tools (Medina-Rivera et al., 2015). It is to note that ANAC13 was removed from the list even though it is classified as an MDM gene because the MDM element is not an exact match in the ANAC13 promoter region (De Clercq et al., 2013). A motif enrichment analysis (*chi*-square test, $p < 0.05$) with treatment specific subsets was performed (Table S8a). The enrichment analysis of DEGs for AA revealed that the motif is significantly enriched across all three species and indicates conserved pathways in monocots and dicots in response to mitochondrial dysfunction and MRR (Figure 5, Table S8). By contrast, the MDM in the promoters of 3AT-responsive DEGs is only conserved in Arabidopsis (p value < 0.001; chi-square test) and barley (p value < 0.05; chi-square test), but not in rice (p value = 0.64). ABA, MV, SA and UV show no MDM enrichment for all three species revealing that the gene induction via the MDM is likely specific to mitochondrial oxidative stress, in agreement with functional studies in Arabidopsis (Ng et al., 2013, De Clercq et al., 2013).

To identify new MDS candidate genes, the gene regulatory network (GRN) of ANAC017 defined by Meng et al. (2019) was analysed for the presence of the MDM (Table S8b). ANAC017 is a regulator in the MRR in Arabidopsis and a direct positive regulator of *AOX1a* (Ng et al., 2013). It directly interacts with the MDM and the promotor of ANAC013, an MDS gene itself (De Clercq et al., 2013). All AtNAC017 GRN genes containing the MDM and that are highly stress responsive to AA and 3AT as well as in at least 3 out of 6 treatments were filtered, in accordance with the MDM enrichment analysis. The final list contained 82 candidate genes that were hierarchically clustered together with the MDS genes (Figure S3). The list of candidate genes in Arabidopsis contains 7 UDP-glycosyltransferases (UGTs), which can attach a sugar molecule to various substrate, including auxin to inactivate signalling. In addition to known MDS gene *UGT74E2* (At1g05680), which plays a role in organelle signalling (Kerchev et al., 2014). The widespread induction of various UGT genes under abiotic and biotic stress indicates that they may be involved in modifying a variety of hormonal signalling pathways under mitochondrial retrograde signalling (Rehman et al., 2018). The presence of a cell wall associated kinase expressed in response to biotic stress (Bot et al., 2019, Meier et al., 2010), links mitochondrial signalling to cell wall, as previously shown for ANAC017 that could restore cell wall growth in the presence of inhibitors to cellulose synthase in an unknown manner (Hu et al., 2016). Other ANACs in the candidate list are ANAC044 (At3g01600), characterised to mediate stress induced cell cycle arrest (Takahashi et al., 2019), ANAC053 (At3g10500) which mediates proteasome stress (Gladman et al., 2016) and ROS production during drought (Lee et al., 2012), and ANAC055 (At3g15500) with a

role in mediating drought responses (Fu et al., 2018). Additionally, three members of the SIMILAR TO RCD-ONE family (SRO2/3/5) are also in the list. Combined these examples position mitochondrial signalling as an important hub for a variety of cellular signalling pathways, consistent with emerging roles for mitochondria in flooding response (Meng et al., 2019), interaction with touch signalling (Xu et al., 2019), and the role of ANAC017, considered the master regulator of mitochondrial retrograde signalling, in regulating growth, senescence and cell wall growth (Meng et al., 2019).

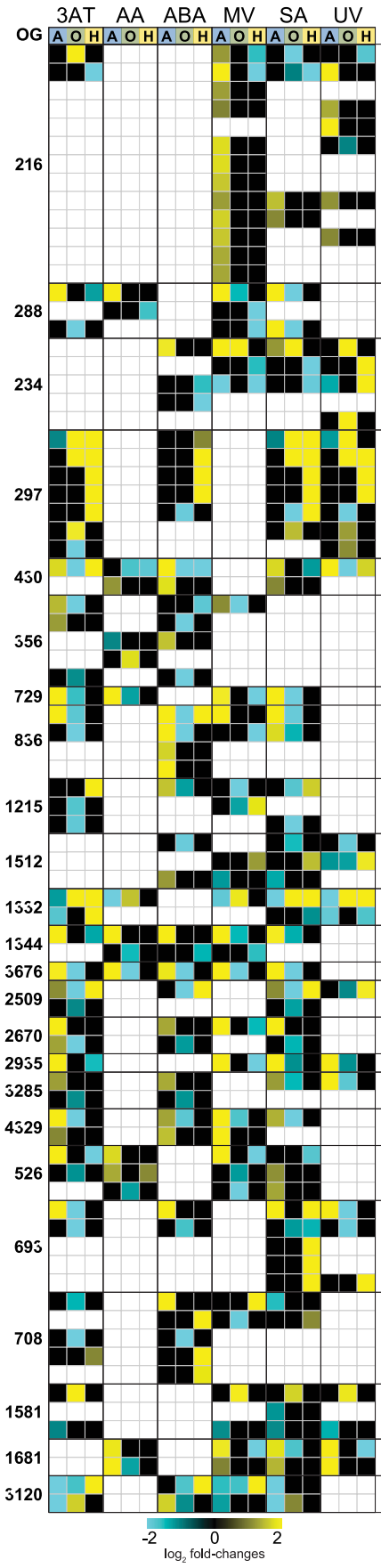
Based on this expanded list of candidates and known MDS genes, the conservation of MDS across the three species was analysed using the inferred OGs (Table S8c). Several OGs were identified that contain genes that also have the MDM and show similar expression patterns across the three species (Figure 5b). This included AOX, which was used as a reference, *SRO2*, 3 and *5*, the alternative dehydrogenase *NDB3*, as well as several glycosyl transferases and Acyl-CoA N-acyltransferase that are present in all three species. Notable is that for barley and rice ABA does not appear to be a major regulator of the MDM-dependent response compared to Arabidopsis (Figure 5b). In rice none of these OGs respond to ABA, while in barley only *NDB3* is negatively regulated by ABA, and only *AOX1a* is slightly up-regulated. This may reflect a divergence in signalling pathways as has been previously reported for biotic and drought stress (Baggs et al., 2020). The MRR marker gene *AtAOX1a* as well as *AOX1a* in rice and barley have previously been reported to contain the MDM which was confirmed in this analysis. These proteins have similar gene expression in response to the different stresses and are all up-regulated in at least 5 out of 6 stress treatments, although in barley *AOX1a* is not responsive to MV (or any other *AOX* gene). Other MDS genes like At*OM66* and Acetyl-CoA N-acyltransferase (At2G32020) are conserved as well and have rice and barley homologs that have the MDM and similar expression pattern (Figure 5b). Novel identified MDS candidates are At*SRO2/3/5* and its homologs in barley and rice. These are members of the plant specific SRO gene family that play important functions in stress responses and development. This family includes RADICAL-INDUCED CELL DEATH 1 (RCD1), a nuclear-localized transcriptional regulator, which was recently shown to suppress the activity of ANAC013 and ANAC017 and increased expression of MDS genes affecting ROS homeostasis in the chloroplasts (Shapiguzov et al., 2019). The corresponding OG with MDS candidates contains OsSRO1c which is highly responsive in all treatments in this study except ABA (Figure 5b). This is in line with a study from by You et al. (2014) that showed OsSRO1c mediated responses to multiple abiotic stresses. OsSRO1c is a direct target gene of OsSNAC1 and plays a role in drought and oxidative stress tolerance by modulating the stress response through interaction with various stress-related proteins (You et al., 2013). The homolog in barley shows the same expression pattern than

Os*SRO1c* and represents an interesting target as there are no studies to date that have analysed the *SRO* gene family in barley. Several other OGs containing new candidate MDS genes show a conserved response across species are At*NDB3* and UDP-glucosyl transferases (UGTs). The role of the latter in modifying hormonal signalling has been outlined, and *NDB3* along with *NDB2* (Sweetman et al., 2019, Senkler et al., 2017) may act with *AOX1a* to form a conserved and complete respiratory chain under stress conditions in the three species (Clifton et al., 2005).

2.3.5 Oppositely responsive genes indicate transcriptomic diversity despite orthology between species

Of the 1661 OGs containing oppositely responsive genes, 102 orthogroups were oppositely responsive in at least three stresses. Of these, 24 orthogroups containing 58, 58 and 51 genes in Arabidopsis, rice and barley, respectively, were oppositely responsive between two species in at least four stresses (Figure 6; Table S9). When the Arabidopsis genes in these two sets of genes were examined for over-represented GO biological processes (Fisher's test, $p<0.05$), the top categories included intracellular signal transduction and response to hormone stimulus in both sets (Table S6b). Interestingly, closer examination of the Arabidopsis genes revealed some similarities to the genes showing cross-species common responses between orthologous genes (Figure 4). Notably, an enrichment (chi-square, $p<0.05$) of cell-to-cell mobile mRNAs was also observed in the oppositely responsive gene set, with nine of the 58 Arabidopsis genes (15% vs. 6.7 % in the genome) also producing known cell-to-cell mobile mRNAs (Thieme et al., 2015) (Figure 6). Examination of the functions of these revealed known roles for many of these genes (indicated in red font) in biotic and abiotic stress in Arabidopsis and rice (Figure 6; Table S7).

A large orthogroup containing 13 out of the 58 Arabidopsis genes (opposite in 4 treatments) encoding disease resistance genes were induced in Arabidopsis following MV, SA and UV treatment, while their orthologous genes were down-regulated in rice and/or barley (Figure 6). Two of these were NB-ARC domain containing disease resistance genes, annotated as CEL-ACTIVATED RESISTANCE 1 (CAR1; At1g50180) and LOCUS ORCHESTRATING VICTORIN EFFECTS1 (LOV1; At1g10920) (Figure 6) and have key roles in defense in Arabidopsis (Table S7b). CAR1 was shown earlier to be a key host immune receptor, responsible for recognising *P. syringae* effectors (Laflamme et al., 2020), while LOV1 elicits a resistance-like response that results in disease susceptibility (Lorang et al., 2012). The loss-of-function of CAR1 and LOV1 resulted in increased susceptibility to *P. syringae* and *Cochliobolus victoriae*, respectively in Arabidopsis (Laflamme et al., 2020, Lorang et al., 2012). Interestingly, the overexpression



OG	At	Os	Hv	Description
	AT1G59218	LOC_Os09g09490	HORVU2Hr1G022620	Disease resistance protein (CC-NBS-LRR class) family
	AT1G58410	LOC_Os10g10360	HORVU5Hr1G072540	Disease resistance protein (CC-NBS-LRR class) family
	AT5G43470	NA	NA	Disease resistance protein (CC-NBS-LRR class) family
	AT1G59620	NA	NA	Disease resistance protein (CC-NBS-LRR class) family
	AT1G58390	NA	NA	Disease resistance protein (CC-NBS-LRR class) family
	AT1G58807	LOC_Os11g41540	NA	Disease resistance protein (CC-NBS-LRR class) family
216	AT1G59124	NA	NA	Disease resistance protein (CC-NBS-LRR class) family
	AT1G53350	NA	NA	Disease resistance protein (CC-NBS-LRR class) family
	AT1G58602	NA	NA	Disease resistance protein (CC-NBS-LRR class) family
	AT5G48620	NA	NA	Disease resistance protein (CC-NBS-LRR class) family
	AT1G58848	NA	NA	Disease resistance protein (CC-NBS-LRR class) family
	AT1G50180	NA	NA	NB-ARC domain-containing disease resistance protein
	AT1G10920	NA	NA	NB-ARC domain-containing disease resistance protein
	AT1G14870	LOC_Os02g36940	HORVU2Hr1G002720	PLANT CADMIUM RESISTANCE 2
288	AT5G35525	LOC_Os03g61500	HORVU5Hr1G115750	PLAC8 family protein
	AT1G14880	LOC_Os10g02300	HORVU5Hr1G115870	PLANT CADMIUM RESISTANCE 1
	AT1G64950	LOC_Os10g36980	HORVU1Hr1G047090	cytochrome P450 family 89 subfamily A polypeptide 5
	AT1G64900	LOC_Os10g37120	HORVU1Hr1G086920	cytochrome P450 family 89 subfamily A polypeptide 2
234	AT2G12190	LOC_Os10g37110	HORVU1Hr1G087200	Cytochrome P450 superfamily protein
	NA	LOC_Os10g37100	HORVU4Hr1G089230	Cytochrome P450 superfamily protein
	NA	LOC_Os10g37160	NA	Cytochrome P450 superfamily protein
	AT4G27710	LOC_Os07g44140	HORVU2Hr1G022080	cytochrome P450 family 709 subfamily B polypeptide 3
	NA	LOC_Os07g23570	HORVU2Hr1G027470	Cytochrome P450 superfamily protein
	NA	LOC_Os07g44110	HORVU2Hr1G027480	Cytochrome P450 superfamily protein
297	NA	LOC_Os07g23710	HORVU3Hr1G056770	Cytochrome P450 superfamily protein
	NA	LOC_Os01g24780	HORVU7Hr1G031730	Cytochrome P450 superfamily protein
	NA	LOC_Os07g44130	NA	Cytochrome P450 superfamily protein
	NA	LOC_Os03g25480	NA	Cytochrome P450 superfamily protein
	AT4G39950	LOC_Os04g08828	HORVU2Hr1G003470	cytochrome P450 family 79 subfamily B polypeptide 2
430	AT2G22330	NA	NA	cytochrome P450 family 79 subfamily B polypeptide 3
	AT4G15800	LOC_Os01g15320	HORVU1Hr1G080640	ralf-like 33
	AT3G05490	LOC_Os01g25540	HORVU3Hr1G035650	ralf-like 22
356	AT3G16570	LOC_Os01g25560	HORVU5Hr1G024410	rapid alkalinization factor 23
	NA	LOC_Os12g35670	NA	rapid alkalinization factor
	NA	LOC_Os02g44940	NA	rapid alkalinization factor
729	AT2G32830	LOC_Os04g10750	HORVU5Hr1G110220	phosphate transporter 1;5
	AT1G22400	LOC_Os02g51900	HORVU6Hr1G078040	UDP-Glycosyltransferase superfamily protein
836	AT1G22380	LOC_Os02g51910	HORVU6Hr1G078110	UDP-glucosyl transferase 85A3
	AT1G22340	NA	NA	UDP-glucosyl transferase 85A7
	AT1G22370	NA	NA	UDP-glucosyl transferase 85A5
	AT5G23750	LOC_Os02g42880	HORVU6Hr1G060700	Remorin family protein
1215	NA	LOC_Os04g45070	HORVU4Hr1G089540	Remorin family protein
	NA	LOC_Os10g36000	NA	Remorin family protein
	AT3G55740	LOC_Os03g44230	HORVU2Hr1G065080	proline transporter 2
1512	AT2G39890	LOC_Os07g01090	HORVU4Hr1G027180	proline transporter 1
	AT2G36590	NA	NA	proline transporter 3
1332	AT1G11700	LOC_Os07g33270	HORVU2Hr1G046550	senescence regulator DUF584
	AT4G21930	NA	HORVU2Hr1G064730	senescence regulator DUF584
	AT1G80820	LOC_Os09g25150	HORVU5Hr1G065330	cinnamoyl coa reductase
1344	AT1G15950	LOC_Os08g34280	HORVU7Hr1G030380	cinnamoyl coa reductase 1
3676	AT4G33420	LOC_Os08g02110	NA	Peroxidase superfamily protein
	AT5G37600	LOC_Os03g12290	HORVU4Hr1G007610	glutamine synthase clone R1
2509	NA	LOC_Os02g50240	HORVU4Hr1G066860	glutamine synthase
	AT1G08940	LOC_Os05g04960	HORVU5Hr1G118530	Phosphoglycerate mutase family protein
2670	AT3G05170	LOC_Os06g01950	NA	Phosphoglycerate mutase family protein
2935	AT3G14990	LOC_Os06g34040	HORVU3Hr1G027700	Class I glutamine amidotransferase-like
	AT2G46620	LOC_Os03g02330	HORVU1Hr1G048340	P-loop containing nucleoside triphosphate hydrolases
3285	NA	LOC_Os10g37500	HORVU4Hr1G088930	P-loop containing nucleoside triphosphate hydrolases
	AT1G21680	LOC_Os03g62370	HORVU5Hr1G119180	DPP6 N-terminal domain-like protein
4329	AT1G21670	NA	NA	DPP6 N-terminal domain-like protein
	AT1G68840	LOC_Os01g04750	HORVU1Hr1G082670	RAV transcirption factor (AtABI5)
526	AT1G25560	LOC_Os01g49830	HORVU3Hr1G064640	RAV transcirption factor (AtEDF1)
	AT3G25730	LOC_Os05g47650	NA	RAV transcirption factor (AtEDF3)
	AT1G10585	LOC_Os01g01840	HORVU3Hr1G000280	bHLH trancription factor
	NA	LOC_Os01g01870	HORVU4Hr1G087580	bHLH trancription factor
693	NA	NA	HORVU3Hr1G000170	bHLH trancription factor
	NA	NA	HORVU3Hr1G000180	bHLH trancription factor
	NA	NA	HORVU3Hr1G000150	bHLH trancription factor
	AT1G56010	LOC_Os06g46270	HORVU2Hr1G103930	NAC trancription factor (AtNAC1)
	NA	LOC_Os12g41680	HORVU5Hr1G011650	NAC trancription factor
708	NA	LOC_Os08g10080	HORVU7Hr1G072670	NAC trancription factor
	NA	NA	HORVU7Hr1G106480	NAC trancription factor
	NA	NA	HORVU7Hr1G106660	NAC trancription factor
	AT3G22780	LOC_Os07g07974	HORVU5Hr1G012950	CPP trancription factor (AtTSO1)
1581	AT4G14770	LOC_Os12g41230	NA	CPP trancription factor (AtTCX2)
	AT3G22760	NA	NA	CPP trancription factor
1681	AT5G50570	LOC_Os11g30370	HORVU5Hr1G076380	SBP transcription factor (AtSPL13A)
	AT5G50670	LOC_Os09g32944	NA	SBP transcription factor (AtSPL13B)
	AT2G20880	LOC_Os04g44670	HORVU6Hr1G060140	ERF transcription factor (AtERF53)
3120	AT4G28140	LOC_Os02g42585	NA	ERF transcription factor (AtERF54)

Figure 6 Oppositely responsive gene expression of orthologous genes to stress in Arabidopsis, rice and barley.

Log2-transformed fold-changes shown as a heatmap for the 24 orthogroups (OGs) that contain differentially expressed genes (DEGs) showing opposite responses (up/down-regulated) in 4 out of 6 stress treatments (3AT = 3-amino-1,2,4-triazole, AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation) for each of the three species (At = Arabidopsis, Os =Rice and Hv = Barley). Arabidopsis genes with known functions (Supplemental Table 7b) (mutants/over-expressors) in development, defence and/or redox homeostasis are indicated in red. Underlining indicates transcription factors.

and suppression of the rice protein LOW SEED SETTING RELATED (OsLSR; LOC_Os10g10360) in the same orthogroup (OG0000216) leads to a constitutively activated immune system (Wang et al., 2016). Similarly, the over-expression of another disease resistance protein (At5g43470) resulted in increased resistance to the cucumber mosaic virus (Sato et al., 2014), while the loss-of-function of two cytochrome p450 family proteins (At4g39950 and At2g22330) and a UDP-Glycosyltransferase superfamily protein (At1g22400) resulted in increased susceptibility to *Alternaria brassicicola* and *P.syringae*, respectively (Nafisi et al., 2007, Carviel et al., 2009). Thus, altering the expression levels of these appear to functionally alter biotic stress responses. Notably, all the aforementioned genes were induced in Arabidopsis, while their orthologous genes in rice and/or barley were decreased in expression (Figure 6).

Alteration in the gene expression of the genes in this set also results in altered abiotic stress responses (Figure 6). The orthogroup OG0002509 contains two glutamine synthetases in rice that are down-regulated in response to ABA, MV, SA and UV while the Arabidopsis and barley homologs in the corresponding group are either up-regulated or unresponsive (Figure 6). Concurrent overexpression of OsGS1;1 and OsGS2 has been shown to enhance the tolerance to osmotic and salinity stress and tolerance to MV induced photo-oxidative stress (James et al., 2018). Similarly, the lack of the Arabidopsis orthologue AtGLN1;1 (At5g37600) leads to impairment of redox homeostasis in chloroplasts of MV-treated leaves (Ji et al., 2019). In addition, the combinatorial loss of ATGLN1;1 and other glutamine synthetases impacts the capacity to tolerate abiotic stresses in Arabidopsis (Ji et al., 2019). In contrast to these upregulated Arabidopsis genes, the gene encoding the cytochrome p450 superfamily protein CYP709B (OG0000297) was down-regulated or unresponsive in Arabidopsis, while its orthologous genes in barley were induced in response to 3AT, SA and UV (Figure 6). Loss-of-function of this gene in Arabidopsis results in increased sensitivity to ABA and salt stress (Mao et al., 2013). The gene encoding the RAV transcription factor (RAV2/ABI3; At1g68840) was induced in Arabidopsis in response to 3AT, AA and MV, while its rice orthologues were downregulated for 3AT, AA and MV and its barley orthologues for 3AT, MV and SA (Figure

6). Interestingly, the loss-of-function of RAV2 has also been shown to alter responses under salt conditions with Arabidopsis *rav2* mutants showing improved seed germination under salt conditions (Fu et al., 2014). Interestingly, AtRAV2 expression is reduced under salt stress (Fu et al., 2014), while one of its rice orthologues (OsRAV2; LOC_Os05g47650) is induced under salt stress (Duan et al., 2016) and has also been implicated as having a functional role in the rice salt stress response (Liu et al., 2020). Similarly, a UDP-glycosyl transferase 85A5 encoding gene in Arabidopsis (At1g22370) is significantly induced in response to 3AT, ABA and SA, while its rice orthologues are down-regulated for the same treatments and its barley orthologue only up-regulated under ABA treatment (Figure 6). This gene is known to be induced under salt stress in Arabidopsis, with ectopic expression in tobacco resulting in improved salt tolerance in transgenic plants (Sun et al., 2013). Thus, alteration in the expression of genes in this subset appears to directly affect abiotic stress responses, with diversity in expression seen for genes with known roles in salt stress tolerance.

Lastly, contrasting expression was observed between Arabidopsis, rice and barley for the genes encoding orthologues of CINNAMOYL COA REDUCTASE (CCR) 1 and CCR 2 in Arabidopsis (AT1G15950 and AT1G80820). and while *CCR1* showed no change in expression, *CCR2* was induced in Arabidopsis under five of the treatments (apart from UV) while their rice and barley orthologues were reduced in expression (Figure 6). CCRs have an important role in lignin biosynthesis and when CCR1 was knocked-out in Arabidopsis, stunted growth and delayed development was observed while CCR1 knockouts in *Medicago truncatula* showed more significant impairment resulting in most plants not surviving (Zhou et al., 2010). Thus, despite orthology, the expression and function of these genes may differ between Arabidopsis, rice and barley indicating a disparity between orthology and expression that must be considered for translational work between species, both in terms of the effects on development and under stress, such as salt stress.

2.3.6 Diversity in transcription factor expression despite orthology

Analysis of genes encoding transcription factors (TFs) responsive to each of the six treatments in Arabidopsis, rice and barley revealed that ERF and NAC TFs were enriched (hypergeometric distribution; p-value< 0.05) in the DEG sets in at least two species across all six stresses (Figure S4, S5a; Table S10). The greatest number of ERFs (Arabidopsis: 32, rice: 22, barley: 26) and NAC TFs (Arabidopsis: 31, rice: 27, barley: 46) induced across all three species was in response to 3AT and UV (Figure S5a). Similarly, WRKY, bHLH, HSF, MYB and C2H2 TFs were significantly enriched ($p<0.05$; Figure S5b; Table S11) in at least two species in response to five of the six treatments. Of these, WRKY factors were

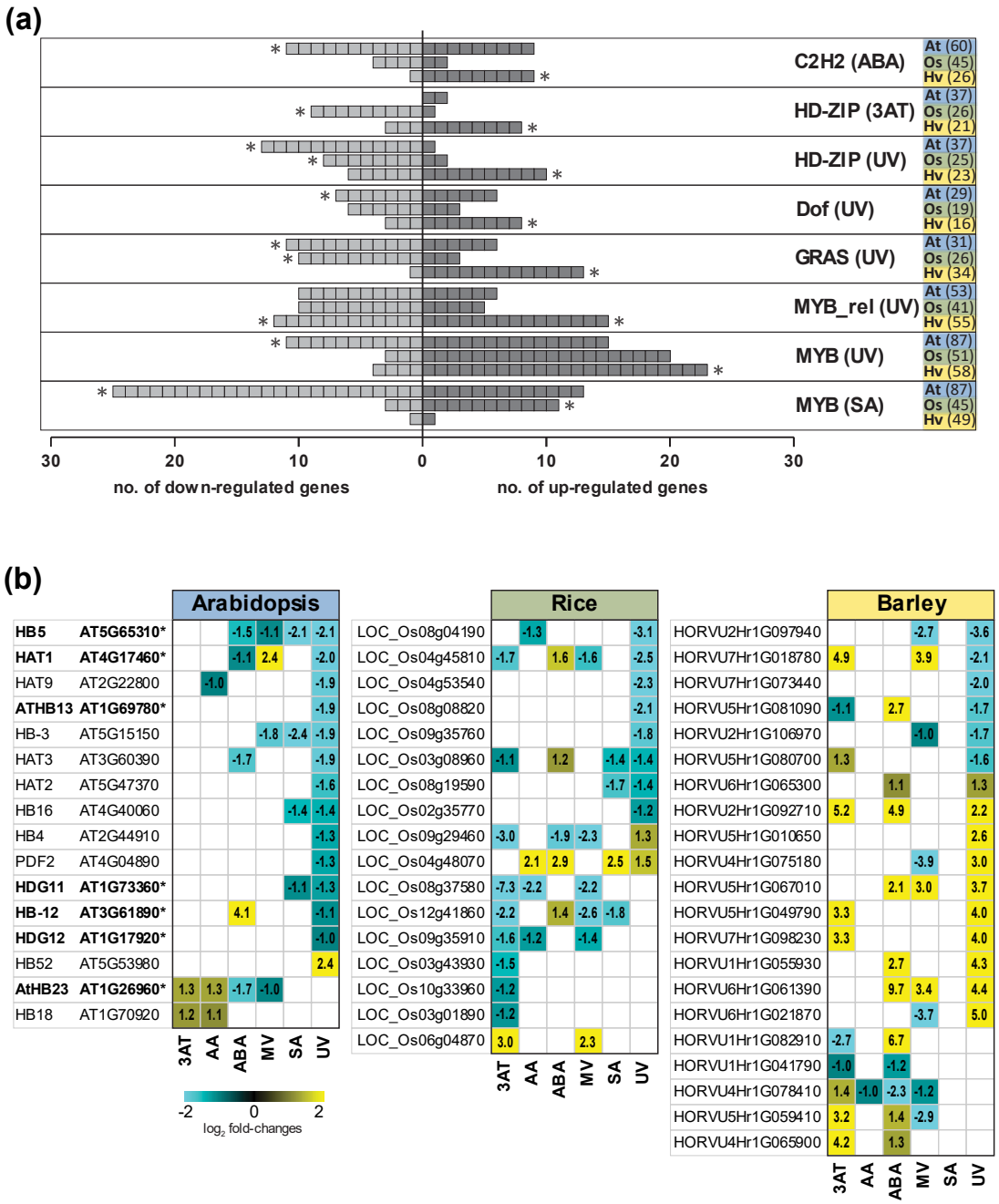


Figure 7 Oppositely responsive transcription factor families.

(a) The number and expression of transcription factors in each family that were significantly enriched ($p<0.05$; indicated by an asterisk) in oppositely responsive gene-sets (up/down-regulated) in Arabidopsis, barley and/or rice. (b) The expression of all homeodomain/homeobox-leucine zipper encoding proteins that were differentially expressed in response to 3AT and/or UV treatment in in all three species. Note Arabidopsis genes annotated with an asterisk indicate genes that have an experimentally confirmed function in development, particularly root and trichome branching (Supplemental Table 7c).

enriched in the up-regulated gene sets, similar to ERFs and NACs (Figure S5b). By contrast, bHLH TFs showed conserved enrichment among down-regulated genes in Arabidopsis and barley in response to ABA, MV and SA, while MYB encoding genes were enriched among the down-regulated genes only in Arabidopsis in response to ABA and

MV (Figure S5b). Given these species-specific differences, we further examined the TF families to identify families that were enriched in oppositely responsive DEGs between species. Five TF families (C2H2, HD-ZIP, Dof, GRAS and MYB) were identified that were enriched in the up-regulated gene sets of one species and the down-regulated gene set (or *vice versa*) of another species in response to the same stress (Figure 7a; Table S12). Notably, all five families were enriched in the up-regulated gene sets in barley, while an enrichment of the same family was observed in the down-regulated gene set(s) in Arabidopsis and/or rice (Figure 7a). For example, in response to ABA the C2H2 family was enriched among the down-regulated genes in Arabidopsis while this family was enriched in the up-regulated genes in barley (Figure 7a).

As TF members in families can complement other TFs in the same family, we examined the expression of all DEGs in each of the five families enriched in the oppositely responsive sets (Figure 7a). This revealed that despite being enriched in oppositely responsive subsets, there are members in the TF families that are up-regulated and down-regulated within each family, making it difficult to ascertain whether the opposite responses represent functional differences. Nevertheless, this examination identified families in which most genes show opposite transcriptomic responses (Figure 7a) and for many of these their responses were conserved across more than one stress (Figure S6). For example, in response to SA and UV, the majority of MYB factors are up-regulated in rice and barley, while most are down-regulated in Arabidopsis and several of these show the same up/down-regulated response across both stresses in each species (Figure S6). Similarly, all but one GRAS family TF are up-regulated in barley in response to UV and similarly to 3AT, while the majority are down-regulated in Arabidopsis and many of these maintain this down-regulation in response to MV (Figure S6). This supports the idea of a conserved stress-responsive regulation of these factors within a single species.

For the homeodomain (HD-ZIPs) family the opposite pattern of regulation between species is observed in response to both 3AT and UV (Figure 7a&b) with HD-ZIPs enriched in the up-regulated genes in barley, down-regulated genes in Arabidopsis (UV only) and down-regulated genes in rice following 3AT and UV treatment (Figure 7a). Closer examination of these HD-ZIP factors revealed that only one HD-ZIP factor in Arabidopsis and two in rice were up-regulated while the most of these were highly induced in response to UV in barley (Figure 7b). In Arabidopsis, of the 14 DEGs encoding HD-ZIPs in response to UV, 13 were up-regulated and six have been experimentally shown to have functionally significant roles in development and/or stress, with a loss-of-function resulting in altered function (Ebrahimian-Motlagh et al., 2017, Roodbarkelari and Groot, 2017, Perotti et al., 2019). Interestingly, the expression of this family in barley revealed 10 of the 16 DEGs encoding HD-ZIPs were up-regulated and five of these by >4-fold (Figure 7b).

2.4 Discussion

Comparison of the transcriptome responses of a model species (Arabidopsis) and two crop species (rice and barley) to six treatments designed to mimic stress responses (Table 1) revealed much needed insight into the level of genetic and transcriptomic conservation between these species. The examination of the responses to hormones (ABA and SA); treatments that cause oxidative stress (3AT, MV); inhibit respiration (AA) or induce genetic damage (UV) in parallel for these three species unveiled similarities and differences in the abiotic stress responsive pathways that are affected (Table 1) revealing a depth of knowledge that is not possible with single treatment/species studies. The use of marker genes ensured the efficacy of the treatment and confirmed comparability between species. This approach revealed commonalities and differences in responses that will provide a roadmap for translation research, helping with the transfer of knowledge gained in a model system to crop species. This study revealed that the responses to treatments (Table 1) were diverse in number, gene ontology, orthology and expression. Analyses of the number of transcripts that responded to the treatments in the different species did not give a trend in terms of specificity or conservation (Figure 1). It was notable that the overlap in response to the various treatments was greater in Arabidopsis than it was for barley and rice, and this may indicate that the regulatory hierarchy in Arabidopsis is more shared than it is in other species (Figure 1).

While these differences can now be tested experimentally, it was notable that the changes in transcript abundance for transcription factors in many families differed fundamentally (Figure 7). Thus, the extensive interaction of signalling networks that have emerged from studies in Arabidopsis may be more limited or different in other species. For example, the two Arabidopsis orthologues to OsMYBS1, which has a role in sugar and hormone mediated signalling in rice were found to play opposite roles to each-other in regulating glucose and ABA signalling in Arabidopsis, indicating distinct regulatory roles of some TFs in these species (Chen et al., 2017). These potential differences have significant implications for the translation into crop species, as under field conditions plants are also exposed to multiple non-optimal conditions (Balfagon et al., 2020, Suzuki et al., 2014). While the response to these multiple conditions may be highly integrated in one species, it is possible they may trigger multiple or even antagonistic parallel pathways in other species. Notably, hormone pathways were enriched among the oppositely responsive sets identified in this study. Given the interaction of anterograde, retrograde and hormone signalling pathways (Balfagon et al., 2020, Hernandez-Verdeja et al., 2020, Medina-Puche et al., 2020), it will be essential to understand these differences for translational.

One difference observed in the response to the treatments were gene ontologies related to protein homeostasis that differed significantly between Arabidopsis, rice and barley. The rate of growth in Arabidopsis is inversely related to protein turnover (Ishihara et al., 2017), and protein synthesis is energetically expensive (Nelson et al., 2014). Thus, the rate of protein turnover and synthesis is a major energy sink in plants, but also indicative of responses to stimuli. Differences in the response to the treatments for protein homeostasis and vesicle trafficking in Arabidopsis (Figure 2A) may point to differences in these processes between plants. Likewise, there were notable differences between Arabidopsis, barley and rice with respect to protein biosynthesis. Thus, both may indicate specificities in protein turnover rates under adverse conditions between the three species, and these may have consequences for growth and development. This facet needs to be further investigated if the principles of energy consumption limiting growth under adverse conditions are to be applied from Arabidopsis to other plants (Salih et al., 2020). It should be noted that in this study the Arabidopsis accession Columbia was used, and given differences between in the rate of protein turnover have been seen in Arabidopsis accessions (Ishihara et al., 2017), this may not necessarily be a reflection of a 'typical' response to stress. Another possible contributing factor is the lifespan differences in Arabidopsis and the two crop species, which impact mRNA and protein turnover.

The approach used in this study to define opposite responses was very conservative, in that it was restricted to orthology and all genes in the orthogroups needed to display the same trend. Therefore, the opposite responses outlined in this study are likely to be an underestimation of the differences between species. This can be seen to some degree when the expression of whole transcription factor families is analysed (Figure 7). The large differences in the number of genes from each family that were up- or down- regulated in abundance in response to the treatment indicates that there were fundamental differences in how genes were regulated. Thus, the common practise of expressing a transcription factor from one species in another species to transfer a trait such as tolerance to a limiting condition may not result in all the desired effects. For example, OsAP2 and OsWRKY24 have been proposed to have opposite roles in rice and Arabidopsis, with these known to be positive regulators involved in increased lamina inclination, grain size and cell elongation in rice, while their overexpression in Arabidopsis resulted in reduced plant size and cell size (Jang and Li, 2018). Similarly, the outcome following over-expression of AtFD and AtFDP in transgenic rice was not as expected compared to that seen in Arabidopsis (Jang et al., 2017). Thus, despite orthology, function and expression can clearly differ between species with differences in response of TFs to treatments observed in closely related monocot species, e.g. for GRAS and HD-ZIP TFs in response to UV. The differences in responses of TF families to stresses may indicate that while the upstream

regulatory network may be different, the resulting responses of conserved target genes are similar. This may limit the utility of promoter motif searching cross-species which is widely used in research on crop species.

Despite similarities in promoter regions and significant orthology between genes, it is possible to observe opposite expression responses such as seen for phototropin genes in Arabidopsis and Brachypodium (Krzeszowiec et al., 2020). Similarly, despite orthology and experimental confirmation of identical function of the PAO/phyllobilin pathway in barley and Arabidopsis, the downstream effects of these proteins differed between the species (Das et al., 2018). Another example of these distinctions can be seen for rice PHYTOCHROME-INTERACTING FACTOR-LIKE1 (OsPIL1), which negatively regulates leaf senescence in rice with *ospil1* mutants senescing earlier than WT, while the opposite was observed for closest homologs of OsPIL1 in Arabidopsis *atpif4* and *atpif5* mutants (Sakuraba et al., 2017). Opposite effects were also seen for the effect of potassium deprivation on jasmonic acid related gene expression (JA) and downstream resistance to herbivorous insects, which again differed between barley and Arabidopsis, despite orthology of JA responsive genes (Davis et al., 2018). These just represent some of the examples of where despite orthology, gene function and phenotypic effects differed between Arabidopsis, rice and barley, highlighting the need for resources such as this study to identify common and distinct responses between species, particularly those across multiple treatments, providing valuable information for further experimental design.

While there were notable differences in the observed species responses in this study, there were also notable similarities. The conserved responses observed between all three species to three or more stimuli revealed that fundamental response pathways have been conserved from perception to response at a gene level (Figure 4). A good example of this was seen with the mitochondrial dysfunctional response, which has been defined in Arabidopsis (De Clercq et al., 2013). In response to AA, and to a lesser degree 3AT, the *cis*-regulatory motif in the promoter region of differentially expressed genes is enriched in all three species (Figure 5a). Furthermore, there are more MDS candidate genes present in Arabidopsis than previously defined. Thus, the mitochondrial regulatory pathway that is controlled by activation of latent ER bound NAC transcription factors and repressed by RCD like proteins is a common theme observed across monocots and dicots. In Arabidopsis, the role of the ANAC017 transcription factor has now expanded as being involved in flooding responses, ageing and senescence and as a growth regulator (Meng et al., 2020). Plants defective in ANAC017 grew and developed more rapidly than wild type controls with as much as 50% additional biomass accumulation, while plant with over-expressed ANAC017 displayed growth retardations (Meng et al., 2020). However, detrimental effects were only observed with higher levels of over-expression. Thus, given

the central role of this transcription factor in stress responses by integrating mitochondrial and chloroplast energy metabolism with environmental conditions, and the conserved nature of this pathway across species, our study exemplifies how natural variation or modification of target genes may be used for agronomic purposes.

The cross-species comparison of transcriptomic responses to the six treatments in this study showed overlap between stresses, more so for Arabidopsis than barley and rice. Over 5000 genes were both orthologous and showing conserved responses in at least two species in response to at least one treatment, indicating conservation across species of the relevant responsive pathways including stress responsive transcription factors such as ERFs, NAC and WRKY TFs. Closer examination of conserved genes showing common responses revealed several genes with known functions in stress response pathways including NAC (Zhao et al., 2018) and ERFs TFs (Zhou et al., 2017) as well as others (red font; Figure 4). While the large overlap in the presence of orthologous genes showing conserved responses was to be expected, it was remarkable that 15-34% of orthologous DEGs between species show opposite transcriptomic responses. Examination of these identified genes with known roles in the biotic and abiotic stress response pathways, with known functions for some of these, whereby alteration in expression resulted in altered phenotypes in Arabidopsis (red font; Figure 6). Thus, despite these genes being orthologous in rice and barley as well, it is possible that the shortlist identified here represents only a subset of genes with divergent regulation, particularly given that the opposite responses were observed following at least four independent treatments. The presence of six orthogroups containing oppositely responsive TFs out the of the 20 that were opposite in at least four treatments indicates diversity in TF gene expression, further supporting the possibility of differential regulation between these species. While this study compared the responses of Arabidopsis, barley and rice to various stimuli, only a single stimulus was used and emerging studies highlight the importance of combination of stresses (Choudhury et al., 2017). The differences observed in the overlapping responses to treatments and opposite responses means that more differences may emerge between species when combined stresses are applied.

2.5 Material and Methods

2.5.1 Plant Material and Growth Conditions

Rice seeds (Oryza sativa L. ssp. Japonica, cultivar Millin) were surface sterilized and germinated on a petri-dish in the dark. After one-week, pre-germinated rice seedlings were transplanted to soil and grown in a growth chamber with a day/night cycle of 12 h/12 h, 29°C/26°C with 350 µE m-2 s-1, and a relative humidity of 65%. Barley seeds (Hordeum vulgare L. ssp. Vulgare, cultivar Commander) were directly sown onto soil and grown in a greenhouse at 21°C. Arabidopsis thaliana (ecotype Columbia-0) seeds were surfaced-sterilized and stratified for 48 h at 4 °C. Plants were grown on soil in a growth chamber with a day/night cycle of 16 h/8 h at 22°C (day)/19°C (night) and 120 µE m-2 s-1.

2.5.2 Stress treatments, tissue collection and RNA isolation

Two week-old Arabidopsis, rice and barley seedlings were sprayed with 2 mM salicylic acid (SA), 1 mM methyl-viologen (MV), 10 mM 3-Amino-1,2,4-triazole (3-AT), 100 mM abscisic acid (ABA) or 50 µM antimycin A with 0.01% Tween20 as a wetting agent until liquid dripped off the leaves. Spraying was repeated after 30 min. Mock control plants were treated in the same way with water and 0.01% Tween20. Leaf samples of rice and barley as well as whole rosette tissue for Arabidopsis were harvested and shock-frozen in liquid nitrogen at given time points after treatments for total RNA extraction. Treatment of rice seedlings with 50 µM AA was performed with 2cm leaf segments floating in 10 mM potassium phosphate buffer (pH 6.8) with 0.01% Tween20 for 3h. Mock treatment was conducted accordingly with water and 0.01% Tween20. Leaf samples were harvested and shock-frozen in liquid nitrogen for RNA-extraction. For total RNA isolation the tissue of 3-4 individual plants was pooled for each of the three biological replicates.

Total RNA was isolated using the SpectrumTM Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer's protocol. On-Column DNase I (Sigma-Aldrich) digestion was performed with the total RNA prior to elution. The quantity and quality of RNA was analysed using a SPECTROstar® (BMG LABTECH, Freiburg, Germany) spectrophotometer and agarose gel electrophoresis.

2.5.3 qRT-PCR

For quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis 1 µg of total RNA was reverse transcribed using the cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s instructions. qRT-PCR was conducted using 50 ng of cDNA and a final primer concentration of 300nM with a SYBRGreen PCRMaster Mix and a QuantStudio 12K flex real-time PCR system (Applied Biosystems). Default settings were

used for the cycle threshold (Ct) value determination. The mRNA levels for each gene were quantified and normalized using two independent housekeeping genes. After each run, a melting curve analysis was performed to verify target-specific product amplification. Gene-specific primer pairs were designed using QuantPrime (Arvidsson et al., 2008) and all primer sequences are listed in Table S13.

2.5.4 RNA-seq

RNA-seq libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit according to the manufacturer's instructions (Illumina) and sequenced on a HiSeq1500 system (Illumina) as 60 bp reads with an average quality score (Q30) of above 95 % and on average 18 million reads per sample. Quality control was performed using the FastQC-software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Transcript abundances as transcripts per million (TPM) and estimated counts were quantified on a gene level by pseudo-aligning reads against a k-mer index build from the representative transcript models (TAIR10 for Arabidopsis, IRGSP-1.0 for rice, IBSCv2 for barley) using the kallisto program with 100 bootstraps (Bray et al., 2016).Only genes with at least 5 counts in a quarter of all samples per genotype were included in the further analysis. The program sleuth with a Wald test was used to test for differential gene expression (Pimentel et al., 2017). Genes were called as differentially expressed with a false discovery rate FDR < 0.05 and a |log2 (fold change)|>1.

2.5.5 Bioinformatic analysis

Orthologues and corresponding orthogroups across Arabidopsis, rice and barley were inferred via OrthoFinder v. 2.3.3 (Emms and Kelly, 2015) with default parameters and MMseqs2 (Steinegger and Soding, 2017) for sequence similarity searches. Protein sequences were retrieved from EnsemblPlants v44 (https://plants.ensembl.org/index.html) for barley (IBSCv2), TAIR (TAIR10 release) for Arabidopsis and IRGSP-1.0 for rice.

Hierarchical clustering and generation of heat maps was performed using the pheatmap R package (Kolde and Kolde, 2015).

2.5.6 Transcription factor enrichment

A complete list of all transcription factor families was obtained from Plant TFDB (Jin et al., 2017). Enrichment of transcription factor was performed using hypergeometric distribution with p-value < 0.05 defined as significant.

2.5.7 PageMan analysis

Analysis of functional categories across species was performed via PageMan (Usadel et al., 2006) using up- and down-regulated differentially expressed genes from all species. Fisher's test for ORA (over-representation analysis) analysis was carried out in PageMan to determine statistically significant over/under representation of genes classified into specific BINS.

2.5.8 GO-term analysis

All Gene Ontology (GO) over-representation analysis was done using the tool at http://geneontology.org/ after selecting the relevant species. The test used is Fisher's test with FDR correction (p-value<0.05).

2.5.9 Motif analysis

In order to do a motif enrichment analysis with stress treatment specific gene subsets in each species, the 2 kb upstream sequence of all genes detected in this study were retrieved using the RSAT retrieve-seq tool (Medina-Rivera et al., 2015). RSAT-dna-pattern tool was used with default parameters to search for any number of occurrences of the pattern (CAAGNNNNNCA[AC]G) within the DNA sequences. To determine the significance of enrichment, a chi-square ($\chi 2$) test was carried out, with p-value < 0.05 marked as significant.

2.5.10 Motif analysis

All NGS data from this study has been submitted to GEO under the accession:

Barley: PRJNA655522

Rice: PRJNA655523

Arabidopsis: PRJNA486068 (Meng et al., 2020)

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Supplemental Figure 1a. Functional categories of up-regulated genes in response to stress across species. Full PageMan (Usadel et al., 2006) output showing over- and under-represented (PageMan - Fisher's test) functional categories in all species for all differentially expressed genes that are upregulated. AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation.

Supplemental Figure 1b. Functional categories of down-regulated genes in response to stress across species. Full PageMan (Usadel et al., 2006) output showing over- and under-represented (PageMan - Fisher's test) functional categories in all species for all differentially expressed genes that are upregulated. AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation.

Supplemental Figure 2. Conserved and distinct stress responses in Arabidopsis, rice and barley. The total number of conserved and opposite OGs for each stress with the numbers that were treatment specific (light grey) and overlapping (dark grey) indicated. 3AT = 3-amino-1,2,4-triazole; AA = antimycin A; ABA = abscisic acid; MV = methyl viologen; SA = salicylic acid; UV = ultraviolet radiation; At = Arabidopsis; Os =Rice; Hv = Barley.

Supplemental Figure 3. Heatmap of MDS candidate genes in Arabidopsis. Hierarchically clustered heatmap of expression values from all ANAC017 gene regulatory network (GRN) genes (Meng et al., 2019) that containing the stringent MDM in their promotor region, are highly stress responsive to AA and or 3AT and differentially expressed in at least 3 out of 6 treatments. Previously characterised MDS genes (De Clercq et al., 2013) are highlighted in red. AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation.

Supplemental Figure 4. Expression of genes encoding transcription factors in Arabidopsis, rice and barley in response to different stress treatments. Numbers of transcription factors (TFs) expressed in the TF families in Arabidopsis (purple), Rice (green) and Barley (yellow) are shown, next to columns indicating the number that were up-regulated (red/pink) or down-regulated (blue/light blue) for that family. (a) 3AT = 3-amino-1,2,4-triazole, (b) AA = antimycin A, (c) ABA = abscisic acid, (d) MV = methyl viologen, (e) SA = salicylic acid, (f) UV = ultraviolet radiation. The number of TFs expressed in the families that were enriched in at least two species across at least two stresses. Note ^ indicates families that are over-represented in oppositely responsive sub-sets.

Supplemental Figure 5. Enrichment of transcription factor families in response to stress across species. Expression of genes encoding transcription factors (TFs) in Arabidopsis (At), rice (Os) and barley (Hv) in response to different stress treatments. a) Number of differentially expressed (up-regulation = dark grey; down-regulation = light grey) TFs with families that were enriched (hypergeometric distribution; p-value< 0.05; Supplemental Table 11) in at least two species across all six stresses. ERF and NAC TF families are visualized. The total number of TFs identified for each family in each species (http://planttfdb.cbi.pku.edu.cn/) is shown in brackets b) Number of differentially expressed TFs with families that were enriched in at least two species across five stresses. WRKY, bHLH, MYB, HSF and C2H2 TF families are visualized. 3AT = 3-amino-1,2,4-triazole, AA = antimycin A, ABA = abscisic acid, MV = methyl viologen, SA = salicylic acid, UV = ultraviolet radiation

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2.9 Supplemental Data

For the purpose of reviewing this thesis the supplemental tables can be found at:

https://cloudstor.aarnet.edu.au/plus/s/5lzTvd2JpChheOF

CHAPTER 3

Functional characterisation of the alternative oxidase isoforms a, c and d in *Arabidopsis thaliana.*

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3.1 Abstract

The cyanide insensitive alternative oxidase (AOX) is encoded by a small gene family in plants. The genes display tissue and developmental differences in expression profiles, and differential post-translation regulation of AOX isoforms by metabolites has been demonstrated in *Arabidopsis thaliana.* The lack of a functional AOX1a protein in Arabidopsis cannot be fully compensated by other AOX isoforms under stress conditions.

To investigate the inability of other AOX isoforms to complement an *aox1a* mutant under stress, the *aox1a* mutant was transformed with constructs that contain different combinations of *AOX1a/c/d* coding sequences under the control of the different native *AOX1a/c/d* promotor regions as well as the constitutively active Cauliflower Mosaic Virus (CaMV) 35S promoter. AOX expression levels on transcript and protein level were compared to survival with antimycin A treatment to determine if the AOX isoforms could complement for a lack of AOX1a. AOX1a, AOX1c and AOX1d are able to support germination and survival in response to antimycin A treatment when those are expressed under the transcriptional control of the *AOX1a* promoter.

Here we show that AOX1c and AOX1d can support germination and survival when treated with antimycin A and complement the lack of AOX1a. Interestingly, this only occurs when their expression is driven by the *AOX1a* promoter region. Preliminary evidence suggests that AOX1a is more efficient at supporting germination and growth compared to AOX1c and AOX1d.

3.2 Introduction

The alternative oxidase (AOX) is the terminal oxidase of the cyanide-resistant alternative respiratory pathway, a characteristic but not unique feature of plant mitochondria and an additional branch of the mitochondrial electron transport chain (mETC) (Millar et al., 2011). In addition to the terminal oxidase cytochrome *c* oxidase, which couples electron transport to ATP synthesis, the non-phosphorylating alternative respiratory pathway reduces oxygen to water without the translocation of protons (Moore and Siedow, 1991). This additional pathway operates at the level of ubiquinone (UQ), bypasses proton pumping complexes III and IV and consequently reduces respiratory energy efficiency by uncoupling ATP-synthesis from electron transport. Traditionally the role of this non-energy-conserving pathway provides metabolic flexibility to plant mitochondria by keeping the ubiquinone pool sufficiently oxidized when the electron flow through the cytochrome pathway becomes limited and inhibits the generation of reactive oxygen species (ROS) (Siedow and Umbach, 2000, Yoshida et al., 2011). In the last decade, mitochondria and chloroplasts have emerged as sensors of environmental conditions using the retrograde signalling pathway, with AOX having a role in maintaining signalling homeostasis (Vanlerberghe, 2013).

The dynamic flexibility of AOX is not only coordinating mitochondrial redox homeostasis but is also important for optimizing photosynthetic performance in the chloroplasts as well as photorespiration under adverse environmental conditions (Vishwakarma et al., 2015, Watanabe et al., 2016). AOX serves as an electron sink for chloroplast-derived excess reducing equivalents that are indirectly transported into mitochondria (Selinski and Scheibe, 2019, Dinakar et al., 2010). AOX protects photosynthetic components against harmful effects of excess light and concomitant overreduction to prevent photoinhibition (Liao et al., 2016, Vishwakarma et al., 2015).

In higher plants, the *AOX* multigene family can be classified into two discrete subfamilies, *AOX1* and *AOX2*, with the latter only being present in dicot species (Considine et al., 2002, Costa et al., 2014). The identification of *AOX2* in early-diverging monocot families, however, suggests an evolutionary scenario with at least partial loss of *AOX2* during speciation events within several monocot orders (Costa et al., 2017). The composition of encoded *AOX* genes of either subfamily is highly diverse in different species indicating functional differences between the different isoforms that is beneficial for plant performance (Selinski et al., 2018a, Costa et al., 2014). This is further supported by spatiotemporal expression pattern as well as isoform specific transcriptional and post-translational regulation as shown in different species like *Arabidopsis thaliana*

(Arabidopsis), *Glycine max* (soybean) and *Cicer arietinum* (chickpea) (Sweetman et al., 2019, Finnegan et al., 1997, Clifton et al., 2006).

In Arabidopsis, the *AOX* multigene family encodes five proteins, *AOX1a/b/c/d* and *AOX2* (Polidoros et al., 2009). *AOX1a* displays the highest expression ubiquitously throughout all developmental stages and tissues and shows the highest changes in transcript abundance in response to stress (Clifton et al., 2005). *AOX1a* is highly responsive to dysfunctions in the mitochondrial-respiratory metabolism, especially dysfunction of the complexes located in the mETC or inhibition of the ATP synthase as well as various biotic and abiotic stresses (Clifton et al., 2006, Vanlerberghe and McIntosh, 1997, Vanlerberghe, 2013, Zarkovic et al., 2005). The induction in response to mitochondrial dysfunction that can be chemically induced by inhibition of the mETC or the tricarboxylic acid (TCA) cycle, makes *AOX1a* the most commonly used indicator to study mitochondrial retrograde responses (MRR) (Zarkovic et al., 2005, Clifton et al., 2005, Rhoads and Subbaiah, 2007).

As AOX activity represents an energetically wasteful process under optimal growth conditions, it is consequently under a very strong repression, mediated by the transcription factor ABI4 that can be lifted by the stress signalling hormone abscisic acid (Giraud et al., 2009). This links the MRR to general regulatory pathways in the cell (Wang et al., 2018). Multiple regulators of AOX have been identified that include NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON (NAC) and WRKY transcription factors as well as CYCLIN DEPENDENT KINASE E1 (CDKE1) and several genes related to auxin signalling (Ng et al., 2013a, De Clercq et al., 2013, Van Aken et al., 2013, Ivanova et al., 2014, Ng et al., 2013b). The transcription factors ANAC013/17 are bound to the endoplasmic reticulum and can migrate into the nucleus to regulate gene expression following proteolytic cleavage that is mediated by mitochondrial retrograde signals (Ng et al., 2013b). Both transcription factors target a set of genes that are highly responsive to mitochondrial dysfunction, named mitochondrial dysfunction stimulon (MDS) genes, that include *AOX1a* and *ANAC013* itself (De Clercq et al., 2013). The nuclear protein RADICAL-INDUCED CELL DEATH1 (RCD1) was shown to supress ANAC013/17 *in vivo* and its inactivation triggers induction of MDS gene expression (Shapiguzov et al., 2019). As RCD1 is linked to chloroplast derived retrograde response pathways it has been proposed to function as an integrator of chloroplast and mitochondrial retrograde signalling pathways (Shapiguzov et al., 2019).

aox1a T-DNA insertion lines with no immunodetectable AOX protein do not show any apparent phenotypic changes under standard growth conditions despite transcriptional rearrangements with significant changes in the basal equilibrium of signalling pathways

related to the antioxidant defence systems (Strodtkötter et al., 2009, Giraud et al., 2008). These metabolic adjustments and induction of alternative pathways could explain unchanged physiological parameters and a wild type like phenotype (Strodtkötter et al., 2009). Exposing *aox1a* knockout (KO) plants to adverse environmental conditions or by chemically inducing mitochondrial dysfunction, however, leads to a stress phenotype. Combined moderate light and drought stress results in notable differences in leaf colour due to high anthocyanin concentrations that are significantly increased compared to the wild type (Giraud et al., 2008). Treatment with antimycin A leads to wilted and necrotic *aox1a* KO plants that have inhibited photosynthesis, increased level of ROS and enhanced membrane leakage (Strodtkötter et al., 2009). The lack of AOX1a cannot be functionally compensated by an increased transcript abundance of *AOX1d* (Strodtkötter et al., 2009, Giraud et al., 2008). To our knowledge it has not been shown if high transcript abundance of AOX1d in response to antimycin A also translates into increased protein abundance in *aox1a* KO mutants. Increased protein abundance of AOX1d has been detected by several authors for *aox1a* mutant lines that are further defective in other genes or pathways. A study using a mutant line that lacks functional AOX1a and is impaired in COX pathway showed increased protein abundance of an AOX isoform other than AOX1a under standard growth conditions (Kühn et al., 2015). In this line, the high protein abundance, presumably AOX1d, based on the transcriptional induction of this isoform, could not compensate the lack of AOX1a.

Several explanations for the lack of functional compensation of AOX1a in response to stress have been proposed including AOX isoform specific differences in translational regulation, tissue- or cell-specific expression, post-translational differences which have been shown for AOX1a, AOX1c and AOX1d or the possible formation of heterodimers between different AOX isoforms (Strodtkötter et al., 2009, Selinski et al., 2018a, Selinski et al., 2018b).

In order to determine if other AOX isoforms can complement for the lack of AOX1a, the *aox1a* mutant was transformed with combinatorial constructs of the of *AOX1a/c/d* coding sequences under the control of the native *AOX1a/c/d* promotor regions as well as the constitutively active Cauliflower Mosaic Virus (CaMV) 35S promoter. AOX expression levels on transcript and protein level were compared to survival with antimycin A treatment to determine if the AOX isoforms displayed different functional characteristics.

3.3 Results

3.3.1 Construction of *AOX* over-expressing lines in the *aox1a*-knockout background

In order to analyse the functional role of the different AOX isoforms in response to mitochondrial dysfunction, the *aox1a* KO background was complemented with the coding sequence of AOX1a, AOX1c or AOX1d, under the control of the 3 kb region 5' upstream of the translational start sites of AOX1a, AOX1c or AOX1d as well as the constitutively active CaMV 35S promoter (Figure 1a). All sequences were amplified and cloned into a binary vector resulting in twelve different constructs that contain all possible combinations of promoter and coding sequences. The different vectors were transformed in the *aox1a* mutant background via *Agrobacterium tumefaciens* mediated transformation and confirmed for homozygosity of the transgene in the T3 generation. All combinations, their names and the number of biological replicates for independent transformation can be found in Table 1. The *AOX1a* 3 kb promoter region contains the coding sequence of another AOX isoform, *AOX1b*, which is located 1505 bp upstream of the translational start site of *AOX1a* (Figure 1b). The promotor region of *AOX1c* contains the coding sequence of the gene *SMR7* and the partial coding sequence of a gene that encodes a Transducin/WD40 repeat-like superfamily protein. Both genes have a reverse gene orientation to *AOX1c*. No gene is located in the 3 kb promotor region upstream of *AOX1d* translational start site in either orientation.

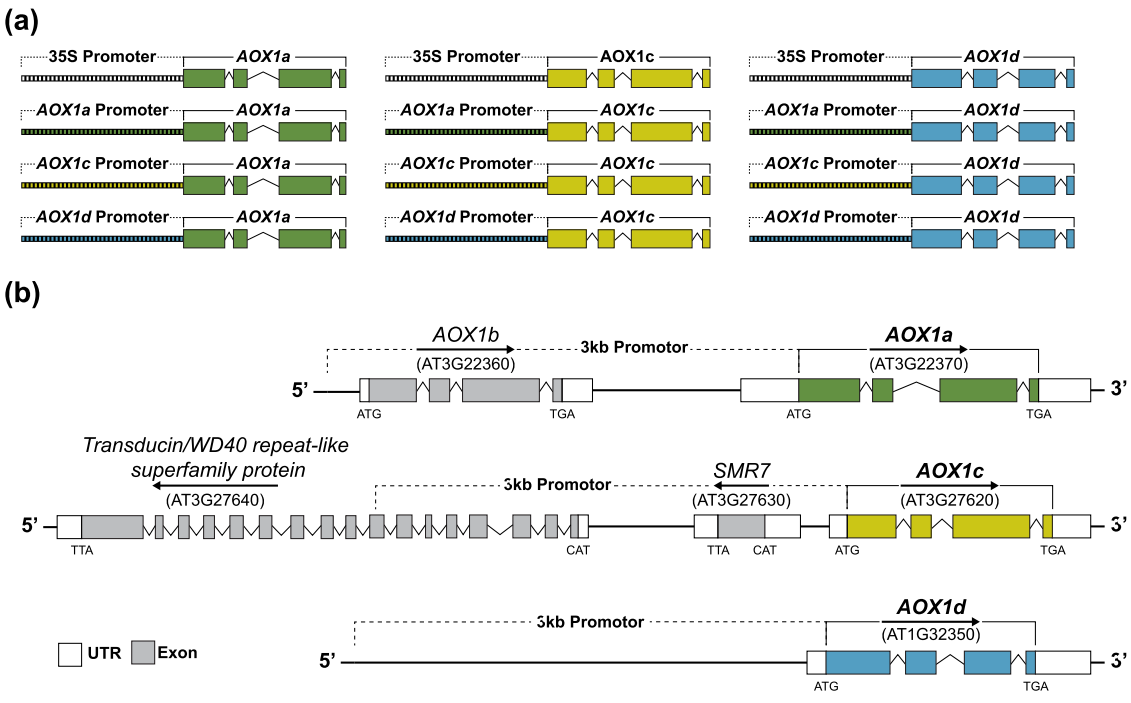


Figure 1. Construction of AOX-chimera and overexpression lines and the gene structure of the corresponding isoforms.

(a) Overview showing the different constructs of AOX1a, AOX1c and AOX1d coding sequences driven by the constitutively active Cauliflower Mosaic Virus (CaMV) 35S promoter or the native 3 kb promotor region of AOX1a, AOX1c and AOX1d. All twelve combinations were cloned into the binary vector pCAMBIA1300 and transformed into Arabidopsis via Agrobacterium tumefaciens-mediated floral dipping. (b) AOX1a, AOX1c and AOX1d gene organization showing gene structure and the 3 kb promotor region including all genes that are located in that genomic region. Gene orientation is indicated by arrows.

3.3.2 AOX transcript abundance

AOX transcript abundance in the complemented *aox1a* lines following treatment with antimycin A to induce mitochondrial dysfunction was determined and compared to mock treatment. Ten-day-old seedlings were spray-treated with antimycin A or control containing solvent only and the transcript abundance of all five *AOX* genes was evaluated 3 h after the treatment. This timepoint represents the maximal induction of *AOX1a* after treatment with antimycin A (Ng et al., 2013a, Van Aken et al., 2016). As reported previously, transcript abundance of *AOX1a* was induced ~40-fold in wild type plants with antimycin A treatment (Figure 2) (Clifton et al., 2005). *AOX1a* transcript abundance and induction were similar to wild type plants in the *aox1b, aox1c, aox1d* and *aox2* mutant backgrounds (Figure 2). For the *aox1a* mutant line (SALK_084897), an increase in *AOX1a* transcript was also observed, but this line does not produce immuno-detectable protein (Giraud et al., 2008). The AOX1a overexpressing line (*AOX1a*-OE) already had increased levels of *AOX1a* and were not further induced by antimycin A treatment.

Finally, for the *rcd1* and *rcd1 aox1a* lines, *AOX1a* was already induced 9-fold in the *rcd-1* line, and with antimycin A treatment this was further increased to ~18-fold compared to wild type untreated. The *rcd1-1 aox1a* double mutant shows a similar induction of *AOX1a* in the control and antimycin A compared to the single knockout line *rcd1-1*. All *aox1a* KO lines complemented with the different *AOX* chimera and over-expressing lines, including the Pro*35S:AOX1a* induced *AOX1a* upon treatment with antimycin A. This is similar to what was observed with the *aox1a* line alone, but the degree of induction differed as may be expected in different lines, e.g. line Pro*AOX1a*:*AOX1d-3* displayed 4-fold reduced levels of *AOX1a* transcript in control conditions, and upon treatment was induced just 4-fold to reach wild type levels.

Transcript abundance of *AOX1b* largely followed a similar pattern to that of *AOX1a* for the control lines (wild type, *aox1a*, *AOX1a-OE*, *aox1b, aox1c, aox1d, aox2, rcd-1*, *rcd-1:aox1a*) in that there was an increase in transcript abundance with antimycin A treatment, and the *AOX1a OE* line not showing any increase. This increase in transcript abundance

for *AOX1b* was also observed in the *35S CaMV* lines driving the expression of *AOX1a*, *AOX1c* and *AOX1d*. Notably all *aox1a* lines complemented with the coding sequence of either *AOX1a, AOX1c* or *AOX1d* under the control of the *AOX1a* promoter displayed high transcript abundance for *AOX1b* in both control and antimycin A treatment. While there was some variation observed in these lines, this increase in transcript abundance varied from greater than 34-fold induction in Pro*AOX1a:AOX1d*-3 to 13,000-fold in Pro*AOX1a:AOX1c*-2 under control conditions, and overall there was little stimulation with antimycin A treatment with the exception of two lines: Pro*AOX1a:AOX1a*-3 and Pro*AOX1a:AOX1d*-3 displayed 90-fold and 34-fold induction in control conditions and 530-fold and 1,400-fold induction, respectively, with antimycin A treatment. These values were still not as high as the induction seen in the other lines even under control conditions. The transcript abundance of *AOX1b* under the controls of either the *AOX1c* or *AOX1d* promoter was similar to the control lines.

Table 1. List of all transgenic mutant lines constructed in this study.
Corresponding names, the genetic background, the specific promotor regions and the coding sequence (CDS) are listed.

Name	Genetic Background	Promoter (*3kb)	CDS
Pro*35S:AOX1a*	*aox1a*	*35S-CaMV*	*AOX1a*
Pro*35S:AOX1c*	*aox1a*	*35S-CaMV*	*AOX1c*
Pro*35S:AOX1d*	*aox1a*	*35S-CaMV*	*AOX1d*
Pro*AOX1a:AOX1a*-1	*aox1a*	*AOX1a**	*AOX1a*
Pro*AOX1a:AOX1a*-2	*aox1a*	*AOX1at*	*AOX1a*
Pro*AOX1a:AOX1a*-3	*aox1a*	*AOX1a**	*AOX1a*
Pro*AOX1a:AOX1c*-1	*aox1a*	*AOX1a**	*AOX1c*
Pro*AOX1a:AOX1c*-2	*aox1a*	*AOX1a**	*AOX1c*
Pro*AOX1a:AOX1d*-1	*aox1a*	*AOX1a**	*AOX1d*
Pro*AOX1a:AOX1d*-2	*aox1a*	*AOX1a**	*AOX1d*
Pro*AOX1a:AOX1d*-3	*aox1a*	*AOX1a**	*AOX1d*
Pro*AOX1c:AOX1a*	*aox1a*	*AOX1c**	*AOX1a*
Pro*AOX1c:AOX1c*	*aox1a*	*AOX1c**	*AOX1c*
Pro*AOX1c:AOX1d*	*aox1a*	*AOX1c**	*AOX1d*
Pro*AOX1d:AOX1a*	*aox1a*	*AOX1d**	*AOX1a*
Pro*AOX1d:AOX1c*	*aox1a*	*AOX1d**	*AOX1c*
Pro*AOX1d:AOX1d*	*aox1a*	*AOX1d**	*AOX1d*

As outlined earlier the *AOX1b* coding sequence is located upstream of *AOX1a* and is therefore within the 3 kb region selected as *AOX1a* promoter. *AOX1b*, however, lacks most of its own promoter region as only 276 bp upstream of the translational start site are within the 3 kb fragment. This might point to a high repression of *AOX1b* when it is present within the natural genomic context, that is derepressed in the transformed lines.

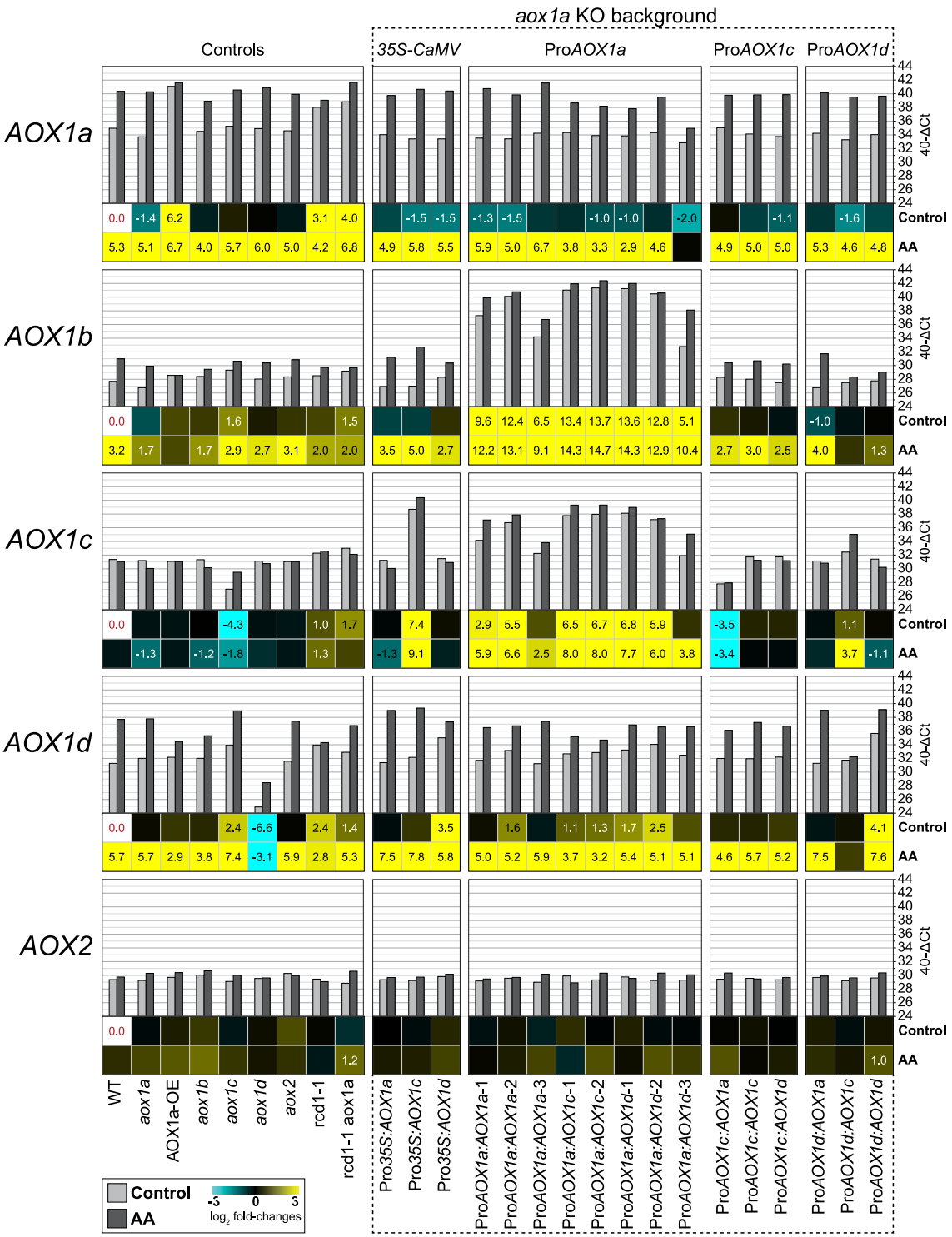


Figure 2. Transcript abundance of AOX isogenes in the various mutant lines used in this study.
To analyse the transcript abundance of the different AOX isogenes, 10-day old seedlings were spray-treated with 50 µM Antimycin A (AA) (or H2O as mock control). After 3 h incubation, total RNA was extracted and relative expression of AOX was measured by quantitative RT-PCR. The bar charts display expression levels that were calculated by comparing the Ct values of the gene of interest with the Ct values of the reference gene Actin 7 (ACT7) (Shu et al., 2013) and subtracting this from the arbitrary cycle number of 40 (40-delta Ct). The heatmap displays $\log_2$ fold changes relative to the wild type control ($2^{-(\Delta\Delta Ct)}$) Values only represent one technical replicate – i.e. 4 seedlings from each line were pooled for RNA isolation. Each line represents an individual transformation event. Primers used are displayed in Supplemental Table S2.

The transcript abundance of *AOX1c* was not induced by antimycin A in the control lines (wild type, *aox1a*, *AOX1a-OE*, *aox1b, aox1c, aox1d, aox2, rcd-1*, *rcd-1:aox1a*), consistent with reports in the literature that it does not respond to mitochondrial dysfunction (Clifton et al., 2005). Transcript abundance of *AOX1c* was reduced in the *aox1c* KO lines. For the *35S CaMV* lines only one line displayed increased levels of expression. As observed with *AOX1b* transcript abundance all the Pro*AOX1a* lines had induced levels of transcripts, the exceptions were Pro*AOX1a:AOX1a*-3 and Pro*AOX1a:AOX1d*-3, that were also the lines with the lowest *AOX1b* transcript abundance in the Pro*AOX1a* lines (albeit still induced). The Pro*AOX1c* and Pro*AOX1d* lines all displayed the expected trend for *AOX1c* transcript abundance, with the Pro*AOX1c:AOX1c* line similar to wild type and the Por*AOX1a:AOX1c* line showing induction with antimycin A.

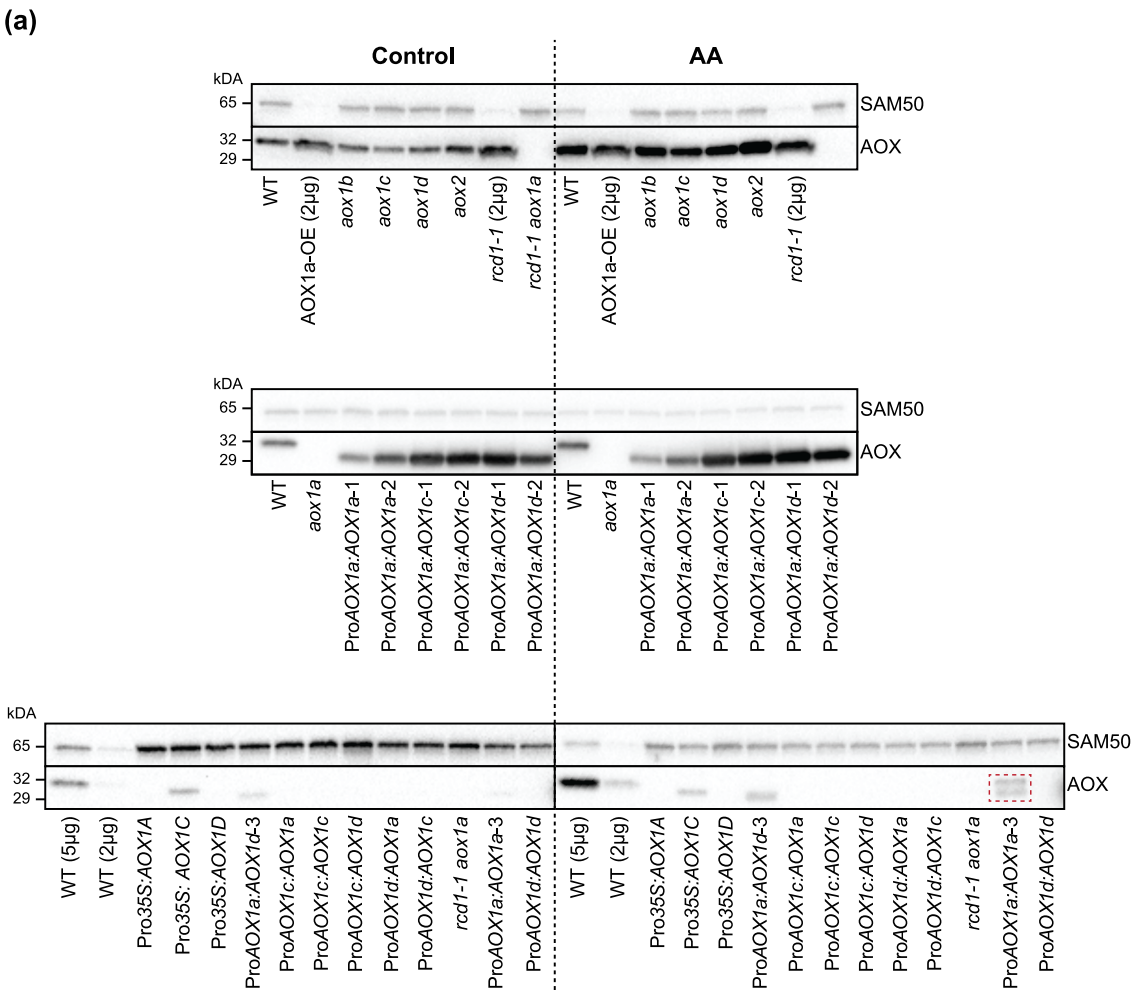
The transcript abundance of *AOX1d* displayed the expected trend in all the lines. In the control lines it was reduced in abundance in the *aox1d* mutant, and displayed induction in the *rcd1* background. It was induced in all the *35S CaMV* lines, Pro*AOX1a* lines, Pro*AOX1c* lines, and Pro*AOX1d* lines, with Pro*AOX1d:AOX1c* the exception where it was expressed at wild type levels and not inducible by antimycin A.

The transcript abundance of *AOX2* does not change in control and antimycin A treatment in any of the lines which is in accordance with its overall very low expression level in leaves and its proposed role in seed maturation and the early stages of germination (Clifton et al., 2006).

In summary the transcript abundance observed with the lines used in this analysis were as expected in most cases. However, a notable exception was the transcript abundance of *AOX1b* and *AOX1c* in the Pro*AOX1a* lines. Irrespective of the coding sequence downstream of the promoter all these lines displayed elevated levels of *AOX1b* and *Aox1c*.

3.3.3 AOX Protein Abundance

To further investigate the role of different AOX isoforms in response to mitochondrial dysfunction, immunoblotting of total protein from isolated mitochondria was performed. Using a monoclonal antibody with affinity for all five AOX isoforms, protein abundance was measured for all lines in response to antimycin A and control conditions (Elthon et al., 1989, Finnegan et al., 1999) (Figure 3a). AOX protein abundance in the wild type increases 3-fold in response to antimycin A treatment (Figure 3). Even though the *AOX1a* transcript abundance in the *aox1a* KO showed a similar level as the wild type in response to treatment with antimycin A and in the mock control, no AOX protein band was detected.


(a)
Control
AA
kDA
65
32
29
SAM50
AOX
WT
AOX1a-OE (2µg)
aox1b
aox1c
aox1d
aox2
rcd1-1 (2µg)
rcd1-1 aox1a
aox1a
ProAOX1a:AOX1a-1
ProAOX1a:AOX1a-2
ProAOX1a:AOX1c-1
ProAOX1a:AOX1c-2
ProAOX1a:AOX1d-1
ProAOX1a:AOX1d-2
WT (5µg)
WT (2µg)
Pro35S:AOX1A
Pro35S: AOX1C
Pro35S:AOX1D
ProAOX1a:AOX1d-3
ProAOX1c:AOX1a
ProAOX1c:AOX1c
ProAOX1c:AOX1d
ProAOX1d:AOX1a
ProAOX1d:AOX1c
rcd1-1 aox1a
ProAOX1a:AOX1a-3
ProAOX1d:AOX1d

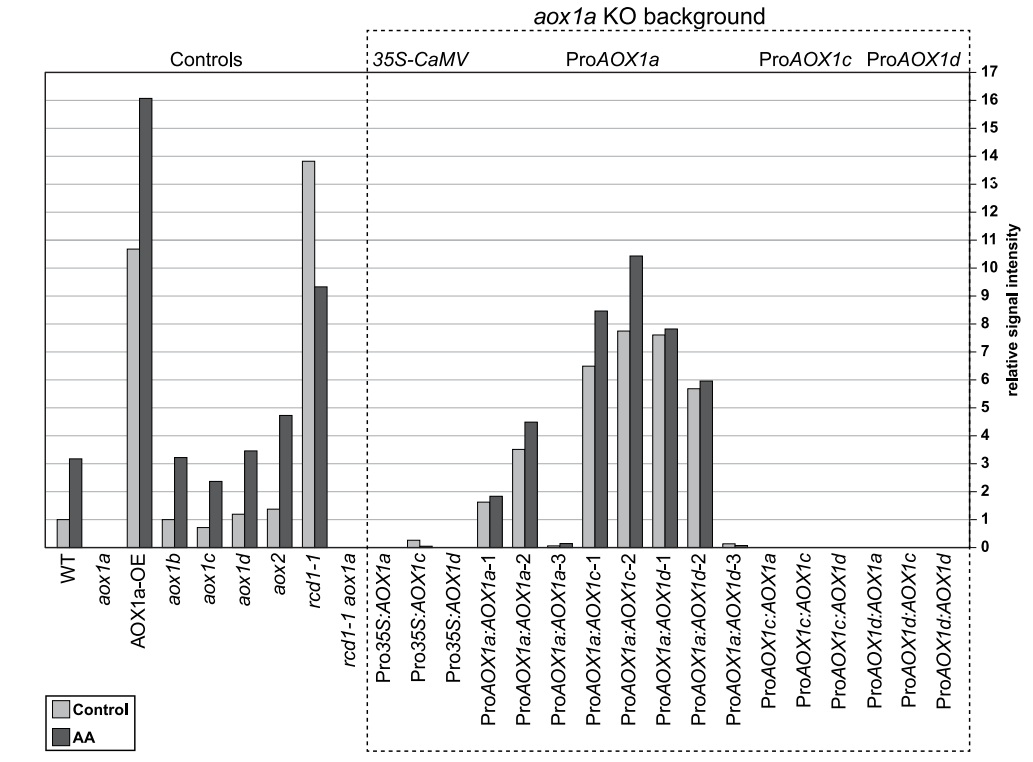

(b)
aox1a KO background
Controls
35S-CaMV
ProAOX1a
ProAOX1c
ProAOX1d
relative signal intensity
Control
AA
WT
aox1a
AOX1a-OE
aox1b
aox1c
aox1d
aox2
rcd1-1
rcd1-1 aox1a
Pro35S:AOX1a
Pro35S:AOX1c
Pro35S:AOX1d
ProAOX1a:AOX1a-1
ProAOX1a:AOX1a-2
ProAOX1a:AOX1a-3
ProAOX1a:AOX1c-1
ProAOX1a:AOX1c-2
ProAOX1a:AOX1d-1
ProAOX1a:AOX1d-2
ProAOX1a:AOX1d-3
ProAOX1c:AOX1a
ProAOX1c:AOX1c
ProAOX1c:AOX1d
ProAOX1d:AOX1a
ProAOX1d:AOX1c
ProAOX1d:AOX1d

Figure 3. Alternative oxidase protein abundance in the lines used in this study.
(a) Expression of AOX protein assessed by immunoblotting in aox1a KO lines complemented with any coding sequence driven by the AOX1a 3 kb promoter in the control and antimycin A (AA) treatment. Specific AOX bands of the complemented lines run slightly lower (29 kDa) compared to wild type (34 kDa) and all control bands including rcd1-1. Protein (10ug total protein if not stated otherwise) of pure isolated mitochondria from different lines 3 h after treatment with antimycin A (50 µM) or mock control treatment was separated in reducing SDS-PAGE and immunoblotted with two different antibodies separately. The antibodies detect either all AOX isoforms or SAM50, the latter a mitochondrial outer membrane protein that was used to visualize protein loading but not for quantification. (b) Quantification of the specific AOX signal intensities obtained by immunoblotting. Quantification was performed using Image Lab software (V.6.0.1, Bio-Rad Laboratories) by comparing the signal density relative to the wild type. Optimal exposure times were chosen to avoid saturated signal intensity of prominent AOX bands. Normalization of the bands to total protein in each lane using stain-free imaging technology (Bio-Rad Laboratories).

This lack of immunodetectable AOX protein as a result of a T-DNA insertion confirms previous studies (Giraud et al., 2008). The AOX1a-OE line shows a 10-fold increased AOX protein abundance under control conditions that is further induced by antimycin A reflecting the increased *AOX1a* transcript abundance in this line measured by qRT-PCR (Figure 3a). All other control lines lacking one of the other four AOX isoforms show a similar protein abundance compared to the wild type with the exception for *aox2* that has a higher abundance of AOX protein in response to antimycin A. AOX protein abundance increased in *rcd1-1*, with increased levels that are comparable to those detected for AOX1a-OE in response to antimycin A and control treatment (Shapiguzov et al., 2019). The same lines used in this study confirm the high AOX protein level in both lines and for both conditions. No AOX protein was detected in the double mutant *rcd1-1 aox1a* before or after the 3 h AA treatment. By contrast, a previous study detected an immunoreactive band with a higher mobility of SDS-PAGE that was proposedly AOX1d, due to its lower molecular weight compared to AOX1a, after treating leaf discs overnight with antimycin A (Shapiguzov et al., 2019).

For the AOX protein isoforms that were driven by the *35S CaMV* promoter, protein levels were very low or undetectable, which does not correlate with the transcript abundance. Pro*35S*:*AOX1a* in the *aox1a*-background does not show any immunodetectable AOX which matches the measured transcript abundance that was unchanged compared to the wild type, yet a protein band is clearly detectable in the wild type (Figure 3a). Pro*35S*:*AOX1c*, however, has highly induced transcript abundance of *AOX1c* that translates into low levels of AOX protein. Compared to the wild type the detected AOX band has a relative signal density of 25% in the control treatment and only 5% following antimycin A treatment (Figure 3b). Even though Pro*35S*-*AOX1d* has induced transcript abundance of *AOX1d*, no AOX protein is detectable for any condition.

The *aox1a*-KO lines complemented with constructs that drive any *AOX1a/c/d* coding sequence under the control of the *AOX1a* promoter express AOX protein with large

differences in protein abundance (Figure 3a and b). In Pro*AOX1a*:*AOX1a*-1 the protein abundance is only slightly higher than the wild type and does not increase in response to antimycin A. Pro*AOX1a*:*AOX1a*-2 protein abundance is much higher with and 3-fold and 4.5-fold increase in the control treatment and in response to antimycin A, respectively. Pro*AOX1a*:*AOX1a*-3, however, has extremely low amount of expressed AOX protein. Both *aox1a* KO lines complemented with the *AOX1c* coding sequence,

Pro*AOX1a*:*AOX1c*-1 and Pro*AOX1a*:*AOX1c*-2, express high levels of AOX protein that is 6-fold higher relative to the wild type in the control treatment and increases in response to antimycin A. Two of the three complementation lines with the *AOX1d* coding sequence under the control of the *AOX1a*, Pro*AOX1a*:*AOX1d*-1 and Pro*AOX1a*:*AOX1d*-2, have a protein abundance almost similar to Pro*AOX1a*:*AOX1c*-1/2 but their protein level does not further increase by antimycin A. Pro*AOX1a*:*AOX1d*-3, however, is similar to Pro*AOX1a*:*AOX1a*-3 with an extremely low amount of expressed AOX protein. While the band in the control treatment is very faint, a double band in the antimycin A treatment is visible. Since pure mitochondrial fractions were used for separation and visualization both bands were used for quantification.

3.3.4 *AOX1a* promotor mediated regulation rescues stress-phenotype regardless of the expressed isoform

Arabidopsis plants lacking the main stress-responsive isoform AOX1a do not show any apparent phenotypic changes under non-stress conditions despite greatly altered transcriptional changes (Giraud et al., 2008, Fiorani et al., 2005). Treatment of *aox1a* knockout plants with antimycin A, inducing mitochondrial dysfunction, leads to wilted plants and necrosis while the leaflets of wild type plants have no visible phenotypic changes (Strodtkötter et al., 2009). To confirm the known phenotype, 10-day old homozygous *aox1a* KO seedlings (growth stage: 1.04; Boyes et al. 2001) were sprayed with antimycin A (Figure 4a). Seventy-two hours after treatment wild type plants do not show any phenotypic changes while the *aox1a* KO line shows necrosis and wilted leaflets as previously reported (Figure 4a & b).

To get a better understanding of these effects, *aox1a* KO plants were analysed under a microscope at different time points (Figure 4b). Twenty-four hours after the initial treatment, the *aox1a* knockout seedlings show a very mild curling of the leaves, mainly of the first two true leaves. Forty-eight hours after treatment this curling becomes more

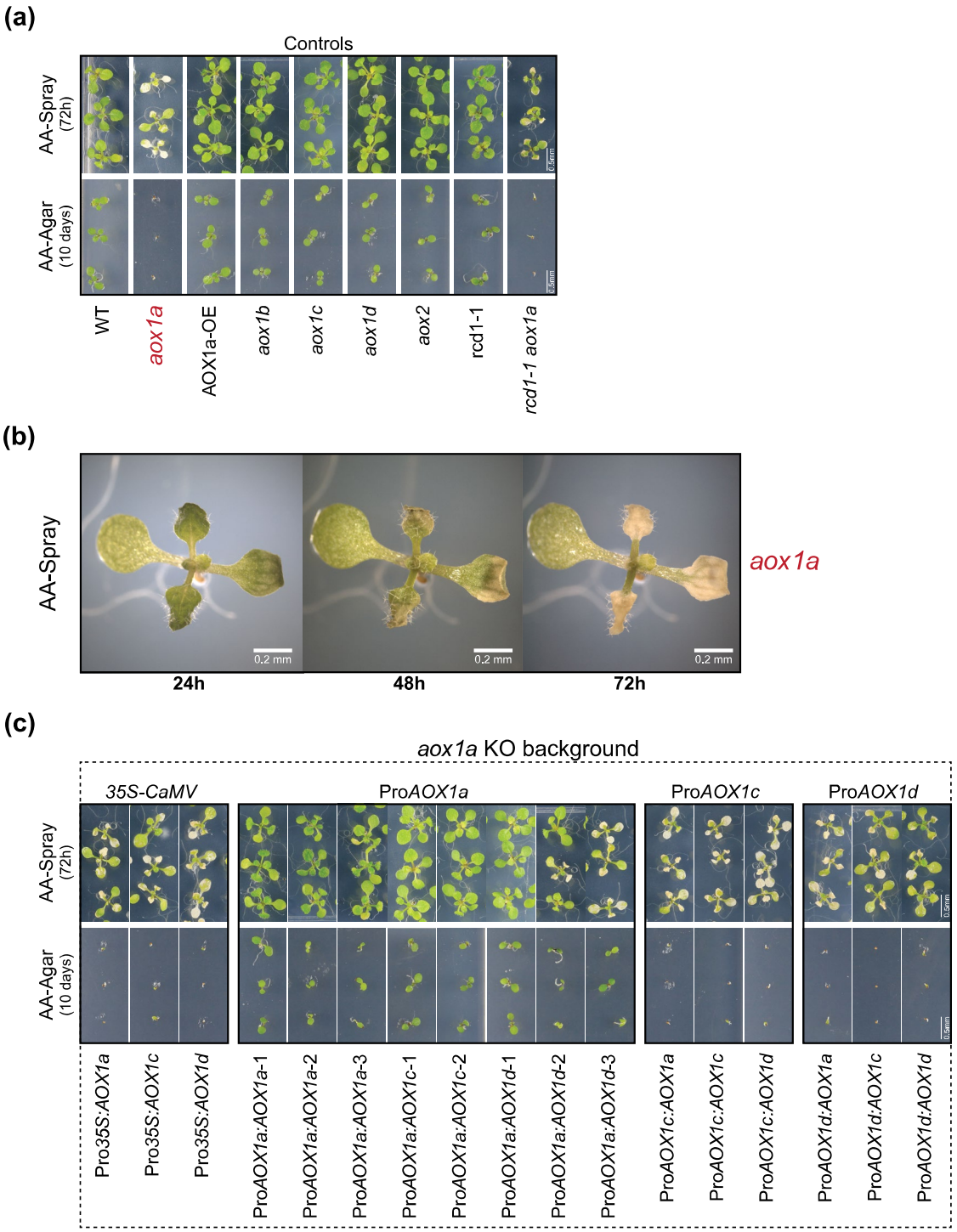


Figure 4. Complementation of the aox1a KO with AOX1a/c or AOX1d coding sequence recovers resistance to chemically induced mitochondrial dysfunction when driven by the native AOX1a promoter.

(a) Ten-day old seedlings of all control lines including wild type (Col-0) and the *aox1a* KO were grown on solid media and sprayed with antimycin A (AA) (50 µM). Pictures were taken 72 h after treatment. The same lines were sown on solid media complemented with AA (50 µM) following stratification in 0.1 % (m/v) agarose solution. Pictures were taken 10 days after sowing. (b) Microscopic images of the aox1a KO phenotype 24 h, 48 h and 72 h after spraying with AA (50 µM). (c) Similar experiment as described previously for all complementation lines in the aox1a KO background.

severe and necrosis of the leaves becomes visible affecting all leaves. Furthermore, large parts of the leaves show chlorosis. This becomes even more severe seventy-two hours after treatment with the majority of the leaves being affected. It must be noted that some leaves do not show any immediate phenotypic changes which could be a consequence of uneven application of antimycin A by spraying.

To characterize the phenotypic response to antimycin A of lines lacking one of the other members of the *AOX* multigene family, single knockout lines for *AOX1b/c/d* and *AOX2* were spray-treated as well. Seedlings of all other *aox* mutant lines do not show any phenotypic changes, which is in line with the wild type (Figure 4a). This is the same for other control lines, *AOX1a*-OE and *rcd1-1*. The *rcd1-1 aox1a* double mutant, however, shows a similar response to the *aox1a* knockout line. The induction of other MDS genes, by inactivation of repressor RCD1 as shown for this line on a transcript level (Shapiguzov et al., 2019) is not sufficient to compensate for the lack of AOX1a.

The effect of chemically induced mitochondrial dysfunction of those lines on germination was investigated by sowing the seeds on solid growth media supplemented with antimycin A. While the wild type seeds germinate normally, the *aox1a* KO stops after the emergence of the radicle (Figure 1a). A correlation between the resistance of seedlings to antimycin A spray and the ability to germinate normally when exposed to antimycin A can be seen for all control lines. Similar to the spray treatment, all single knockouts of the other AOX isoforms as well as *AOX1a*-OE and *rcd1-1* germinate comparable to the wild type while *rcd1-1 aox1a* stops after radicle emergence.

Under stress-conditions the gene expression of *AOX1d,* which encodes for the second stress-responsive isoform in Arabidopsis, is induced but cannot functionally compensate the lack of AOX1a (Strodtkötter et al., 2009, Kühn et al., 2015). Different explanations have been discussed including protein amount, post-translational fine regulation or cell specific localisation (Selinski et al., 2018a, Selinski et al., 2018b, Strodtkötter et al., 2009). To get a better understanding all *aox1a* KO complementation lines were exposed to antimycin A and sown on solid growth media supplemented with antimycin A (Figure 4c).

Pro*35S-AOX1a* did not show any induction of *AOX1a* gene expression or any other isoform (Figure 2) and no protein was detected via immunoblotting (Figure 3). Consequently, this line is not resistant to spray-treatment with antimycin A and the germination is inhibited (Figure 4). Even though the presence of the transgene was confirmed via PCR using promotor and coding sequence specific primer pairs (Supplemental Table S2), the transgene might be silenced due to multiple copies or by positional effects. Pro*35S-AOX1c*, however, showed induced expression of *AOX1c* (Figure 2) and a small amount of protein was detected in response to antimycin A and the

control treatment (Figure 3). Despite the expressed AOX1c protein, this line is not able to germinate and shows the antimycin A stress-phenotype (Figure 4). Thus, the AOX1c protein in this line not able to compensate the lack of AOX1a which might be due to the low amount of expressed protein which is only 26% relative to the wild type in the control conditions and only 5% in response to antimycin A. Pro*35S-AOX1d* shows induction of *AOX1d* on the transcript level (Figure 2) which does not translate into protein as no AOX band is visible by immunoblotting (Figure 3). In line with Pro*35S-AOX1a* this line is not resistant to spray-treatment with antimycin A and germination is inhibited (Figure 4).

All Pro*AOX1a*:*AOX1a* complementation lines can functionally compensate the lack of AOX1a (Figure 4). Despite the varying levels of immunodetected AOX1a protein (Figure 3), with Pro*AOX1a*:*AOX1a*-3 showing lower levels than the wild type, a small amount of *AOX1a* protein seems to be sufficient for complementation. Immunoblotting revealed a very high protein abundance of AOX1c in the lines Pro*AOX1a*:*AOX1c*-1 and Pro*AOX1a*:*AOX1c-2*, both lines can compensate the lack of AOX1a (Figure 3 and 4). Pro*AOX1a*:*AOX1d*-1 can also rescue the antimycin A stress induced phenotype and shows a normal germination in the presence of antimycin A (Figure 3). This indicates that regardless of the isoform the transcriptional regulation mediated by the *AOX1a* promoter leads to sufficient amount of protein, in this study AOX1c and AOX1d, to compensate the lack of AOX1a.

Differences can be seen for the two other Pro*AOX1a*:*AOX1d* lines. Pro*AOX1a*:*AOX1d*-2 and Pro*AOX1a*:*AOX1d*-3 do show a mild antimycin A stress induced phenotype with the latter being more severe (Figure 4). A similar trend can be seen for the germination experiment, with both lines having some seeds with inhibited germinating (Supplemental Figure S2). The ability to compensate the lack of AOX1a seems to correlate with the amount of AOX1d protein as Pro*AOX1a*:*AOX1d*-3 expressed very low levels of AOX1d (Figure 3). This could also indicate that post-translational fine regulation by metabolites, as shown to be different for oxaloacetate (OAA) between AOX1a and AOX1d (Selinski et al., 2018a), might play a role when the AOX1d protein level is very low. It is unclear which isoform leads to the observed compensation in all complementation lines that are driven by the *AOX1a* promoter given the AOX antibody cannot distinguish between the different isoforms. A commonality of these lines is the extremely high induction of *AOX1b* and high induction of *AOX1c* on the transcript level.

None of the other lines in the *aox1a* background that were complemented with constructs containing the different AOX coding sequences driven by the *AOX1c* and *AOX1d* promoter can rescue the antimycin A stress-phenotype and germination arrest. No protein could be detected for any of these lines which explains the lack of compensation.

3.4 Discussion

The overall aim of this set of experiments was to determine if any of the three AOX isoforms complement the lack of AOX1a under mitochondrial stress, specifically after treatment with antimycin A. The answer obtained was yes, in that it was clearly observed that both AOX1c and AOX1d could support germination and survival when treated with antimycin A. This only occurred, however, when expression was driven by the *AOX1a* promoter region. When the expression of any isoform was driven by either the *AOX1c* or *AOX1d* promoter region, no AOX protein was detected in these lines and they consequently did not survive antimycin A treatment. In these lines, however, not only AOX transcript was detected but it was further induced by antimycin A treatment. When the expression of the three isoforms was driven by the *35S* CaMV promoter, no survival was observed. It is to note, that the AOX1a OE line from a previous study (Umbach et al., 2005) did produce substantial amounts of AOX1a transcript and protein, and supported survival of antimycin A treatment. Importantly this over-expressing line was constructed in a wild type background and consequently contains endogenous AOX1a. Over-expressing lines for AOX have also been constructed for *Nicotiana benthamiana* (tobacco) and *Solanum tuberosum* (potato) in wild type backgrounds (Vanlerberghe et al., 1994, Hiser et al., 1996), but to our knowledge, no AOX over-expressing line has been constructed in an Arabidopsis *aox1a* mutant background. The reason that no AOX protein accumulated in these lines is unclear but may relate to post-transcriptional regulation of AOX.

While it was evident, that either AOX1a, AOX1c or AOX1d could support the germination and survival of plants when treated with antimycin A and when expressed at sufficient levels, there were some discrepancies observed that require further investigation. Three lines in this study expressed a low amount of AOX protein, namely Pro*35S*:*AOX1c*, Pro*AOX1a:AOX1a*-3 and Pro*AOX1a:AOX1d*-3. Only the Pro*AOX1a:AOX1a*-3 displayed a survival phenotype. The Pro*35S*:*AOX1c* line constitutively expressed as much immunodetectable protein as the Pro*AOX1a:AOX1a*-3 line following antimycin A treatment, but it did not support germination or survival on antimycin A. AOX transcript abundance in line Pro*AOX1a:AOX1d*-3 was less and the germination was delayed following treatment with antimycin A. This line further showed necrotic lesions in response to antimycin A (Figure 4 and Supplemental Figure S1 & S2). Thus, while all three isoforms can support survival of antimycin A treatment, preliminary evidence from this study suggests that AOX1a does it most efficiently. Due to the fact, that this is based on a limited number of lines at this stage, it is unclear, if these results relate to a threshold amount of protein, and/or any tissue/cell specific expression of that protein. While AOX1c protein was expressed in greater amounts than AOX1a, there may be a yet unknown tissue or

cell specific enrichment as they were expressed under different promoters. To draw the definite conclusion that some isoforms can support growth or survival more efficiently on antimycin A compared to others, the analysis of a greater number of lines with limiting amount of AOX isoforms under different AOX promoters would be required.

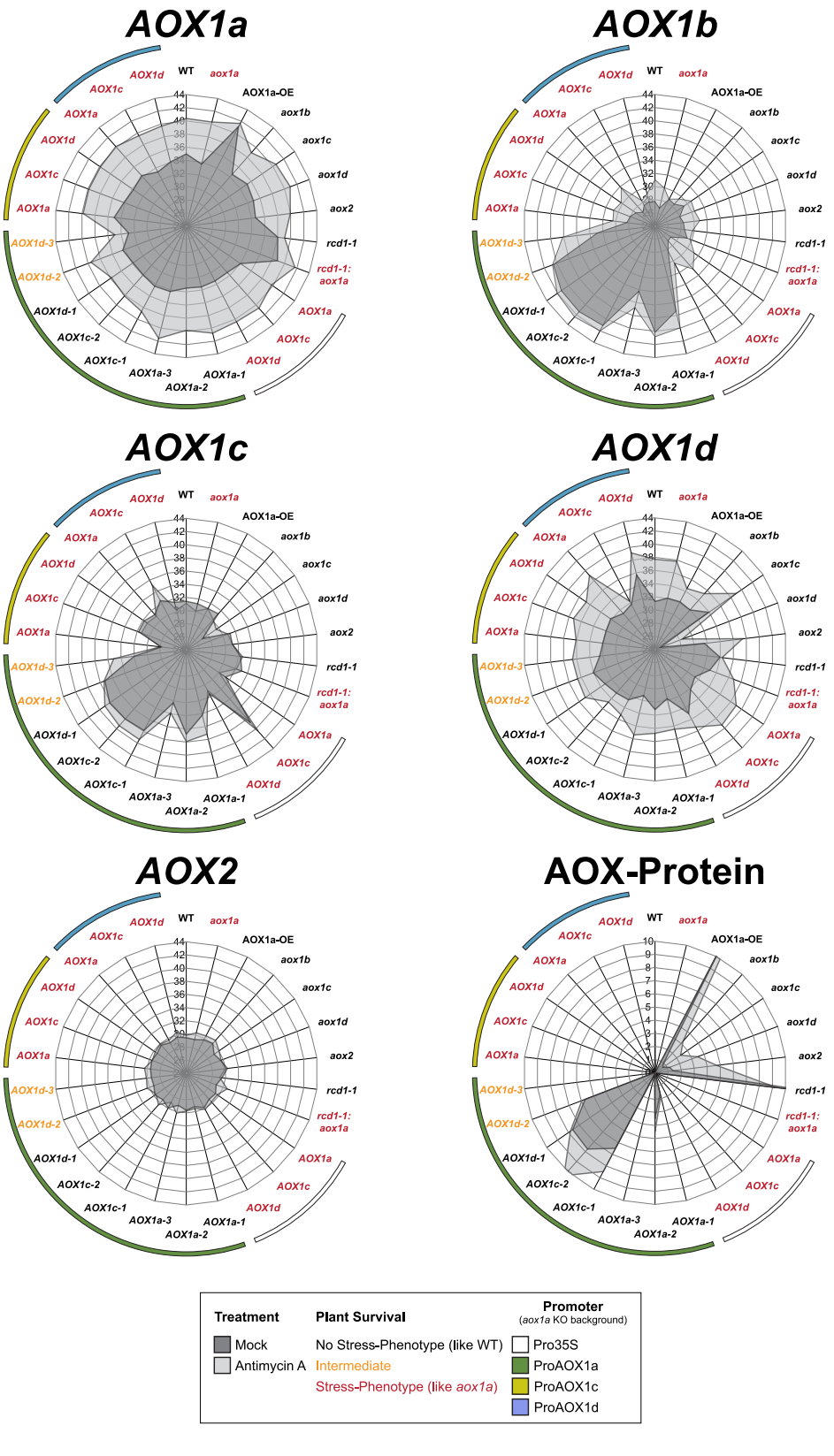


Figure 5. Radar plots summarizing AOX gene expression and protein abundance in *aox1a KO* complementation lines in response antimycin A.

Overview showing gene expression data (Figure 2) and relative protein abundance (Figure 3) of all *aox1a* KO complementation lines and controls that were spray-treated with 50 µM antimycin A (AA) (or H_2O as mock control). Gene expression values were calculated by comparing the Ct values of the gene of interest with the Ct values of the reference gene Actin 7 (ACT7) (Shu et al., 2013) and subtracting this from the arbitrary cycle number of 40 (40-delta Ct). The radar plot showing AOX protein displays relative AOX signal intensities (fold-change compared to the WT control) obtained via immunoblotting. Plant survival was scored according to Figure 4 and Supplemental Figure 1 & 2. The intermediate phenotype germinates in the presence of AA but shows the AA stress phenotype after spray treatment.

Additional observations were raised in this study that warrant further investigation. The transcript abundance of *AOX1b* was highly induced in all *aox1a* KO lines (log_2 fold-changes up to 14.7 corresponding to 27,000-fold compared to the wild type) when the *AOX1a* promoter was used to drive expression (Figure 2 & 5). The *AOX1b* coding sequence is located 1,505 bp upstream of the translational start site of *AOX1a* and is therefore located within the 3 kb *AOX1a* promoter region (Figure 1). This *per se* should not mean that transcript abundance of *AOX1b* would be highly induced but may indicate that the expression of *AOX1b* is strongly repressed *in vivo*. Since this was observed in independent lines, it is highly unlikely a result of the insertion of a foreign gene in a highly expressed gene coding region. While the high *AOX1b* transcript abundance maybe explained by its location within the *AOX1a* promoter region, a similar increase in gene expression can be detected for *AOX1c* (Figure 2). Besides an increased transcript abundance in the overexpressing line Pro*35S:AOX1c*, in response to antimycin A as well as the control treatment relative to the wild type, all *aox1a* lines complemented with any AOX coding sequence driven by the 3kb *AOX1a* promoter show increased transcript abundance for *AOX1c* (Figure 2 & 5). This correlates with the *AOX1b* transcript abundance of the same lines. It is unclear why the transcript abundance of *AOX1c* increases in these lines.

The high transcript abundance for both *AOX1b* and AOX1c in these lines suggests that this may be a result of the transformation of the *aox1a* line with the 3 kb *AOX1a* promoter sequence. While the underlying mechanism is unclear, further investigation to determine the integrity of the inserted DNA and the expression of full-length transcripts for *AOX1b* and *AOX1c* in these lines is required.

The transcript abundance of *AOX1d* has previously been shown to increase in *aox1a* KO lines in response to antimycin A (Strodtkötter et al., 2009), which is in line with the results of this study. Under control conditions, the *AOX1d* transcript level is not induced in the wild type and the *aox1a* KO (Figure 2 & 5). This confirms that *AOX1d* is a stress-responsive isoform and that the induction is not caused by the lack of AOX1a protein

under normal growth conditions. Furthermore, induction can be detected in all lines in response to antimycin A except for Pro*AOX1d:AOX1c*.

It has previously been proposed, that AOX1d is not able to compensate the lack of AOX1a, based on increased transcript abundance. This was further supported by induced protein of an isoform other than AOX1a in mutant lines lacking AOX1a in combination with other defects (Kühn et al., 2015, Konert et al., 2015). This induction on the protein level, however, has never been shown for the single *aox1a* KO in control conditions or in response to antimycin A. In this study no AOX protein band can be detected, either in the *aox1a* KO nor in any other line that shows highly induced *AOX1d* transcript level. In fact, even for the two lines that have increased *AOX1d* transcript not only in response to antimycin A but also in the control treatment, Pro*35S:AOX1d* and Pro*AOX1d:AOX1d*, no AOX protein band can be detected in pure mitochondrial fractions by immunoblotting (Figure 3).

Table 2. Molecular mass of AOX isoforms in Arabidopsis.

The molecular mass of the individual AOX isoforms lacking the mitochondrial transit peptide was predicted using a science gateway molecular mass calculator (http://www.sciencegateway.org/tools/proteinmw.htm). The TargetP-2.0 Server (http://www.cbs.dtu.dk/services/TargetP/) was used to predict the presence of a N-terminal presequence for each AOX protein sequence.

AOX Protein (without transit peptide)	Predicted Molecular Weight (kDa)
AOX1a	33.45
AOX1b	32.59
AOX1c	32.85
AOX1d	31.6
AOX2	37.47

Based on the analyses carried out in this study the overall question arises, why, as observed in many instances, a high transcript level is not accompanied by an increase in protein. While there are many levels of regulation for AOX expression, the transcript induction in general leads to induction of AOX protein. This has been observed in a variety of studies in Arabidopsis, tobacco, potato and soybean (Umbach et al., 2005, Vanlerberghe et al., 1994, Hiser et al., 1996). The only difference between these studies and the data presented here is the difference in the genotypic background. While the studies above all used a wild type background, including the AOX overexpressor lines, this study used the *aox1a* background. Observed changes in thousands of transcripts in

an *aox1a* background (Giraud et al., 2009) may result in *AOX* transcripts not being translated or mis-expressed. An alternative explanation is that the *aox1a* knock-out line expresses residual, non-functional, transcripts as observed in this study. As this transcript is non-productive and contains the T-DNA sequence (qRT-PCR primer bind in exon 4 behind T-DNA insert; Supplemental Figure 3), it may trigger targeted breakdown of *AOX* transcript via a microRNA silencing mediated mechanism. As the AOX genes display high sequence identity, even at a nucleic acid level, all transcripts may be targeted. Thus, while transcript abundance can be detected, it would be interesting to establish if full length transcripts are stable. Therefore, it would be worth carrying out full length transcript analyses for all *AOX* genes in these lines to allow for a more conclusive interpretation of results.

All AOX protein in this study was detected using a monoclonal antibody raised against a recombinant soybean AOX protein and shows a single band with the exception of Pro*AOX1a:AOX1d-3* which displays two distinct bands (Figure 3a). As immunoblotting was performed with pure mitochondrial fractions, both bands were used for relative quantification of the detected signal. Interestingly, all detected bands of complemented *aox1a* KO lines run at around 29 kDa, compared to the bands of all control lines including wild type, AOX1a-OE, *aox1b/c/d*, *aox2* and *rcd1-1* that have the expected size of around 32 kDa (Figure 3a). Several studies report a lower AOX band that can be detected in several mutant lines lacking *AOX1a* in combination with other genes or mETC pathway complexes (Kühn et al., 2015, Konert et al., 2015). Increased AOX1d protein level was proposed by Kühn et al. (2015) based on increased *AOX1d* transcript abundance and the detection of increased AOX protein other than AOX1a under standard growth condition. The line used in that study lacks functional AOX1a and is further impaired in the COX pathway. The detected AOX bands from purified mitochondria of wild type and the mutant line (*aox1a:rpoTmp)* grown under standard growth conditions are of similar size (32 kDa). Two different bands were detected in a study using a mutant line defective in a specific regulatory B′γ subunit of the protein phosphatase 2A (PP 2A) (Konert et al., 2015). The faint and lower band (29 kDa) detected in pure mitochondrial fractions was identified to be AOX1d specific by using a combination of immunoblotting, data-dependent acquisition and selected reaction monitoring mass spectrometry techniques. AOX1d has the smallest predicted molecular weight of all AOX isoforms with 31.6 kDa which would fit to the results by Konert et al. (2015). Overall, the variation of the predicted molecular masses between the different isoforms of the AOX1 subfamily in Arabidopsis is very small (Table 2).

The AOX specific bands detected in this study (Figure 3a), especially the lower bands at 29 kDa in the *aox1a* complementation lines indicate that the interpretation of detected AOX bands is very challenging. A good example represents the *AOX1c* overexpressing

line Pro*35S:AOX1c* that only shows upregulation of *AOX1c* on the transcript level and has a faint band at 29 kDa (Figure 3a). Based on the gene expression, which must be interpreted carefully, and the lack of AOX1a in the mutant background, it is highly likely that this line is specific for AOX1c protein.

This is different for the lower AOX bands with very high signal intensities relative to the wild type in response to antimycin A and the control treatment detected for the complementation lines Pro*AOX1:AOX1a-1/2*, Pro*AOX1:AOX1c-1/2* and Pro*AOX1:AOX1d-1/2*. These lines have been complemented with the *AOX1a* promoter region that contains the *AOX1b* coding sequence. As outlined earlier, the transcript abundance of *AOX1b* in these lines is extremely induced which indicates that the high protein abundance could be AOX1b protein. This is further supported by the fact, that there is a correlation between the transcript and protein level with these lines and the two lines Pro*AOX1:AOX1a/d-3* that have very low relative signal intensities and also show the lowest level of AOX1b transcript induction (Figure 3). A similar correlation in those lines can be seen between transcript and protein abundance of AOX1c. Consequently, there is strong evidence that the detected AOX band with very strong signal intensities in the complementation lines could be specific for AOX1c or a combination of both AOX1b and AOX1c isoforms.

The binding site of the monoclonal antibody, most widely used for the immunodetection of AOX, is located in the C-terminal end of the protein and has been localised to the sequence RADEAHHRDVNH (Finnegan et al., 1999). This sequence is highly conserved in AOX isoforms from different plant species but is also present in fungi (*Aspergillus niger*), algae (*Chlamydomonas sp.*) or protozoa (*Trypanosoma brucei*) with small differences compared to the consensus plant sequence (Finnegan et al., 1997). Besides Arabidopsis, multiple AOX bands haven been detected in several other species using isolated mitochondria like *Sauromatum guttatum* (voodoo lily) (Elthon and McIntosh, 1987), soybean (Finnegan et al., 1997) and *Cicer arietinum* (chickpea) (Sweetman et al., 2019). Comparison of immunodetected protein bands of samples from different tissues and at different developmental stages allowed for the differentiation of the AOX isoforms in some legume species (Sweetman et al., 2019, Finnegan et al., 1997).

While the multiple bands expressed in other species, especially in soybean and chickpea, can be ascribed to different genes, it is unclear if mobility on gels is a good indication of the Arabidopsis isoforms. This is based on the following evidence:

1) When screening these transgenic lines for AOX protein abundance initially crude mitochondrial preparations were used. These were obtained using low and high-speed centrifugation steps, but without sucrose or Percoll purification steps. With

such mitochondria we routinely detected a protein band at 29 kDa, likely representing cross reaction with a non-mitochondrial protein. In samples of crude mitochondria or whole-leaf tissue extracts the amount of AOX protein is very small compared to the total amount, requiring different protein loading and elongated exposure times for immunoblots.

2) Using the same transgenic line to prepare mitochondria on two separate occasions a single and double band upon immunoblotting was detected.

We conclude from these preliminary results, that crude extracts cannot be used to judge the abundance of mitochondrial proteins due to the cross reaction with an unknown protein. However, it is possible that AOX proteins undergo post-translational modification that may affect their mobility. While the modification is not known, it is tempting to suggest that this may be phosphorylation as AOX has been reported to interact with the protein phosphatase 2A (PP 2A) (Konert et al., 2015). The phosphorylation or lack of it may affect the stability of AOX proteins and may account for the discrepancy between protein and transcript abundance observed in this study. The transgenic lines in this study, with other mutant lines encoding the protein phosphatase 2A (PP 2A) would be ideal material to test this hypothesis.

3.5 Material and Methods

3.5.1 Plant Material

Arabidopsis thaliana Columbia-0 (Col-0) was used as the wild-type control for all experiments in this study. Homozygous T-DNA insertion lines for *aox1a* (SALK_084897) (Giraud et al., 2009) ;*AOX1a*-OE (Umbach et al., 2005); *aox1b* (SALK_040620); *aox2* (SALK_014733); *rcd1-1* (Overmyer et al., 2000) and *rcd1-1 aox1a* (Brosche et al., 2014) were obtained.

3.5.2 Growth Conditions and Stress Treatments

Arabidopsis plants were grown in growth chambers at 22°C under 120 mmol m^{-2} s^{-1} light in a 16-h light/8-h dark photoperiod either on soil or Gamborg's B5 medium (PhytoTechnology, Austratec) containing 3.21 g/L Gamborg's B5 salts (Austratec) supplemented with1 % (m/v) sucrose, 2 mM MES hydrate (Sigma-Aldrich), and 0.9% (w/v) Difco agar (BD Biosciences). The pH was adjusted to 5.8. All seeds were surface sterilized and stratified for 48 h before being transferred to growth chambers.

Stress treatments were performed on 10-day-old Arabidopsis seedlings with four rosette leaves (Stage 1.04;Boyes et al. (2001)) on B5 medium by spraying the plants with 50 µM antimycin A complemented with 0.1% (v/v) Tween 20 (Sigma-Aldrich) or a corresponding water solution (mock control). Plant tissue for qRT-PCR was harvested 3 h after stress treatment and shock-frozen in liquid nitrogen for downstream analysis. Plants were also germinated on B5 medium supplemented with 50 µM AA following stratification in 0.1% (m/v) agarose for 3 days.

3.5.3 Generation of Transgenic Arabidopsis Plants

The 3 kb promotor fragments upstream of their translational start codon and the full-length coding sequences of *AOX1a* (At3g22370), *AOX1c* (At3g27620) and *AOX1d* (At1g32350) were amplified from wild-type (Col-0) cDNA via PCR. Different DNA fragments of the *AOX1a/c/d* coding sequences as well as fragments of the native 3 kb *AOX1a/c/d* promotor regions as well as the fragment of the constitutive promotor cauliflower mosaic virus *35S* (CaMV *35S*) were cloned into the binary destination vector pCAMBIA1300 via Gibson assembly. All vectors were transferred into the *aox1a* mutant background by Agrobacterium mediated floral dipping (Clough and Bent, 1998)). Homozygous transgenic lines were selected via resistance to hygromycin-B (15 µg/ml) (Harrison et al., 2006) and

screened for homozygosity of the T-DNA insert (Alonso and Stepanova, 2003) and the transgene with specific primer pairs. The full list of transgenic lines as well as all cloning and genotyping primers used in this study are listed in Supplemental Table S2.

3.5.4 qRT-PCR

For quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis 1 µg of total RNA was reverse transcribed using the cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's instructions. qRT-PCR was conducted using 50 µg of cDNA and a final primer concentration of 300 nM with a SYBR Green PCR Master Mix and a QuantStudio 12K flex real-time PCR system (Applied Biosystems). Default settings were used for the cycle threshold (Ct) value determination. The mRNA levels for each gene were quantified and normalized using ACT7 (AT5G09810) as reference gene. After each run, a melting curve analysis was performed to verify target-specific product amplification. If not stated otherwise, gene-specific primer pairs were designed using QuantPrime (Arvidsson et al., 2008). All primer sequences used in this study are listed in Supplemental Table S2.

3.5.5 Isolation of Mitochondria

For the isolation of mitochondria, seeds were surface sterilized, stratified for 48 h and sown on solid media. Plants were grown for 2 weeks and pure mitochondria isolated as described previously (Lyu et al., 2018). Fractions were stored at -80°C and maintained on ice when in use.

3.5.6 Immunodetection

Proteins of different mitochondrial fractions were resolved by SDS-PAGE using stain-free gels (Bio-Rad, Sydney) and transferred to a Hybond-C extra nitrocellulose membrane (Bio-Rad, Sydney). Immunodetection was performed as previously described (Wang et al., 2012). The intensities of bands of interest were quantified using the Image Lab software and normalized to protein loading determined via stain-free technology (Bio-Rad, Sydney). Antibodies used were raised against AOX (Elthon et al., 1989) and SAM50 (Carrie et al., 2010).

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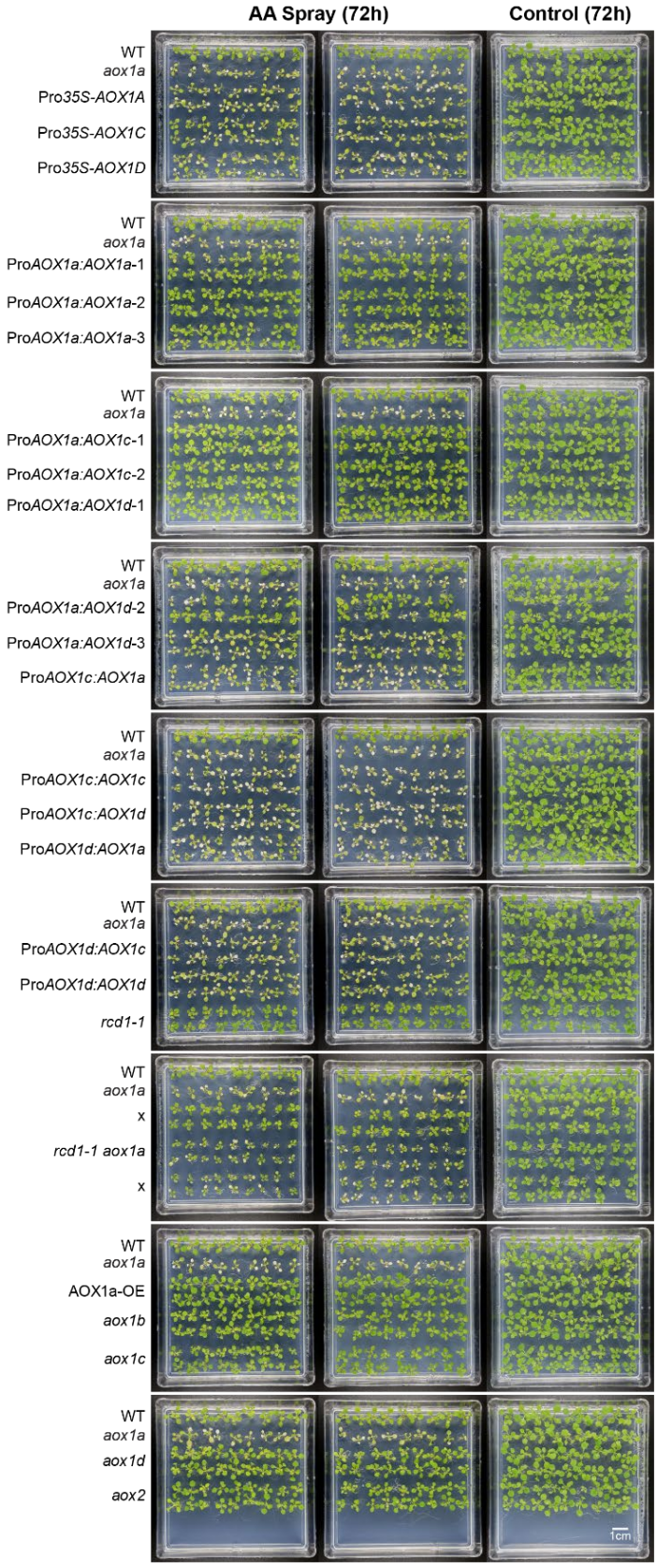
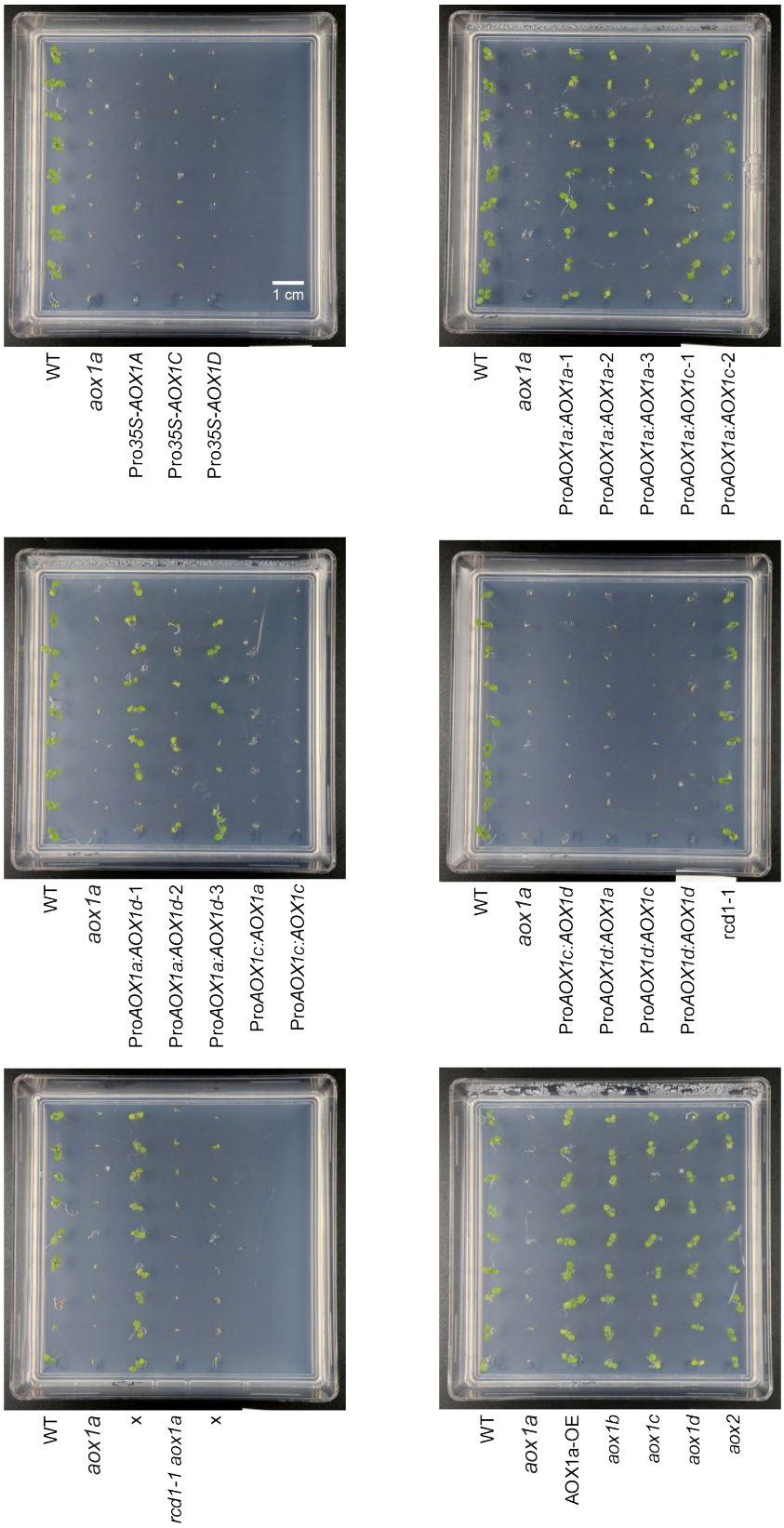


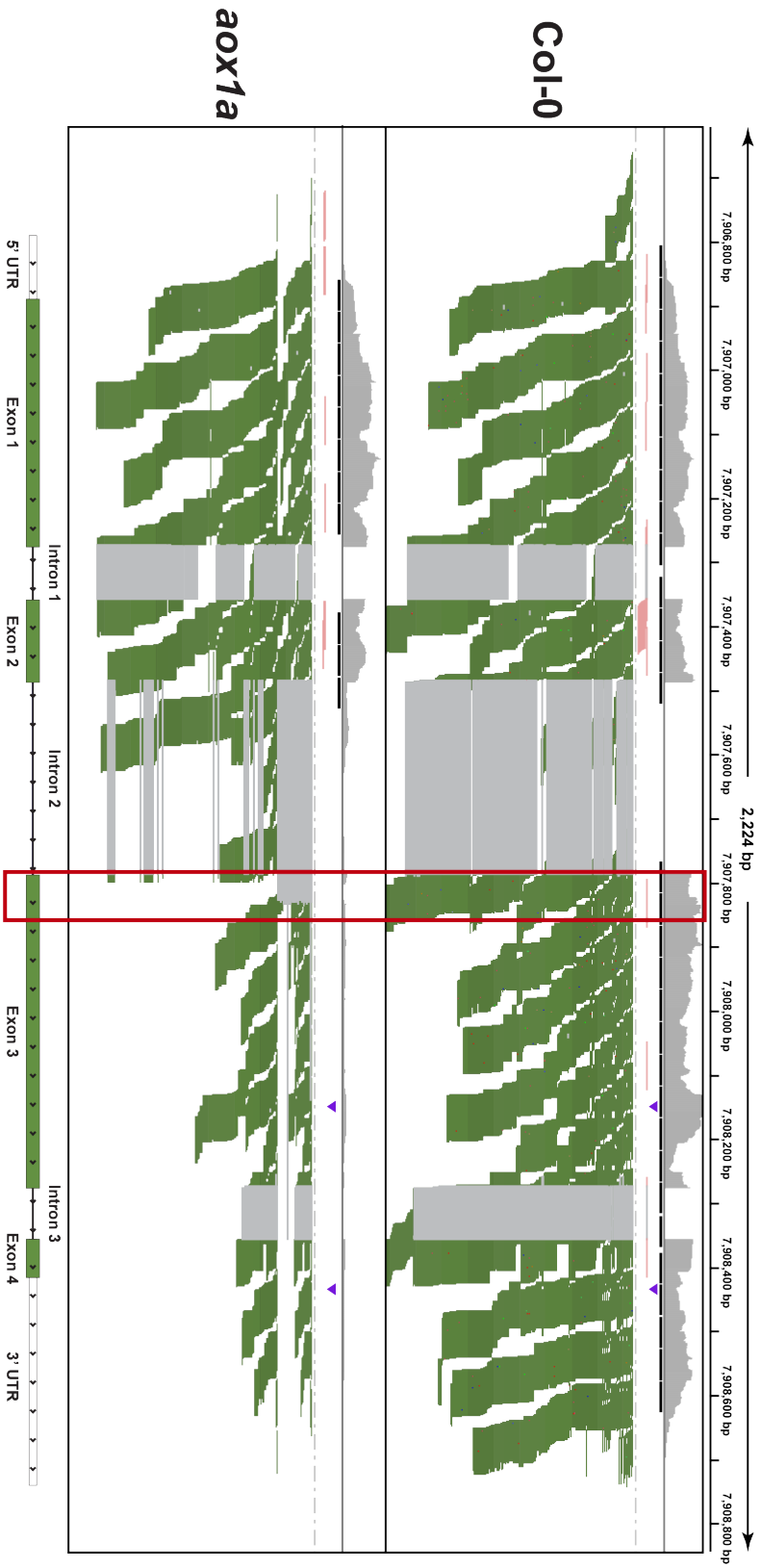
Supplemental Figure 1. Spray-treatment of *aox1a* complementation lines.

10-day old seedlings of all control lines including WT (Col-0) and the aox1a-KO were grown on solid media and sprayed with antimycin-A (50 µM). Pictures were taken 72 h after treatment.



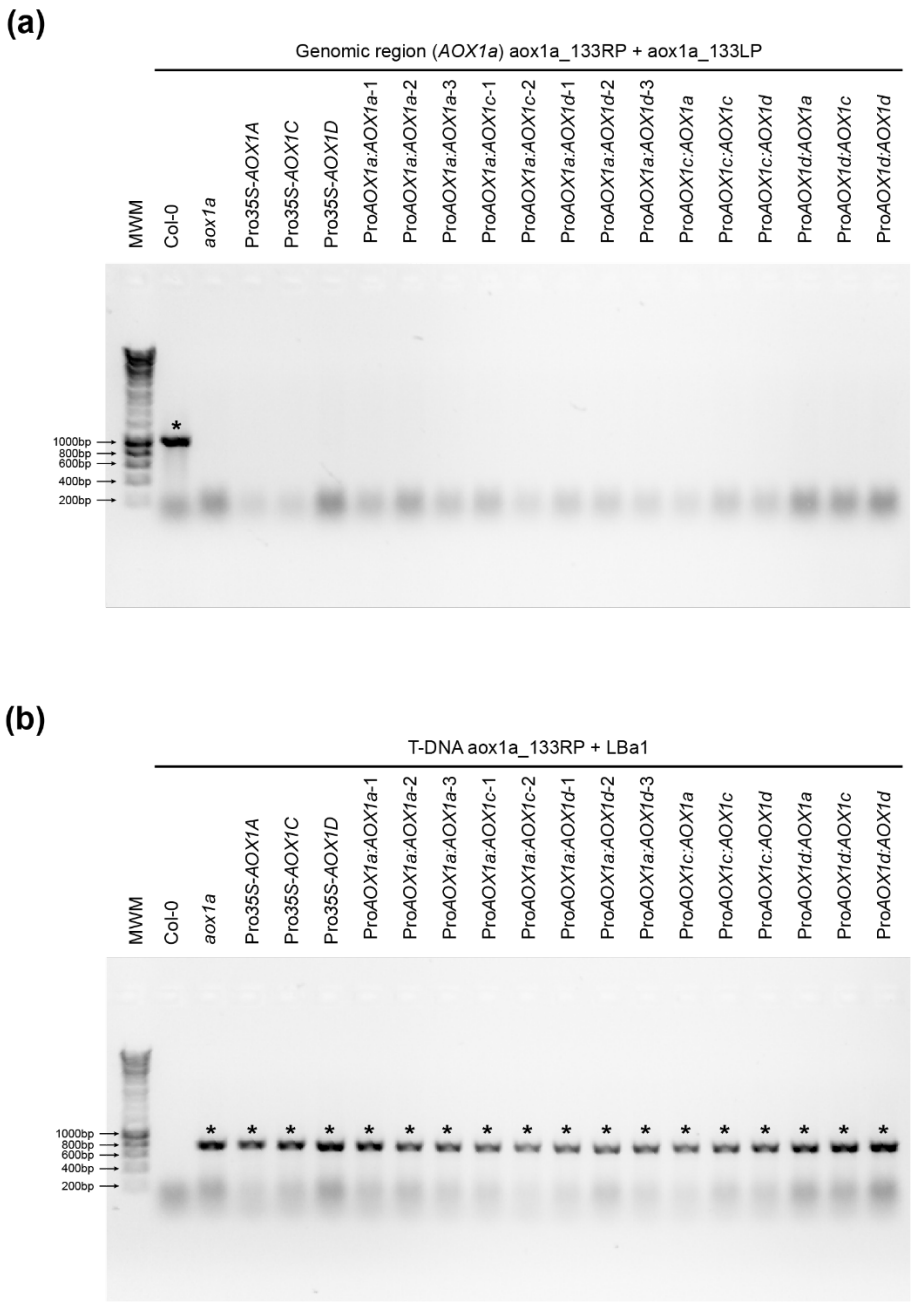
Supplemental Figure 2. Germination of aox1a complementation lines in the presence of antimycin A.

Arabidopsis seedlings were grown on solid growth media supplemented with antimycin-A (AA) (50 µM). Pictures were taken 10 days after sowing. Seeds were stratified in 0.1% (m/v) Agarose for 3 days prior to sowing.



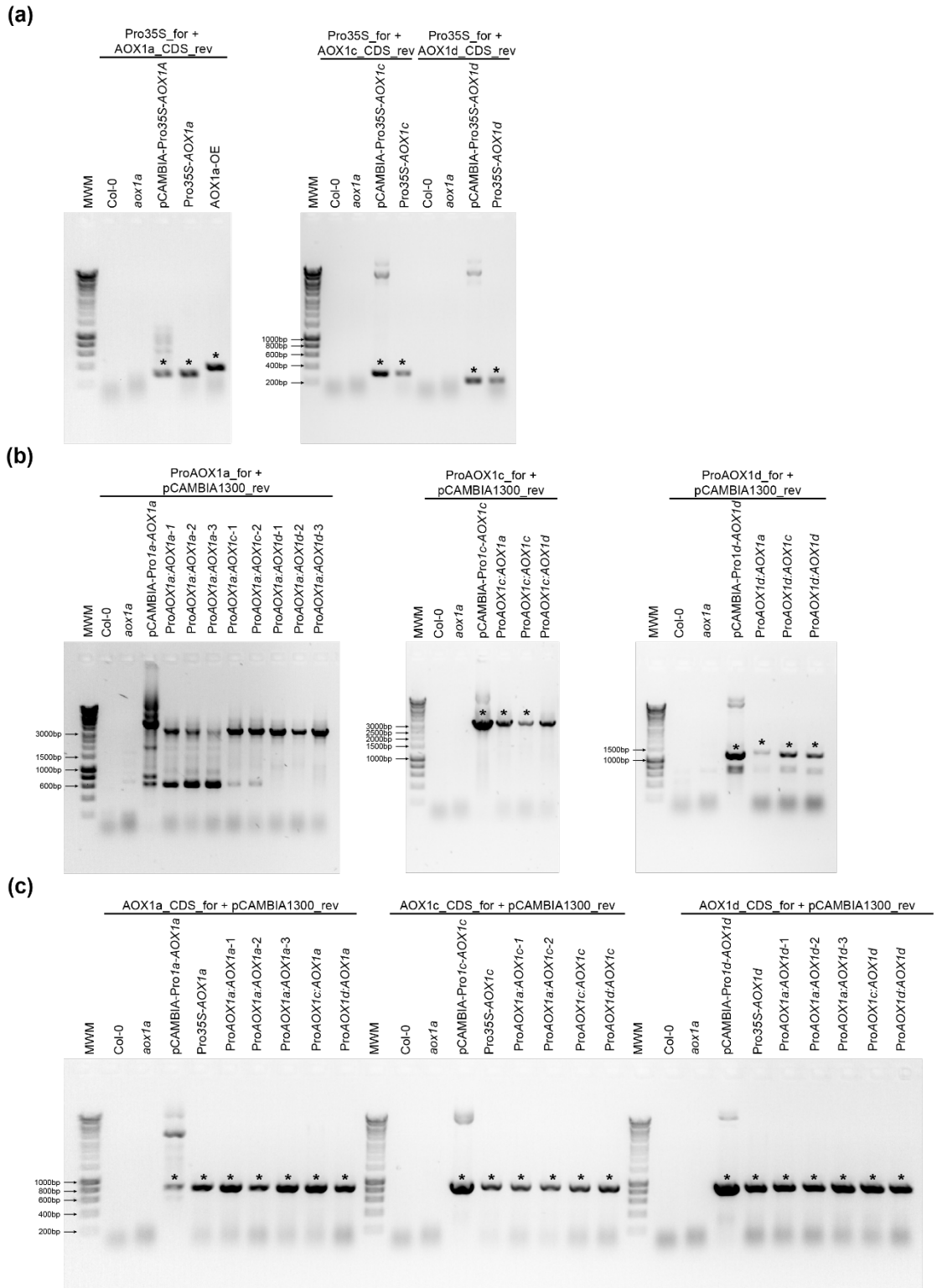
Supplemental Figure 3. Localisation of T-DNA insert in aox1a mutant line.

Confirmation of the location of the T-DNA insert in the aox1a KO (SALK_084897). RNAseq reads for the *aox1a* mutant line used in this study were mapped to the reference genome and suggest that the T-DNA is located in the 5' region of exon 3 due to a lack of overlapping reads in this region (highlighted with red box) when compared to the WT. Fewer read counts were detected following the proposed insertion site. This confirms the location published previously (Watanabe et al., 2008, Giraud et al., 2008, Strodtkötter et al., 2009)



Supplemental Figure 4: Genotyping for homozygosity of T-DNA insert.

All mutant lines in the *aox1a* mutant background were screened for homozygosity with PCR reactions using specific primer for the (a) genomic region of *AOX1a* and the (b) T-DNA insert. All specific primer sequences can be found in Supplemental Table S2. The corresponding specific bands are indicated with an asterisk ("*"). MWM, molecular weight marker.



Supplemental Figure 5: Genotyping of all *aox1a* complementation lines for the presence of the transgene.

All transgenic Arabidopsis lines were confirmed with PCR reactions using primer pairs specific for the (a) transgenic 35S-CaMV and (b) native 3 kb AOX1a/c/d promoter regions as well as the corresponding (c) AOX1a/c/d gene coding sequences. All primer sequences can be found in Supplemental Table S2. The corresponding specific bands are indicated with an asterisk ("*"). MWM, molecular weight marker.

Supplemental Table 1: AOX gene expression data obtained via qRT-PCR.

The data corresponds to the visualization in Figure 2. ACT7 (Shu et al., 2013) was used as a reference gene.

	40- delta Ct												2^(-delta delta Ct)									
	AOX1a		AOX1b		AOX1c		AOX1d		AOX2		AOX1a		AOX1b		AOX1c		AOX1d		AOX2	
Sample	Control	AA	Control	AA	Control	AA	Control	AA	Control	AA	Control	AA	Control	AA	Control	AA	Control	AA	Control	AA
WT (Col-0)	35.0	40.4	27.7	31.0	31.4	31.1	31.3	37.7	29.4	29.8	0.0	5.3	0.0	3.2	0.0	-0.3	0.0	5.7	0.0	0.5
aox1a	33.7	40.3	26.8	29.9	31.2	30.1	32.0	37.8	29.3	30.3	-1.4	5.1	-0.9	1.7	-0.2	-1.3	0.1	5.7	-0.1	0.8
AOX1a-OE	41.1	41.6	28.6	29.9	31.1	31.1	32.2	34.5	29.8	30.4	6.2	6.7	0.9	0.9	-0.2	-0.3	0.6	2.9	0.3	1.0
aox1b	34.5	38.9	28.4	29.5	31.4	30.2	32.0	35.3	30.1	30.7	-0.4	4.0	0.7	1.7	0.0	-1.2	0.5	3.8	0.7	1.3
aox1c	35.3	40.6	29.4	30.7	27.0	29.5	33.9	38.9	29.1	30.0	0.4	5.7	1.6	2.9	-4.3	-1.8	2.4	7.4	-0.3	0.6
aox1d	34.9	40.9	28.0	30.4	31.1	30.8	24.9	28.5	29.6	29.7	0.0	6.0	0.3	2.7	-0.2	-0.6	-6.6	-3.1	0.2	0.3
aox2	34.6	39.9	28.4	30.9	31.1	31.1	31.6	37.4	30.3	30.0	-0.3	5.0	0.6	3.1	-0.3	-0.3	0.1	5.9	0.9	0.6
rcd1-1	38.0	39.1	28.5	29.8	32.3	32.6	34.0	34.3	29.5	29.1	3.1	4.2	0.8	2.0	1.0	1.3	2.4	2.8	0.1	-0.3
rcd1-1 aox1a	38.8	41.7	29.2	29.7	33.0	32.2	32.9	36.8	28.9	30.7	4.0	6.8	1.5	2.0	1.7	0.8	1.4	5.3	-0.5	1.2
Pro35S-*AOX1a*	34.0	39.7	27.0	31.2	31.3	30.1	31.4	39.0	29.4	29.7	-0.9	4.9	-0.8	3.5	-0.1	-1.3	-0.1	7.5	0.0	0.3
Pro35S-*AOX1c*	33.4	40.7	27.0	32.7	38.8	40.5	32.2	39.4	29.3	29.8	-1.5	5.8	-0.7	5.0	7.4	9.1	0.6	7.8	-0.1	0.4
Pro35S-*AOX1d*	33.4	40.4	28.3	30.4	31.5	31.0	35.0	37.3	29.9	30.2	-1.5	5.5	0.6	2.7	0.2	-0.4	3.5	5.8	0.5	0.8
ProAOX1a:*AOX1a*-1	33.5	40.8	37.3	40.0	34.2	37.2	31.7	36.5	29.2	29.5	-1.3	5.9	9.6	12.2	2.9	5.9	0.2	5.0	-0.2	0.1
ProAOX1a:*AOX1a*-2	33.4	39.8	40.2	40.8	36.8	37.9	33.2	36.8	29.6	29.7	-1.5	5.0	12.4	13.1	5.5	6.6	1.6	5.2	0.2	0.3
ProAOX1a:*AOX1a*-3	34.2	41.6	34.2	36.8	32.3	33.9	31.2	37.4	29.0	30.2	-0.6	6.7	6.5	9.1	0.9	2.5	-0.3	5.9	-0.4	0.8
ProAOX1a:*AOX1c*-1	34.3	38.6	41.1	42.0	37.8	39.4	32.7	35.2	29.9	28.9	-0.5	3.8	13.4	14.3	6.5	8.0	1.1	3.7	0.5	-0.5
ProAOX1a:*AOX1c*-2	33.9	38.2	41.4	42.5	38.0	39.4	32.9	34.7	29.4	30.4	-1.0	3.3	13.7	14.7	6.7	8.0	1.3	3.2	0.0	1.0
ProAOX1a:*AOX1d*-1	33.9	37.8	41.3	42.1	38.2	39.0	33.2	36.9	29.8	29.6	-1.0	2.9	13.6	14.3	6.8	7.7	1.7	5.4	0.4	0.2
ProAOX1a:*AOX1d*-2	34.3	39.5	40.5	40.7	37.2	37.4	34.0	36.6	29.3	30.4	-0.5	4.6	12.8	12.9	5.9	6.0	2.5	5.1	-0.1	1.0
ProAOX1a:*AOX1d*-3	32.8	35.0	32.8	38.2	31.9	35.1	32.5	36.6	29.3	30.1	-2.0	0.1	5.1	10.4	0.6	3.8	0.9	5.1	-0.1	0.7
ProAOX1c:*AOX1a*	35.0	39.8	28.3	30.4	27.8	28.0	32.0	36.1	29.5	30.4	0.2	4.9	0.6	2.7	-3.5	-3.4	0.5	4.6	0.1	1.0
ProAOX1c:*AOX1c*	34.1	39.8	28.0	30.7	31.8	31.3	32.0	37.3	29.6	29.5	-0.7	5.0	0.3	3.0	0.4	-0.1	0.4	5.7	0.2	0.1
ProAOX1c:*AOX1d*	33.8	39.9	27.5	30.2	31.8	31.2	32.2	36.7	29.4	29.7	-1.1	5.0	-0.2	2.5	0.4	-0.1	0.7	5.2	0.0	0.3
ProAOX1d:*AOX1a*	34.2	40.2	26.8	31.7	31.2	30.9	31.3	39.0	29.7	29.9	-0.6	5.3	-1.0	4.0	-0.2	-0.5	-0.2	7.5	0.3	0.5
ProAOX1d:*AOX1c*	33.3	39.5	27.5	28.3	32.5	35.1	31.7	32.3	29.3	29.7	-1.6	4.6	-0.2	0.6	1.1	3.7	0.2	0.7	-0.2	0.3
ProAOX1d:*AOX1d*	34.0	39.7	27.8	29.0	31.4	30.2	35.6	39.1	29.7	30.4	-0.8	4.8	0.0	1.3	0.1	-1.1	4.1	7.6	0.2	1.0

Supplemental Table 2: Primer sequences.

List of all primer and the corresponding sequences used for cloning via Gibson assembly, genotyping and qRT-PCR in this study. The qRT-PCR housekeeping gene (ACT7) is highlighted with an asterisk ("*").

(a)

Cloning Primer (Gibson assembly)

Promoter	Forward	Reverse	Combination
Pro35S	TATGACCATGATTACGAATTCAGATTGTCGTTTCCCGCCTTCAGT	TGGCTCCACCGCGAGTTATCATCATTACCATGGTCAAGAGTCCCCCGTGT	1
Pro35S		AGCGACGGAGTAATGTAGTGATCATTACCATGGTCAAGAGTCCCCCGTGT	2
Pro35S		TGCGGTAAATCGATCTGTAGGACATTACCATGGTCAAGAGTCCCCCGTGT	3
ProAOX1a	TATGACCATGATTACGAATTCACCAGCCAAGTCTATAGAAAAAAG	TGGCTCCACCGCGAGTTATCATCAT TGTTTCAAATCGGAAAAAGTGAAAT	4
ProAOX1a		AGCGACGGAGTAATGTAGTGATCATTGTTTCAAATCGGAAAAAGTGAAAT	5
ProAOX1a		TGCGGTAAATCGATCTGTAGGACATTGTTTCAAATCGGAAAAAGTGAAAT	6
ProAOX1c	TATGACCATGATTACGAATTCTACTTTTACATCTTAAATCCCACA	TGGCTCCACCGCGAGTTATCATCAT TTTTCGGATTCAAGGGAGATTTGTT	7
ProAOX1c		AGCGACGGAGTAATGTAGTGATCAT TTTTCGGATTCAAGGGAGATTTGTT	8
ProAOX1c		TGCGGTAAATCGATCTGTAGGACAT TTTTCGGATTCAAGGGAGATTTGTT	9
ProAOX1d	TATGACCATGATTACGAATT CTCGAGCAAGGCATGGAAGAGACAG	TGGCTCCACCGCGAGTTATCATCAT GAGTAACAACAGTTGGGGTGTGATG	10
ProAOX1d		AGCGACGGAGTAATGTAGTGATCAT GAGTAACAACAGTTGGGGTGTGATG	11
ProAOX1d		TGCGGTAAATCGATCTGTAGGACAT GAGTAACAACAGTTGGGGTGTGATG	12

CDS	Forward	Reverse	Combination
AOX1a_CDS	ATGATGATAACTCGCGGTGGAGCCA	AACGACGGCCAGTGCCAAGCTTCAATGATACCCAATTGGAGCTGGA	1
AOX1c_CDS	ATGATCACTACATTACTCCGTCGCT	AACGACGGCCAGTGCCAAGCTTCAGTGATATCCTATAGGAGCTGGA	2
ProAOX1d	ATGTCCTACAGATCGATTTACCGCA	AACGACGGCCAGTGCCAAGCTTTAATGATATCCAATAGGAGCGGA	3
AOX1a_CDS	ATGATGATAACTCGCGGTGGAGCCA	AACGACGGCCAGTGCCAAGCTTCAATGATACCCAATTGGAGCTGGA	4
AOX1c_CDS	ATGATCACTACATTACTCCGTCGCT	AACGACGGCCAGTGCCAAGCTTCAGTGATATCCTATAGGAGCTGGA	5
AOX1d_CDS	ATGTCCTACAGATCGATTTACCGCA	AACGACGGCCAGTGCCAAGCTTTAATGATATCCAATAGGAGCGGA	6
AOX1a_CDS	ATGATGATAACTCGCGGTGGAGCCA	AACGACGGCCAGTGCCAAGCTTCAATGATACCCAATTGGAGCTGGA	7
AOX1c_CDS	ATGATCACTACATTACTCCGTCGCT	AACGACGGCCAGTGCCAAGCTTCAGTGATATCCTATAGGAGCTGGA	8
AOX1d_CDS	ATGTCCTACAGATCGATTTACCGCA	AACGACGGCCAGTGCCAAGCTTTAATGATATCCAATAGGAGCGGA	9
AOX1a_CDS	ATGATGATAACTCGCGGTGGAGCCA	AACGACGGCCAGTGCCAAGCTTCAATGATACCCAATTGGAGCTGGA	10
AOX1c_CDS	ATGATCACTACATTACTCCGTCGCT	AACGACGGCCAGTGCCAAGCTTCAGTGATATCCTATAGGAGCTGGA	11
AOX1d_CDS	ATGTCCTACAGATCGATTTACCGCA	AACGACGGCCAGTGCCAAGCTTTAATGATATCCAATAGGAGCGGA	12

(b)

Genotype Screening Primer

Name	Sequence (5'-3')	SALK_ID
aox1a_133RP	TTCTTTGGATTTTTGCAGCC	SALK_084897
aox1a_133LP	TGATCAGTAACACACGATTTTAAGC	SALK_084897
LBa1	TGGTTCACGTAGTGGGCCATCG	
Pro35S_for	CGCAAGACCCTTCCTCTATATAAG	
AOX1a_CDS_rev	TCTCCCAGAGTGATCGTG	
AOX1c_CDS_rev	TCACGCCCCAATAACTAA	
AOX1d_CDS_rev	TTTCTGGCTGGTTATTCC	
ProAOX1a_for	ATGTTTGGACTAATCTTGCC	
ProAOX1c_for	GGATCTAGCTACATTGAGT	
ProAOX1d_for	GCCTAACTCTTGTAGCTCTA	
pCAMBIA1300_rev	TGTAAAACGACGGCCAGT	
AOX1a_CDS_for	GACGCGAATCAGAAGAAAAC	
AOX1c_CDS_for	AATATTTTAGGGTTCCGGCAG	
AOX1d_CDS_for	GGACATCTCATTAGCCACTTG	

(c)

qRT-PCR

Gene	AGI	Sequence (5'-3')	Reference
AOX1a_for	At3g22370	GATTACTGGAGGCTTCCTGCTG	
AOX1a_rev	At3g22370	CACGACCTTGGTAGTGAATATCAG	
AOX1b_for	At3g22360	TGGAGACTTGAAGCTGATGCG	
AOX1b_rev	At3g22360	CACGACCTTGGTAATGAATATCGG	
AOX1c_for	At3g27620	GCAACGCTTCGTGATGTTGTC	
AOX1c_rev	At3g27620	CATGACCTTGGTAGTGAATATCGG	
AOX1d_for	At1g32350	CTTTCACAACCCAAATGGTACG	
AOX1d_rev	At1g32350	GCCTCTTCTTCTAAGTATCCAGTG	
AOX2_for	At5g64210	GGAGATTGCCTAAAGATGCTACG	
AOX2_rev	At5g64210	CCTTGATTGCGAATGTCAGAAG	
ACT7*	At5g09810	CTTGATCTTCATGCTGCTAGGT	Shu et al., 2013
ACT7*	At5g09810	GGAAACATCGTTCTCAGTGGT	Shu et al., 2013

CHAPTER 4 - GENERAL DISCUSSION

Global organisations like the United Nations and national governments frequently remark that food production must increase by ~ 70% to feed the world population by 2050 (Alexandratos and Bruinsma, 2012, Ray et al., 2013, Tester and Langridge, 2010). This statement ignores that increasing food production requires an increased input of finite resources, such a phosphate fertiliser, and energy inputs in terms of fuel (Qaim, 2020). Apart from the fact that such increases in productivity may not be sustainable they often come at a cost that is not affordable to everyone. Thus, food security means sufficient food at an affordable price. While increasing production is one strategy to increase food production, limiting losses is another important one (Hickey et al., 2019, Gilliham et al., 2017). The latter comes with the advantage that it does not require additional inputs of finite resources and is sustainable in that it allows increases in profits for growers. Finally, increases in production must span the scales of large, industrialised farms to the small farms that are the majority in many countries (Lammerts van Bueren et al., 2018, Bradshaw, 2017). A common theme in production is yield loss due to abiotic and biotic stresses, that impact across all scales of farming irrespective of technology and farming methods. It is estimated that flooding and drought combined accounts for more than 70 % of the reduction in harvest as shown for the U.S. (Bailey-Serres et al., 2012). Biotic pests such as rust fungi can lead to 5.47 million tonnes of wheat loss each year with a value of 1 US$ billion equivalents (Beddow et al., 2015).

Research in plant biotic pests have resulted in understanding of how bacterial and fungal pathogens impact yield, and breeding programs develop varieties resistant to emerging pests to prevent large losses, e.g. rust fungi in wheat (Figueroa et al., 2018). While for biotic stresses the introduction of a single resistance gene if often sufficient for sufficient resistance, abiotic stress tolerance is often complex and more multi-layered. Thus, the development of plant varieties that can maintain current yields but with greater tolerance to drought, heat, salinity etc is an ongoing challenge in plant breeding (Zhang et al., 2018).

4.1 Arabidopsis as a model species

Arabidopsis is by far the best-studied and most important plant model species and its analysis has significantly shaped the landscape in fundamental plant research at a genetic, molecular and systems levels. Besides its favourable traits that make genetic approaches applicable, several key events in the Arabidopsis research paved the way for this agronomically unimportant weed to become the best investigated model plant to date (Buell and Last, 2010, Koornneef and Meinke, 2010). Especially the initiative that led to the first fully sequenced genome in plants in the year 2000 represents a crucial milestone

and marks the transition of Arabidopsis from a genetic to a genomic model system (Meinke et al., 1998, Buell and Last, 2010). Currently more than 30,000 researchers around the globe are working with Arabidopsis directly or utilize the wide range of resources that are based on Arabidopsis related research which underlines the extraordinary position of this model plant in the plant research community (International Arabidopsis Informatics, 2019). This community is highly collaborative and established comprehensive online databases and stock centres to provide and share seeds and information resources that further accelerated the research. As a result, a wide range of tools and analytical methods were development and optimized that are not only applicable to Arabidopsis but also to other plant species (Provart et al., 2016).

Despite its limitations as a model and clearly existing molecular and morphological differences to agronomically relevant plant species, the unparalleled knowledge of molecular, cell, and evolutionary biology in Arabidopsis serves researchers as a reference for comparison in translational research focussing on agronomically relevant crops (Woodward and Bartel, 2018). Translational research describes the application of knowledge gained via fundamental research to agronomic improvement, but also the knowledge transfer between different crop species (Ronald, 2014, Jacob et al., 2018). As a result of advances in high-throughput NGS technologies whole-genome sequences are available for the majority of important crop species including rice (Kawahara et al., 2013), wheat (International Wheat Genome Sequencing et al., 2018), maize (Schnable et al., 2009) and barley (Mascher et al., 2017) and allow for comparative analyses between different species. Moreover, the development of bioinformatic tools to analyse and integrate datasets in Arabidopsis resulted in sophisticated methods beneficial for the analysis of crop plants with more complex genomes and consequently more complex problems. The application of genomics in plant breeding has greatly accelerated and consequently there is now a shortage of markers. In fact, the bottleneck in plant improvement is linking traits obtained via genomic approaches to the phenotype in the field (Furbank and Tester, 2011). In this context, accurate phenotyping and the measurement of a wide range of plant growth parameters is crucial for successful crop breeding and the development of platforms that combine high-resolution phenotyping, automated data collection and computational have the potential to accelerate the development of new crop varieties (Tanger et al., 2017, Shakoor et al., 2017). The field of high-throughput phenotyping technologies that are not limited to certain plant species and allow for a rapid and precise phenotypic assessment under field conditions are advancing rapidly and may be able to accelerate breeding efforts (Shakoor et al., 2017).

4.2 Translational Research

In the past, the maximisation of crop yields has been the focus of crop-breeding programmes rather than efforts that aim to improve stress tolerance, especially abiotic stress tolerance (Gilliham et al., 2017). In order to engineer crop species with enhanced stress tolerance it is crucial to understand the complexity of abiotic stress responses in plants from the initial perception to changes in gene expression and the induction of stress-response pathways (see Chapter 1). Breeding plants with enhanced abiotic stress resistance, however, is challenging as plants experience combinations of multiple stresses in their natural habitats that differ in duration and severity. Even when these challenges can be solved, the process of translating fundamental research into commercially released crop varieties without yield penalties is very time-consuming and can take 10 to 15 years (Gilliham et al., 2017). This vast time scale from the initial discovery to commercialisation but also infrastructural or budgetary restraints as well as difficulties in the classical research evaluation that is based on research output, represent limiting factors in translational research (Nelissen et al., 2014, Gilliham et al., 2017).

Overall, translational research has a huge potential to impact plant productivity and has only just started. Notable examples of translation of fundamental knowledge from laboratories to crop improvement include aluminium tolerance, water use efficiency and salt tolerance (Gilliham et al., 2017).

The comparative analyses presented in chapter 2 links the transcriptomic responses between the dicot species Arabidopsis with the two monocot species rice and barley which are both agronomically important cereals. This study was designed to simply ask what is the same and what differs between species. While there many examples in the literature documenting responses to stress in various species, an overall picture of what is conserved and what is different is still not clear. Given that genomic sequences are available for crop species, the question arises what the targets for precision editing and synthetic biology approaches are. The aim of the cross-species transcriptome study was to provide a roadmap that supports this decision making, predict the outcome of translation and select suitable targets.

On an evolutionary scale, the species used for the analysis are separated by 140-150 million years, when the group of monocots branched off from dicots (Chaw et al., 2004). To overcome this evolutionary distance and the complexity that arises from highly dynamic gene evolution, orthology was chosen as a basis for comparison. More specifically, orthogroups that contain orthologous and paralogous genes, which represents the logical extension of orthology to multiple species and is a frequently used unit of comparison (Emms and Kelly, 2019).

This study clearly revealed conserved and opposite responses to stress across species. These outcomes can be considered as conservative, given that the comparison was based on orthologous genes that are defined to retain ancestral function across distinct species (Altenhoff et al., 2012). It may even be expected that all processes would be conserved, and yet many processes at various levels show opposite responses. The non-conserved nature of some responses explained some previous studies where expression of genes from one species did not achieve the desired or predicted function when expressed in another species (Nelissen et al., 2014).

Both the conserved and opposite responses are very valuable knowledge for translation research. This knowledge can now be applied to the crop species with a much deeper understanding of how response may impact growth, development and ultimately yield. It allows researchers working with a model system to identify regulatory targets that have the potential to solve a problem in a crop species to predict the outcomes and even optimise the response. In this context, the diversity gained in the Arabidopsis 1000 genome project may be leveraged to understand how the particular pathway may vary (Weigel and Mott, 2009). The effects of such variation can then be tested in crop species. Additionally, stacking of conserved processes in response to multiple environmental conditions could be a possibility (Wani et al., 2016). Understanding how different signalling pathway interact and how they can be synergistic or antagonistic, may allow for simultaneous optimization of tolerance to multiple adverse abiotic conditions, rather than going through individual breeding programs. The diverse processes are also useful for translational research and could be utilized due to differential activation of a pathway between species and serves as an indicator for choosing candidate genes as well.

Interestingly, the results obtained in chapter 2 revealed not only differences between Arabidopsis and the two monocot species rice and barley, but also differences between the monocot species themselves. With an increasing number of crop species now being the target for genomics studies, the public availability of these datasets will provide a valuable resource to compare more closely related species.

Taken together, it is fundamental in translational research to understand what needs to be targeted and this study provides a start of this knowledge base. While synthetic biology in plants is still at a very early stage, such data sources will be invaluable in designing approaches to rationally engineer plants.

4.3 From Gene to Function

While examining responses of genes to various stimuli gives a picture of how signalling pathway operate in plants, it does not demonstrate conserved function. Thus, orthologous proteins do not necessarily have identical function. The AOX gene family in Arabidopsis represents a good example to illustrate that changes in gene expression in response to stress do not necessarily lead to enhanced stress tolerance or plant survival. AOX1a and AOX1d are both highly stress-responsive isoforms with increased transcript abundance in response to various stress conditions, while the other isoforms of this multigene family are unresponsive (Clifton et al., 2006). As shown in chapter 3 this was confirmed for all AOX isoforms in response to chemically induced mitochondrial dysfunction. Based on increased transcript abundance of AOX1d in response to mitochondrial dysfunction as well as increased level of AOX protein other than AOX1a in mutant lines, post-translational regulation or cell specific expression were postulated (Selinski et al., 2018).

The results in this study show, that no functional protein can be detected via immunoblotting despite the transcriptional induction of AOX1d. This explains why AOX1d cannot compensate the lack of the main stress-responsive isoform and MRR marker AOX1a under stress conditions. Differences in the post-translational fine regulation, however, might play a role and require a certain level of functional AOX1d protein.

The results in this study further show that the stress-phenotype shown for plants lacking AOX1a can be complemented by other isoforms, AOX1c and AOX1d, when driven by the AOX1a promoter. This redundant functionality of related genes not only highlights the need to dissect the regulatory properties within gene families but represents a target for synthetic biology and the engineering of plants with improved performance (Florez-Sarasa et al., 2020).

Overall, this study shows that while conserved processes in terms of gene regulation can be observed, ultimately it is necessary to determine the functionality of these responses at a protein level. Subtle differences in protein function may exist and this cannot be predicted from gene sequence or expression patterns alone.

4.4 Future Studies

While plant production has kept up with the ever increasing demand for food the rate of increase in production is decreasing in the last decade which highlights the need for basic plant research to be translated to increase crop production and to ensure sustainability (Bradshaw, 2017, Weiner, 2017). While traditional breeding methods allow diversity between varieties to be selected, modern genetics and genomics now allow variability between species to be selected and transferred. This represents both an

exciting and challenging time for plant biology. The need to increase plant production needs to be balanced with an approach that ensure integrity in the production system so that the genetics of the production are known and understood. While ultimately social issues will decide what is and is not acceptable, science must define what is and is not possible (Weiner, 2017). The ability to be able to transfer desirable traits, especially in terms of abiotic stress tolerance from one species to another represents an opportunity to dramatically increase plant production in a sustainable manner (Gilliham et al., 2017). In addition to developing plants for traditional agriculture, it will be vital to develop plants for food production in emerging environments not traditionally used, such as urban and vertical farming (Benke and Tomkins, 2017). Currently these systems are in their infancy, but without knowledge of plant growth and development, varieties of plants more suitable to alternative growing may be developed from the beginning which might be faster than the reverse approach. The ‘re-domestication’ of tomato using genome editing is an example of how it is possible to go back in time and re-select for traits in a precision manner, and maintain beneficial traits that may have been lost in the hundreds or thousands of years with traditional domestication (Zsogon et al., 2018). However, our understanding of the dynamic expression of the genome will need to be more fully developed before this kind of approaches are routine.

While this study looked at the transcriptional responses between species it was limited in many ways, only protein coding genes were examined and the importance of microRNA and long-coding RNA was ignored, but it was not feasible to incorporate them into this study. Furthermore, the study selected a single tissue for each species at a single time point but developmental studies with multiple tissues are required to obtain a complete picture. In addition, the emergence of cell specific transcriptomes and how cell lineage is defined represents an important factor that needs to be incorporated to understand whole plant responses to stress (Libault et al., 2017). Beyond transcriptomes, changes in epigenomes, proteomes, metabolomes etc need to be understood in order to fully model plants responses to variable conditions.

Despite the fact that there will always be a desire for more data and resolution to increase the accuracy of predictions, current technology and approaches have accelerated our understanding of how plant respond to the environment and we are at the exciting stage that allows a knowledge transfer between species.

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