The role of autophagy in plant mitochondrial retrograde signalling

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This thesis is presented for the degree of Doctor of Philosophy
(Biochemistry) of the University of Western Australia

ARC Centre of Excellence in Plant Energy Biology
School of Molecular Sciences

January 2020
Thesis declaration

I, Martyna Broda, certify that:

This thesis has been substantially accomplished during enrolment in this degree.

This thesis does not contain material which has been submitted for the award of any other degree or diploma in my name, in any university or other tertiary institution.

In the future, no part of this thesis will be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of The University of Western Australia and where applicable, any partner institution responsible for the joint-award of this degree.

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The following approvals were obtained prior to commencing the relevant work described in this thesis: OGTR (2113) and NLRD (RA/5/1/373).

Third party editorial assistance was provided in preparation of this thesis by my supervisors, Professor A. Harvey Millar, Dr Olivier Van Aken and Chun Pong (Alex) Lee.

Technical assistance was kindly provided by Dr Adriana Pruzinska in the research of plant senescence. Further assistance have been provided by Dr Brendan O’Leary for determining starch levels in Arabidopsis mutants described in Chapter 3, Dr Markus Schwarzländer by providing support, roGFP2 constructs and training to ratiometric analysis using roGFP2 fusions described in Chapter 4.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

Signature: 

Date: 31/01/2020
Abstract

Control of gene expression is a very dynamic process with implications on cell and plant development, as well as abiotic and biotic stress responses. Evolution of semiautonomous organelles, chloroplasts and mitochondria, required establishment of underlying mechanism(s) responsible for communication between nuclear, mitochondrial and chloroplast genomes. To this day however, important aspects of these mechanisms remain unknown. In the past two decades, several molecular components that contribute to mitochondrial retrograde signalling have been identified, but it remains unclear how they are cooperatively involved in conveying the information from mitochondria to nucleus. In this thesis, current knowledge about how one of these components, the ER-bound transcription factor ANAC017, mediates communication between mitochondria and nucleus has been extended to show how senescence and autophagy contribute to its mode of action.

A collection of different ANAC017 mutants and overexpression lines was gathered to resolve the impact of ANAC017 on plant senescence. It was shown that overexpression of ANAC017 led to accelerated senescence and a highly inducible autophagy pathway in 5-week old plants. ANAC017 target genes displayed a transient induction of expression upon senescence induction. Moreover, ANAC016, a very close homolog of ANAC017 displayed the same transient pattern of expression as ANAC017 target genes. The ANAC016 promoter contains a binding site for ANAC017. Together, this implies a role for ANAC016 downstream of ANAC017 in plant senescence. This helps to resolve conflicting reports of how ANAC017 and ANAC016 act to influence plant senescence in Arabidopsis thaliana.

Analysis of accelerated senescence in ANAC017 overexpression lines led to the discovery of similar senescing phenotypes in autophagy-deficient mutants and prompted an investigation into the role of autophagy in plant mitochondrial retrograde signalling. Results obtained demonstrate that blocking of autophagy resulted in the suppression of antimycin A (AA) stimulated retrograde signalling in Arabidopsis plants and cell cultures. Moreover, induction of autophagy in cell cultures primed them for elevated responses to AA. These results suggest that the autophagy pathway is involved in the activation of retrograde signalling and perhaps ANAC017 itself. Investigation into the membrane topology of ANAC017 then revealed that both its N- and C-terminal ends are located in the ER lumen, indicating a more complex
process is required to release it to the nucleus than previously thought. Together, these results suggest a novel model for ANAC017 activation and the involvement of autophagy in plant mitochondrial retrograde signalling.
# Table of contents

## Contents

Thesis declaration .......................................................................................................................... iii  
Abstract ...................................................................................................................................... iv  
Table of contents ........................................................................................................................ vi  
List of figures ............................................................................................................................... ix  
List of tables ............................................................................................................................... xii  
Acknowledgements ...................................................................................................................... xiv  
Authorship declaration: co-authored publications ................................................................. xv  
Abbreviations ............................................................................................................................. xviii  

### Chapter 1

Structure and function of mitochondria and chloroplasts ......................................................... 1  
Evolution of semi-autonomous organelles and their impact on cell signalling ...................... 2  
Organellar post-translational response to abiotic stresses ..................................................... 4  
Stress-specific transcriptional regulation of organellar proteins ........................................... 8  
  - Transcription factors involved – are they known to be induced by stress or specific environmental conditions? ....................................................................................................................... 8  
  - Transcriptional regulation of mitochondrion- and chloroplast-encoded genes .............. 9  
  - Coordinating gene expression between nucleus and organelles .................................. 10  
Retrograde signalling .................................................................................................................. 10  
  - Chloroplast retrograde signalling ............................................................................... 11  
  - Mitochondrial retrograde signalling ........................................................................... 12  
The role of ANAC017 as a transcription factor coordinating chloroplast and mitochondrial communication with nucleus ......................................................... 15  
Autophagy, Mitophagy and links to stress signalling ............................................................. 20  
Aims of this study ....................................................................................................................... 24  
References .................................................................................................................................. 26  

### Chapter 2

Abstract ........................................................................................................................................ 42  
Mitophagy as a type of autophagy ............................................................................................. 45  
  - What is the evidence for mitophagy in plants? ................................................................. 46  
  - The mechanism of mitophagy in plant and non-plant systems .................................... 47  
  - Mitochondrial membrane autophagy receptors in plants ............................................. 52  
  - Role for rhomboid and other proteases ......................................................................... 54  
  - The role of mitophagy in signalling .............................................................................. 55  
  - Role of mitophagy during senescence .......................................................................... 56  
Conclusions ................................................................................................................................. 59  
References .................................................................................................................................... 60  
Supplementary material ............................................................................................................. 71
Abstract ......................................................................................................................... 77
Introduction .................................................................................................................... 80
Results ............................................................................................................................. 82
  Overexpression of ANAC017 leads to increased senescence rate .................................. 82
  Exploration of genome-wide transcriptional responses during leaf senescence ............ 85
  Autophagy and cell death-related genes become hyper-activated in ANAC017 OE lines after induction of senescence ................................................................. 91
  Hormone signalling is affected in ANAC017 mutants ................................................... 93
  Core ANAC017-regulated genes are transiently ‘super-induced’ during senescence in ANAC017 OE lines ................................................................. 93
  ANAC017 and ANAC017-regulated genes are induced during the later stages of natural senescence .................................................................................................. 95
Discussion ....................................................................................................................... 96
  Harmonising conflicting reports on ANAC016 and ANAC017 function during senescence .............................................................. 96
  ANAC017 overexpression triggers time-dependent responses in mitochondrial and chloroplast functions 98
  Evidence for a link between ANAC017 function and autophagy .................................. 99
Conclusions .................................................................................................................... 100
Methods .......................................................................................................................... 101
  Plant material and growth conditions .......................................................................... 101
  Antimycin A treatment ................................................................................................. 101
  RNA extraction, cDNA synthesis, qRT-PCR ................................................................. 101
  Senescence assay ......................................................................................................... 102
Accession numbers ....................................................................................................... 103
Acknowledgements ....................................................................................................... 103
References ...................................................................................................................... 104
Supplementary figures .................................................................................................. 110
Supplementary tables .................................................................................................... 116
Chapter 4 ...................................................................................................................... 118
Abstract ....................................................................................................................... 120
Introduction .................................................................................................................... 121
Results ............................................................................................................................. 122
  Accelerated senescence phenotype in ANAC017 overexpression lines is similar to autophagy-deficient mutants .......................................................... 122
  Autophagy-deficient mutants have reduced retrograde signalling capability .............. 124
  The kinetics of mitochondrial retrograde signalling responses are affected upon chemical inhibition or induction of autophagy pathway .......................... 127
  The C- and N-terminal end of ANAC017 are located in the endoplasmic reticulum lumen .............................................................................................................. 131
Discussion .................................................................................................................... 134
Future prospects .......................................................................................................... 137
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Materials and Methods</td>
<td>138</td>
</tr>
<tr>
<td>Plant material</td>
<td>138</td>
</tr>
<tr>
<td>Senescence assay</td>
<td>138</td>
</tr>
<tr>
<td>ANAC017 topology analyses- ratiometric imaging</td>
<td>138</td>
</tr>
<tr>
<td>Tunicamycin resistance phenotyping</td>
<td>139</td>
</tr>
<tr>
<td>Retrograde signalling responses in Arabidopsis suspension cell cultures</td>
<td>139</td>
</tr>
<tr>
<td>Antimycin A treatment on Arabidopsis seedlings</td>
<td>139</td>
</tr>
<tr>
<td>RNA isolation, cDNA synthesis and qRT-PCR analysis</td>
<td>139</td>
</tr>
<tr>
<td>References</td>
<td>140</td>
</tr>
<tr>
<td>Supplementary figures</td>
<td>144</td>
</tr>
<tr>
<td>References</td>
<td>144</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>145</td>
</tr>
<tr>
<td>Retrograde signalling in organelle biogenesis</td>
<td>146</td>
</tr>
<tr>
<td>Autophagy- a regulatory pathway of mitochondrial composition</td>
<td>146</td>
</tr>
<tr>
<td>Emerging link between transcriptional regulation of retrograde signalling, senescence and autophagy</td>
<td>148</td>
</tr>
<tr>
<td>Future directions</td>
<td>150</td>
</tr>
<tr>
<td>References</td>
<td>152</td>
</tr>
<tr>
<td>Appendices</td>
<td>157</td>
</tr>
<tr>
<td>Appendix 1</td>
<td>158</td>
</tr>
<tr>
<td>Appendix 2</td>
<td>171</td>
</tr>
<tr>
<td>Appendix 3</td>
<td>186</td>
</tr>
</tbody>
</table>
List of figures

Chapter one

Figure 1 Mitochondrial electron transport chain (mETC).
Figure 2 Structure and function of photosystems
Figure 3 Identification of ANAC017 as a regulator of AOX1a.
Figure 4 Localization of ANAC017 in plant cell.
Figure 5 Proposed model for convergence of ANAC017- and PAP-dependent signalling pathways.
Figure 6 Micro- and macroautophagy.
Figure 7 Mechanism of autophagosome formation.

Chapter two

Figure 1 A Putative Model of the Mechanisms of Mitophagy in Plants.
Figure 2 Regulation and role of mitophagy in plants.
Box 1 Figure 1 Gene expression of ATG genes during dark-induced and developmental senescence.

Chapter three

Figure 1 Overexpression of ANAC017 leads to accelerated senescence
Figure 2 Exploration of RNA-seq data from dark-induced Senescence assay in Col-0a, anac017-1, ANAC017 OEa and OEc plants.
Figure 3 Time and localization-dependent transcriptional
responses based on RNA-seq data.

Figure 4  GO term enrichment in A. *transient* and B. *sustained* type responses that are common between *ANAC017 OEa* and *OEc*.

Figure 5  Heat map of DEGs that belong to autophagy pathway, during the dark-induced senescence assay in ANAC017-related genotypes.

Figure 6  Core ANAC017-regulated genes are differentially expressed in dark-induced and natural senescence.

Supplementary Figure 1  Functional comparison of ANAC017 and ANAC016 in mitochondrial retrograde signalling responses.

Supplementary Figure 2  Binding motifs.

Supplementary Figure 3  2-way ANOVA test significance in senescence assay.

Supplementary Figure 4  Extended dark-induced senescence time-course on Col-0a, anac017EMS, anac017-1 and anac016.

Supplementary Figure 5  A heat map of A. DEGs involved in biosynthesis, degradation and signalling of ethylene, salicylic acid (SA), jasmonic acid (JA), gibberellins, auxins and cytokinins. B. DEGs previously published to be involved in senescence.

Supplementary Figure 6  Expression of ANAC017 controlled genes over
the dark-induced senescence time-course in Col-0a plants.

Chapter four

Figure 1  
ANAC017 overexpressing plants display Accelerated senescence similar to autophagy-deficient mutants.

Figure 2  
Retrograde signalling responses in anac17EMS and autophagy-deficient mutants compared to Col-0, upon stimulation with Antimycin A and analysed using qRT-PCR.

Figure 3  
Mitochondrial retrograde signalling responses in PSB-D cell cultures treated with wortmannin prior AA application.

Figure 4  
Kinetics of mitochondrial retrograde signalling responses induced with 50µM AA, after pre-treatment with tunicamycin.

Figure 5  
ANAC017 overexpressing plants are more resistant to ER stress and autophagy inducer tunicamycin.

Figure 6  
ANAC017 topology analysis in the endoplasmic reticulum.

Figure 7  
Autophagy- a model mechanism for mitochondrial retrograde signalling

Supplementary Figure 1  
Arabidopsis rhomboid proteases in secretory pathway
List of tables

Chapter two

Table 1  Conservation of Mitophagy Components in Plants with Yeast and Animals

Table 2  Arabidopsis mitochondrial proteins containing an ATG8-Interacting Motif.

Chapter three

Supplementary table 1  RNA-seq data for Col-0a, anac017-1, ANAC017 OEc and OEc during dark-induced senescence time-course.

Supplementary table 2  Sums of counts of technical replicates obtained for each of the biological samples analysed by RNA-seq.

Supplementary table 3  GO Term analyses of DEGs in different types of time-dependent responses in dark-induced senescence time-course for Col-a, anac017-1 and commonly expressed genes between ANAC017 OEa and OEc.

Supplementary table 4  GO Term analyses of DEGs in different types of time-dependent responses in dark-induced senescence time-course for commonly expressed genes between ANAC017 OEa and OEc.

Supplementary table 5  A summary of experimental design used in this and previous studies concerning role of
ANAC017 in senescence.

Supplementary table 6 Primer sequences used in the qRT-PCR analyses.

Chapter four

Table 1 Collection of different autophagy-deficient mutants used in this study.
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## Authorship declaration: co-authored publications

This thesis contains work that has been published and/or prepared for publication.

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<tbody>
<tr>
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<tr>
<td>Student contribution to work:</td>
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<td>Appendix 1</td>
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I, A. Harvey Millar, certify that the student’s statements regarding their contribution to each of the works listed above are correct.

As all co-authors’ signatures could not be obtained, I hereby authorise inclusion of the co-authored work in the thesis.

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Abbreviations

AA  antimycin A
ABI4 Abscisic acid insensitive 4
ADP adenosine diphosphate
AIM ATG8-interacting motif
ANAC Arabidopsis NAC domain-containing protein
AOX1a alternative oxidase 1a
APX ascorbate peroxidase
ATG AuTophaGy
ATP adenosine triphosphate
CAT catalase
CK2 casein kinase 2
CoRR colocation (of gene and gene product) for redox regulation of gene expression
CRS chloroplast retrograde signalling
DHA dehydroascorbic acid
EMS ethyl methanesulphonate
ER endoplasmic reticulum
ERMES ER-mitochondria encounter structure
ETC electron transport chain
FtsH filamentous temperature sensitive H
FUNDC1 FUN14 domain-containing protein 1
GR glutathione reductase
GSH glutathione
GUN genomes uncoupled
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<td>HR</td>
<td>hypersensitive response</td>
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<tr>
<td>IMM</td>
<td>inner mitochondrial membrane</td>
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<tr>
<td>Lhcb</td>
<td>light-harvesting chlorophyll a/b-binding</td>
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<tr>
<td>MAMs</td>
<td>mitochondria-associated membranes</td>
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<td>MCU</td>
<td>mitochondrial calcium uniporters</td>
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<tr>
<td>MDS</td>
<td>mitochondrial dysfunction stimulon</td>
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<tr>
<td>mETC</td>
<td>mitochondrial electron transport chain</td>
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<td>MFA</td>
<td>monofluoroacetate</td>
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<td>MORF2</td>
<td>multiple organellar RNA editing factor 2</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MV</td>
<td>methylviologen, also known as paraquat</td>
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<td>NADH</td>
<td>nicotinamide adenine dinucleotide (NAD) hydrogen (H)</td>
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<td>NEP</td>
<td>nuclear encoded RNA polymerase</td>
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<td>outer mitochondrial membrane protein of 66kDA</td>
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<td>PAP</td>
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<tr>
<td>PPR</td>
<td>pentatricopeptide repeat</td>
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<td>plastid transcription active chromosome</td>
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<td>rao</td>
<td>regulator of alternative oxidase</td>
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Chapter 1

General Introduction
Structure and function of mitochondria and chloroplasts

Mitochondria and chloroplasts are semi-autonomous organelles, which retained part of their genomes. They both feature inner and outer membranes and electron transport chains. In addition, chloroplasts contain a third membrane system, thylakoids.

The mitochondrial electron transport chain (mETC) is embedded within the inner mitochondrial membrane (IMM) and consists of 5 protein complexes. Complex I (NADH dehydrogenase) oxidizes NADH, formed in the mitochondrial matrix during TCA cycle, to NAD⁺ while reducing ubiquinone (UQ) to ubiquinol (UQH₂) (Figure 1). A second pathway for UQ reduction leads via Complex II (succinate dehydrogenase) in the mitochondrial membrane. Succinate dehydrogenase (SDH, Complex II) is a bridge between the mETC and the Krebs cycle in the matrix. It oxidizes succinate to fumarate during which UQ is reduced to UQH₂ (Figure 1). From UQH₂ electrons are transferred to cytochrome c, located in the mitochondrial intermembrane space, by Complex III (cytochrome bc1 oxidoreductase) (Figure 1). Cytochrome c, in turn is a donor of electrons for cytochrome c oxidase (Complex IV) in the mitochondrial inner membrane (Figure 1). During the process of electron transfer via complexes I, III and IV, protons are translocated from the mitochondrial matrix into the intermembrane space, generating a gradient of protons (H⁺) that is used by ATP synthase (Complex V) for oxidative phosphorylation of ADP to form ATP (Figure 1).

During the process of electron transfer through the mitochondrial and chloroplast ETC, an unavoidable production of reactive oxygen species (ROS), superoxide (O₂⁻⁻), occurs. In mitochondria, the majority of O₂⁻⁻ is produced by Complex I and Complex III, and to some degree Complex II when their electron transfer centres are highly reduced (citations; also Belt, Plant Physiol 2017). During a stress that impacts mitochondria and disrupts the ETC, overproduction of ROS can oxidize lipids and proteins, which in turn can lead to damage and/or increase of their turnover. To avoid this, plants have both antioxidant defences such as O₂⁻⁻ dismutase and the ascorbate-glutathione cycle to remove ROS as well as additional ETC enzymes: alternative oxidase (AOX), additional NADH dehydrogenases and uncoupling proteins (UCP) that can lower the production of ROS. AOX and alternative NAD(P)H dehydrogenases avoid proton gradient generation while still aiding in the electron flow within mETC. Under some circumstances this can limit O₂⁻⁻ production, and subsequent dismutation to H₂O₂ (Catania et al., 2019, Maxwell et al., 1999).
Unlike the mETC, the chloroplast electron transport chain does not reside in the inner envelope membrane of chloroplasts, but in the third membrane system, the thylakoid membranes. Similar to the mitochondrial ETC, electrons flow through chloroplast Photosystems I and II (Figure 2A) is a series of reduction and oxidation reactions (called Z-scheme, Figure 2B, that end with NADPH synthesis and is coupled through a proton gradient to ATP synthesis. The chloroplast process of ATP and NADPH production is a light-dependent reaction. Afterwards this reducing and phosphorylating potential is used to convert carbon dioxide and water into sugars during light-independent reactions, also known as the Calvin cycle.

The chloroplast electron transport chain process starts with light energy being captured by antenna complexes Lhcb. This excites an electron of water which is then transferred to the P680 centre of Photosystem II. This is followed by excitation of chlorophyll which reduces pheophytin and further transfers electrons onto ubiquinone A, ubiquinone B and plastoquinone. Next, electrons are transferred onto a 2Fe-2S centre of cytochrome b_{6}f complex via Rieske protein onto cytochrome f. Following the reduction of cytochrome f, plastocyanin is reduced and excites the P700 centre of PSI, where a series of electron transfers is carried out via chlorophyll, phylloquinone, iron-sulphur clusters and onto ferredoxin. During the last step, ferredoxin carries electrons to ferredoxin reductase, which reduces NADP$^+$ to NADPH. Similarly to the mETC, during electron transfer through photosystems, a proton

Figure 1. Mitochondrial electron transport chain (mETC). Figure adapted from (Grant, 2020).
gradient \((H^+)^\) is generated and used by ATP synthase in oxidative phosphorylation of ADP to ATP.

Figure 2. Structure and function of photosystems. A. Structure of photosynthetic electron transport chain. Figure adapted from (Hou, 2011). B. Z-scheme of electron transfer via photosystems and their redox potential. Figure adapted from (Orr and Govindjee, 2013).

**Evolution of semi-autonomous organelles and their impact on cell signalling**

According to the endosymbiosis theory, a cyanobacteria (Kern *et al.*, 2020, Lawrence *et al.*, 2019) and α-proteobacteria (Roger *et al.*, 2017, Williams *et al.*, 2007) were engulfed by a
larger archaeal host (Spang et al., 2015, Spang et al., 2019, Zaremba-Niedzwiedzka et al., 2017), where they were transformed into chloroplasts and mitochondria respectively through millions of years of adaptation. These organelles then became semi-autonomous as the result of a gradual transfer of their genomes to the host nucleus. In the model plant Arabidopsis thaliana, only 57 genes remain within the mitochondrial genome (Unseld et al., 1997). These genes encode rRNAs, tRNAs and some membrane components of the mETC, while the majority of the components for building mitochondria (about 2000 proteins) are encoded in the nucleus (Millar et al., 2005, Murcha et al., 2014). rRNA genes have been particularly useful in evolutionary studies of the mitochondrial genome. Sequencing analyses of genes encoding rRNAs in mitochondria have been found to be evolving slowest of all the mitochondrial genes and it is thanks to this that researchers were able to pinpoint the origin of mitochondrial genome to α-proteobacteria (Yang et al., 1985). Moreover, modern mitochondria and bacteria share similar mechanisms for protein synthesis, utilizing N-formyl methionyl tRNA during the process, which further confirms the endosymbiotic theory of origin (Fridovich, 1974, Gray, 2015).

Similarly to the mitochondrial genome, only a small number (87) of genes remained within the chloroplast Arabidopsis genome while the rest of the proteome is encoded by nuclear genes (Sato et al., 1999). These numbers can vary between species and have been widely used as molecular markers for studies of evolution and species identification (de Vere et al., 2015, Kubo and Newton, 2008, Tonti-Filippini et al., 2017).

There are a couple of hypotheses suggesting why mitochondria and chloroplasts have retained their genomes and not transferred all their genes to the nucleus. One of the theories concerns the gene transfer from mitochondria or chloroplasts to nucleus itself. It suggests that genetic code disparity and the hydrophobicity of certain proteins, which would complicate protein import from the cytosol to the mitochondria, are powerful barriers to overcome (Bullerwell et al., 2000, Jacobs, 1991).

The CoRR (colocation of gene and gene product) for redox regulation of gene expression) hypothesis takes into consideration the redox state of proteins encoded in their organellar genomes (Allen, 2003). In this hypothesis, proteins encoded within organellar genomes and are the components of electron transfer chains (mETC, photosystems). CoRR was initially based on the function of chloroplasts, where a kinase senses the reduced state of
plastoquinone and phosphorylates itself, which in turn will activate a pathway of regulation of organellar gene expression. It is argued that this regulation requires almost instantaneous and unrestricted control of protein synthesis and therefore it is favoured for the genes to remain in the organellar genome (Allen, 1993, Allen, 2003, Allen, 2015).

Organellar post-translational response to abiotic stresses

Due to their sedentary lifestyle, plants are exposed to a variety of stresses from the physical environment (such as high light, salinity or temperature changes), and from other organisms including pathogens, animals and even other plants. In response, they have developed response systems to adjust their metabolism and increase stress tolerance. In this process, ROS are commonly formed and can act as signalling molecules triggering changes in gene expression and protein translation for stress recovery. There are 3 main types of ROS in plants: singlet oxygen ($^1$O$_2$), hydrogen peroxide (H$_2$O$_2$) and O$_2^-$:

Singlet oxygen production is linked to chloroplast function. Chloroplasts generate energy via the electron transport chain during photosynthesis. Singlet oxygen is generated when chlorophyll becomes over-exited when light hits the light harvesting complex on photosystem II (PSII) (Allen et al., 2011). A consequence of excessive $^1$O$_2$ formation is photodamage to the PSII complexes. Previously, it was also shown that $^1$O$_2$ can influence nuclear gene expression (Pesaresi et al., 2006). Therefore, it was thought to be involved in the communication between chloroplasts and the nucleus (Galvez-Valdivieso and Mullineaux, 2010, Page et al., 2017). In addition, $^1$O$_2$ is known to contribute to programmed cell death (Kim and Apel, 2013, Kim et al., 2012) and/or antioxidant production, which can influence resistance to pathogens in fruits (Decros et al., 2019).

Hydrogen peroxide is formed in chloroplasts, peroxisomes or mitochondria from the O$_2^-$ in an enzymatic reaction catalysed by superoxide dismutase (SOD) with iron, zinc or copper in its catalytic centre (McKersie et al., 2000, Xing et al., 2013, Xing et al., 2015). In excessive amounts, hydrogen peroxide has also been shown to cause programmed cell death in plants (Klessig et al., 2000, Pathirana et al., 2017). During biotic stress, such as attack by biotrophic pathogens, hydrogen peroxide can trigger the hypersensitive responses (HR) to cause localized cell death and prevent pathogens from spreading (Bittner et al., 2017, Bozhkov and Lam, 2011). Given that hydrogen peroxide is formed mainly in energy producing organelles,
it has been studied for its direct role in retrograde signalling pathways. It was shown that a burst of ROS was present in many mitochondrial retrograde signalling studies published to date, implying it could be a requirement for signalling to occur. However, monofluoroacetate (MFA), a TCA cycle inhibitor, was shown to induce mitochondrial retrograde signalling without oxidative stress (Ng et al., 2013b, Umbach et al., 2012, Van Aken et al., 2016a). Nevertheless, bursts of ROS during retrograde signalling are not specific enough to trigger such organelle-specific responses (Moller and Sweetlove, 2010). For example, H$_2$O$_2$ can be produced in both chloroplasts and mitochondria, but the responses it triggers differ. H$_2$O$_2$ from chloroplasts triggers nuclear gene expression of photosynthesis-associated nuclear genes (PhANGs) while H$_2$O$_2$ from mitochondria triggers different genes including AOXs (Oelmuller et al., 1986, Pogson et al., 2008, Van Aken et al., 2016a, Wang et al., 2018).

There are two ways for the cell to deal with ROS production: prevention and detoxification. In mitochondria alternative NADH dehydrogenases, AOXs in mETC and UCP play a role in the prevention of ROS generation. Each of those act a different stage of the electron transfer through mETC. Alternative NADH (NDA) and NADPH (NDC) dehydrogenases present on the matrix side of the IMM have 10x higher $K_{m}$, which would suggest that they compete with Complex I for the substrate only in high NADH concentration conditions (Rasmusson et al., 2008). Moreover, external set of alternative NADH and NADPH dehydrogenases are present on the outer surface of IMM and they can be calcium dependent or independent (Møller, 1997, Rasmusson et al., 2008). In either case, they allow for the oxidation of internal and external pools of NADH or NADPH without the proton transfer through the membrane. Similarly, AOX bypass Complex III and IV and also does not produce proton gradient through the membrane. In Arabidopsis, five isoforms of AOX exist: AOX1a-d and AOX2, with AOX1a being used as a marker for mitochondrial retrograde signalling marker and AOX1d used as a marker for senescence. In standard (non-stressed) conditions, AOX are present at a very small protein level and highly expressed only upon stress induction, such as using AA. By bypassing proton transfer through the membrane, AOX and NAD(P)H dehydrogenases can prevent the ROS production in mitochondria and alter ATP rate production, reducing it by 60 and 30%, respectively (Rasmusson et al., 2008, Vanlerberghe, 2013).

A third and largely different layer of prevention of excessive ROS formation comes from the presence of uncoupling protein (UCP). UCPs are upregulated during oxidative stress (Begcy et
environmental

Various environmental conditions?

Transcription factors involved

Stress activity

however

detoxified

oxygen

peroxisomes, SOD are

Another reduction of ascorbic oxygen, interaction work scavengers as (SOD), responsible also prevention preventing mitochondrial metabolism towards respiration and oxygen consumption, therefore preventing excessive ROS formation (Begcy et al., 2011, Echtay et al., 2002).

Prevention of ROS generation is only partially effective and therefore within cells there are also mechanisms in place to detoxify them from ROS: enzymatic and non-enzymatic. Enzymes responsible for ROS scavenging include, but are not restricted to, Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Reductase (GR) and peroxidases, while antioxidants, such as ascorbic acid, carotenoids, α-tocopherol and glutathione make a pool of non-enzymatic scavengers (Das and Roychoudhury, 2014). Both, enzymes and antioxidants are required to work together to effectively remove harmful ROS from the cell. A good example of this interaction can be ascorbate peroxidase (APX). It catalyses reduction of H₂O₂ to water and oxygen, while utilizing ascorbic acid as a reducing agent (Koussevitzky et al., 2008). Oxidized ascorbic acid (dehydroascorbic acid, DHA) is recycled back to its reduced form with the help of GR which uses NADPH to reduce GSSH to glutathione (GSH) as an intermediate step in reduction of DHA (Trivedi et al., 2013).

Another two very important enzymes in ROS scavenging are SOD and catalase (CAT). There are three main isoforms of SOD, based on the metal cofactor bound with the protein: Mn-SOD (present in mitochondria), Fe-SOD (present in chloroplasts) and Cu/Zn-SOD (present in peroxisomes, chloroplasts and cytosol). SODs catalyse reaction in which O₂⁻⁻ is dismutated into oxygen and hydrogen peroxide (Boguszewska et al., 2010, Mittler, 2002). H₂O₂ is further detoxified into oxygen and water by a catalase. CAT has a much higher activity in the cytosol, however in recent years presence of organellar isoforms has been confirmed with a lower activity (Mhamdi et al., 2010).

**Stress-specific transcriptional regulation of organellar proteins**

*Transcription factors involved – are they known to be induced by stress or specific environmental conditions?*

Various studies identified a role for numerous transcription factors in plant adaptation to environmental stresses and implicating transcriptional regulation to be very important in
acquiring resistance to abiotic stresses. For example, DREB1a in rice (Oryza sativa) and Arabidopsis confers resistance to drought, high light and cold (Dubouzet et al., 2003, Maruyama et al., 2004). WRKY46 was also shown to increase salt stress tolerance in plants (Lv et al., 2020). Moreover, NAC transcription factors have also been shown to increase drought tolerance in plants (Nakashima et al., 2012). Knowledge of which transcription factor increases which tolerance can have a great biotechnological potential in agriculture. It could be used for breeding purposes of crops with higher abiotic stress tolerance, however it needs to be carefully considered. Increased levels of NAC transcription factors have been shown to accelerate senescence in plants which can result in a decrease in crop yield (Borrill et al., 2019, Gregersen et al., 2013, Ma et al., 2018).

Transcriptional regulation of mitochondrion- and chloroplast-encoded genes

Chloroplasts and mitochondria contain their own genomes. They also retained their own transcription mechanism, however it is not fully independent from the nucleus. Transcription is carried out by RNA polymerases and there are two main types that carry that task in organelles: nuclear encoded RNA polymerase (NEP) and plastid encoded RNA polymerase (PEP). NEP is a T3/T7 bacteriophage type polymerase (Lerbs-Mache, 2011). There are three isoforms of NEP polymerases, named RpoTp, which functions in chloroplasts only, RpoTm, which functions in mitochondria and RpoTmp which carries out transcription in both chloroplasts and mitochondria (Yu et al., 2014). In mitochondria, disturbances to the function of RpoTmp was shown to result in decreased transcript abundance of NAD6 and COX1 transcripts which lead to a decrease in Complex I and IV abundance in mETC (Kuhn et al., 2009). Since RpoTmp is responsible for transcription of genes encoded in mitochondrial genome, this would suggest that mRNA levels of organelle encoded genes will co-determine the overall protein level of assembled complexes (Kuhn et al., 2009).

In chloroplasts, another, plastid encoded RNA polymerase (PEP) also plays a role. PEP is a multimeric, eubacterial-type polymerase (Liere et al., 2011). There are differences between polymerases in the set of genes they transcribe. NEP transcribes mostly genes encoding housekeeping genes and components of the transcriptional and translational apparatus, which includes PEP, while PEP transcribes genes encoding photosynthesis-related genes (Liere et al., 2011). PEP is a part of a much larger complex, containing 50 different proteins, most of which are encoded within the nuclear genome, called plastid transcription active
chromosome (pTAC) (Melonek et al., 2012) and which play a role in chloroplast transcription processes.

**Coordinating gene expression between nucleus and organelles**

Coordination of communication between different organelles is very important for biogenesis and survival processes of the cell. Due to the presence of independent organellar genomes (mitochondrial and chloroplast) within the respective organelles, coordination of gene expression between chloroplast and nucleus as well as mitochondria and nucleus has been investigated in the past (Deng and Gruissem, 1987, Giege et al., 2005). In both cases, researchers have found that mitochondrial and chloroplast gene expression is independent of nuclear transcription and that the regulation of organelle function exists at the post-transcriptional level (Deng and Gruissem, 1987, Giege et al., 2005, Leon et al., 1998, Van Aken et al., 2016b).

**Retrograde signalling**

With so few of the genes essential for chloroplast or mitochondrial function actually encoded within their respective organellar genomes, it is of great importance that a system is in place to balance synthesis of nuclear - and organelle-encoded proteins for the major machinery of photosynthesis and respiration, especially important during plant development and adaptation processes to environmental stresses. Intracellular communication (signalling) via message transfer within a plant cell can be achieved by anterograde signalling (from the nucleus to organelles) and retrograde signalling (from organelles to the nucleus). Anterograde signalling involves gene expression and translation of organellar proteins that is initiated by an external stimulus and is coordinated with the targeted organelle to maintain appropriate stoichiometry of proteins required to build each protein complex (Jung and Chory, 2010, Ng et al., 2014, Wang et al., 2018). Retrograde signalling, however, controls nuclear gene expression in response to messages initiated in the organelle that are conveyed to the nucleus. Most retrograde signals are generated during the development of organelles, particularly during seed germination when bulk biogenesis of chloroplasts and mitochondria occurs (Giege et al., 2005, Jung and Chory, 2010). During vegetative and floral development, retrograde signals are generated in response to abiotic or biotic stimuli and carry information
about the energetic status of organelles to the nucleus (Ishiga et al., 2017, Karpinska et al., 2017, Wang et al., 2018).

**Chloroplast retrograde signalling**

Chloroplast retrograde signalling (CRS) can be a result of biotic or biotic stresses influencing the function of chloroplasts. The main role of chloroplasts is to absorb light, which is then transformed into chemical energy in the forms of NADPH, ATP and sugars. Plants have developed protective mechanisms to prevent damage to light harvesting complexes during light-induced stresses. It was observed that high light induces bursts of reactive oxygen species (ROS), which in turn leads to changes in the expression of approximately 700 nuclear-encoded chloroplast components (Rossel et al., 2007). In addition to ROS signalling, it was shown that under high light both mitochondria and chloroplasts accumulate SAL1, a phosphatase that is able to hydrolase a phosphate group from 3’-phosphoadenosine 5’-phosphate (PAP) as its direct target (Estavillo et al., 2011). It was shown that SAL1 can act as an oxidative stress sensor and that it can form a dimer upon high light stress which in turn reduces its activity (Chan et al., 2016). In the absence of SAL1, PAP starts to accumulate and has the ability for exoribonuclease (XRN) activity inhibition, which results in the decrease in mRNA stability and high drought resistance, indicating its role in oxidative stress-induced retrograde signalling in plants (Chan et al., 2016, Van Aken and Pogson, 2017). SAL1 has also been shown to regulate hypocotyl elongation and flowering time, which indicates that retrograde signalling is used in the developmental switch from vegetative growth to reproductive phase (Feng et al., 2016, Kim and von Arnim, 2009).

Another well studied group of proteins that is involved in chloroplast retrograde signalling is the GENOMES UNCOUPLED (GUN) family. In Arabidopsis, 6 loci have been identified to encode for GUN proteins. Despite numerous studies over the past 3 decades, the role of GUN1 in chloroplast retrograde signalling was only recently hinted on. GUN1-6 have been shown to be involved in tetrapyrrole biosynthesis (Larkin et al., 2003, Mochizuki et al., 2001, Shimizu et al., 2019, Strand et al., 2003). Tetrapyrroles are metabolic compounds build from four pyrrole groups linked either in the cyclic or linear fashion and are central in chlorophyll formation. Recently, GUN1 has been reported to bind tetrapyrrole heme which reduces chlorophyll synthesis and as a result can alter chloroplast-to-nucleus retrograde signalling (Shimizu et al., 2019). GUN1 was first identified during a screen of Arabidopsis mutants using
norflurazon, an inhibitor of phytoene desaturase (carotenoid biosynthetic enzyme) (Susek et al., 1993) which blocks the expression of photosynthesis-associated nuclear genes (PhANGs) in wild-type plants (Oelmuller et al., 1986). GUN1 belongs to a family of proteins containing pentatricopeptide repeat (PPR), which also contain a small muteS-related (SMR) domain (Koussevitzky et al., 2007). Surprisingly, GUN1 does not bind RNAs as other known PPR proteins (Tadini et al., 2016), but it can interact with MORF2 (multiple organellar RNA editing factor 2) that belongs to a larger group of proteins which form the “editosome” (Zhao et al., 2019). The interaction between GUN1 and MORF2 is required for the editing of 11 transcripts, leading to a conclusion that GUN1 targets a specific set of RNAs and can control specific part of retrograde signalling (Zhao et al., 2019). Moreover, through interaction with a chloroplast chaperone cpHSC70-1, GUN1 can regulate protein import into chloroplasts and play a role in de-etiolation processes (Wu et al., 2018, Wu et al., 2019). GUN1 is present at high protein levels in very young seedlings when chloroplasts are only developing (Wu et al., 2018), providing strong evidence for the role of retrograde signalling in chloroplast biogenesis.

Mitochondrial retrograde signalling

Downstream effects of how altered protein content/function or environmental changes impact the transcriptional, translational and post-translational processes in the plant cell have been extensively studied in the last three decades (Giraud et al., 2012, Leister, 2012, Schwarzlander et al., 2012). Application of mETC inhibitors, such as antimycin A (AA, complex III inhibitor), reverse and forward genetics, or combinations of all three are the most common approaches in mitochondrial retrograde signalling studies. It was as early as 1980s, when discovery of alternative components in mETC took place, which included AOX1a. From then, knowledge has vastly advanced, showing an ability of AOX1a in ROS generation prevention (Purvis and Shewfelt, 1993) and naming AOX1a as a marker for mitochondrial stress and hypersensitive responses in plants (Lacomme and Roby, 1999). Similar to plastid retrograde signalling, bursts of ROS and Ca^{2+} are often observed upon the induction of mitochondrial retrograde signalling (Leister, 2012, Schwarzlander et al., 2012). It was previously argued that this burst of ROS and/or Ca^{2+} would not be specific enough (Moller and Sweetlove, 2010), because the same ROS are produced upon different types of stresses. There is a clearly a distinct transcriptional difference between the responses that are triggered by specific stresses (Gujjar et al., 2014). Therefore, identification of protein components, such as
transcription factors, is necessary to further characterise the mechanisms involved in mitochondrial retrograde signalling.

ABSCISIC ACID INSENSITIVE 4 (ABI4) is a transcription factor that has been identified to be involved in the regulation of nuclear gene expression of AOX1a (Giraud et al., 2009). It was shown that ABI4 binds to the promoter of photosynthesis-related genes (Koussevitzky et al., 2007) and that decreased levels of ABI4 correlate with elevated levels of AOX1a (Clifton et al., 2005, Giraud et al., 2009).

In addition to ABI4, other transcription factors have been identified as regulators of mitochondrial retrograde signalling via AA application. These include ANAC017 (Ng et al., 2013b), and ANAC013 which works downstream of ANAC017 (De Clercq et al., 2013, Van Aken et al., 2016a). Some members of the WRKY transcription factor family have also been linked to mitochondrial retrograde signalling (Van Aken et al., 2013, Vanderauwera et al., 2012). WRKY transcription factors bind to the promoters of genes containing the W-box, (T)TGAC(C/T) (Rushton et al., 1995). It was reported that WRKY40 and WRKY60 are involved in both chloroplast and mitochondrial retrograde regulation (Van Aken et al., 2013). WRKY40 was shown to inhibit mitochondrial retrograde gene expression stimulated by AA (Van Aken et al., 2013), while WRKY60 was shown to stimulate mitochondrial dysfunction stimulon (MDS) genes (Van Aken et al., 2013).

The role of post-transcriptional and post-translational regulation on mitochondrial retrograde signalling has also been considered. It was shown that changes in mitochondrial transcription trigger retrograde signalling (Niazi et al., 2019). Since changes to mitochondrial redox status can be a trigger for retrograde signalling, it is expected that generation of ROS or Ca²⁺ flux will act as messengers (Moller, 2001). Moreover, phosphorylation of mitochondrial enzymes could also contribute to induction of mitochondrial retrograde signalling (Djajanegara et al., 2002, Hartl and Finkemeier, 2012). Furthermore, posttranslational modifications can affect the redox state and precede transcriptional activation of biosynthetic processes in imbibed seeds (Moller et al., 2020, Nietzel et al., 2020). Examples of protein families involved in the phosphorylation processes are Target of Rapamycin (TOR) and Sucrose non-fermenting-related kinase 1 (SnRK1) and both might be involved in mitochondrial retrograde signalling (Hartl and Finkemeier, 2012). It has been established that TOR acts as a sensor for nutrient availability and energy metabolism and it is proposed to be involved in the maintenance of
mitochondrial respiration (Dobrenel et al., 2011, O'Leary et al., 2019, Schieke and Finkel, 2006), therefore it could possess the ability to affect mitochondrial retrograde signalling. SnRK1 has been shown to mediate communication between organelles via phosphorylation of transcription factors that regulates gene expression or which proteins are located in mitochondria (Wurzinger et al., 2018), making it a natural target for studies of its role in retrograde signalling.

**Mitochondrial retrograde signalling markers**

In mitochondrial retrograde signalling studies, researchers monitor the status of MDS markers. These marker genes include AOX1a (At3g22370) (Dojcinovic et al., 2005, Zarkovic et al., 2005), Uridine diphosphate glycosyltransferase 74E2 (UGT74E2, At1g05680) (Kerchev et al., 2014) up-regulated by oxidative stress (UPOX, At2g21640) (Ho et al., 2008, Uggalla, 2017, Van Aken et al., 2009b) and outer mitochondrial membrane protein of 66kDA (OM66/BCS1, At3g50930) (Zhang et al., 2014) . The mETC is prone to overreduction due to its fixed stoichiometry between electron transport and ATP synthesis. Increase in alternative ETC bypasses, for example by expression of AOX1a, provides an alternative way for energy dissipation and prevention of reactive oxygen burst (Selinski et al., 2018). It is the first line of defence for the plant cell when it’s exposed to a variety of stresses, such as salinity, high temperature, drought or pathogen attack which can in extreme cases lead to programmed cell death (Van Aken et al., 2009a, Wanniarachchi et al., 2018). Upon overexpression of AOX, decrease in H₂O₂ production and induction of autophagy associated with reduced levels of programmed cell death, are often observed (Liu et al., 2014, Zhu et al., 2018).

While AOX1a is the classical marker to follow mitochondrial retrograde signalling responses, UGT74E2 has among the highest induction levels among MDS genes (Kerchev et al., 2014). UGT74E2 is an auxin UDP-glucosyltransferase, overexpression of which leads to perturbation in auxin signalling (Kerchev et al., 2014, Tognetti et al., 2010). Auxins are a group of plant hormones that stimulate plant growth, therefore during stress conditions, the focus of the cell and mitochondria is shifted from auxin-regulated growth towards cell survival. In *Arabidopsis*, AA triggers the expression of UGT74E2 from nearly non-detectable to high levels, often causing a ~70 or higher relative fold change in transcript abundance, which can be reduced by the pre-application of auxins (Kerchev et al., 2014). This implies that there is an antagonistic relationship between auxin and mitochondrial signalling and the transcriptional
analyses of UGT74E2 mutants confirm this relationship (Ivanova et al., 2014, Kerchev et al., 2014, Tognetti et al., 2010).

Two additional MDS marker genes for quantifying mitochondrial stress responses at the transcriptional level are UPOX and OM66/BCS1. To date, the function of UPOX remains unknown (Shapiguzov et al., 2019, Van Aken et al., 2009b). Both UPOX and AOX1a promoters contain an ANAC017 binding motif within ~1000bp region upstream of the start codon (Uggalla, 2017), suggesting they might be co-expressed under stress conditions by related mechanisms. Salicylic acid (SA) can also induce the expression of UPOX, as well as OM66 (Uggalla, 2017, Zhang et al., 2014). OM66 overexpressing plants have been shown to have increased resistance to Pseudomonas syringae, however it showed also a higher level of necrosis upon inoculation with the necrotrophic fungus Botrytis cinerea (Zhang et al., 2014).

Overlapping signalling pathways mentioned before, still require more comprehensive work in order to fully understand their potential and limitations as well as regulatory components. Mechanisms of how a message is conveyed between different cell compartments are yet to be identified, however data obtained in the past few years as well as continuous development of techniques can shed new light into retrograde signalling in plants.

The role of ANAC017 as a transcription factor coordinating chloroplast and mitochondrial communication with nucleus

AA has already been used in studies of retrograde control in animal tissues since the 1980s (Matsumura et al., 1986) and it was thus only a matter of time before it was applied to study this process in plant systems as well. AA is a mitochondrial complex III inhibitor, which inhibits electron flow through the mETC and induces a wide range of retrograde responses, including the synthesis of AOX1a on a transcript and protein level that compensate for the inhibitor action of AA (Zhang et al., 2017). To understand how the message of faulty electron transport chain function is conveyed to the nucleus, AA was applied to identify regulators of expression of AOX1a, as a proxy for mitochondrial retrograde signalling (Ng et al., 2013b). Forward genetic screening of Arabidopsis ethyl methanesulphonate (EMS) mutagenised plants expressing luciferase under the control of the AOX1a native promoter has identified several loss of functions mutants, rao1/cdke1 (Ng et al., 2013a), rao2-1 and rao2-2 (Ng et al., 2013b) (Figure 3B), that failed to induce luciferase activity (Figure 3A). Point mutations in rao2-1 and
rao2-2 were mapped to a single gene, NAC domain containing protein 17 (ANAC017, At1g34190). The induction of AOX1a expression upon 3-hour of AA treatment was also lower in T-DNA insertion mutants for ANAC017 (anac017-1 SALK_022174, anac017-2 SALK_044777), confirming that insufficient induction of mitochondrial retrograde signalling is a result of mutations in the ANAC017 gene (Figure 3B and 3C).

Figure 3. Identification of ANAC017 as a regulator of AOX1a. A. Luminescence of 2-week old seedlings treated with 50µM AA in Col:LUC, rao2-1 and rao2-2 plants. B. ANAC017 gene structure and position of point mutations in rao2-1 and rao2-2 EMS mutants and T-DNA insertion in anac017-1 and anac017-2 lines. C. AOX1a transcript level in Col:LUC, rao2-1, anac017-1 and anac017-2 2-week old seedlings treated with 50µM AA or mock treatment (deionized water). Adapted from (Ng et al., 2013b).

ANAC017 is a 62kDa transcription factor and it is predicted to contain a transmembrane domain near the C-terminal end (523-548aa). Since ANAC017 regulates the expression of nuclear-encoded AOX1a, this protein was expected to reside within the mitochondrial
membrane. However, experimental evidence showed that ANAC017 was targeted to the endoplasmic reticulum (ER) (Figure 4A) (Ng et al., 2013). Moreover, upon stress induction (for example with AA), the N-terminal end of ANAC017 translocates to the nucleus, while the C-terminal end remains in the ER membrane (Figure 4B) (Ng et al., 2013b). Tosyl phenylalanyl chloromethyl ketone (TPCK), an inhibitor of rhomboid proteases, has been shown to inhibit AA-induced expression of AOX1a (Ng et al., 2013b). This indicates that proteolytic cleavage of the N-terminus of ANAC017 is required to enable its function as a transcription factor in the nucleus.

**Figure 4.** Localization of ANAC017 in plant cell. A. ANAC017-GFP colocalises with ER-RFP marker used as a control in transiently transformed onion cells. B. Onion cells were transiently transformed with RFP-ANAC017-GFP and treated with 50µM AA. Pictures were taken 3h post treatment with 60x objective. Upon stress induction N-terminal end of ANAC017 translocates to nucleus (marked with red, picture in the centre) while GFP fused to C-terminal end of ANAC017 remained in the membrane of ER. Adapted from (Ng et al., 2013b).

ANAC017 is closely related to several other transcription factors, including ANAC013 and ANAC016 (Ng et al., 2013b). ANAC013 has been shown to be involved in mitochondrial retrograde signalling and ANAC017 to bind to the promoter of ANAC013 regulating its
expression. This indicates that the role of ANAC013 is dependent upon and downstream of ANAC017 (De Clercq et al., 2013). Low expression of AOX1a in anac017 knock-out mutant (Ng et al., 2013b) is thus most likely a compensation effect from ANAC013. There has been very limited study of how ANAC016 is involved in retrograde signalling, however it will be addressed further in this thesis (Chapter 3).

More recently, it has been shown that the activity of ANAC017 and ANAC013 decreases when they interact with radical-induced cell death 1 (RCD1) (Shapiguzov et al., 2019). Lack of RCD1 (rcd1) induces ROS-dependent mitochondrial retrograde signalling to the nucleus and the expression of MDS genes. The accumulation of $O_2^-$ is increased in the rcd1 mutant and chloroplast functions are compromised upon treatment with methyl viologen (MV, also known as paraquat) in the light (Shapiguzov et al., 2019). Out of 66 nuclear-encoded genes that were defined as markers for the flu mutant, MV or O$_3$ treatment (Gadjev et al., 2006), transcript abundance of 34 of those genes are regulated by ANAC017 (Ng et al., 2013b). While roots of ANAC017 knock-out mutants are more sensitive to MV treatment compared to wild type, ANAC017 overexpressing lines are more tolerant to the same treatment with less suppressed root growth compared to wild type (Van Aken et al., 2016a). Altogether, these data suggest that ANAC017 is involved in both mitochondrial and plastid retrograde signalling.

*Model for ANAC017 mediated communication with nucleus and integration of signals from mitochondria and chloroplast*

Organelle dysfunction can lead to a burst of ROS and affects metabolism of the entire cell, so it needs to be controlled. Therefore, it has been proposed that mitochondria and chloroplasts communicate with each other to prevent excessive ROS formation and optimise metabolism. The mitochondrial stress marker AOX has been shown to accumulate upon a high light stress (Blanco et al., 2014). Moreover, plants treated with norflurazon had greater mitochondrial DNA and transcript levels (Hedtke et al., 1999). Vice versa, it was shown that dysfunction in mitochondria can change biosynthesis of lipids in chloroplasts (Xu et al., 2008).

It has been also shown that disturbances in the function of chloroplasts can impact expression of genes encoding mitochondrial proteins and vice versa (de Souza et al., 2017, Upadhyay and Srivastava, 2019, Van Aken et al., 2016a, Van Aken and Pogson, 2017), leading to the
conclusion that communication between these two organelles is also retrograde signalling dependent.

Recently it was also proposed that PAP- and ANAC017-dependent signalling pathways converge to suppress excessive signalling caused by external stresses that can affect both types of energy-producing organelles (Van Aken and Pogson, 2017) and which would lead to programmed cell death (PCD).

Figure 5. Proposed model for convergence of ANAC017- and PAP-dependent signalling pathways. In general upon stress applied on mitochondria or chloroplasts via electron transport chain (ETC) inhibition or high light/drought respectively, a burst of hydrogen peroxide causes inhibition of SAL1 and accumulation of PAP. Through unidentified mechanism PAP can translocate to nucleus and inhibit exoribonucleases (XRN) and in turn affect mRNA stability and gene expression. In other part of this this model, oxidative stress is sensed by the endoplasmic reticulum (ER) which can recruit ANAC017 to change gene expression of stress responsive genes. Adapted from (Van Aken and Pogson, 2017).

In the proposed model (Figure 5), upon oxidative stress in mitochondria or chloroplasts, ROS inhibit SAL1, which in turn causes accumulation of PAP. PAP can then translocate to nucleus, where it can inhibit exoribonucleases (Dichtl et al., 1997, Hirsch et al., 2011), which then affects gene expression of ANAC017/PAP target genes (Crisp et al., 2018, Van Aken and Pogson, 2017).
ANAC017 and its role in senescence

Senescence, or aging is an integral part of every being and generally is understood as a deterioration of processes within the cell and tissue. In plants, senescence can be clearly observed by the yellowing of leaves. Plant senescence can be developmental (correlated to plant age) or stress induced and allows for recycling of nutrients (Guiboileau et al., 2010). In the field of plant science, developmental senescence is often argued to be the preferred way to study naturally occurring processes within the cell (Gan and Amasino, 1997), however it carries some disadvantages. Natural senescence is stretched out over a longer period of time, compared to induced senescence (weeks vs days) and therefore induced senescence is often chosen to study the molecular effects of genetic mutations or abiotic stresses on plant performance (Keech et al., 2007). In recent years, a possible role for ANAC017 in plant senescence has also been investigated, however results obtained from 3 different studies came to 3 different conclusions (Kim et al., 2018, Kim et al., 2013, Meng et al., 2019).

In 2013, researchers analysed the role of ANAC016 in Arabidopsis thaliana knock-out mutant and overexpressing lines. Due to the high similarity of ANAC016 with ANAC017, they performed senescence analyses on anac017 knock-out mutants, and they concluded that ANAC017 has no positive nor negative role in dark-induced senescence (Kim et al., 2013). On the contrary, other researchers identified a negative role for ANAC017 during natural senescence (Kim et al., 2018). To add to the confusion, newly published research identified a positive role for ANAC017 during natural senescence (Meng et al., 2019). Thus, the contradicting information on the role of ANAC017 in plant senescence is currently unresolved.

It was shown before that accelerated senescence can reduce yield (Borrill et al., 2019, Gregersen et al., 2013, Ma et al., 2018), making resolution of the role of ANAC017 in senescence of potential importance for applied research. This will be discussed in great detail in Chapter 3.

Autophagy, Mitophagy and links to stress signalling

Autophagy is a vesicular, self-recycling process within a cell. In normal conditions, autophagy aids in turnover of cellular components, such as lipids, amino acids or even proteins when they are damaged for example oxidized due to their function. However, the rate of autophagy is severely increased under stress conditions, such as nutrient depletion or during aging.
(senescence). This raises the possibility that autophagy is a participant in the initiation or decay of cellular signalling events associated with stress and aging.

The term ‘autophagy’ was used for the first time by Christian de Duve in 1963 (AVS de Reuck and Camron, 1963), however it wasn’t until the 1990s when Yoshinori Ohsumi identified the components involved in the process (Mizushima et al., 1998, Takeshige et al., 1992, Tsukada and Ohsumi, 1993). Since then, our understanding of the process has grown significantly. Animal autophagy is quite different to yeast and plant autophagy. Autophagosomes fuse with lysosomes, acidic vesicles in which the degradation takes place, whereas in yeast and plants, once autophagosomes are formed, they fuse with the vacuole, forming autophagic bodies where the degradation takes place (Minina et al., 2014).

Autophagy can be divided into two main types, micro- and macroautophagy. In yeast and plants, during microautophagy, the vacuole engulfs part of the cytosol, containing amino acids, lipids and proteins and directly degrades the content of the autophagic body, skipping the process of autophagosome formation (Figure 6). On the contrary, macroautophagy involves autophagosome formation, translocation to vacuole, fusion and degradation and recycling. Macroautophagy can be further divided into subtypes, depending on the type of cargo that is being engulfed by the double membrane to form the autophagosome: endophagy, autophagy of endoplasmic reticulum; chlorophagy, autophagy of chloroplasts; mitophagy, autophagy of mitochondria, etc (Figure 6).
The process of autophagosome formation and eventually degradation of carried cargo requires several steps: induction, membrane delivery, nucleation, maturation, tonoplast docking and digestion (Figure 7). During the induction phase, Target of Rapamycin (TOR) gets inhibited which leads to dephosphorylation of the ATG1/13 complex components, which allows for its assembly and translocation to the pre-autophagosomal structure (PAS). After docking of the ATG1/13 complex in the PAS, a double membrane starts to form with the aid of ATG9 protein that recruits lipids into a newly forming structure. Addition of phosphatidylinositol-3 phosphate (PI3P) to PAS, during nucleation allows for recruitment of other ATG proteins to the membrane. This growing double membrane recruits ATG8-PE, which decorates the mature autophagosome. Upon the closure of the membrane, the autophagosome translocates towards the vacuole, where it docks into the membrane of the vacuole and fuses with it. Single membrane vesicles inside the vacuole, now called autophagic bodies can be degraded and the basic components of the original cargo can be recycled and reused in building new cell compartments, protein complexes or even new membranes.

In Arabidopsis, there are 9 different isoforms of ATG8, named ATG8a-i. Its lipidated form, ATG8-PE plays a crucial role in cargo recognition. It binds to the ATG8-interacting motif (AIM) within the protein sequence of the receptor and since it decorates mature membrane of
autophagosome, researchers use it as a marker to observe relative autophagosome rates forming in the cell.

In recent years, a new role for autophagy in turnover of RNA has also been emerging (Frankel et al., 2017, Goetz and Wilkinson, 2017, Guo et al., 2014, Orvedahl et al., 2011), however the vast majority of the evidence comes from mammalian and yeast studies. In the classical view, RNA degradation is performed by ribonucleases (RNases), however it was shown that amino acid starvation or nitrogen starvation induced bulk RNA degradation in rat liver, yeasts and in Arabidopsis tissues (Floyd et al., 2015, Huang et al., 2015, Mortimore et al., 1989).

Therefore, it could be suggested that autophagy has an additional role in cellular signalling, either through the release of signalling factors, the selective removal of organelles engaged in active signalling processes or the depletion of RNA therefore dissipating anterograde or retrograde signals.
Figure 7. Mechanism of autophagosome formation. (a) initiation and ATG1/13 complex; (b) lipid (membrane) delivery; (c) nucleation; (d) expansion and maturation; (e) tonoplast docking and fusion of outer autophagosomal membrane with vacuole; (f) digestion. Figure adapted from (Li and Vierstra, 2012).

**Aims of this study**

Based on the literature analysis conducted above, I have resolved to focus on three aims to this thesis, as noted below:
1. Given the potential of selective autophagy to influence plant signalling processes, I aimed to undertake a thorough review of the evidence for mitophagy in plants and performed analysis to identify possible targets for ATG8 cargo recognition (Chapter 2).

2. In the light of published research linking ANAC017 function with plant senescence, I aimed to resolve the conflicting information by performing comparative analyses of mutants collected from different research groups (Chapter 3).

3. On the foundation of correlative data collected by Dr Olivier Van Aken and Dr Adriana Pruzinska that plants overexpressing ANAC017 display a similar phenotype to autophagy-deficient mutants, I aimed to investigate if there was a causative link between autophagy and plant mitochondrial retrograde signalling (Chapter 4).

The combined evidence from this thesis aims to better understand the mechanism of mitochondrial retrograde signalling in plants and in the future provide a new means of modulating stress responses in plants.
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Chapter 2

Mitophagy: a mechanism for plant growth and survival
Mitophagy: a mechanism for plant growth and survival

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Abstract
Mitophagy is a conserved cellular process important for the autophagic removal of damaged mitochondria to maintain a healthy mitochondrial population. Mitophagy appears to occur also in plants and has roles in development, stress response, senescence and programmed cell death. However, many of the genes that control mitophagy in yeast and animal cells are absent in plants, and no plant proteins marking defunct mitochondria for autophagic degradation are yet known. New insights implicate general autophagy-related proteins in mitophagy, affecting the senescence of plant tissues. Mitophagy control and its importance for energy metabolism, survival, signalling and cell death in plants are discussed. Furthermore, we suggest mitochondrial membrane proteins containing ATG8-interacting motifs, which might serve as mitophagy receptor proteins in plant mitochondria.
**Mitophagy as a type of autophagy**

Autophagy is the process of controlled recycling of cellular contents and organelles to promote cell survival or redistribute nutrients. In normal cellular conditions autophagy may recycle components that accumulate for example oxidative damage (Xiong et al., 2007), but its rate can drastically be increased under a variety of stress conditions, senescence and cell death (Liu et al., 2009, Marino et al., 2014, Minibayeva et al., 2012). During autophagy, portions of the cytoplasm are captured in vesicles (*autophagosomes*; see glossary) and degraded in lysosomes (animals) or the vacuole (*Saccharomyces cerevisiae* and plants) (Minina et al., 2014).

Many cellular organelles have been described to undergo autophagy, including the endoplasmic reticulum (ER), the nucleus, mitochondria (*mitophagy*) and chloroplasts (*chlorophagy*) (Okamoto, 2014, Sakuraba et al., 2014, Spitzer et al., 2015). This review will particularly focus on mitophagy in plants. We define this as the process of mitochondrial degradation through autophagy-related processes, not the role of mitochondria during general autophagy. Mitochondria are crucial for energy metabolism, biosynthesis, regulation of cell death and are also involved in stress response and intracellular signalling (Millar et al., 2008, Ng et al., 2014, Van Aken and Van Breusegem, 2015). A key component of mitochondrial function is the electron transport chain (ETC), which despite its beneficial roles is also a major source of reactive oxygen species (ROS) production that can lead to oxidative damage (Huang et al., 2016). Moreover, dysfunctional mitochondria consume cytosolic ATP, resulting in energy losses (Gomes and Scorrano, 2013). Therefore, the controlled removal of dysfunctional or superfluous mitochondria by mitophagy is important for maintaining a healthy mitochondrial population. In *C. elegans* mitophagy is involved in coordination of mitochondrial biogenesis, recycling of Fe-S clusters during Fe starvation and has implications for longevity and ageing (Palikaras et al., 2015, Schiavi et al., 2015).

Moderate rates of autophagy thus promote cell survival, while excessive autophagy can lead to cell death in most organisms, including in plants (Minina et al., 2013a, Reumann et al., 2010, Yoshimoto et al., 2009). In plants, mitochondria and chloroplasts are both subject to autophagy, and several AuTophagy (ATG) genes have been implicated in these processes (Li et al., 2014, Sakuraba et al., 2014, Spitzer et al., 2015). However, the molecular mechanisms
of mitophagy in plants and how the process or the key components are differentiated from chlorophagy need to be investigated further.

**What is the evidence for mitophagy in plants?**

Different types of autophagy have been described in plants based on ultrastructural observations (van Doorn and Papini, 2013). During microautophagy the vacuolar membrane engulfs a portion of the cytoplasm and buds off, forming a membrane-bound vesicle inside the vacuole. By contrast, macroautophagy takes place outside the vacuole by formation of double-membrane autophagosomes. Another autophagy-related phenomenon in plants has been termed mega-autophagy or mega-autolysis, which refers to the extensive breakdown observed at the end of developmental programmed cell death (PCD), but it is debated whether this is a true form of autophagy (van Doorn, 2011, van Doorn and Papini, 2013). Plant specific types of autophagy involving the chloroplasts are also reported to occur. Small vesicles called RuBisCO-containing bodies (RCBs) move from the chloroplasts to the vacuole, before the whole organelle moves, in order to quickly recycle RuBisCO (a major nitrogen sink in plants), in a process requiring AutoPhaGy-related (ATG) protein ATG5 and involving ATG8 (Ishida et al., 2008). Interestingly, chloroplasts have also been reported to perform autophagic tasks by engulfing portions of the cytoplasm and degrading this content inside the chloroplast (Filonova et al., 2000, van Doorn et al., 2011).

The occurrence of mitophagy in yeast and animals is well-established, however this field of study in plants is still in its early stage. An early study reported mitochondria being enclosed in a double-membrane structure similar to ER during autophagy in mung bean (Vigna radiata) (Toyooka et al., 2001). These autophagosome-like structures containing mitochondria were observed to fuse with lytic vacuoles. Numerous autophagosomes enclosing mitochondria have been described after one day of tracheary element differentiation in xylem (Kwon et al., 2010). Wertman and colleagues reported that aggregates of mitochondria can be observed inside the vacuole during later stages of developmental PCD in the lace plant (Aponogeton fenestralis) (Wertman et al., 2012). A study characterising accelerated cell death 5 mutants reported mitochondrial ROS formation and the presence of mitochondria in autophagosomes (Bi et al., 2014). Also Minibayeva and colleagues demonstrated both intact and partly degraded mitochondria in the vacuole after methyl viologen treatment of wheat roots (Minibayeva et al., 2012). However, another study criticized this claim and suggests the
authors were not observing mitophagy, but rather mitochondria in cytoplasmic strands (van Doorn and Papini, 2013). The observation of mitochondria in vacuoles and lytic vesicles should nevertheless be taken cautiously as evidence for mitophagy, as direct analysis of organelle degradation kinetics is required to conclude that autophagy is selective towards a certain type of organelle.

Recently, it was reported that during senescence mitochondrial proteins and mitochondrial vesicles were degraded by autophagy (mitophagy) in Arabidopsis (Arabidopsis thaliana) (Li et al., 2014). Studies of mitochondrial protein degradation rate have been performed in Arabidopsis cell cultures (Nelson et al., 2013) and Arabidopsis plants (Li et al., 2017b), and both reveal a basal rate of mitochondrial protein removal from plant cells of approximately 5-10% per day, analogous to the rates of mitochondrial turnover in some yeast and mammalian cells (Kim et al., 2012, Pratt et al., 2002). Loss of the Lon1 mitochondrial matrix protease in plants led to an increase in mitochondrial turnover for a large number of respiratory-related proteins that could indicate induction of mitophagy (Li et al., 2017a). Chloroplasts have even been observed to invaginate mitochondria to degrade them internally, as an alternative means of mitophagy (Ragetli et al., 1970), however to the date no independent reports of this phenomenon exist. In summary, it appears a number of studies in plants have observed processes analogous to mitophagy and provide evidence that this is an actively controlled process (Box 1).

**The mechanism of mitophagy in plant and non-plant systems**

Both non-selective and selective autophagy (such as mitophagy) can be divided into phases: 1) initiation, 2) recognition of cargo, 3) nucleation and phagophore (the double membrane that encloses cytoplasmic components during macroautophagy) formation, 4) autophagosome maturation, 5) delivery of cargo and finally 6) degradation in the vacuole (in yeast and plants) or in lysosomes (in mammals). These processes are tightly controlled by signalling pathways, which involve ATG proteins, membrane structures and marker proteins, as well as regulation of degradation systems (i.e. vacuoles or lysosomes). Furthermore, post-translational modifications play a role in recruiting and targeting the autophagy complexes (see Box 1 and Figure 1).
Figure 1. A Putative Model of the Mechanisms of Mitophagy in Plants. Upon imposition of stress, mitochondria send a signal of an unknown nature which inhibits target of rapamycin (TOR) kinase. Inhibition of TOR allows the formation of active ATG1/13 complex by dephosphorylation, in conjunction with ATG11 and ATG101, and the complex is then recruited to the surface of mitochondria. A putative receptor present on the outer or inner mitochondrial membrane (which may become exposed upon outer-membrane rupture) interacts with the ATG1/13 complex and induces pre-autophagosomal structure (PAS) formation. The autophagosome is decorated with ATG8–phosphatidylethanolamine (ATG8–PE) adducts, leading to delivery and degradation of mitochondria in the plant vacuole.

Non-plant systems

The initiation of a mitophagosome (an autophagosome engulfing a mitochondrion) requires targeting of mitochondria for degradation and the formation of an initial isolation membrane. In yeast, mitophagy involves the mitochondrial proteins ATG32 or ATG33 (Kanki
et al., 2009, Okamoto et al., 2009). ATG32 is located in the mitochondrial outer membrane and acts as a receptor, recruiting other ATG proteins which are essential for the initial isolation membrane formation. ATG32 recruits ATG8 and ATG11 is phosphorylated by CK2 (casein kinase 2) to stabilise the ATG32-ATG11 interaction (Aoki et al., 2011, Kanki et al., 2013). Together with the core ATG proteins, the ATG32-ATG11 complex generates the isolation membrane to engulf a mitochondrion (Okamoto, 2014). In yeast, ATG11 is part of the ATG1/13 complex along with ATG101, ATG17 and the yeast specific proteins ATG29 and ATG32 (Ryabovol and Minibayeva, 2016, Thompson and Vierstra, 2005).

In mammals, at least two distinct mitophagy pathways exist. One pathway that occurs in mammalian cells involves hypoxia- or uncoupling-induced phosphorylation of the outer mitochondrial membrane protein FUNDC1 by the ATG1 homologue ULK1, resulting in mitophagy (Wu et al., 2014). A second pathway in mammalian systems is dependent on the mitochondrial transmembrane potential and is affected in Parkinson’s disease. In healthy mitochondria, the kinase PINK1 is partially imported through the TOM complex and across the mitochondrial inner membrane (IMM) in a Δψm-dependent manner. There, PINK1 is degraded by pre-senilin associated rhomboid-like protease PARL (Jin et al., 2010). In damaged mitochondria where Δψm is reduced, PINK1 remains active at the outer mitochondrial membrane (OMM) where it phosphorylates the E3 ubiquitin ligase Parkin. Parkin can then ubiquitinylate multiple proteins on the mitochondrial surface including voltage-dependent anion channel VDAC1 (Geisler et al., 2010), which eventually leads to core ATG protein recruitment and mitophagy. Although PINK1/parkin are not present in plant genomes (Table 1), there might be functional analogies with plant FRIENDLY in Arabidopsis thaliana (Logan et al., 2003). FRIENDLY1 is an ortholog of Clueless in Drosophila melanogaster, and deletion of either protein causes a severe clustering of mitochondria within the cytoplasm. As Clueless is required for mitophagy in concert with Parkin (Cox and Spradling, 2009, Wang et al., 2016), this topic needs to be further investigated in plants.

The origin of the autophagosomal membrane is still under investigation but significant evidence exists that autophagosomes can arise from plasma membrane, Golgi, endosomes, as well as from ER and/or mitochondrial membranes (Chan and Tang, 2013, Hailey et al., 2010, Lamb et al., 2013, Mueller and Reski, 2015). In mammalian systems, ER-mitochondria contact sites may be of key importance for autophagosome formation during starvation, involving
recruitment of ATG14 and ATG5 proteins. Mitophagy is observed at ER-mitochondria contact sites via mitochondria-associated membranes (MAMs) that derive from the ER (Hamasaki et al., 2013, Senft and Ronai, 2015). Disruption of these MAMs can inhibit autophagosome formation. The ER may thus provide the platform for autophagosome formation, with the mitochondria contributing other components required for the process (Hamasaki et al., 2013). These ER-mitochondria contact sites are maintained, for example by the ER-mitochondria encounter structure (ERMES) complex in yeast (Kornmann et al., 2009). It was shown that under starvation conditions mitochondrial and autophagosomal membranes becomes continuous (Hailey et al., 2010), although the reason behind this starvation specificity remains unclear. It is also known that mitochondria and ER share contact sites that are required for mitophagy in yeasts (Bockler and Westermann, 2014).

*Plants*

Although some of the mechanisms described above may conceptually be similar in plants, there is very little conservation of mitophagy regulators between yeast/animals and plants. Most of the core ATG genes are conserved in plants (Meijer et al., 2007, Reumann et al., 2010), but the specific players in mitophagy are largely absent in the *Arabidopsis thaliana* genome (Table 1), such as yeast ATG32. Also key mammalian regulators such as DCT-1 (BNIP3, related to BCL-2) that act as mitophagy receptors in mammals, are not present in plant genomes. Recently, a homolog of yeast ATG11 (and animal FIP200) has been found in *Arabidopsis thaliana* and has been proven to be involved in mitophagy under nitrogen-starvation conditions (Li et al., 2014, Li and Vierstra, 2014). The sequence homology between yeast and plant ATG11 is however low (20% identity), with AtATG11 containing traces of both ATG11 and ATG17 domains (see Table 1). In Arabidopsis, ATG11 interacts directly with ATG8 (homologous to mammalian LC3), ATG13 and ATG10 (Li et al., 2014). The interaction between ATG11 and ATG1 is indirect and led by ATG13 (Fig. 1). ATG11 is thought to help link the ATG1/13 complex and to promote the delivery of vesicles to the vacuole, however ATG11 is not completely essential for the assembly of the autophagic bodies (Li et al., 2014). ATG11 is also thought to be involved in the dynamic turnover of the ATG1/ATG13 kinase complex during nutrient starvation (Li et al., 2014, Suttangkakul et al., 2011).
Table 1. Conservation of Mitophagy Components in Plants with Yeast and Animals

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Animals</th>
<th>Arabidopsis thaliana</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATG1</td>
<td>ULK1/ATG1</td>
<td>AtATG1a-d</td>
<td>Core ATG protein</td>
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<tr>
<td>ATG5</td>
<td>ATG5</td>
<td>AtATG5 (At5g17290)</td>
<td>Core ATG protein</td>
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<tr>
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<td>ATG7</td>
<td>AtATG7 (At5g45900)</td>
<td>Core ATG protein</td>
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<tr>
<td>ATG8</td>
<td>ATG8/LC3</td>
<td>9 proteins AtATG8a-i</td>
<td>Core ATG protein</td>
</tr>
<tr>
<td>ATG11</td>
<td>ATG11</td>
<td>AtATG11 (At4g30790)</td>
<td>ATG11 contains traces of ATG11 and ATG17 domains</td>
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<tr>
<td>ATG13</td>
<td>ATG13</td>
<td>ATG13a-b</td>
<td>Core ATG protein</td>
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<td>ATG14</td>
<td>ATG14</td>
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<td>ATG protein</td>
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<td>ATG32</td>
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<td>Receptor for mitophagy</td>
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<td>ATG33</td>
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<td>-</td>
<td>-</td>
<td>ERMES-complexes</td>
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<tr>
<td>MDM34</td>
<td>-</td>
<td>-</td>
<td>ERMES-complexes</td>
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<td>Similar to UBQ/HEL1</td>
<td>PARKIN</td>
<td>UBQ/ARIADNE</td>
<td>E3 ubiquitin ligase</td>
</tr>
<tr>
<td>?</td>
<td>PINK1</td>
<td>?</td>
<td>Conservation in MAPKKK protein, only +/- 100 amino acids of S81</td>
</tr>
<tr>
<td>-</td>
<td>FUNDC1</td>
<td>-</td>
<td>Very low similarity to AtWHY3 ssDNA-binding protein (E-value 1.3)</td>
</tr>
<tr>
<td>PCP1</td>
<td>PARL</td>
<td>AtRBL12 and AtRBL10</td>
<td>Rhomboid-like proteases (AtRBL12 is mitochondrial, AtRBL10 is plastidic)</td>
</tr>
<tr>
<td>-</td>
<td>BNIP3/DCT-1</td>
<td>-</td>
<td>Receptor for mitophagy, involved in cell death and mitochondrial biogenesis</td>
</tr>
<tr>
<td>Nma111 (nuclear)</td>
<td>Omi/HTRA2/PARK13</td>
<td>AtPARK13 (At5g27660)</td>
<td>Mitochondrial serine protease</td>
</tr>
</tbody>
</table>

Also ATG7 is important during senescence-induced mitophagy (Li et al., 2014). ATG7 is an E1-like enzyme which mediates the conjugation of ATG8 with phosphatidylethanolamine (PE) and of ATG12 with ATG5, resulting in the formation of ATG8-PE and ATG5-ATG12 complexes (Doelling et al., 2002). The ATG5-ATG12-ATG16L complex (an E3-like enzyme) is responsible for lipidation of ATG8 by PE (Fujita et al., 2008, Ryabovol and Minibayeva, 2016). The ATG8-PE adduct decorates the mature autophagosomal membrane (Fig. 1), making it a good marker for the observation of autophagosome formation also in plants (Doelling et al., 2002, Liu and Bassham, 2012, Ryabovol and Minibayeva, 2016).

With regards to autophagosome membrane formation, ATG5 and ATG8 are recruited during phagophore formation in close association with the endoplasmic reticulum (ER) (Le Bars et
Recently, the autophagy protein ATG9 has been shown to be important in the regulation of autophagosome membrane progression from the ER (Zhuang et al., 2017). The loss of function atg9 mutants in Arabidopsis displayed unusual tubular structures extending from the ER upon induction of autophagy. This phenomenon has not been observed in other autophagy mutants. Loss of ATG9 in Arabidopsis does not affect ATG8 conjugation onto the autophagosomal membrane, indicating a role for ATG9 downstream of initial ATG8 recruitment. The role of ATG9 in this process currently appears to be unique to Arabidopsis (and perhaps other plant systems), and stands opposite to other organisms where loss of function atg9 mutants fail in autophagosome formation (Yamamoto et al., 2012).

**Mitochondrial membrane autophagy receptors in plants**

Based on our current knowledge, it appears that specific proteins on the mitochondrial surface act as markers for degradation and recruit the autophagy machinery to a specific mitochondrion. In yeast, ATG32 is an OMM receptor involved in tagging mitochondria for autophagosomal degradation, however to date there are no proteins on the plant mitochondrial surface that have been experimentally confirmed to have a similar function. Recently, a bioinformatic tool was developed to predict proteins that may interact with ATG8 (Xie et al., 2016), a core protein of autophagy machinery. Based on the set of experimentally-determined OMM proteins in Arabidopsis (Duncan et al., 2011), a predicted ATG8-interacting protein set [62] for Arabidopsis comprises 12 proteins including cytochrome b reductase, hexokinase 1, translocases of the OMM (TOM20s and TOM40) and voltage-dependent anion channel VDAC2 (Table 2). Mitochondrial protein import plays an important role in control of autophagy in animal systems, so perhaps a similar phenomenon occurs in plants explaining the presence of TOM20/40 in this list (Jin et al., 2010). Furthermore, VDAC1 ubiquitination by Parkin is a crucial step in marking mitochondria for autophagy (Geisler et al., 2010) in animals, and also hexokinase plays an autophagy-promoting role via the TOR pathway (Roberts et al., 2014), potentially explaining these proteins being in the predicted ATG8-interacting protein set.

**Table 2.** Arabidopsis mitochondrial proteins containing an ATG8-Interacting Motif.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Description</th>
<th>ATG8-interacting motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>At2g01460</td>
<td>P-Loop containing AAA+ ATPase with uridine kinase domain</td>
<td>HDDFSSL (570)</td>
</tr>
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</table>
**Inner mitochondrial proteins**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1G07180</td>
<td>Internal alternative NAD(P)H-ubiquinone oxidoreductase A1; NDA1</td>
<td>IDEWMRV (365)</td>
</tr>
<tr>
<td>At2G20800</td>
<td>External alternative NAD(P)H-ubiquinone oxidoreductase B4; NDB4</td>
<td>TDEWLRV (359)</td>
</tr>
<tr>
<td>At2G29990</td>
<td>Internal alternative NAD(P)H-ubiquinone oxidoreductase A2; NDA2</td>
<td>IDEWMRV (363)</td>
</tr>
<tr>
<td>At2G43400</td>
<td>Electron transfer flavoprotein-ubiquinone oxidoreductase; ETFQO</td>
<td>YEEFQKL (364)</td>
</tr>
<tr>
<td>At4G05020</td>
<td>External alternative NAD(P)H-ubiquinone oxidoreductase B2; NDB2</td>
<td>TDEWLRV (354)</td>
</tr>
<tr>
<td>At5G52840</td>
<td>NADH-ubiquinone oxidoreductase-related</td>
<td>EEDWEMI (71)</td>
</tr>
<tr>
<td>At1G17530</td>
<td>Translocase of inner mitochondrial membrane 23; TIM23-1</td>
<td>DDVWTSV (135)</td>
</tr>
<tr>
<td>At1G20350</td>
<td>Translocase of inner mitochondrial membrane 17-1; TIM17-1</td>
<td>EDPWNSI (87)</td>
</tr>
<tr>
<td>At1G72750</td>
<td>Translocase of inner mitochondrial membrane 23-2; TIM23-2</td>
<td>DDVWTSV (136)</td>
</tr>
<tr>
<td>At2G26140</td>
<td>ATP-dependent zinc metalloprotease FTSH4</td>
<td>EETFGL (138)</td>
</tr>
<tr>
<td>At2G37410</td>
<td>Translocase of inner mitochondrial membrane 17-2; TIM17-2</td>
<td>EDPWNSI (87)</td>
</tr>
<tr>
<td>At3G98580</td>
<td>Mitochondrial ADP/ATP carrier; AAC1</td>
<td>DFGGSL (137)</td>
</tr>
<tr>
<td>At5G11690</td>
<td>Translocase of inner mitochondrial membrane 17-3; TIM17-3</td>
<td>EDPWNSI (87)</td>
</tr>
<tr>
<td>At5G25450</td>
<td>Cytochrome bd ubiquinol to cytochrome c oxidase</td>
<td>DDLYDPL (36)</td>
</tr>
<tr>
<td>At5G53170</td>
<td>ATP-dependent zinc metalloprotease FTSH 11</td>
<td>EEMFVGV (432)</td>
</tr>
</tbody>
</table>

**AT1G36650**

<table>
<thead>
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>CoA transporter</td>
<td>FYIYEEL (209)</td>
</tr>
<tr>
<td>ATPase F1 complex, α subunit protein</td>
<td>LIYDDL (538)</td>
</tr>
<tr>
<td>NADH-ubiquinone oxidoreductase-related</td>
<td>RTRIDFEV (12)</td>
</tr>
<tr>
<td>ATP synthase D chain, mitochondrial; ATPQ</td>
<td>RRAFDEV (41)</td>
</tr>
<tr>
<td>NADH-ubiquinone oxidoreductase</td>
<td>YNFEDV (195)</td>
</tr>
<tr>
<td>Metabolite transporter, substrate carrier</td>
<td>LVYFYDEV (315)</td>
</tr>
<tr>
<td>Folate transporter 1; FOLT1</td>
<td>FTAYEEL (183)</td>
</tr>
<tr>
<td>ATP synthase subunit 1</td>
<td>LIYDDL (268)</td>
</tr>
<tr>
<td>Alternative NAD(P)H-ubiquinone oxidoreductase C1</td>
<td>EYDVLWL (192)</td>
</tr>
<tr>
<td>Gamma carbonic anhydrase 3; GAMMA CA3</td>
<td>DTEYDSV (249)</td>
</tr>
<tr>
<td>Gamma carbonic anhydrase 1; GAMMA CA1</td>
<td>VIEFEKV (224)</td>
</tr>
<tr>
<td>NADH-ubiquinone oxidoreductase B18 subunit</td>
<td>KCEYELV (60)</td>
</tr>
<tr>
<td>Gamma subunit of mitochondrial ATP synthase; ATP3</td>
<td>NVEFDAL (190)</td>
</tr>
</tbody>
</table>

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**AT2G33040**

<table>
<thead>
<tr>
<th>Description</th>
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</tr>
</thead>
<tbody>
<tr>
<td>elongated mitochondria ELM1 protein of unknown function (DUF1022)</td>
<td>HDEFAAL (248)</td>
</tr>
<tr>
<td>DGD1 SUPPRESSOR 1, DGS1, galactoglycerolipid biosynthesis</td>
<td>TDEWDLV (558)</td>
</tr>
<tr>
<td>TraB family protein</td>
<td>GEDFVHI (18)</td>
</tr>
<tr>
<td>Hexokinase AtHXK1</td>
<td>DELFNFI (141)</td>
</tr>
<tr>
<td>VDAC2 voltage dependant anion channel</td>
<td>DDIFFCCL (49)</td>
</tr>
<tr>
<td>NADH:cytochrome B5 reductase 1 AtCBR1</td>
<td>NVTYDDI (191)</td>
</tr>
<tr>
<td>Wavy-growth WAV2 prolyl oligopeptidase</td>
<td>NLIYEDI (51)</td>
</tr>
<tr>
<td>Adenosine 5’-monophosphate deaminase AtAMPD FAC1</td>
<td>MSEWDLQ (521)</td>
</tr>
<tr>
<td>Translocase of the outer mitochondrial membrane TOM20-2</td>
<td>TADFERL (5)</td>
</tr>
<tr>
<td>Translocase of the outer mitochondrial membrane TOM20-3</td>
<td>ETETFDR (4)</td>
</tr>
<tr>
<td>Translocase of the outer mitochondrial membrane TOM40</td>
<td>PVPYEEL (31)</td>
</tr>
</tbody>
</table>

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**AT5G12290**

<table>
<thead>
<tr>
<th>Description</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH:Cytochrome B5 reductase 1 AtCBR1</td>
<td>NVTYDDI (191)</td>
</tr>
<tr>
<td>Wavy-growth WAV2 prolyl oligopeptidase</td>
<td>NLIYEDI (51)</td>
</tr>
<tr>
<td>Adenosine 5’-monophosphate deaminase AtAMPD FAC1</td>
<td>MSEWDLQ (521)</td>
</tr>
<tr>
<td>Translocase of the outer mitochondrial membrane TOM20-2</td>
<td>TADFERL (5)</td>
</tr>
<tr>
<td>Translocase of the outer mitochondrial membrane TOM20-3</td>
<td>ETETFDR (4)</td>
</tr>
<tr>
<td>Translocase of the outer mitochondrial membrane TOM40</td>
<td>PVPYEEL (31)</td>
</tr>
</tbody>
</table>
Prohibitin 2 was identified as an IMM receptor in animal systems, which interacts with LC3/ATG8 (Wei et al., 2017). This interaction requires the rupture of the OMM first, which occurs during Parkin-mediated autophagy (Chan et al., 2011, Wei et al., 2017). Amongst Arabidopsis IMM proteins (213 IMM proteins based on SUBA4 (Hooper et al., 2017) and Uniprot (Apweiler et al., 2004)) another set of 36 predicted ATG8-interacting proteins [62] can be identified (Table 2). This list includes various ETC, ATP synthase and Translocon of IMM (TIM) proteins, FtsH4/11 proteases (Zhang et al., 2017), mitochondrial calcium uniporters MCU1/6 (Teardo et al., 2017), metabolite transporters, but not prohibitins. However, ATG8 interaction motifs [62] were not identified in animal PHB2 either, so a role for plant prohibitins in mitophagy should not be ruled out. As loss of prohibitins in plants results in a range of mitochondrial defects, mitochondrial swelling and retrograde signalling responses, it will be interesting to find if mitophagy is affected in prohibitin mutants and mitochondrial mutants in general (Piechota et al., 2015, Van Aken et al., 2016, Van Aken et al., 2007).

Role for rhomboid and other proteases

Rhomboid proteases are a specific group of conserved sequence-specific intramembrane proteases (Chan and McQuibban, 2013). Mitochondrially targeted members are involved in PCD and autophagy in animal systems. For instance, PARL prevents apoptosis by activation of Omi1 and by preserving cristae structure to prevent cytochrome c release (Chao et al., 2008). It is also involved in suppressing mitophagy by cleaving PINK1 (Chan and McQuibban, 2013).
Plant genomes also encode rhomboid-like proteases (e.g. 15 putatively in Arabidopsis), and some rhomboid proteases are also present in the mitochondrial and chloroplast membranes (Kmiec-Wisniewska et al., 2008, Knopf et al., 2012). Chloroplast rhomboids may be involved in maturation of Tic40 import component (Karakasis et al., 2007). However, no drastic phenotypes have been observed in single or double mutants of AtRBL8 and AtRBL9, with some partial sterility defects in atrbl8 plants that may be attributed to decreased expression of jasmonic acid (JA) synthase *allene oxide synthase* (Thompson et al., 2012). Thus, no significant evidence exists that chloroplast or mitochondrial rhomboid proteases play an active role in plant autophagy or PCD.

An Arabidopsis mitochondrial protease (AtPARK13) with similarity to animal Park13/Omi/HtrA (a substrate of autophagy-activator PINK1) has been reported to have a role in thermotolerance (Basak et al., 2014). The authors suggest it can directly cleave substrates, but no involvement in autophagy per se has been proven.

Yeast iAAA/FtsH-like proteases are involved in cleaving the C-terminal of mitophagy receptor ATG32, thereby stimulating autophagy potentially by improving the interaction of ATG32 with ATG11 (Wang et al., 2013). In Arabidopsis, loss of mitochondrial protease Ftsh4 caused severe leaf senescence, cell death, and increased autophagy levels (Zhang et al., 2017). ATG5 and ATG8 were required for autophagosome formation and the senescence phenotype of *ftsh4* mutants. Crossing of *ftsh4* with salicylic acid (SA) signalling-deficient mutants reversed the senescence and autophagy phenotypes, suggesting an important role of SA. Also a role for WRKY transcription factors was suggested.

Several other mitochondrial proteases are induced at a transcript level during conditions that are linked with autophagy induction, such as senescence (C1pB4, all three ClpX’s, and C1pB2), dark treatment (Lon2) and nitrogen starvation (C1pP2 and metacaspase MC3) (Lin and Wu, 2004, Liu et al., 2008, Roberts et al., 2012, Sanmartin et al., 2005). It will therefore be interesting to determine in the future if these proteases are involved in mitophagy induction or progression in plants.

*The role of mitophagy in signalling*

The communication from mitochondria to nucleus has been studied intensively in plants and some components in this retrograde signalling have been identified (Ng et al., 2014).
Understanding how this communication is coordinated might be a key to understand the outcomes of different cellular responses and their link to autophagic processes in plants. Generic ROS signalling in cells is likely not specific enough to induce targeted nuclear transcriptional changes in response to specific organelle defects, rather, receptors of specific ROS signals might be needed (Moller and Sweetlove, 2010). One possibility in the case of mitochondria is the ROS-dependent induction of the unfolded protein response (UPRmt), which has been studied in non-plant systems (Haynes and Ron, 2010). The precise mechanisms of UPRmt in plants are only beginning to be understood (Wang and Auwerx, 2017), but it seems plausible that mitophagy could be involved. For instance the mammalian mitochondrial deacetylase sirtuin SirT3 is a regulator of both UPRmt and mitophagy (Liang et al., 2013, Papa and Germain, 2014). It is thought that SirT3 helps to sort moderately stressed mitochondria from irreversibly damaged ones. From previous studies it is known that a similar unfolded protein response occurs in the endoplasmic reticulum (UPRER) and can activate formation of autophagosomes in plants (Liu et al., 2012, Pu and Bassham, 2013, Senft and Ronai, 2015). ER and mitochondria interact through junctions on the ER membrane (Bockler and Westermann, 2014), and significant evidence exists that autophagosomal membranes can be derived from both ER (Chan and Tang, 2013, Zhuang et al., 2017) and mitochondria (at least in non-plant systems, as discussed above) (Chan and Tang, 2013). Mitophagy may also take part in retrograde signal suppression, for instance by removing damaged organelles that may be sending out stress signals. At least in animal systems, suppression of mitophagy results in retrograde signalling that regulates mitochondrial biogenesis (Palikaras et al., 2015). The role of mitochondria in oxidative stress-induced autophagy in plants has been previously reviewed (Minibayeva et al., 2012), further highlighting specific areas of research that are needed to understand the impact of mitophagy on plant mitochondrial function and signalling.

*Role of mitophagy during senescence*

Most Arabidopsis mutants lacking autophagy-related genes have no clear early developmental phenotypes, except *atg6* mutants that have pollen germination defects (Reumann et al., 2010). However, lack of autophagy often results in accelerated senescence in Arabidopsis (Avila-Ospina et al., 2014, Li et al., 2014, Sakuraba et al., 2014, Wada et al.,
2009). Furthermore, dark induced senescence causes chlorophagy, which requires ATG4, although no abnormal whole-plant senescent phenotypes were observed in atg4a4b-1 Arabidopsis mutants (Wada et al., 2009). Chlorophagy is of major importance for nitrogen recycling as 80% of cellular nitrogen is held in the chloroplasts (Wada et al., 2009), as well as during recovery from UV-induced damage (Izumi et al., 2017). Chloroplasts are degraded much earlier than mitochondria during senescence. Mitochondria are possibly retained longer for recycling nitrogen via NH₄⁺ by glutamate dehydrogenase (Avila-Ospina et al., 2014). As sugars are depleted rapidly in a senescing leaf and amino acids need to be recycled, glutamate and branched chain amino acids such as lysine can feed electrons into the mitochondrial electron transport chain, keeping metabolism and nutrient recycling going (Araujo et al., 2010, Avila-Ospina et al., 2014, Avin-Wittenberg et al., 2015). A recent study showed that during dark-induced senescence concentrations of most amino acids increased, but this was less pronounced in atg mutants (Barros et al., 2017). On the other hand, TCA cycle intermediates such as citrate were more abundant and dark respiration rate was higher in atg mutants than in WT plants. The atg mutant plants responded to dark-induced senescence by increasing transcripts of alternative mitochondrial respiration pathway enzymes ETF/ETFQO. This suggests a metabolic reorientation when autophagy is disrupted, and that the lack of protein degradation in atg mutants slowed the generation of amino acids used as alternative substrates for respiration (Barros et al., 2017).

After one day of dark-induced senescence a significant increase in ROS production by mitochondria and peroxisomes has been observed that lasted throughout senescence (Rosenwasser et al., 2011), possibly reflecting the heightened activity of these organelles during senescence. In contrast, chloroplast ROS levels dropped after 1 day and gradually returned to basal levels over the course of senescence (Keech, 2011). Based on this, it could be speculated that mitochondria (and perhaps peroxisomes) are the main players that allow complete recycling of cell content and potentially lead to cell death at the end of plant senescence. In agreement, plant mitochondria keep moving actively around the cell (Keech, 2011) and maintain their function (Chrobok et al., 2016) until the last stages of senescence when chlorophyll is already largely degraded. This implies that cell survival through mitochondrial metabolic function until the last moments of senescence is crucial to maximise nutrient remobilisation (Chrobok et al., 2016) (Figure 2). When the time for cell death in plants
has arrived, it is unclear how the PCD threshold is reached, and if mitochondria and their autophagic removal play an active role (Box 2). It is possible that mitochondrial degradation is the final step in completion of senescence, or alternatively that they simply run out of substrates to maintain cellular viability. One explanation for the observed accelerated senescence phenotype in plant mitophagy mutants *atg11* (*Li et al., 2014*) may be that high activity and observed ROS production of mitochondria in senescing leaves requires adequate mitochondrial quality control and the removal of damaged organelles. When these damaged organelles accumulate they lose optimal functionality resulting in premature senescence. In agreement, *ATG11* transcripts are gradually upregulated during leaf senescence in Arabidopsis, peaking during the final stages (Box 1 Figure I).

Figure 2. **Regulation and role of mitophagy in plants.** Conditions like natural aging and stress can lead to the induction of senescence and may be associated with mitochondrial damage. Depending on circumstances, this may lead to increased bulk autophagy or specific mitophagy. Autophagy/mitophagy may help the plant with efficient recycling of nutrients from senescent or damaged tissues, or allow tissue survival. At the end of senescence or during extreme stress conditions mitophagy may contribute to cell death. Mitophagy may also play a role during developmental cell death. Retrograde signalling can be induced by mitochondrial stress, which may contribute to prevention of cell death (Van Aken and Pogson, 2017). Images for senescent leaf and lightning were obtained freely from www.freepik.com.
Autophagy and potentially mitophagy may also play a role in ageing and lifespan extension in plants. Low light conditions can induce lifespan extension via caloric restriction in Arabidopsis, and autophagy supports this extended lifespan by efficient recycling of contents, (Minina et al., 2013b). Also in animals, it is thought that a decline in mitophagy and thus mitochondrial quality control may contribute to aging (Diot et al., 2016, Palikaras et al., 2015).

Conclusions

Current evidence suggests that mitophagy occurs in plants both during normal development and under conditions such as prolonged darkness and oxidative stress (Figure 2). At present, only limited experimental information is available on how mitophagy contributes to suppressing premature senescence in plants, and whether mitophagy and plant PCD are linked (Li et al., 2014, Minina et al., 2014, Yoshimoto et al., 2009). An emerging model suggests that mitochondria are needed to allow efficient recycling and remobilisation of nutrients for instance in senescent leaves (Figure 2). This might put significant pressure on mitochondrial energy systems, thus requiring efficient removal of damaged and ‘worn-out’ organelles. If this turnover mediated by mitophagy is inhibited, the plants may senesce without complete remobilisation of nutrients. Thus, removing damaged, potentially ROS-overproducing energy organelles may promote cell survival, and may contribute to the natural turnover of ageing mitochondria. During stress, it appears that ROS such as superoxide may be a signal that triggers autophagy to remove organelles that are engaged in excessive ROS production (Minibayeva et al., 2012).

Mechanistically, we understand only a little about how mitophagy in plants is executed. Many of the core ATG protein components appear to be conserved in plants, but we have virtually no evidence of how individual plant mitochondria are marked for removal by autophagy. We hope that the list presented in Table 2 will be a useful resource for guiding such studies in the future. There is a need for further development of mitophagy tools in plants such as reporter lines and antibodies against proteins that are specifically degraded in plant mitochondria by autophagy (Bassham, 2015, Klionsky et al., 2016). We also have very little understanding of how plant mitophagy could be involved in regulating cellular processes outside of senescence, such as general tissue maintenance, gamete development, developmental processes that involve cell removal, and whether mitophagy plays a role in stimulating or quenching stress-related signalling pathways in plants.
References


FILONOVA, L. H., BOZHKOV, P. V., BRUKHIN, V. B., DANIEL, G., ZHIVOTOVSKY, B. & VON ARNOLD, S. 2000. Two waves of programmed cell death occur during formation and


MULTIVESICULAR BODY PROTEIN1 regulates the autophagic turnover of plastids in Arabidopsis. Plant Cell, 27, 391-402.


Supplementary material

Box 1

As mitochondria are a significant source of ROS, they are likely to be targets of autophagy in stress conditions in plants (Huang et al., 2016). Autophagy is thought to be induced by the plant hormone salicylic acid via NPR1 to act as a negative feedback loop repressing senescence and programmed cell death (Yoshimoto et al., 2009, Zhang et al., 2017, Zhou et al., 2015). Oxidative stress triggered by ETC inhibitors such as antimycin A (AA) or methylviologen (MV) was found to induce high levels of plant autophagy (Minibayeva et al., 2012). This effect could be overcome by exogenous addition of antioxidants. A more detailed investigation of the impact of the ETC inhibitors myxothiazol, AA or potassium cyanide (KCN) on yeasts has confirmed that AA and KCN can induce autophagy as reported by Minibayeva and colleagues in plants (Deffieu et al., 2013, Minibayeva et al., 2012). However, Deffieu and co-workers claimed that AA and KCN induced nonspecific autophagy rather than mitophagy, whereas myxothiazol induced autophagy to a lesser extent. Like AA, myxothiazol blocks complex III, but it is thought to result in far less superoxide formation than AA. These results suggest that autophagy is a response to ROS formation itself, rather than energy organelle inhibition. Also conditions such as hypoxia, mitochondrial uncoupling and loss of ΔΨm are known triggers for mitophagy in animal systems, but have not been studied extensively in plants.

Posttranslational modification of proteins such as ubiquinylation, phosphorylation and acetylation seems to be important in the regulation of mitophagy levels in the eukaryotic cell (Eiyama and Okamoto, 2015, Shiba-Fukushima et al., 2012). It is already known that dephosphorylation of Arabidopsis ATG1 and ATG13 plays crucial role in the formation of the ATG1/13 complex (Suttangkakul et al., 2011), which is required for autophagosome formation (Box 1 Figure) (Ryabovol and Minibayeva, 2016, Thompson and Vierstra, 2005).
Figure 1. Gene expression of ATG genes during dark-induced and developmental senescence. The transcripts of many genes encoding AuTophaGy related proteins are induced by senescence. The left data set represents dark induced senescence (columns represent number of days) (van der Graaff et al., 2006). The right dataset represents natural developmental senescence of whole plants (Breeze et al., 2011) sampled from day 19 to day 39 of growth, either 7 h into the light period (AM) or 14 h into the light period (PM). Some ATG genes show very rapid induction (e.g. ATG8B), while others show more gradual induction patterns (e.g. ATG7). Some ATG genes also seem to display diurnal expression patterns (e.g. AtTSPO) Colour scale indicates fold change of mRNA expression relative to the first time point of the respective data set; grey fields indicate that the gene was not represented on the CATMA microarrays.

Two starvation conditions are widely used as triggers in autophagy studies in plants: nitrogen starvation and carbon starvation (Aubert et al., 1996, Li et al., 2014). Nitrogen starvation seems to be a trigger for the induction of mitophagy in plants (Li et al., 2014) and yeast (Deffieu et al., 2013). The carbon status and sugar levels may also play a role in plant autophagy. Environmental changes like the intensity of light, access to water and temperature influence the level of carbohydrate supply. Aubert et al. suggest that the supply of mitochondria with respiratory substrates, and not the decrease of sucrose and hexose phosphates, controls the induction of bulk autophagy in plant cells starved in carbohydrates (Aubert et al., 1996). Altogether, nutrient homeostasis of the cell and the respiratory status
of mitochondria are linked, and likely important in deciding between bulk autophagy and selective autophagic processes like mitophagy.

**Box 2**

Mitophagy has been studied as a mechanism to improve cell survival by removal of damaged component or recycling nutrients (Kubli and Gustafsson, 2012), but excessive levels of autophagy could tip the balance towards cell death (Catanzaro et al., 2015, Minina et al., 2014). A key mechanism that affects autophagy appears to be mitochondrial fragmentation. A highly fragmented mitochondrial pool is more easily degraded by mitophagy, while a highly aggregated mitochondrial pool may be more resistant (Catanzaro et al., 2015). The fact that plant mitochondria aggregate early during cell death (Scott and Logan, 2008) may contribute to a failing of mitophagy to rescue the cell.

The role of autophagy in plant PCD is however not very well understood. A recent study demonstrated that during developmental PCD of suspensor cells in Norway Spruce a metacaspase- and autophagy dependent pathway is used, but in their absence a mitochondrial PCD pathway was observed (Minina et al., 2013). However, in rice starchy endosperm PCD, mitochondrial membrane permeabilisation and caspase-like activity preceded cell death, suggesting mitochondrial PCD and autophagy are not necessarily mutually exclusive during plant developmental PCD (Kobayashi et al., 2013). Some studies even suggested that mitochondria undergoing permeability transition (MPT) become targeted for autophagy, so widespread MPT inside a cell following pro-death signals may trigger cell death by excessive removal of mitochondria by autophagy (Lemasters, 1998). Arabidopsis mutants in the mitochondrial protease Ftsh4 displayed increased senescence, PCD and autophagy. Crossing with *atg5* or *atg8* mutants reduced PCD levels, suggesting that autophagy stimulated the senescence PCD phenotype (Zhang et al., 2017). In agreement, many of the Arabidopsis ATG genes are transcriptionally regulated during leaf senescence (van der Graaff et al., 2006) (Figure 1 in Box 1). Wertmann and colleagues described macro- and mega autophagy during lace plant PCD (Wertman et al., 2012). Many autophagic vesicles were being formed during early PCD stages. These vesicles contained organelle aggregates which often co-stained with mitochondria already during early stages of PCD stages. These aggregates migrated to the vacuole in late stage PCD, suggesting mitophagy is part of the PCD process.
Autophagy is necessary for PCD in developmental tracheary element formation in the xylem (Kwon et al., 2010). Mitophagy was observed during the first day of tracheary element induction with brassinolide/H$_3$BO$_3$, while a brassinosteroid-insensitive mutant did not show this process, indicating the involvement of phytohormones. Finally, autophagy may also play a role in plant immunity and pathogen-induced PCD, a process potentially downstream of catalase function, linking ROS production with autophagy-dependent PCD (Hackenberg et al., 2013, Munch et al., 2015, Teh and Hofius, 2014). It thus seems that in plants autophagy may be both a suppressor and stimulator of PCD processes.

References


Chapter 3

Increased expression of ANAC017 primes plants for accelerated senescence
Increased expression of ANAC017 primes plants for accelerated senescence

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Short title: Elevated ANAC017 expression accelerates senescence

One-sentence summary

Increased expression of ANAC017 in Arabidopsis thaliana primes plants for accelerated senescence, induction of autophagy and cell death via a transient ‘super-induction’ of ANAC017, ANAC016 and their target genes.

Author contributions:


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Abstract

Recent studies in *Arabidopsis thaliana* have reported conflicting roles in leaf senescence for ANAC017, a transcription factor regulating mitochondria-to-nuclear signalling, and its closest homolog ANAC016. By synchronising senescence in leaves of knock-out and overexpressing mutants from these contrasting studies, we demonstrate that elevated ANAC017 expression consistently causes accelerated senescence and cell death. A time-resolved transcriptome analysis revealed that senescence-associated pathways such as autophagy are not constitutively activated in ANAC017 overexpression lines, but require a senescence-stimulus to trigger accelerated induction. ANAC017 transcript and ANAC017-target genes are constitutively upregulated in ANAC017 overexpression lines, but surprisingly show a transient ‘super-induction’ one day after senescence-induction. This induction of ANAC017 and its target genes is observed during the later stages of age-related senescence, indicating the ANAC017 pathway is also activated in natural senescence. Finally, our analyses show that ANAC016 likely acts downstream of and is transcriptionally controlled by ANAC017 in a feed-forward loop during senescence.
Introduction

Plants lead sessile lifestyles in their vegetative growth phase and must therefore continuously adapt to changes in the environment, including abiotic or biotic stresses. Complex networks of gene expression underlie these stress responses (Coolen et al., 2016), (Zhang et al., 2019). Intracellular signalling pathways are often divided into two main types: anterograde signalling, when information originates from the nucleus and is transferred to other compartments of the cell (e.g. chloroplasts or mitochondria); and retrograde signalling which originates in the autonomous organelles and is transferred to the nucleus (Woodson and Chory, 2008). This tight communication between organelles is important for proper maintenance of cellular homeostasis and stress responses (Jones, 2019, Kwasniak et al., 2013, Wu et al., 2019). To study such signalling pathways, researchers have used naturally-occurring triggers (e.g. light intensity) to induce specific responses, as well as chemical inhibitors and genetic approaches. Commonly used inhibitors that trigger chloroplast or mitochondrial retrograde signalling are paraquat, also known as methyl viologen, or antimycin A (AA), respectively.

Such studies have led to the identification of transcription factors and other proteins that are involved in retrograde signalling. More specifically for plant mitochondria-to-nucleus or chloroplast-to-nucleus signalling, a group of NAC domain containing transcription factors, including ANAC017 and ANAC013, was shown to play an important role during chemical inhibition of mitochondrial and chloroplast function (De Clercq et al., 2013, Ng et al., 2013, Van Aken et al., 2016a). It was also determined that ANAC017 plays a role during developmentally controlled mitochondrial retrograde signalling (Van Aken et al., 2016b), (Ng et al., 2013). ANAC017 is localised in the endoplasmic reticulum (ER) membrane, and upon treatment for example with antimycin A, the N-terminal end of ANAC017 is cleaved from the membrane and is thought to translocate to nucleus. There it recognizes a CT[T/C]GXXXXXCA[A/C]G-related motif (Ng et al., 2013, O'Malley et al., 2016) in promoters of stress responsive genes and regulates their expression (Ng et al., 2013). Knock-out mutants of ANAC017 show strongly repressed mitochondrial retrograde signalling, which can be compensated during later stages (starting around 9 hours post stress induction) by other transcription factors like ANAC013 (Ng et al., 2013, Van Aken et al., 2016a, Van Aken et al., 2016b). A recent study showed that a nuclear protein, Resistant to Cell Death 1 (RCD1)
interacts with and represses ANAC013 and ANAC017, by which it is able to regulate both mitochondrial and chloroplast communication with the nuclear transcriptional apparatus (Shapiguzov et al., 2019). Interestingly, overexpression of ANAC017 in gain-of-function mutants leads to increased resistance to ER-stress (Chi et al., 2017).

An involvement of ANAC017 in plant senescence has also been proposed, but its exact role and its connection to retrograde signalling of organelle function remains unclear. Two recent independent studies have reported opposing developmental senescence phenotypes of ANAC017 mutants. Kim et al. (Kim et al., 2018) showed that an ANAC017 overexpressing line displayed a slower rate of leaf senescence, while knock-out mutants exhibited a faster senescence phenotype, which suggests that ANAC017 is a negative regulator of natural senescence. On the contrary, overexpression of ANAC017 reported by Meng et al 2019 resulted in faster leaf senescence than control plants, which suggests a positive role of ANAC017 in senescence. ANAC017 (At1g34190) is a neighbouring gene to ANAC016 (At1g34180) and several studies have compared their contrasting role in dark-induced leaf senescence (Kim et al., 2013, Sakuraba et al., 2016, Sakuraba et al., 2015). ANAC016 was described as a positive regulator of leaf senescence based on dark-induced senescence assays of detached leaves, whereas anac016 knock-out mutants retained dark green colour and appeared to be healthy, while OE lines were heavily senescing after 4 days in darkness compared to WT (Kim et al., 2013). In the same study, dark induced senescence of the anac017 knock-out mutant showed the same rate of senescence as wild-type plants and it was reported that ANAC017 did not play a role in senescence (Kim et al., 2013). Thus far the ambiguity between these papers hasn’t been addressed and remains unresolved. Delayed senescence can result in higher crop yield, while premature senescence phenotypes have been shown to reduce the yield in crops (Borrill et al., 2019, Gregersen et al., 2013, Ma et al., 2018). Therefore, unravelling the true role of ANAC017 in senescence could potentially be used in the selective breeding of crop plants.

In this study, we used a different experimental approach to determine the role of ANAC017 in plant senescence. By darkening only individual leaves, while keeping the rest of the plant in optimal conditions for plant growth, we analysed dark-induced senescence in a synchronised way, while maintaining systemic communication, mimicking partial shadowing by a neighbouring plant. By comparison of different ANAC017 mutants that were used in
multiple studies (this study, (Kim et al., 2018, Meng et al., 2019, Van Aken et al., 2016a), together with anac016-2 (Kim et al., 2013) we provide clear evidence that overexpression of ANAC017 positively regulates leaf senescence, and in fact regulates the expression of ANAC016. Using a time-course approach in individually darkened leaves (IDL) coupled with RNA-seq we are able to provide detailed information on the expression response of ANAC017 targets and the rate of senescence in different lines.

Results

Overexpression of ANAC017 leads to increased senescence rate

To resolve the disputed senescing phenotypes of different ANAC017 knock-out mutants and overexpressing lines we collected mutants from a number of studies, and produced additional independent lines (Fig. 1A-B). ANAC016 has been published previously as a positive regulator of senescence (Kim et al., 2013, Sakuraba et al., 2016, Sakuraba et al., 2015) The ANAC016 gene (At1g34180) is located next to ANAC017 (At1g34190) on the Arabidopsis genome and is the closest paralog to ANAC017, with 71% sequence identity (Suppl. Fig. 1A). We therefore tested the role of ANAC016 during retrograde signalling responses. We used Antimycin A (AA) as a mitochondrial stress inducer on anac016 knock-out plants and tested the expression of Alternative Oxidase 1a (AOX1a), a classical marker for mitochondrial stress responses. We were able to show that retrograde signalling responses are no different in anac016 knock-out mutants compared to Col-0a plants, while the anac017EMS knock-out mutant displays almost complete abolition (Suppl. Fig. 1B).
Figure 1. **Overexpression of ANAC017 leads to accelerated senescence.** A. Expression levels of ANAC017 and ANAC016 in a range of ANAC017 knock-out and overexpressing genotypes determined by qRT-PCR. B. Phenotype of analysed genotypes in individually darkened leaf senescence time-course at indicated time points. C. Chlorophyll measurements during senescence time-course at indicated time points. D. Ion leakage measurements taken during dark-induced senescence time-course. E. Starch levels presented as the glucose equivalents at end of day and end of night. n=6. Student’s t-test was used to simplify visualisation of statistical differences between WT and each genotype at a given time point. * p<0.1, ** p<0.05, ***p<0.01, full 2-way ANOVA results are shown in Suppl. Fig. 3.
We tested all lines used in the study for the gene expression levels of ANAC017 (Fig. 1A). Consistent with previously published findings, knock-out mutants of ANAC017 had less transcript, with higher significance in the anac017EMS mutant line compared to Col-0a plants. Interestingly, highest overexpression of ANAC017 was observed in OEb and OEc lines (20 and 34 fold change, respectively), which display the same phenotype as OE2 and OE3 published by Meng et al. (2019). Since ANAC016 was previously published as a positive regulator of senescence, we also analysed its transcript level in 5-week-old plants. ANAC016 transcript was undetectable in anac016 mutant plants. Interestingly, overexpression of ANAC017 results in significantly increased expression of ANAC016 in all OE lines (Fig. 1A).

We considered if the expression of ANAC016 could be regulated by ANAC017 or the other way around. The ANAC016 promoter region contains a putative ANAC017-binding motif (Ng et al., 2013, O'Malley et al., 2016) (Suppl. Fig. 2A) within 1.5 kb of the transcriptional start site. ANAC016 has been found to bind a nearly identical motif as ANAC017, C[TG]TGXXCA[A/C]GXA, termed the mitochondrial dysfunction motif (MDM) (O'Malley et al., 2016) (Suppl. Fig. 2B). A different study identified ANAC016 to bind to a completely different binding motif (ANAC16BM) (Sakuraba et al., 2015), GATTGGAT[A/T]CA (Suppl. Fig. 2C). Neither ANAC16BM nor clear MDM motifs are found in the promoter region of ANAC017. Thus, together with the increased expression of ANAC016 in ANAC017 overexpression lines, this suggests that ANAC016 probably is a target for expression regulation by ANAC017.

We then analysed leaf senescence phenotype in individually darkened leaves 6 and 7 of 5-week-old plants grown under long day (LD) conditions at day 0, 1, 2, 3 and 4 post treatment. It was obvious that three out of four ANAC017 overexpressing lines (Oefa-OEc) displayed visible yellowing of leaves already at day 2 compared to both Col-0 controls (Fig. 1B). The ANAC017 OX (Kim et al., 2018) line displayed senescence a day later. All ANAC017 overexpression lines had lost turgor by day 4. In contrast, both anac017 and anac016 knock-out mutants remained green and retained turgor, with no visible differences to Col-0a and Col-0b plants over the full senescence time-course. To quantitatively characterize the differences in those lines, we analysed the level of chlorophyll and ion leakage across the cell membrane. 2-way ANOVA analysis showed time-dependent differences within all analysed genotypes, however no genotype-dependent difference between anac017EMS, anac017-1 and anac016 knock-out mutants compared to wild-type plants (Fig. 1C). A genotype and
genotype:time effect was confirmed for all overexpressing lines compared to Col-0a and Col-0b plants (Fig. 1C). Overexpression of ANAC017 resulted in a clear decrease in chlorophyll content in all overexpression lines by day 2, and this difference with Col-0 plants increased further at later stages of the senescence time-course (Fig. 1B-D). Consistent with the loss of chlorophyll and turgor, ion leakage of cell membranes increased dramatically in all four OE lines over the time course (Fig. 1D).

As effects of ANAC017 or ANAC016 loss of function may not become clear until after day 4, we repeated the senescence assay and extended the time course until day 7 post senescence induction. Using a 2-way ANOVA analysis we found that there is no consistent genotype-dependent difference between Col-0a and anac016 or both anac017 knock-outs used in the study (Suppl. Fig. 4), regarding visible senescence and chlorophyll loss.

ANAC017 OE lines had previously been reported to contain lower levels of starch (Meng et al., 2019), but no quantitative data was provided in that study. It could be speculated that dysfunction in starch synthesis or degradation might affect senescence phenotypes in ANAC017-related genotypes. We tested the amount of starch in 5-week-old plants at the end of the day and end of night, which would reveal whether these genotypes have the same synthesis and degradation capability as Col-0 plants. Our results show that there is no difference in starch content between any of the lines at the end of the day (Fig. 1E), which indicates that they were all competent in starch synthesis. Also, all lines were able to degrade the majority of their starch during a night period which indicates that they were competent in starch degradation. Anac016 and anac017-1 knock-out and two out of four ANAC017 overexpression plants had lower levels of starch at the end of the night than Col-0 (Fig. 1E), however, the accelerated senescence phenotype was not correlated with starch content or synthesis or degradation rate.

*Exploration of genome-wide transcriptional responses during leaf senescence*

To further analyse how knocking-out or overexpressing of ANAC017 influences transcriptional responses, we performed RNA-seq analyses. For this, leaf 6 and 7 were pooled together and analysed at day 0, 1 and 3 of the previously described senescence time-course for Col-0a, anac017-1 and two overexpressing lines OEa and OEc, chosen because they display contrasting developmental phenotypes but both show accelerated senescence. Generally,
transcript counts were mapped and a minimum of 21243 transcripts were detectable in each of the 36 samples (Suppl. table 1), with an average of about 24 million counts per sample (Suppl. table 2). Exploration of the RNA-seq data revealed that the Col-0a and anac017-1 transcriptomes were relatively similar to each other, as were both overexpressing lines (Fig. 2A). By contrast, there was a much greater dissimilarity between the overexpressor and Col-0a/anac017-1 groupings and this dissimilarity increased throughout the time-course (Fig. 2A). 

At day 0 of the senescence time-course, anac017-1 had in total 1205 differentially expressed genes compared to Col-0a, the majority of which (714) were downregulated (Fig. 2B). Much larger numbers of differentially expressed genes were obtained for OE lines compared to Col-0a, 4178 and 4504 for OEa and OEc respectively (Fig. 2B). These numbers were similar to those obtained by (Meng et al., 2019), however they increase by almost 3 fold and 2 fold at day 1 and day 3 of the senescence time-course, respectively.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>anac017-1 vs Col-0a</td>
<td>Up-regulated</td>
<td>491</td>
<td>441</td>
</tr>
<tr>
<td></td>
<td>Down-regulated</td>
<td>714</td>
<td>283</td>
</tr>
<tr>
<td>OEa vs Col-0a</td>
<td>Up-regulated</td>
<td>3455</td>
<td>9058</td>
</tr>
<tr>
<td></td>
<td>Down-regulated</td>
<td>723</td>
<td>1030</td>
</tr>
<tr>
<td>OEc vs Col-0a</td>
<td>Up-regulated</td>
<td>2687</td>
<td>9185</td>
</tr>
<tr>
<td></td>
<td>Down-regulated</td>
<td>1817</td>
<td>1378</td>
</tr>
<tr>
<td>OEa vs OEc</td>
<td>Up-regulated</td>
<td>198</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>Down-regulated</td>
<td>1150</td>
<td>269</td>
</tr>
</tbody>
</table>
We then determined if there are sets of genes that respond differently over time in the four tested genotypes. Based on the gene expression patterns, we divided the senescence responses into 4 types (Fig. 3): late responses, where transcripts were significantly up- or down-regulated only at day 3 post darkening; early responses, where transcripts were significantly elevated or decreased at day 1, with no further change at day 3 compared to day 1; transient responses, where transcripts were up- or down-regulated at day 1, and at day 3 post darkening the level of transcripts are recovering toward their original level; and finally sustained responses, where significantly changed transcripts were observed at day 1 post treatment, and continued in the same direction on day 3.

Next, we focused our attention to genes where transcription was significantly changed in both overexpressing lines in the same way. Early type responses had the smallest number of genes with changed expression (313 genes). The majority of genes showed late type responses (1197), followed by transient (816) and sustained (812) responses. We then analysed whether products of differentially expressed genes (DEGs) in those 4 groups were targeted to specific subcellular locations (Fig. 3B). Large differences could be observed for transcripts encoding proteins located in the mitochondrion and plastid. Most of the DEGs encoding mitochondrial proteins belonged to late down and transient up groups, while DEGs encoding plastid proteins were mostly in the late down and sustained down responses.

We then widened our search and looked at gene ontology (GO) enrichment in genotype-specific groups (Suppl. table 3) and identified clusters of GO terms that were specific to transient or sustained responses occurring in both ANAC017 OE lines (Suppl. table 4). In transient responses GO term enrichment were found for ribosomal biogenesis RNA processing and mitochondrial function processes (mitochondrial transport and organisation, import into mitochondria) (Fig. 4A); these GO terms represented 68% of all transiently expressed genes, and contained mostly upregulated genes. Sustained response gene sets displayed GO term enrichment for catabolism of tetrapyrrole and chlorophyll, photosynthetic processes and organisation of chloroplasts (Fig. 4B), which is consistent with results from
subcellular localization studies and most of the genes in these groups showed sustained down-regulation.

Figure 3. Time and localization-dependent transcriptional responses based on RNA-seq data. A. Four types of responses in RNA-seq dataset defined by p_adj value and fold change, presented on figures above appropriate Venn diagrams. Up-regulated transcripts (top pane, red), down-regulated transcripts (bottom pane, blue). B. Frequency of sub-cellular localization of DEG products within each type of response described above, common between
ANAC017 OEa and OEc. Asterisks represent statistical significance (Fisher’s exact test) *p<0.1, ** p<0.05, ***p<0.01.
Figure 4. **GO term enrichment** in A. *transient* and B. *sustained* type responses that are common between *ANAC017 OEa* and *OEc*. For visualisation purposes (log2 transformed) fold change values represent mean of *ANAC017 OEa* and *OEc* fold change.
Autophagy and cell death-related genes become hyper-activated in ANAC017 OE lines after induction of senescence

Interestingly, genes that show up-regulation in sustained responses were enriched in GO terms for autophagy related components and cell death. These results are in agreement with the senescence assay which shows that ANAC017 OE lines consistently senesce faster than Col-0, anac017 and anac016 lines. We therefore looked for further evidence of transcriptional induction of the autophagy pathway. We identified all the key components in regulation and formation of autophagosomes in our RNA-seq data, with the exception of 2 genes: LST8-2 (At2g22040), expressed only in silique and seeds, and ATG8D (At2g05630). In contrast to previous findings (Meng et al., 2019), autophagy-related genes (ATGs) were not constitutively up-regulated in ANAC017 overexpressing lines before the induction of senescence (Day 0). However, they were induced much more strongly in both ANAC017 OE lines at day 1 and/or day 3 of the dark-treatment (Fig. 5, Suppl. table 1). In agreement, the GO category ‘pre-autophagosomal structure’ was overrepresented among sustained UP genes in both ANAC017 OE lines (Suppl. table 3). In conclusion, the ANAC017 OE lines have normal expression of ATG-related genes, but after one day of darkening the ATG-pathway is strongly induced and continues to rise at day 3.
Figure 5. Heat map of DEGs that belong to autophagy pathway, during the dark-induced senescence assay in ANAC017-related genotypes. Lowest and highest expression values for each gene have been normalised between 0 and 1.
**Hormone signalling is affected in ANAC017 mutants**

Previous reports identified differential expression of genes related to jasmonic and/or salicylic acid hormone signalling in ANAC017 mutant plants (Kim et al., 2018, Meng et al., 2019) which are known to induce senescence. We therefore screened our RNA-seq data of induced senescence for genes associated with metabolism and signal transduction of hormones which can induce (SA, JA, and ethylene) (He et al., 2002, Morris et al., 2000, Qiu et al., 2015) or restrain senescence (gibberellins, auxins, cytokinins) (Hu et al., 2017, Serova et al., 2019, Xiao et al., 2015). Genes associated with metabolism and signal transduction of jasmonic acid, ethylene and salicylic acid were significantly up-regulated in ANAC017 OE lines compared to either Col-0a or anac017-1 lines (Suppl. Fig. 5A, Suppl. table 1). Negative regulation of senescence through cytokinins, auxins and/or gibberellins also seemed to be up-regulated in ANAC017 OE lines (Suppl. Fig. 5A, Suppl. table 1). Therefore, we further analysed genes found downstream of hormonal pathways and which were shown to be involved in senescence, including SAGs, YLS7, and YLS9, PAD4 (James et al., 2018, Jin et al., 2018, Vogelmann et al., 2012, Yoshida et al., 2001). The majority of those genes were inducible over the senescence time-course either at day 1 or day 3 in both ANAC017 OE lines (Suppl. Fig. 5B, Suppl. table 1). This, together with the expression of genes associated with autophagy, cell death and metabolism of JA, SA and/or ethylene is in agreement with faster senescence in ANAC017 OE lines.

**Core ANAC017-regulated genes are transiently ‘super-induced’ during senescence in ANAC017 OE lines**

Previous research of ANAC017-regulated genes focused on identification of genes that were positively regulated by ANAC017, and could no longer be induced by Antimycin A or in double mutant backgrounds of anac017 with mitochondrial function mutants (Ng et al., 2013, Van Aken et al., 2016b). It is also known that upon chemical induction, ANAC017 controlled genes are only highly expressed for a period of time and then return to their level in the original state (Ng et al., 2013, Van Aken et al., 2016b). We wanted to see whether we could identify these genes in one of the described types of dark-induced responses in ANAC017 overexpressing lines. The vast majority of ANAC017 core genes previously identified were strongly and constitutively induced in both ANAC017 OE lines at day 0. Interestingly, despite these constitutively high expression levels, most of the ANAC017 core target genes (53% in
OE3.8 and 63% in OE9) were found in the transient up category (Fig. 6A, Suppl. table 1). This indicates that despite the already high expression levels of ANAC017-dependent genes at day 0, a very transient senescence-triggered ‘super-induction’ is observed at day 1, which decreases again by day 3.

Figure 6. Core ANAC017-regulated genes are differentially expressed in dark-induced and natural senescence. A. Expression of ANAC017, ANAC016 and core ANAC017-regulated genes during the dark-induced senescence assay. Lowest and highest expression values for each gene have been normalised between 0 and 1. B-C. Expression of ANAC016, ANAC017, representative ANAC017-controlled genes and core autophagy-related genes based on publically available microarray data (32). NS- natural senescence; expression in leaves with different stages of yellowing (expressed as percentage) during natural senescence; DIS- dark-induced senescence on attached leaves; DET- dark-incubated detached leaves. Heat map colour scale represents fold changes normalised to the first time point within each sample group.
ANAC017 and ANAC017-regulated genes are induced during the later stages of natural senescence

The accelerated senescence phenotype of the ANAC017 OE lines raises the question whether the ANAC017 pathway is also active during senescence in wild type plants. Our RNA-seq data set focused on the first three days after dark-induction of senescence, and at this stage the wild type plants are still not showing clear visual signs of senescence. As can be seen from Fig. 6A, most of the ANAC017-regulon genes are not induced in Col-0a even at day 3 (Fig. 6A, Suppl. Fig. 6), with the exception of UGT74E2 and AOX1d that are starting to come up by day 3. To assess whether the ANAC017-dependent genes are differentially expressed in wild type plants over longer senescence periods, we analysed their expression in a publicly-available microarray time course experiment covering natural senescence, prolonged dark-induced senescence on attached leaves, and dark-incubated detached leaves (Fig. 6B) (van der Graaff et al., 2006). In line with a positive role of ANAC017 during senescence, many of its classic target genes such as AOX1a, UGT74E2, OM66 and ANAC013 show a gradual increase in expression during natural and dark-induced senescence. During dark-induced senescence in attached leaves (the condition most similar to our experiments), ANAC017-target genes like AOX1a and ANAC013 are showing clear sustained induction from day 6 to 9, and some even earlier at day 2 to 4 (e.g. UGT74E2 and AOX1d). In dark-incubated detached leaves, the induction of many ANAC017-dependent genes can be observed as early as two days. Also during natural senescence, ANAC017-dependent genes are gradually but strongly induced, especially when leaf yellowing is becoming visible (Fig. 6B).

Interestingly, ANAC017 and ANAC016 transcripts themselves are ‘super-induced’ at Day 1 during dark-induced senescence in the overexpression lines. (Fig. 6A). Moreover, ANAC017 and ANAC016 are gradually induced during the later stages of natural senescence in wild type plants, peaking when chlorosis can be visually detected (Fig. 6C). It can thus be inferred that the ANAC017 pathway is naturally induced during the later stages of senescence.
Discussion

Harmonising conflicting reports on ANAC016 and ANAC017 function during senescence

Three previous studies have presented ANAC017 as having contrasting roles in senescence. Kim et al (Kim et al., 2013) concluded that ANAC017 plays no role in senescence but its closest homolog, ANAC016, is a positive regulator of senescence. In contrast, Kim et al. (Kim et al., 2018) demonstrated that mutants lacking either ANAC017, ANAC082 or ANAC090 senesce faster during aging, which indicated that they were negative regulators of natural senescence. More recently, Meng et al (Meng et al., 2019) instead suggested that ANAC017 has a positive role in natural senescence because overexpressing ANAC017 accelerates developmental leaf aging. It is difficult to explain why different studies come to varying conclusions with regards to the role of ANAC017 during senescence; but differences in growth stages of plants, conditions of their growth and experimental treatments (e.g. detached leaves vs intact plants, dark induced senescence vs natural senescence) used in these studies are possible factors that contribute to differences in the observed mutant phenotypes (Suppl. Table 5).

The relationship between the functions and binding sites of ANAC016 and ANAC017 during senescence and mitochondrial signalling has been unclear based on the available literature. As ANAC016 and ANAC017 are the most similar proteins in the Arabidopsis NAC transcription factor family based on sequence homology, it is likely that they operate in a similar way with regards to their protein activity and which promoters they bind on the genome. This is supported by the fact that ANAC013 and ANAC053 are more divergent in sequence to ANAC017 than ANAC016, and act redundantly to ANAC017 (De Clercq et al., 2013, Van Aken et al., 2016a). Notably, there is also a discrepancy in the literature concerning the DNA binding site preference of ANAC016. A previous study suggested the existence of a non-ANAC017 like binding motif (ANAC16BM; GATTGGATTCA) (Sakuraba et al., 2015). In contrast, ANAC016 was identified by a yeast one-hybrid screen using the same mitochondrial dysfunction motif (MDM, CTTGxxxxCA(A/C)G) from the AOX1a and UGT74E2 promoters that identified ANAC017 and ANAC013 (De Clercq et al., 2013). Furthermore, a genome-wide chromatin immunopurification screen using ANAC016 as bait clearly identified many of the same promoters as ANAC017 and ANAC013, which are part of the ANAC017-controlled MDM regulon (O'Malley et al., 2016). The consensus motif for ANAC016 identified in this ChIP-seq experiment is also nearly identical to the ANAC017-binding motif identified by ChIP-seq.

96
(O’Malley et al., 2016), which was independently confirmed by electromobility shift assays, yeast-one hybrids and ChIP-qPCR (De Clercq et al., 2013, Ng et al., 2013). However the ANAC016BM (Sakuraba et al., 2015) was not found to be enriched in the genome-wide ChIP-seq study (O’Malley et al., 2016). Overall, independent studies from two different labs have now shown that ANAC016 binds to the same MDM motif as ANAC017 and ANAC013 using a variety of experimental approaches, whereas ANAC16BM-binding has not been independently confirmed.

With both ANAC016 and ANAC017 implicated in senescence, their individual roles now need to be clarified. Summarising our findings and others’, it seems clear that overexpression of ANAC016 or ANAC017 both result in similar fast-senescence phenotypes. In contrast, senescence phenotypes in the anac016 and anac017 knock-out plants are - at least in our hands - very subtle. Although ANAC016 and ANAC017 are very similar in amino acid sequence, there are significant differences in the regulation of their own transcripts. It was previously shown that ANAC017 transcripts are orders of magnitude more abundant in the cell than ANAC016 transcripts (Van Aken et al., 2016a) in young in vitro grown seedlings. This was also observed in the present RNA-seq data set, where ANAC017 transcripts are 46 times more abundant than ANAC016 transcripts at Day 0 in Col-0. Though ANAC017 transcript abundance is stable during mitochondrial dysfunction (Van Aken et al., 2016a), during natural senescence ANAC017 transcripts are clearly induced towards the later stages, when chlorosis is beginning to be observed. Also ANAC016 was identified as a senescence up-regulated gene with >three-fold upregulation from day 31 after sowing onward (Breeze et al., 2011, Podzimska-Sroka et al., 2015). In our study, ANAC017 transcripts were stable during the 3 days in Col-0, while ANAC016 is in the late up category, being upregulated approx. 4.5-fold at Day 3 in Col-0.

Interestingly, ANAC016 transcripts were already upregulated 5-9 fold in the ANAC017 OE lines at Day 0. Furthermore, ANAC016 and ANAC017 themselves show the same transient ‘super-induction’ up to 50-60x at Day 1 and decline at Day 3, as observed for e.g. AOX1a and UGT74E2. The ANAC016 promoter contains a putative ANAC017 binding motif, suggesting that ANAC016 is in fact under the control of ANAC017, and perhaps even in a self-amplifying loop such as ANAC013 (De Clercq et al., 2013). During direct mitochondrial inhibition with Antimycin A, the retrograde induction of AOX1a is nearly completely abolished in anac017 mutants, whereas anac016 mutants respond like wild type. This could be easily explained by
the much lower abundance of ANAC016 compared to ANAC017, suggesting that ANAC017 is the dominant protein active in the signalling pathway, with smaller contributions of ANAC013 and ANAC016.

Therefore, we propose a model where ANAC017 is activated transcriptionally and post-translationally during the later stages of leaf senescence, thereby increasing the expression of ANAC013, ANAC016 and the other MDM regulon genes such as AOX1a and UGT74E2. Most likely the activation of ANAC017 is triggered by increasing stress on mitochondrial function during the late senescence process. This may also explain why anac017 and anac016 knock-out lines have no or subtle visual phenotypes during senescence, as they only operate towards the end of senescence when leaf yellowing is already visible (Fig. 6B). Most likely, the transcript profiles of ANAC016 OE and ANAC017 OE lines are very similar as they are overexpressing nearly the same protein, and we strongly suspect that the MDM genes are also highly expressed in ANAC016 OE lines. Unfortunately, we were not able to obtain the previously-published ANAC016 OE lines (Kim et al., 2013) to verify this hypothesis.

**ANAC017 overexpression triggers time-dependent responses in mitochondrial and chloroplast functions**

Our RNA-seq analyses identified four different types of responses. Among sustained responses were genes involved in chloroplast biogenesis and function. It was previously shown that chloroplasts are first in line for degradation and recycling processes in order to maintain mitochondrial and, in general, cellular function (Avila-Ospina et al., 2014, Chrobok et al., 2016, Law et al., 2018, Peterson and Huffaker, 1975). In the anac017-1 mutant and Col-0a plants chloroplast-related genes show early type responses, while in both overexpressing lines show a steady (sustained) decline in expression. That is consistent with the observed faster yellowing of leaves in the ANAC017 OE lines.

An overrepresentation of genes encoding mitochondrial proteins shows a very different pattern. Several mitochondrial function categories are transiently upregulated in ANAC017 OE lines, including mitochondrial organisation, transport and protein import. This indicates that the accelerated senescence is underpinned by a rapid boost in mitochondrial biogenesis/maintenance and activity, most likely to sustain the rapid recycling of nutrients observed in the following two days of the time course. Conversely, mitochondrial functions
are also overrepresented in the *late down* category, indicating that mitochondrial functions are among the last to be switched off during the final moments before complete senescence and cell death.

As ANAC017 is a key regulator of mitochondrial function during stresses, and mitochondria are crucial to bring senescence and nutrient recycling to a good end, it is not surprising that the ANAC017 pathway is activated during the later stages of senescence. Presumably, this is needed to deal with the increasing stress that later-stage senescence may pose on plant mitochondria. Additionally, it was proposed previously that the ANAC017 pathway may be involved in the suppression of cell death (Van Aken and Pogson, 2017). It could thus be argued that the ANAC017 pathway initially delays cell death to allow maximum recovery of nutrients. One explanation for the *accelerated senescence* in ANAC017 OE lines could be that the high expression of the ANAC017 target genes, which also appears to occur during the latest moments of natural senescence, tips the balance towards death. Such careful balances between survival and cell death can also be seen for instance during other process such as autophagy (Das *et al*., 2012). How exactly overexpression of ANAC017 affects the cellular balance, ultimately leading to accelerated senescence will require extensive further work.

**Evidence for a link between ANAC017 function and autophagy**

A previous report observed transcriptional up-regulation of the autophagy pathway in 5-week-old ANAC017 OE lines and described it as a constitutive up-regulation (Meng *et al*., 2019). The controlled conditions and extensive time course in our data set allowed us to show that ATG genes are not-constitutively induced in ANAC017 OE lines. In contrast, they are increasing in expression in the ANAC017 OE lines only after a senescence-inducing trigger, leading to *sustained up* type responses in ANAC017 OE lines, and most likely contributing to the accelerated senescence and cell death. It will be very interesting to unravel how exactly the ANAC017 regulon can pre-dispose the plants to undergo such fast senescence. The timing of induction of ANAC017-regulated genes during the later stages of senescence matches very closely to the transcriptional upregulation of many autophagy-related genes (Fig. 5, Fig. 6B) (Broda *et al*., 2018, van der Graaff *et al*., 2006). This further suggests that in a natural context the ANAC017 and autophagy signalling pathways are synchronised during senescence. Whether there are common signals underlying their activation, or whether one pathway induces the other, is currently unclear.
Conclusions

In conclusion, our analyses suggest that activation of the ANAC017-pathway is a naturally-occurring phenomenon during the later stage of senescence. From a regulatory perspective, it appears likely that ANAC017 is in fact an upstream regulator of ANAC016 during senescence. ANAC016 may then in turn co-activate the same genes as ANAC017, strengthening the induction as observed for ANAC013. As the DNA binding motifs and target genes of ANAC016 and ANAC017 appear to be very similar (De Clercq et al., 2013, O’Malley et al., 2016), the similarity of the fast-senescence phenotypes observed when either gene is overexpressed can easily be explained. Our results also show that overexpression of ANAC017 and its target genes in itself is not sufficient to accelerate senescence, but that an additional senescence-inducing signal (dark-induction or developmental ageing) is needed to expedite senescence, autophagy, and ultimately cell death. It thus appears that ANAC017 activation predisposes or ‘primes’ plants for accelerated senescence.
Methods

Plant material and growth conditions

*Arabidopsis thaliana*, ecotype Col-0 from our lab was used as a control for all experiments and here named Col-0b. anac017EMS was previously published by Ng S. et al., 2013 (Ng *et al.*, 2013); OEa was previously published by Van Aken O. et al, 2016 (Van Aken *et al.*, 2016a); anac017-1 and ANAC017 OX line were obtained from Kim H.J. et al. 2018 (Kim *et al.*, 2018); anac016 (SALK_074316) was obtained from Arabidopsis Biological Resource Center. OEb and OEc were generated in Col-0a by floral dipping using 35S expression vector pB7WG2 (Karimi *et al.*, 2002).

All plants were stratified for 3 days in 4°C and grown for 5 weeks in soil consisting of soil, perlite and vermiculate mixture in 4:1:1 ratio under long day conditions (LD, 16h light, 8h darkness, 100 µmol m⁻² s⁻¹) at 22°C.

Antimycin A treatment

Antimycin A treatment was performed as previously described (Broda and Van Aken, 2018). In general, 50 µM Antimycin A was used to spray 2-week old seedlings from a distance of about 20 cm using spray bottle. Samples were collected at indicated time-points, snap frozen in liquid nitrogen and stored at -80°C until use.

RNA extraction, cDNA synthesis, qRT-PCR

Tissue subjected for analysis were snap-frozen in liquid nitrogen and stored at -80°C until use. Tissue was then ground using a bead mill and total RNA was isolated using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, STRN250-1KT) with On-Column DNase treatment (Sigma-Aldrich, DNASE70) following the manufacturer’s instructions. 500 ng of total RNA was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-rad, 1708890) (Broda and Van Aken, 2018). cDNA was further diluted and used for quantitative real-time PCR using QuantiNova SYBR green PCR kit (Qiagen, 208056). A list of primers used for the qRT-PCR can be found in Suppl. table 6. Two housekeeping genes were used for normalization: UBQ10 and AKT2 (Czechowski *et al.*, 2005) and analysed using geometric averaging of multiple control genes (Vandesompele *et al.*, 2002).
**Senescence assay**

Plants were grown for 5 weeks in soil in standard long day growth conditions. Plants were randomized throughout the tray, shelf position and room position to compensate for environmental factors. Leaf 5 was collected for starch and ANAC017 and ANAC016 gene expression analysis. Leaf 6 and 7 was used for chlorophyll and ion leakage analysis.

For starch analysis leaf 5 was collected at the end of day and end of night time point and tissue was snap frozen in liquid nitrogen and kept at -80°C until further use. Tissue was ground using a bead mill and incubated with 80% ethanol at 90°C for 3 min with maximum agitation. Samples were then centrifuged for 10 min at 20 000 g. This step was repeated 3 times. The pellet was then used for starch enzymatic assays, using a protocol described previously (O’Leary et al., 2017, Smith and Zeeman, 2006).

Leaf 6 and 7 were covered with aluminium foil for dark induced senescence and harvested at indicated time points. On each day, pictures were taken of all leaves using the same camera objective and automatic exposure. For illustration purposes, representative leaves were selected using the Quick Selection tool in Photoshop and placed on a uniform black background. After pictures were taken, chlorophyll was measured using a SPAD meter (SPAD-502Plus, Konica Minolta). Each leaf was measured multiple times in different positions on the leaf blade. Leaf 6 and 7 were then combined for ion leakage measurement. Ion leakage was described previously (Guo and Gan, 2006) and applied with minor changes. Leaves were cleaned of soil residues, placed in 50 mL falcon tube with 20 mL dH₂O, and placed on a rocker for 30 min with gentle rocking. Initial measurement was made using a HI98192 meter (Hanna Instruments). Leaves were then boiled and cooled down to room temperature and a second measurement was taken. Ion leakage is presented as a percentage of initial over the final measurement.

RNA-seq library preparation and differential gene expression analysis

Individually darkened leafs 6 and 7 from day 0, 1 and 3 were collected and pooled together from Col-0 a, anac017-1 (SALK), OE3.8 and OE9 from senescence assay for total RNA isolation using same procedure as described above.

For RNA-seq library preparation, total RNA was treated with Ambion Turbo DNase (ThermoFisher Scientific, AM1907) and quantified using Qubit™ RNA BR Assay Kit (Invitrogen,
500 ng of RNA was then used for library preparation using TruSeq Stranded Total RNA with Ribo-Zero Plant Kit (Illumina, RS-122-2401) and TruSeq RNA UD Indexes for up to 96 samples (Illumina, 20022371). Samples were sequenced using a HiSeq1500 with SBS kit v3 for 50 cycles (Illumina, FC-401-3002). Alignment of reads was performed against the TAIR10 annotation using STAR (Dobin et al., 2013). On average 24 million reads per sample were mapped (Suppl. table 2). Counts were assigned to genes using summarizedOverlaps and analysis of differentially expressed genes was performed with the EdgeR package (McCarthy et al., 2012, Robinson et al., 2010). Transcripts were considered differentially expressed if FDR ≤ 0.05 and FC ≥ 2 or FC ≤ -2. Raw RNA-seq data files are available from ArrayExpress with accession number E-MTAB-8478.

Enrichment of gene ontology analysis was performed using AgriGO v2 (Tian et al., 2017). Graphical representation of GO terms was performed using the GOplot package in R (Walter et al., 2015).

Accession numbers
RNA-seq data files are available from ArrayExpress with accession number E-MTAB-8478.

Acknowledgements
We thank Prof. Hong Gil Nam and Dr Sunhee Kim (Daegi Gyeongbuk Institute of Science and Technology) for kindly providing transgenic lines. We thank Soodeh Tirnaz for laboratory assistance.


GUO, Y. & GAN, S. 2006. AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J, 46, 601-12.


Supplementary figures

A. Protein sequence alignment of ANAC017 and ANAC016.

B. Expression of AOX1a, a marker for mitochondrial retrograde signalling, during Antimycin A time-course. Asterisks (*) represent a statistical significance of a genotype in comparison to its own 0 time point. Hash sign (#) represents statistical significance of genotype at indicated time point compared to same time point in Col-0. * or # p≤0.1, ** or ## p≤0.05, *** or ### p≤0.01

Supplementary figure 1. Functional comparison of ANAC017 and ANAC016 in mitochondrial retrograde signalling responses.

A. Protein sequence alignment of ANAC017 and ANAC016.

B. Expression of AOX1a, a marker for mitochondrial retrograde signalling, during Antimycin A time-course. Asterisks (*) represent a statistical significance of a genotype in comparison to its own 0 time point. Hash sign (#) represents statistical significance of genotype at indicated time point compared to same time point in Col-0. * or # p≤0.1, ** or ## p≤0.05, *** or ### p≤0.01
**Supplementary figure 2. Binding motifs.** A. ANAC017 binding motif based on Chip-Seq analyses (O’Malley et al., 2016). B. ANAC016 binding motif from Chip-seq analyses (O’Malley et al., 2016). C. ANAC016 binding motif as found by Sakuraba et al., 2015. Image taken from (Sakuraba et al., 2015).
Supplementary figure 3. 2-way ANOVA test significance in senescence assay. A. 2-way ANOVA significance of SPAD chlorophyll measurements taken over the course of 4 days of dark-induced senescence. B. 2-way ANOVA significance of ion leakage measurements taken over the course of 4 days of dark-induced senescence.
Supplementary figure 4. Extended dark-induced senescence time-course on Col-0a, *anac017EMS*, *anac017-1* and *anac016*. A. Phenotype of analysed genotypes in individually darkened leaf senescence time-course at indicated time points. B. Chlorophyll level (top panel) and ion leakage (bottom panel) of leaves during extended senescence time-course at indicated time-points. * p≤0.1, ** p≤0.05, *** p≤0.01.
Supplementary figure 5. A heat map of A. DEGs involved in biosynthesis, degradation and signalling of ethylene, salicylic acid (SA), jasmonic acid (JA), gibberellins, auxins and cytokinins. B. DEGs previously published to be involved in senescence.
**Supplementary figure 6.** Expression of ANAC017 controlled genes over the dark-induced senescence time-course in Col-0a plants.
References


Supplementary tables

Supplementary tables can be found in the folder supplied with this thesis.

Supplementary table 1. RNA-seq data for Col-0a, anac017-1, ANAC017 OEs and OEc during dark-induced senescence time-course.

Supplementary table 2. Sums of counts of technical replicates obtained for each of the biological samples analysed by RNA-seq. Supplementary table 3. GO Term analyses of DEGs in different types of time-dependent responses in dark-induced senescence time-course for Col-a, anac017-1 and commonly expressed genes between ANAC017 OEs and OEc.

Supplementary table 4. GO Term analyses of DEGs in different types of time-dependent responses in dark-induced senescence time-course for commonly expressed genes between ANAC017 OEs and OEc.

Supplementary table 5. A summary of experimental design used in this and previous studies concerning role of ANAC017 in senescence.

Supplementary table 6. Primer sequences used in the qRT-PCR analyses.
Chapter 4

Links between autophagy and retrograde signalling
Mitophagy: a mechanism for plant growth and survival

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Abstract

Plant mitochondria must respond to stress applied from both within or outside of the cell in a timely manner. In response to stress, mitochondria trigger retrograde signalling, however the mechanism of action is not known, although several components have been identified. In this work, a novel aspect of mitochondrial retrograde signalling is presented, demonstrating that autophagy is involved in the signalling process. Increased expression of the mitochondrial retrograde regulation (MRR) component ANAC017 was shown previously to prime Arabidopsis for accelerated senescence. Here, we demonstrate that this senescing phenotype is very similar to the senescence phenotype in autophagy-deficient mutants. To confirm the role of autophagy in plant MRR, we show that genetic or chemical inhibition of the autophagy pathway results in the suppression of retrograde signalling in Arabidopsis. Conversely, induction of autophagy with ER stress stimulator tunicamycin results in an elevated mitochondrial retrograde response. ANAC017 has been shown to be anchored into the endoplasmic reticulum (ER) and needs to be released to allow transcriptional activation in the nucleus. As the involvement of autophagy in MRR has potential implications for the activation mechanism of ANAC017, we studied its ER membrane insertion topology. Results presented here demonstrate that both the N-terminal and C-terminal of ANAC017 are located in the ER lumen, in contrast to previous assumptions that the N-terminal transcription factor domain of ANAC017 is protruding into the cytosol. This work thus presents a new model for the activation mechanism of ANAC017 and a stimulating role for autophagy in mitochondrial signalling in plants.
Introduction

Over the past three decades, cellular signalling has been widely studied in a variety of organisms including yeasts, worms, flies, animals and plants. The situation in plants is arguably more complicated due to the presence of additional autonomous organelles, plastids. In every cell, signalling can be divided in two main groups: extracellular and intracellular signalling, with the second one having two main subgroups, anterograde and retrograde signalling (De Clercq et al., 2013, Leister, 2012, Meng et al., 2019). Anterograde signalling can be defined by communication of the nucleus with other compartments of the cell, and retrograde signalling as a reverse process (Woodson and Chory, 2008). Plastid retrograde signalling is the most studied intracellular communication in the plants, whereas mitochondrial communication is not as well understood. Moreover, multiple studies have shown that communication of chloroplasts with mitochondria must occur as well, however this is even less understood (Li et al., 2017, Schwarzlander et al., 2012, Van Aken et al., 2016, Van Aken and Pogson, 2017). Some of the components involved in mitochondrial retrograde regulation (MRR) have been identified, yet the mechanism of action and how they interact with other cellular processes remains largely unknown. A good example is ANAC017, a transcription factor that was found during the screen for mutants that are impaired in mitochondrial retrograde signalling (Ng et al., 2013). It was shown that ANAC017 resides in the membrane of the endoplasmic reticulum (ER) and upon mitochondrial stress induction, the N-terminal end is cleaved from the membrane by suspected rhomboid proteases and translocates to the nucleus where it regulates the expression of mitochondrial stress responsive genes, AOX1a among them (Ng et al., 2013). It is not known how the stress from mitochondria is transferred to ER to cause the cleavage of ANAC017 from the membrane. It is also not known, what triggers rhomboid proteases to cleave the N-terminal end from ER. Therefore, there must be a barrier that safeguards ANAC017 from the action of proteases and also a mechanism that enables the triggering of this cleavage. One of the cellular processes that may be involved in this regulation is autophagy and its use of ER membrane.

Autophagy is a self-recycling process, more widely studied in response to poor nutrient availability. It involves vesicle-based transportation of cargo that can be specific, like mitochondria (mitophagy) (Broda et al., 2018), chloroplasts (chlorophagy) (Zhuang and Jiang, 2019) or even ER (endophagy), (Nakatogawa, 2020) among others. It was also shown that the
endoplasmic reticulum can contribute its membrane for autophagosome formation (Chan and Tang, 2013). When autophagy is triggered, a pre-autophagosomal structure (PAS) is formed and draws cargo to the ATG8 protein, a receptor that decorates the membrane of autophagosomes. ATG8 recognises specific motifs in proteins present on the targeted organelle that are destined for degradation (Johansen and Lamark, 2019, Noda et al., 2010). Based on this it can be hypothesised that autophagy could contribute to either enabling of signal transduction or for the recovery of stress when an ER-based process is required for the signalling.

Here we show that the senescence phenotype of ANAC017 overexpressing lines shares similarity with autophagy-deficient mutants. We also show that, analyses of retrograde signalling responses in autophagy mutant plants, and chemically induced inhibition or stimulation of autophagy show that autophagy is an important process in mitochondrial retrograde signalling in plants. To explore the implications this may have on the activation mechanism of ANAC017, the topology of ANAC017 has also been analysed. Moreover, our analysis show for the very first time that both N- and C- terminal ends of ANAC017 face the ER lumen.

**Results**

*Accelerated senescence phenotype in ANAC017 overexpression lines is similar to autophagy-deficient mutants.*

Previous research identified that overexpression of ANAC017 primes plants for accelerated senescence (Chapter 3; Broda M. et al., 2019, under review). A faster senescence phenotype is also a characteristic for plants deficient in autophagy (Avila-Ospina et al., 2014, Yoshimoto et al., 2009). We therefore performed a senescence assay on ANAC017 overexpressing and knock-out lines in parallel with two representative autophagy deficient mutants, using detached leaves incubated on petri dish plates with wet Whatman paper, in complete darkness. At indicated time-points, pictures were collected, and chlorophyll was measured using a SPAD meter. Leaves from each plate were then combined to assess ion leakage. In line with previously observed accelerated senescence in darkened attached leaves (Chapter 3), our results showed that ANAC017 overexpressing plants yellowed significantly faster than Col-0 leaves, yet similarly to autophagy deficient mutants (Figure 1A-B). We observed similar
rates of ion leaking in ANAC017 OE and autophagy deficient mutants using conductivity as a measure for cell integrity (Figure 1C). Moreover, 2-way ANOVA analysis revealed that ANAC017 OEa and atg7-2 are not statistically different to each other, especially at day 3 after dark treatment.

Figure 1. ANAC017 overexpressing plants display accelerated senescence similar to autophagy-deficient mutants. A. Pictures of 4-week old leaves detached and incubated in complete darkness on wet Whatman paper. B. Chlorophyll level in detached dark induced senescing leaves. C. Conductivity of detached dark incubated leaves. Letters represent 2-way ANOVA statistical significance with Tukey post hoc test.
**Autophagy-deficient mutants have reduced retrograde signalling capability**

To elucidate whether there is a link between autophagy and plant mitochondrial retrograde signalling, we assessed how dysfunction of the autophagy pathway affected the response to antimycin A (AA), an inhibitor of complex III of the ETC in mitochondria and inducer of MRR. Therefore, we monitored transcriptional responses of MRR target genes to treatment with 50μM AA in a representative set of autophagy-deficient mutants with defects at different stages of autophagosome formation (Table 1). Samples were collected at indicated time-points over a 12 h time course and analysed using qRT-PCR (Figure 2).

Table 3. Collection of different autophagy-deficient mutants used in this study.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Encoded protein</th>
<th>Involved in</th>
<th>Mutant</th>
<th>Previously published in</th>
</tr>
</thead>
<tbody>
<tr>
<td>At5g17920</td>
<td>ATG5</td>
<td>Extension of autophagosome</td>
<td>atg5-1; SAIL_129_B07</td>
<td>(Yoshimoto et al., 2009)</td>
</tr>
<tr>
<td>At5g45900</td>
<td>ATG7</td>
<td>Extension of autophagosome</td>
<td>atg7-2; SALK_057605</td>
<td>(Zhang et al., 2008)</td>
</tr>
<tr>
<td>At4g16520</td>
<td>ATG8f</td>
<td>Expansion of autophagosome</td>
<td>atg8f; SALK</td>
<td>NA</td>
</tr>
<tr>
<td>At2g31260</td>
<td>ATG9</td>
<td>nucleation</td>
<td>atg9-3; SALK_128991</td>
<td>(Shin et al., 2014)</td>
</tr>
<tr>
<td>At4g30790</td>
<td>ATG11</td>
<td>Initiation of autophagosome formation</td>
<td>atg11-1; SAIL_1166_G10</td>
<td>(Li and Vierstra, 2014)</td>
</tr>
<tr>
<td>At3g62770</td>
<td>ATG18a</td>
<td>Membrane elongation</td>
<td>atg18-1; GABI_651D08</td>
<td>(Lai et al., 2011)</td>
</tr>
</tbody>
</table>

Our results suggest that autophagy-deficient mutants have reduced levels of induction of transcriptional responses to AA by about 30-50% compared to Col-0 plants, especially at the earlier at early stages of the time course (3-6 h) post AA treatment (Figure 2). The response was intermediate to Col-0 and anac017EMS plants, which are almost completely abolished in
the induction of transcription of mitochondrial dysfunction markers at the earlier time points (Figure 3). The observed slight expression induction of those genes at the late stages post AA treatment could be explained by the presence of other transcription factors, such as ANAC013, which acts downstream of ANAC017 and which can take over the role of missing ANAC017 (Figure 3, anac017EMS, 9-12h post antimycin A treatment) (De Clercq et al., 2013, Van Aken et al., 2016). The obtained results indicate the involvement of autophagy in mitochondrial retrograde signalling, especially at the early stages of induction.
Figure 2. Retrograde signalling responses in anac17EMS and autophagy-deficient mutants compared to Col-0, upon stimulation with Antimycin A and analysed using qRT-PCR. Student’s t-test was used to simplify visualisation of statistical differences. Hash indicates statistically significant change in the gene expression within Col-0 at a marked time-point compared to Col-0 0h; asterisks indicate statistically significant difference within analysed mutants compared to Col-0 at marked time-point. Error bars represent standard error. #p≤0.05, ##p≤0.01, ###p≤0.001, *p≤0.05, **p≤0.01, ***≤0.001
The kinetics of mitochondrial retrograde signalling responses are affected upon chemical inhibition or induction of autophagy pathway

To further confirm the results obtained from autophagy-deficient mutants, a chemical inhibition of autophagy using wortmannin was performed. Wortmannin is an inhibitor of PI 3-kinase VPS34, involved in the autophagosome formation during nucleation process. Inhibition of VPS34 stops the recruitment of ATG proteins into the membrane and as a consequence maturation of autophagosomes. PSB-D cell cultures were pre-treated with 30µM wortmannin for 3h, before the induction of retrograde signalling responses with 50µM antimycin A. DMSO was used as a control for the solvent utilized to dissolve wortmannin. Samples were collected at indicated time points and mitochondrial dysfunction stimulon (MDS) gene expression levels were analysed using qRT-PCR.

Our results show that wortmannin reduced the ability of Arabidopsis cell cultures to respond to antimycin A (Figure 4, left panel) and results in similar expression levels as anac017EMS presented on Figure 2. This effect was very clearly observed in the MDS target genes, UPOX and UGT74E2. For AOX1α, wortmannin triggered a similar induction in the cell cultures as observed in DMSO + AA, indicating that the general cellular stress caused by wortmannin can already induce AOX1α expression, which is known to respond to a wide range of stresses beyond mitochondrial signals(Ho et al., 2008). This confirms previous results from atg mutants that autophagy is required for normal mitochondrial retrograde signalling in Arabidopsis (Figure 2).
Figure 3. Mitochondrial retrograde signalling responses in PSB-D cell cultures treated with wortmannin prior AA application. Relative MDS expression analyses in Arabidopsis PSB-D cell cultures pre-treated with 30µM wortmannin for 3h prior application of AA. Letters represent 2-way ANOVA analyses with Tukey post-hoc test. Error bars represent standard error.
To assess whether induction of autophagy could conversely induce stronger or earlier transcriptional response to AA, we applied AA after 3h of pre-treatment of cell cultures with 0.15 µg/ml tunicamycin, an inducer of ER stress and autophagy. Samples were collected before the treatment with tunicamycin (-3h), 3h post tunicamycin pre-treatment (0h) when AA was applied and every 3h post antimycin A application, for the duration of 12h. Transcripts were analysed using qRT-PCR analyses. Our results show that tunicamycin prompts faster and/or stronger responses to AA over the 12h time-course for AOX1a and UPOX at 2-3 h post AA treatment (Figure 4).
Figure 4. Kinetics of mitochondrial retrograde signalling responses induced with 50µM AA, after pre-treatment with tunicamycin. Letters represent 2-way ANOVA analyses with Tukey post-hoc test. Error bars represent standard error.
To determine if ANAC017 mutants showed any longer term phenotypes of resistance or susceptibility to the induction of autophagy, we assessed their growth in vitro on MS plates with 0.3 µg/ml of tunicamycin (Tm) (Liu et al., 2012). Our results suggest that both ANAC017 overexpressing plants used, OEA and OEB respectively, are more resistant to tunicamycin than WT or autophagy mutants (Figure 5).

![Figure 5. ANAC017 overexpressing plants are more resistant to ER stress and autophagy inducer tunicamycin. Plant were grown in vitro for 3 weeks under long day conditions.](image)

The C- and N-terminal end of ANAC017 are located in the endoplasmic reticulum lumen

The ANAC017 transcription factor has been previously shown to reside in the ER membrane and be cleaved upon stress to translocate to nucleus to regulate the expression of stress
responsive genes (Ng et al., 2013). However, the topology of ANAC017 in the ER membrane has not been studied and therefore how simple the cleavage and movement of ANOC017 to the nucleus is unclear. Bioinformatic analysis using ARAMEMNON (Schwacke et al., 2003) shows that ANAC017 is predicted to have one clear transmembrane helix that spans across the membrane (Figure 6A). As a consensus, ANAC017 is predicted to have its N-terminal end protruding into the ER lumen and C-terminal in the cytosol. To test this hypothesis, confocal microscopy-based ratiometric imaging analysis was performed using transgenic lines stably expressing roGFP2 fused to either end of ANAC017, as previously described (Brach et al., 2009, Schwarzlander et al., 2008). This method allows for non-invasive analyses of protein topology, thereby preventing stress induction (e.g. during protoplast preparation or agrobacterium infiltration) which would otherwise have caused the translocation of the N-terminal end to the nucleus. roGFP2 is a redox sensitive variant of GFP with GFP with two excitation peaks (at 405nm and 488nm) that reflect the nature of the two adjacent, surface-exposed cysteine residues – a high 405/488nm fluorescence ratio corresponds to a highly oxidised state in which the two cysteine form a disulphide bridge, whereas a low 405/488nm fluorescence ratio corresponds to a highly reduced state in which the two cysteine remain reduced. It has been shown using roGFP that the ER lumen is more oxidising than the cytosol (Brach et al., 2009; Schwarzlander et al., 2008). By monitoring the oxidation state of ANAC017-roGFP2 and roGFP2—ANAC017 lines, it is possible to determine the topology of ANAC017 in ER in vivo.

Roots of 14-day old transgenic seedlings were visualised under a confocal microscope. Consistent with previous findings in tobacco leaves (Brach et al., 2009), ratios of fluorescence excited with 405nm to fluorescence gathered upon excitation with 488nm (ratio 405/488nm) for SEC22-roGFP and for roGFP-SEC22 measured in Arabidopsis roots a almost identical and here were used as controls for oxidized and reduced roGFP2 state (Figure 2B). Surprisingly, both ANAC017-roGFP2 and roGFP2-ANAC017 have high 405/488 ratios, very similar to SEC22-roGFP2 control, indicating that both termini of ANAC017 are located in a relatively more oxidised environment, i.e. most likely in the ER lumen (Figure 6B). The simplest explanation of this is that ANAC017 contains two transmembrane helices and that the ANAC017 transcription factor domain is located in the ER lumen. However, there is a lack of bioinformatic evidence supporting the presence of two transmembrane (Figure 6A). More
likely, ANAC017 is a monotopic membrane protein or a peripheral membrane protein in which the predicted “transmembrane” alpha-helix integrates into or interacts with the lumen side of the ER membrane.

Figure 6. ANAC017 topology analysis in the endoplasmic reticulum. A. Bioinformatic analyses of ANAC017 membrane topology using ARAMEMNON (http://aramemnon.uni-koeln.de). Direction is described in relation to cell cytosol (“inside”) (Hofmann and Stoffel, 1993). B. Ratiometric imaging analyses of ANAC017 tagged with redox sensitive roGFP2. SEC22 was used as a known control as previously described (Brach et al., 2009).
Discussion

In a previous study (Chapter 3, Broda M. et al., 2019, under review), analyses of senescence phenotypes in ANAC017 mutant plants have shown that overexpression of ANAC017 accelerates senescence. Here, comparative analyses with autophagy-deficient mutants revealed that the accelerated senescence is very similar to atg mutants. In previous studies, the N-terminal end of ANAC017 was identified to be cleaved from the ER membrane by rhomboid proteases (Ng et al., 2013), but researchers have not yet experimentally determined the topology of ANAC017 in the ER membrane. Here, it was shown that both ends of ANAC017 are in an oxidising environment. All of the bioinformatic prediction tools for ANAC017 topology predict only one transmembrane domain. Therefore it can be argued that ANAC017 displays a monotopic protein characteristics (Allen et al., 2019).

Building upon the topology of ANAC017 and the similarity of senescence phenotypes with autophagy-deficient mutants, it was hypothesized that autophagy is part of the process of retrograde signalling responses. To test this, retrograde signalling responses were tested in three different scenarios: autophagy-deficient mutant plants, in cell cultures pre-treated with an autophagy inhibitor (wortmannin) and in cell cultures with chemically induced autophagy using tunicamycin.

These experiments showed that inhibition of autophagy reduced the retrograde signalling responses by 30-50% in atg mutants (Figure 3) or even completely abolished them in wortmannin treated cell cultures (Figure 4). On the contrary, induction of autophagy in Arabidopsis cell cultures reversed that effect and showed a stronger and/or earlier retrograde response (Figure 6-7). Mutation in specific single genes encoding autophagy-related protein, causes complete lack of autophagosome formation (Thompson et al., 2005). Since autophagy deficiency in atg mutants only reduced the strength of the response to AA, but not completely eliminates it, we could speculate that autophagy is likely not the only component in the MRR induction.

Previously, studies identified only transcription-related components that are involved in the signal transduction from mitochondria to nucleus, among which ANAC017 was identified. However, to date, no knowledge exists on the physical transduction of the signal. Most likely the rhomboid protease responsible for N-terminal cleavage of ANAC017 belongs to the secretory pathway and faces cytosol (Supplementary figure 1) and therefore, there is a need
for the N-terminus of ANAC017 to be exposed to cleavage. Furthermore, the cleaved TF domain of ANAC017 would need to be in the cytosol to allow it to migrate it to the nucleus. This would favour a membrane-based mechanism for that exposure. Results presented here suggest that autophagy might be the missing link. This would make a cellular process that has previously not been implicated in retrograde signalling, a part of the signalling mechanism.

A model for autophagy involvement in mitochondrial retrograde signalling can be proposed based on available evidence (Figure 7). In normal conditions, ANAC017 resides in the ER membrane, with both of its ends facing the ER lumen (Figure 7A). Antimycin A treatment of plants induces mitochondrial stress which in turn triggers autophagy (Figure 7B) (Minibayeva et al., 2012). It has been shown before that the ER can be a membrane source for autophagosome formation (Chan and Tang, 2013), and therefore it is possible that autophagosome formation during mitochondrial stress responses allows for inversion of the membrane orientation and exposure of ANAC017 into the cytosol (Figure 7C). This allows for the release of the ANAC017 N-terminus and its translocation into the nucleus (Figure 7D) where it recognizes genes of the MDS, binds to their promoters and induces their expression. A double membrane autophagosome then translocates to the vacuole, where it fuses with the tonoplast (Figure 7E) and becomes an autophagic body. During the last steps, degradation of the autophagic body occurs and allows for recycling of individual components, like amino acids and lipids back to the cytosol where they can be used in de novo synthesis of new proteins and compartments (Figure 7F-G). Such a turnover system would also explain how AA-induced gene expression peaks at 3-6h and then can completely revert to basal levels by 12h.
Figure 7. **Autophagy - a model mechanism for mitochondrial retrograde signalling.** ANAC017 contains 2 transmembrane domain, spanning ER membrane with both N- and C-terminal end of ANAC017 located in ER lumen. Induction of mitochondrial stress, e.g. by Antimycin A application triggers autophagosome formation, which allows for the N-terminal end of ANAC017 to be exposed to cytosol, which allows for its cleavage by rhomboid proteases and translocation to nucleus to regulate MDS gene expression. Remaining autophagosome translocates near vacuole where it fuses with tonoplast and becomes autophagic body. In the last step, degradation and recycling of individual components back to the cell occurs.
Future prospects

The observation of autophagosome formation in plants treated with Antimycin A is interesting correlative data for a link between retrograde signalling and autophagy (Minibayeva et al., 2012, Rakhmatullina et al., 2016). However, proving the model of interaction of autophagy with mitochondrial retrograde signalling requires evidence that the two share space and time during stress responses. To confirm this, co-localization of ANAC017 with autophagosomes upon AA treatment will have to be determined in the future. An alternative approach would be to study co-localization of ANAC017 with autophagosomes (currently underway). Moreover, future studies should include confirmation of ANAC017 monotopic topology in the ER membrane, by carbonate extraction followed by western blotting as well as identification of rhomboid protease responsible for ANAC017 cleavage.
Materials and Methods

Plant material

Arabidopsis thaliana, ecotype Col-0 was used as a control for all experiments. anac017EMS was previously published by Ng S. et al., 2013 (Ng et al., 2013); OEa was previously published by Van Aken O. et al, 2016 (Van Aken et al., 2016), OEb and OEc were described in Chapter 3. All seeds were stratified for 3 days in 4°C and grown in vitro for 2 weeks on plates containing full strength MS media or for 4 weeks on soil consisting of soil, perlite and vermiculate mixture in 4:1:1 ratio for senescence assay. Plants were grown under long day conditions (LD, 16h light, 8h darkness, 100 µmol m⁻² s⁻¹) at 22°C.

PSB-D Arabidopsis thaliana suspension cell cultures (Menges and Murray, 2002) were subcultured weekly by transferring 20ml of 1-week old culture into new flask containing 100ml modified MSMO media and grown in complete darkness with rotary shaking 200rpm for 3 days before use. MSMO media was modified by addition of 0.5g/l of MES.

Senescence assay

Leaf 6 and 7 of 4 week old plants grown under long day conditions were harvested and placed on petri dish with a wet Whatmann paper and placed in complete darkness. Leaf yellowing was monitored daily using SPAD meter measurements and ion leakage analysis.

ANAC017 topology analyses- ratiometric imaging

Constructs encoding for roGFP2-SEC22 (reduced control), SEC22-roGFP2 (oxidized control), roGFP2-ANAC017 (N-terminal) and ANAC017-roGFP2 (C-terminal) and containing attB sites were synthesized by IDT and cloned using Gateway cloning were stably transformed into Arabidopsis and plants were analysed by confocal microscopy. Topology analyses of ANAC017 were performed using roGFP2 probe fused to N- or C-terminal end of ANAC017 as previously described by (Brach et al., 2009). roGFP2-SEC22 was used as a known reduced control, SEC22-roGFP2 was used as an oxidized control. Images were collected using Nikon A1RMP confocal microscope with 60x WI objective. Image analyses were performed using Ratio Redox Analysis Software (Fricker, 2016). Assay as performed on roots in Arabidopsis stable transformants.
Tunicamycin resistance phenotyping

Seeds were sown onto MS media containing 0.3µg/ml tunicamycin. Equal volume of DMSO was used as a solvent control. Seeds were stratified in darkness at 4°C for 3 days prior germination in LD growth conditions for 3 weeks.

Retrograde signalling responses in Arabidopsis suspension cell cultures

Arabidopsis suspension cell cultures were pre-treated with 30µM wortmannin or 0.3µg/ml tunicamycin for 3h prior application of Antimycin A. Samples were then collected at indicated time-points, centrifuged at max speed for 30sec, snap frozen in liquid nitrogen and stored at -80°C until use.

Antimycin A treatment on Arabidopsis seedlings

Antimycin A treatment on seedlings was performed as previously described (Broda and Van Aken, 2018). In general, 50 µM AA was used to spray 2-week old seedlings. Spray treatment was described in detail by (Van Moerkercke et al., 2019). Samples were collected at indicated time-points, snap frozen in liquid nitrogen and stored at -80°C until use.

RNA isolation, cDNA synthesis and qRT-PCR analysis

Tissue subjected for analysis was ground using bead mill and RNA was isolated using Spectrum™ Plant Total RNA kit (Sigma-Aldrich, STRN250-1KT) with On-Column DNase treatment (Sigma-Aldrich, DNASE70). cDNA synthesis was performed using iScript cDNA synthesis kit (Bio-rad, 1708890) and further diluted prior qRT-PCR analysis using QuantiNova SYBR green PCR kit (Qiagen, 208056). UBQ10 was used as a housekeeping gene for data normalization (Czechowski et al., 2005).
References


Supplementary figures

RBL15 (At3g58460)

RBL13 (At3g58460)

RBL7 (AT4G23070)

Supplementary figure 1. *Arabidopsis rhomboid proteases in secretory pathway*. RBL15, RBL13 and RBL7 predicted with active proteolytic activity (Adamiec et al., 2017) and their consensus topology within the membrane based on ARAMEMNON bioinformatic analyses (http://aramemnon.uni-koeln.de) (Schwacke et al., 2003).

References


Chapter 5

General discussion
Throughout this thesis, mitochondrial retrograde signalling is considered a process with an underlying mechanism that to this day remained largely elusive. In the literature, it is commonly described as a process of conveying a message from mitochondria to the nucleus. Sending such a message requires both specificity and movement in the cell, from one place to another. Further identification of components involved in sending this message is one step forward in the studies of the mechanism. A mechanism involves a set of components that work together, but the presence of each component isn’t enough. There is a need for a set of relays that allow for the components to work together and move the message within the cell. Rather than to only continue to look for specific molecular components, I have chosen to investigate how cellular processes like senescence and autophagy can be part of this mechanism. In so doing I attempt to further integrate retrograde signalling into what we know about the cascading interactions of plant mitochondrial biogenesis, autophagy, and maintenance during developmental processes like senescence and plant growth performance.

**Retrograde signalling in organelle biogenesis**

It is believed that retrograde signalling plays a role not only in stress recovery, but also in biogenesis of organelles. In plants, a large set of biogenesis-related processes is triggered in seeds upon imbibition. In a classical view, mitochondria and chloroplasts need to be formed from their respective proto-organelles present in seeds. Since a lot of components of mature mitochondria need to be synthesised de novo, premitochondria are required to inform the nucleus about their current state and what genes need to be transcribed (Law et al., 2014, Leister, 2012). However, very recent studies concerning the topic of metabolic activation in seeds consider the basic levels of ATP, redox and metabolites (Nietzel et al., 2020) and reveal that it may not be the case that a pool of functional mitochondria need to be already present in seeds, but that biogenic processes are very rapid and driven by the wider redox status of the seed (Nietzel et al., 2020, Paszkiewicz et al., 2017, Raveneau et al., 2017).

**Autophagy- a regulatory pathway of mitochondrial composition**

In mammalian cells, mitochondrial biogenesis has been linked to autophagy. Lack of the autophagy pathway was shown to reduce the mitochondrial composition of the cell, most likely due to insufficient biogenesis of mitochondria (Liu et al., 2019). In plants, induction of
autophagosome formation by AA (Minibayeva et al., 2012) hints toward the role of autophagy in organelle function and a potential link to retrograde signalling. Two main types of autophagy are present in cells: selective and non-selective (Kissova et al., 2007, Li and Vierstra, 2012). For selective autophagy, a recognition of a targeted cargo is required. Comparative analyses of autophagy-linked proteins throughout different species show lack of many homologs in Arabidopsis, at least at the level of sequence similarity. However, functional homologs are possible. For example, it was shown that prohibitin 2 in mammalian inner mitochondrial membrane can be a receptor for ATG8 interaction (Wei et al., 2017). Based on sequence homology between mammalian prohibitin 2 and prohibitins from Arabidopsis, it is possible that they are functional homologs (Chapter 2, (Broda et al., 2018)). With mitophagy being the security guard between survival and cell death, identification of possible targets for selective mitophagy in plants, could have a great potential in future applications of nutrient assimilation and increasing yield in crops (Broda et al., 2018, Kellner et al., 2017).

Autophagy deficiency, can lead to increased levels of senescence, which in plants, can be observed by visible yellowing of the leaf. Senescence is regulated on multiple levels, which includes regulation of gene expression (Woo et al., 2016), and leads to programmed cell death as the last step. Identification of the components involved in this process is therefore very important.

Due to programmed cell death being tightly linked to mitochondrial function, the role of mitochondria in plant senescence needs to be considered. Many studies identified mitochondria to remain active in senescing cells until very late stages (Keech et al., 2007, Ruberti et al., 2014, Zottini et al., 2006). Since senescence is a process that occurs within the whole cell and leaf tissue, cellular compartments need to be considered as well. It was shown in senescing leaves that chloroplasts are the first source of nutrients to be recycled into other organelles, specifically their components are delivered to energy producing organelles, peroxisomes and mitochondria, which leads to metabolic reprogramming to maintain energy homeostasis and essential organelar function for as long as possible in senescing tissues (Chrobok et al., 2016, Ruberti et al., 2014). Even though eventually the number of mitochondria starts to decrease, respiration rate remains stable in senescing leaves (Chrobok et al., 2016) or can even increase (Collier and Thibodeau, 1995). This major reprogramming
of metabolism requires signalling to the nucleus to regulate gene expression and initiate a metabolic switch. Analysis of transcription factors and their role in senescence is thus of significant importance to understand this signalling event.

**Emerging link between transcriptional regulation of retrograde signalling, senescence and autophagy**

Several studies have been published analysing transcription factors belonging to the NAC family (Bengoa Luoni et al., 2019, Kim et al., 2018, Kim et al., 2013, Li et al., 2018) with the majority of the studies recognizing accelerated leaf senescence by the ectopic presence of the transcription factor in question (Bengoa Luoni et al., 2019). Due to a number of studies with contradictory results on the role of ANAC017 in plant senescence (Kim et al., 2018, Kim et al., 2013, Meng et al., 2019), I have carried out comparative studies of mutants from different sources to resolve its true role. I showed that accelerated senescence was linked to overexpression of ANAC017 in four independent lines, by predisposing plants to accelerated senescence (Chapter 3, Figure 1). However, the lack of ANAC017 in Arabidopsis plants did not markedly delay senescence. This might be due to functional compensation by other transcription factors. ANAC016 is the transcription factor with the highest sequence similarity to ANAC017 (Chapter 3, Supplementary Figure 1A) and was shown to be a positive regulator of senescence (Kim et al., 2013, Sakuraba et al., 2016, Sakuraba et al., 2015). My analyses of the ANAC016 promoter region showed that ANAC017 has the potential to regulate ANAC016 expression (Chapter 3, Supplementary Figure 2), but it is very likely that ANAC017 is not the only regulator responsible for senescence. In the absence of ANAC017, senescence can still be regulated by other transcription factors, such as ANAC016. It could be speculated that transcriptional activation of senescence was pre-existing in ANAC017 OE lines, allowing for a much faster starting point of senescence. It is also likely, that accelerated senescence caused by overexpression of ANAC017 has an underlying metabolome changes. Slightly lower levels of starch in analysed mutants at the end of night time-point presented here (Chapter 3, Figure 3E) could indicate metabolic switch, in which branched-chain amino acids could be a source for ATP production as previously observed by (Law et al., 2018). However, further metabolome analyses would be required to confirm this hypothesis.

Overexpression of ANAC017 leads not only to accelerated senescence. RNA-seq analyses revealed that stimulation of the expression of genes involved in both mitochondrial and
chloroplast biogenesis at the early stages of senescence occurred in both OE lines analysed (Chapter 3, Figure 3A). Moreover, the expression of mitochondrial function genes was downregulated at later stages of senescence (Chapter 3, Figure 3B), which is in agreement with available literature stating that mitochondria are the last to be recycled during senescence (Chrobok et al., 2016, Law et al., 2018). It is believed that mitochondria are degraded during the last stages of senescence (Peterson and Huffaker, 1975) due to their being the last line of defence in cells before they go down a pathway of programmed cell death. Since overexpression of ANAC017 accelerates recycling processes, such as autophagy, it can be argued that ANAC017 signalling delays premature cell death in plants (Van Aken and Pogson, 2017).

Accelerated senescence phenotypes in ANAC017 OE lines were also shown to be very similar to those observed in autophagy-deficient mutants (Chapter 4, Figure 1), indicating a potential link between retrograde signalling and autophagy. Additional evidence supporting this link comes from our RNA-seq analyses, which showed high induction of autophagy at late stages of induced senescence in OE lines (Chapter 3, Figure 5, Figure 6B). This led to more detailed study of the possible role of autophagy in mitochondrial retrograde signalling. Indications regarding the involvement of autophagy in retrograde signalling are already known from the literature. AA or MV, classical inducers of retrograde signalling, have been shown to induce autophagosome formation (Minibayeva et al., 2012), hinting at this role; however this was not investigated in the context of retrograde signalling. Moreover, studies showing autophagosome formation look at autophagosomes formed after several hours post-induction, without monitoring autophagosome formation over time (Li et al., 2014, Minibayeva et al., 2012). From data presented in this thesis, it is clear that autophagy deficiency leads to a partial block of the retrograde response to antimycin A (Chapter 4, Figure 3-4). Given the unexpected luminal orientation of ANAC017 in the ER (Chapter 4, Figure 6), release of ANAC017 into the cytosol requires a membrane-based mechanism. Autophagy may play a dual role in the process. On one hand autophagy might bring an answer as to how ANAC017 is released into the cytosol (Chapter 4, Figure 7), on the other, since it is a recycling process, perhaps it aids the cell with stress recovery and prevention of excessive signalling to the nucleus.
Finally, retrograde signalling processes are important not only for functioning of the organelle in question, but for the overall performance of the whole plant. It was previously shown that ANAC017 can be responsible for response to both chemical stimulation of retrograde signalling as well as during retrograde signalling triggered by genetic defects (Van Aken et al., 2016). Lack of retrograde responses can lead to reduced biomass, especially at later stages of plant development (Van Aken et al., 2016), or susceptibility to environmental stresses (Wang et al., 2018). Overexpression of WRKY15, a transcription factor that acts as a repressor of mitochondrial retrograde signalling, can lead to a reduced salt stress tolerance in Arabidopsis (Vanderauwera et al., 2012). Deficiency in mitochondrial retrograde signalling usually results in the lack of induction of AOX expression (Dojcinovic et al., 2005, Selinski et al., 2018, Wanniarachchi et al., 2018) which can lead to decreased drought and biotic stress susceptibility (Dahal et al., 2017, Vanlerberghe, 2013). Interestingly, reduced levels of AOXs decrease photosynthetic activity in plants (Vanlerberghe et al., 2016), which further shows the importance of retrograde signalling in overall plant performance.

**Future directions**

In my proposed model for mitochondrial retrograde signalling (Chapter 4 figure 7), induction of retrograde signalling and autophagosome formation can lead to recycling of components or organelles neighbouring the induction site. This may involve targeted engulfment of mitochondria, preventing “over-signalling” to the nucleus under non-induced conditions and removing a portion of possibly damaged mitochondria from the cell. In the future studies, analysis of possible mitochondrial targets that interact with ATG8 presented here (Chapter 2, Table 2) will be of big importance. Information acquired from this could potentially lead to targeted applications for induction of mitophagy and downstream effects, such as faster stress recovery.

Crucial to resolving the model of retrograde signalling will be observation how soon after application of AA, autophagosomes start to form and whether the co-localization of autophagosomes can be observed together with ANAC017. My attempt using previously published microscopy staining of autophagosomes, LysoTracker Red (Minibayeva et al., 2012) or monodansylcadaverine (MDC) (Contento et al., 2005) of RFP-ANAC017-GFP or GFP-ATG8a plants have proved to be ineffective in nitrogen starved plants, a known condition for autophagosome formation induction (data not shown). Therefore, a new approach consisting
of either another fluorescent protein compatible with both RFP and GFP or western blot analysis would be of great importance.

Moreover, future studies will involve analyses of metabolome changes in ANAC017 overexpressing plants undergoing accelerated senescence over time-course to understand the underlying cause of accelerated senescence in those plants.
References


Appendices
Chapter 7

Studying Retrograde Signaling in Plants

Martyna Broda and Olivier Van Aken

Abstract

Cellular homeostasis requires precise communication between various types of organelles. In particular, the communication between nucleus and semiautonomous organelles, mitochondria and chloroplasts, has received widespread attention. Communication from nucleus to other organelles is known as anterograde signaling, whereas communication from mitochondria or chloroplasts to the nucleus is known as retrograde signaling. Here we discuss methods used to study retrograde signaling in Arabidopsis thaliana. These methods may also be modified to study retrograde signaling in other plant species.

Key words Mitochondria, Chloroplasts, Retrograde signaling, Antimycin A, Methylviologen, Plants

1 Introduction

For the past two decades, a great effort has been put into understanding intra-cellular communication between different organelles, however only relatively little is known [1]. Two models of communication between nucleus and chloroplasts and/or mitochondria have been identified. Anterograde signaling stands for messages being sent from the nucleus, whereas retrograde signaling stands for messages being sent to nucleus. A very common practice in the studies of retrograde signaling is the use of chemicals, usually inhibitors that target the electron transport chain in chloroplasts and mitochondria, including methylviologen and antimycin A, respectively, which leads to stress induction. This will result in feedback signaling from the damaged organelle to the nucleus, where nuclear gene expression is modified [2–7]. Therefore, the analysis of specific marker genes and their expression patterns has become a reliable tool to study retrograde signaling in a dynamic way. Various other retrograde pathways have been described in the past, particularly for chloroplast retrograde signaling [8–10]. These pathways are often also studied using markers for gene expression, such as light-harvesting complex subunits (e.g., LHCΒ2.4). Here, we present a detailed step-by-step protocol
to study retrograde signaling research in Arabidopsis using antimycin A and methylviologen, which are thought to trigger bursts of reactive oxygen species such as superoxide and hydrogen peroxide, leading to retrograde signaling [11, 12].

# Materials

## 2.1 Media and Solutions

1. MS media: dissolve 4.3 g Murashige–Skoog basal salt mixture (MS), 1 mL Gamborg B5 vitamin mix, 20 g sucrose and 0.5 g MES (2-(N-morpholino)ethanesulfonic acid) in 850 mL milliQ water. Adjust pH to 5.7 with 1 M KOH. Adjust final volume to 1 L, add 8 g agar and autoclave. Pour media into sterile plastic petri dishes. This should be done in a sterile laminar flow cabinet.

2. Antimycin A: to prepare a 50 mM stock solution of antimycin A (AA), dissolve 13.72 mg of antimycin A in 500 μL of ethanol. Add 500 μL of 50 mM AA stock solution to 500 mL of MilliQ water and add 3–5 drops of Tween 20 (approx. 1 drop per 100 mL), mix well. Addition of Tween 20 improves the uptake of chemicals by improving the contact with leaves. The use of freshly prepared stocks is recommended, but stock solution can be stored for a few days at −20 °C.

3. Methyl viologen: to prepare a 50 mM stock solution of methyl viologen (MV), dissolve 6.4 mg of MV in 500 μL of water. Add 500 μL of 50 mM MV stock solution to 500 mL of MilliQ water and add 3–5 drops of Tween 20 (approx. 1 drop per 100 mL), mix well. Addition of Tween 20 improves the uptake of chemicals by improving the contact with leaves. The use of freshly prepared stocks is recommended, but stock solution can be stored for a few days at −20 °C.

## 2.2 Chlorine Gas Sterilization and Seed Sowing

1. Bleach (12% NaClO).
2. 32% HCl.
3. Laminar flow bench.
4. Sterile pipette tips.
5. 0.2% agarose.
6. 1.5 mL Eppendorf tubes.
7. Sterile plastic petri dishes (see Note 1).
8. Leukopor or equivalent porous tape.

## 2.3 Spray Treatment and Sample Collection

1. Spray bottle.
2. MilliQ water.
3. Antimycin A or methylviologen and appropriate mock solution.
5. Tweezers.
6. 1.5 mL Eppendorf tubes.
7. 3 mm metal beads for grinding.
8. Liquid nitrogen.
9. Storage boxes.

2.4 Tissue Grinding
1. Retsch Mixer Mill MM 400 or equivalent, alternatively mortar and pestle.
2. Liquid nitrogen.

2.5 RNA Extraction and Quantification
1. Spectrum™ Plant Total RNA Kit (Sigma) (see Note 2).
2. On-column DNase (Sigma).
3. Eppendorf tubes.
4. ThermoBlock.
5. NanoDrop or any UV-compatible spectrophotometer for RNA quantification.

2.6 RNA Quality Check and cDNA Synthesis
2.6.1 RNA Quality Check
1. 1X TBE buffer.
2. Ethidium bromide.
3. Agarose.
4. Loading dye.
5. 100 bp marker.
6. Molecular grade water.

2.6.2 cDNA Synthesis
1. iScript™ cDNA Synthesis Kit (Bio-Rad).
2. Thermocycler.
3. Sterile 0.2 mL PCR tubes.
4. Molecular grade water.
5. Sterile filter pipette tips.

2.7 qRT-PCR (See Note 3)
1. QuantiNova SYBR Green PCR mix or comparable reaction mix.
2. DNA oligonucleotide primers for marker and housekeeping genes.
3. Molecular grade water.
4. 384-well plate.
5. Sealing foil.
6. Centrifuge compatible with multiwell plates.
7. Roche LightCycler 480 or comparable instrument.
3 Methods

The steps starting from growing the plants to quantitative analyses of transcripts are presented in Fig. 1.

To avoid contamination while growing plants on MS media plates, seeds should be sterilized prior to sowing. An aliquot of seeds should be put into a 1.5 or 2 mL Eppendorf tube and placed into a rack with the lid open to allow gas exchange (adjust seed amount to number of plants needed, do not exceed 100 μL of seeds per tube). A rack with tubes should be placed in the container that would allow sealing the volatile gas during the sterilization, such as a desiccator or vacuum bell. The container should be placed in a fume hood for safety. Pour 50 mL of bleach into a beaker and place it next to the seeds. As quick as possible add 1.5 mL of 32% HCl into the bleach and seal the container. Sterilize seeds overnight (4 h may be sufficient depending on contamination level of the...
seeds). After sterilization, place the opened tubes with seeds under the flame hood for at least 2–3 min to ensure toxic gas escape, then move tubes to sterile laminar flow bench to air out further and sow.

Sow the seeds on MS plates with a pipette and seal the plates with Leukopor tape. This can be facilitated by suspending the seeds in a sterile 0.2% agarose solution (autoclave and allow to cool down to room temperature prior to sowing) and pipetting with a 20 pipette (the end of the sterile tip may need to be removed to allow seeds to pass) (see Note 1). Be careful not to disturb the surface of media on the plates. Stratify the seeds for 2–3 days by keeping them in the cold room (see Note 5). After stratification, place all the plates horizontally in the growth room in random order with long day conditions (22 °C and 100 μmol s⁻¹ m⁻² light) and allow your plants to grow for 2 weeks. Every 3–4 days randomize the plates to keep equal conditions for all used genotypes at various spots on the same shelf. Be gently when moving the plates to avoid mechanical stress responses.

When studying retrograde signaling plants in older than 2 weeks, seeds can be sown straight on soil. Prepare the soil mixture and place it in pots. Water the pots with appropriate amount of water and sow 3–5 seeds per pot. Close the pots with a transparent lid. Stratify for 2–3 days in cold room. Place the pots in the growth room under long-day conditions. Keep the lid on for a week, then lift it slightly for another 5–7 days. Weed out the excess of plants, leaving one in the center of a pot.

3.2 Spray Treatment with Retrograde Signaling Inducers and Sample Collection

Two week old seedlings of Arabidopsis are often used in the study of retrograde signaling. Very gently segregate the plates in order of genotypes and time point (see Figs. 2 and 3) and take off the tape from around the plates. Avoid excessive vibration of the plates and plants to minimize transcriptional responses caused by mechanical stimulation.

Using a spray bottle, spray all plants with either 50 μM AA, or 50 μM MV, or mock solution (water with Tween 20), starting with the ones that will be collected at the latest time point (see Note 6). After the appropriate time of treatment, collect the seedlings/leaves using tweezers into a 1.5 mL Eppendorf tube containing a metal bead and snap freeze in liquid nitrogen. Store samples at −80 °C until further use.

3.3 Tissue Grinding and Aliquoting the Samples

Grind the tissue using The Mixer Mill MM 400. Make sure that all tubes containing your samples contain a metal bead. While grinding make sure that the tissue won’t defrost, by cooling down the Mixer Mill holders and racks with liquid nitrogen. Grind the tissue until fine powder for max 30 s at a time.

Aliquot the samples into fresh sterile 1.5 mL Eppendorf tubes, about 30 mg each (up to 90 mg if difficult tissue). Do not allow samples to defrost.
Fig. 2 Relative mRNA abundance of *HSP17.6 CII* obtained with qRT-PCR analyses of seedlings sprayed with methylioglozen for 3 h. For mock treatment, samples were collected after 3 h of spray treatment with water and Tween 20. Statistical analyses performed using Student’s t-test show that mock treatment does not have a significant impact on the expression of gene of interest (*HSP17.6 CII*). In contrast, spray treatment with methylioglozen induces gene expression of *HSP17.6 CII* in wild-type plants (Col-0). 3 h post treatment. Mutants in transcription factor ANAC017 display severely reduced response to MV [14]. Black hash indicates statistically significant change in the gene expression within Col-0 at a marked time-point compared to Col-0 0 h; colored asterisks indicate statistically significant difference within ana017-1 compared to Col-0 at marked time-point. Error bars represent standard error. #p ≤ 0.05, ##p ≤ 0.01 *p ≤ 0.05, **p ≤ 0.01

3.4 Isolation of RNA

Using Spectrum™

Plant Total RNA Kit
with on-Column

DNase Treatment

The protocol for isolation of total RNA is based on the procedure developed by Sigma. When working with RNA, keep the working space clean. Use only sterile pipette tips (preferably filter tips) and Eppendorf tubes. All the steps are performed at room temperature, unless otherwise stated.

1. To the aliquoted and frozen tissue, add 500 μL of freshly prepared lysis buffer (see Note 7). Vortex at max speed for 30 s.
2. Incubate samples at 56 °C for 4 min.
3. Centrifuge the samples at max speed for 3 min (see Note 8).
4. Avoiding the pellet, transfer the lysate to a filtration column (blue ring) placed in the collection tube and centrifuge samples for 1 min at max speed

Fig. 3 Relative mRNA abundance of four marker genes (*ADX1a, AtOM66, UPOX, UG774E2*) for mitochondrial signaling in 14 days old Arabidopsis seedlings upon stress treatment with antimycin A. Samples were collected at indicated time points, 0 h being untreated samples. Statistical analyses were performed using Student’s t-test. Black hash indicates statistically significant change in the gene expression within Col-0 at a marked time-point compared to Col-0 0 h; colored asterisks indicate statistically significant difference within ana017-1 compared to Col-0 at marked time-point. Error bars represent standard error. #p ≤ 0.05, ##p ≤ 0.01 *p ≤ 0.05, **p ≤ 0.01
Fig. 3 (continued)
5. Add 500 μL of binding solution into the filtrate and mix by pipetting five times (see Note 9).
6. Transfer 700 μL of the mixture into a binding column (red ring) placed in the collection tube and centrifuge 1 min at max speed. Discard the flow-through.
7. Repeat the step 6 with the remaining mixture from step 5.
8. Apply 300 μL of Wash Solution 1 onto the center of binding column and centrifuge 1 min at max speed.
9. Discard the flow-through and place the column back into the collection tube.
10. Apply 80 μL of the freshly prepared DNase mix (see Note 10) onto the center of binding column and let it incubate for 15 min at room temperature.
11. Wash column with 500 μL of Wash Solution 1 and spinning down the samples at max speed for 1 min.
12. Discard the flow-through and place the column back into the collection tube. Add 500 μL of Wash Solution 2 on the column and spin it down 30 s at max speed.
13. Discard the flow-through and dry the collection tube gently by tapping it on a tissue. Place the column back in the collection tube and centrifuge 1 min at max speed.
14. Being careful not to wet the membrane on the column, transfer the column into a fresh 1.5 mL tube.
15. Add 50 μL of the elution buffer directly on the center of the membrane and incubate at room temperature for 2 min. Centrifuge 1 min at max speed. RNA is extracted. Place the tubes containing RNA on ice and measure its concentration using a NanoDrop or equivalent. Store RNA samples at −80 °C until further use.

3.5 RNA Quality Check and cDNA Synthesis

3.5.1 RNA Quality Check

While working with RNA it is important to make sure that the samples did not degrade before proceeding with the experiment. Quality check of RNA can be done by electrophoretic separation of the extracted RNA on 1.2% agarose gel containing ethidium bromide or equivalent.

1. Mix RNA by flicking the side of the tube and transfer 2 μL of RNA into a sterile PCR tube.
2. Add 3 μL of molecular grade water (DNase-, RNase-, and Proteinase-free water).
3. Add 1 μL of RNase-free loading dye and mix by pipetting.
4. Apply all 6 μL into the wells of the prepared agarose gel.
5. Run the electrophoresis at about 70–100 mV until the dye band will be about halfway through the length on the gel (30 min should suffice). Visualize the gel under UV light.
3.5.2 cDNA Synthesis

1. Into fresh and sterile PCR tubes, pipette 500 ng of the extracted RNA samples. Adjust the volume to 5 µL with molecular grade water, if the volume of RNA is less than 5 µL. This can be done in 96-well PCR plates to increase throughput.

2. Prepare master mix as follows (per sample):

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x iScript reaction mix</td>
<td>2 µL</td>
</tr>
<tr>
<td>iScript reverse transcriptase</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Molecular grade water</td>
<td>2.5 µL</td>
</tr>
</tbody>
</table>

3. Add 5 µL of the master mix to the RNA. Mix gently and briefly spin down the tubes.

4. Set up the thermocycler as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>42 °C</td>
<td>30 min</td>
</tr>
<tr>
<td>85 °C</td>
<td>5 min</td>
</tr>
<tr>
<td>4 °C</td>
<td>on hold</td>
</tr>
</tbody>
</table>

After the cDNA synthesis, samples can be frozen and stored at −20 °C until further use.

3.6 qRT-PCR Analysis of Retrograde Signaling Using Known Marker Genes

3.6.1 Primer Design

A wide range of online tools for qRT-PCR primer design exists. Usually primers are designed to amplify a product between 75 and 300 bp. *Universal ProbeLibrary Assay Design Centre* (Roche) allows to design primers for the gene of interest in different organisms either by sequence or gene ID. A list of commonly used housekeeping and marker genes for studying retrograde signaling in *Arabidopsis thaliana* are shown in Table 1.

Prepare the primers in master stocks (100 µM or 200 µM). Working concentration of primers is 20 µM of premixed forward and reverse primers.

Prepare master mix (for 1 sample) (*see Note 11*) by mixing:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x QuantiNova STBR green PCR master mix</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>Primers (20 µM)</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>Molecular grade water</td>
<td>1.25 µL</td>
</tr>
</tbody>
</table>

Dilute prepared cDNA four times (up to ten times) using molecular grade water with 1x final concentration of yellow dye (*see Note 12*).
Pipette 4 μL of Master Mix into the appropriate wells, if possible using a repeat pipette, such as the Eppendorf Multipette, and spin it down at 200 × g for 10–15 s. Precool the plate before loading, and keep the plate on ice while loading. For qRT-PCR applications, technical duplication or triplication of each reaction is preferred to improve reliability.

Pipette 1 μL of diluted cDNA into the appropriate wells on the plate. Spin down the plate regularly to avoid evaporation (e.g., every 24–48 wells). When the plate is loaded, spin it down and seal it with a transparent foil. Make sure that all wells are completely sealed by pressing the foil on the plate. Vortex the plate at 1200 rpm for 30 s and centrifuge again. Load the plate into the LightCycler 480, set up the program and run it.

Program used in qRT-PCR analyses is set up as follows:

<table>
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<tr>
<th>Program name</th>
<th>Number of cycles</th>
<th>Temperature (°C)</th>
<th>Hold</th>
<th>Ramp rate (°C/s)</th>
<th>Analyses</th>
</tr>
</thead>
<tbody>
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<td>Denaturation</td>
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<td>95</td>
<td>2 min</td>
<td>4.8</td>
<td>None</td>
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<tr>
<td>Amplification</td>
<td>45</td>
<td>95, 60</td>
<td>5 s, 10 s</td>
<td>4.8, 2.5</td>
<td>Quantification, Melting</td>
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<tr>
<td>Melting curve</td>
<td>1</td>
<td>95, 65, 95</td>
<td>10 s, 1 min, Continuous</td>
<td>4.8, 2.5, 0.11, acquisition 5°C</td>
<td>Melting curves</td>
</tr>
<tr>
<td>Cooling</td>
<td>1</td>
<td>40</td>
<td>1 s</td>
<td>2.5</td>
<td>None</td>
</tr>
</tbody>
</table>

3.6.3 Statistical Analyses and An Example of Collected Data

Collected $C_p$ values (e.g., crossing point) for genes of interest are normalized using housekeeping gene $C_p$ values, for instance using GeNorm [13] or comparable calculators (http://download.genequantification.info/). To obtain fold-change values, the normalized relative expression values (based on housekeeping genes) can be renormalized for instance to the average of wild-type untreated samples (e.g., Col-0 untreated, time point 0), which are set as 1. If absolute quantification of transcript abundance is required, a standard dilution series can be set up, preferably using the same master mix on the same PCR plate as the samples of interest. A template for the standard curve can be prepared by performing a standard PCR reaction (e.g., 50 μL) using the primers of the gene of interest on an appropriate cDNA template. The PCR product can be extracted from a low-melting point agarose gel and quantified (e.g., using PicoGreen or Qubit assays—ThermoFisher). A 10x dilution series can then be run in parallel to create a standard curve.

An example of results using methylviologen or antimycin A is presented in Figs. 2 and 3.
Table 1
Primer sequences used for qRT-PCR

<table>
<thead>
<tr>
<th>AGI</th>
<th>Name</th>
<th>Marker for:</th>
<th>Primer sequence</th>
</tr>
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<tr>
<td>Az5g25760</td>
<td>UBC</td>
<td>Housekeeping</td>
<td>LP CTCCGACTCAGGGATCTTCTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP TTTGTGCCATTGAATGGACCC</td>
</tr>
<tr>
<td>Az3g22370</td>
<td>AOX1a</td>
<td>Mitochondrial</td>
<td>LP GACCGTCCGTAAGGTTTCCG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP CTCTGTATTGGGCTGTCCTCCT</td>
</tr>
<tr>
<td>Az2g21640</td>
<td>UPOX</td>
<td>Mitochondrial</td>
<td>LP CCGAGAACCCGCAAAAACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP CCTTCTTTCCAACTGCGCTC</td>
</tr>
<tr>
<td>Az1g05680</td>
<td>UGT74E2</td>
<td>Mitochondrial</td>
<td>LP TAATCTCTTTCACACTTCATATCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP ACAACAAAACACTGTCAGACAC</td>
</tr>
<tr>
<td>Az3g50930</td>
<td>AzOMe6</td>
<td>Mitochondrial</td>
<td>LP TGCGAGACCCGACGTATG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP ACCCTCCGGATTTGCT</td>
</tr>
<tr>
<td>Az5g12830</td>
<td>HSP7.6A</td>
<td>Chloroplast</td>
<td>LP CTTCAAGACCTTACATGCGAGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP CCTGATAACCGTACCTGCT</td>
</tr>
<tr>
<td>Az5g12020</td>
<td>HSP7.6CII</td>
<td>Chloroplast</td>
<td>LP ACCCTCAGCGTTTACATGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RP CGATGACGCTACCGGTC</td>
</tr>
</tbody>
</table>

4 Notes

1. The size of the petri dishes can be adapted to the number of samples to be analyzed and the amount of tissue required for further analysis. We routinely use 6 cm diameter plates from Sarscett (Cat. No. 82.1135.500). Alternatively, soil-grown plants can be treated similarly to in vitro grown plants.

2. Other methods for plant RNA extraction can be used, including other column-based kits, TRIzol, and comparable products. If non-column extraction methods are used, ThermoFisher DNA-free Turbo DNase treatment is recommended to remove unwanted DNA contamination.

3. Many platforms, PCR reagents and detection methods are available for real-time quantitative PCR. Please adapt the procedure and materials depending on the available facilities and equipment.

4. Soak seeds in 0.2% agarose solution around 15 min prior sowing them on plate. This concentration of agarose helps with the separation of seeds while sowing. Leukopor tape allows gas exchange; however, it decreases the possibility of random contamination of plates significantly.
5. Stratification of seeds can be carried out either before sowing them on MS plates by soaking them in the 0.2% agarose and keeping them in the cold room, or after sowing them by keeping the plates in the cold room.

6. When collecting samples after short time of spraying the seedlings with retrograde signaling inducer, such as antimycin A, the seedlings will be wet. While collecting the samples with tweezers, place seedlings gently on a tissue to allow it to soak up the moisture. This will help with the process of grinding the tissue into a powder prior RNA preparation.

7. To prepare Lysis buffer add 10 μL of β-mercaptoethanol to 990 μL of Lysis Solution. This step should be performed under the fume hood.

8. At this stage, start defrosting DNase digestion buffer for On-column DNase treatment.

9. If total RNA yield is expected to be low, add an additional 250 μL of binding solution (total of 750 μL of binding solution) and mix by pipetting.

10. DNase mix for 1 sample: to 70 μL of DNase digestion buffer add 10 μL of On-column DNase I and mix gently by inverting until homogenous solution or gentle pipetting. DNase is susceptible to physical denaturation so do not vortex.

11. When preparing Master Mix, make it with 8% excess. This will allow to use a repeat pipettor without running out of mix for the last few wells.

12. A 100x concentrated yellow dye is provided with the Bio-Rad Quantinova kit that can be added to the cDNA samples to improve visual inspection. Prepared Master Mix will have a light blue color when using the Bio-Rad Quantinova master mix. During pipetting of diluted yellow cDNA template into the blue master mix, the resulting mixture will become green which allows for visual inspection of pipetting.

Acknowledgments

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References


Appendix 2

Neofunctionalization of Mitochondrial Proteins and Incorporation into Signaling Networks in Plants

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Abstract

Because of their symbiotic origin, many mitochondrial proteins are well conserved across eukaryotic kingdoms. It is however less obvious how specific lineages have obtained novel nuclear-encoded mitochondrial proteins. Here, we report a case of mitochondrial neofunctionalization in plants. Phylogenetic analysis of genes containing the Domain of Unknown Function 295 (DUF295) revealed that the domain likely originated in Archaea. The C-terminal DUF295 domain is usually accompanied by an N-terminal F-box domain, involved in ubiquitin ligations via binding with ASK1/SP1-type proteins. Due to gene duplication, the gene family has expanded rapidly, with >94 DUF295-related genes in Arabidopsis thaliana alone. Two DUF295 family subgroups have uniquely evolved and quickly expanded within Brassicaceae. One of these subgroups has completely lost the F-box, but instead obtained strongly predicted mitochondrial targeting peptides. We show that several representatives of this DUF295 Organellar group are effectively targeted to plant mitochondria and chloroplasts. Furthermore, many DUF295 Organellar genes are induced by mitochondrial dysfunction, whereas F-box DUF295 genes are not. In agreement, several Brassicaceae-specific DUF295 Organellar genes were incorporated in the evolutionarily much older ANAC017-dependent mitochondrial retrograde signalling pathway. Finally, a representative set of DUF295 T-DNA insertion mutants was created. No obvious aberrant phenotypes during normal growth and mitochondrial dysfunction were observed, most likely due to the large extent of gene duplication and redundancy. Overall, this study provides insight into how novel mitochondrial proteins can be created via “intercompartamental” gene duplication events. Moreover, our analysis shows that these newly evolved genes can then be specifically integrated into relevant, pre-existing compensation networks.

Key words: mitochondria, evolution, retrograde signalling, stress, neofunctionalization.

Introduction

Mitochondria are membrane-bound organelles with crucial roles in eukaryotic cells, including ATP production, Fe/S clusters, the Krebs cycle, and many other metabolic functions. The endosymbiotic theory proposes that mitochondria are derived from ancestral bacteria that were engulfed by a host cell, probably of archaean origin (Spang et al. 2015). The exact lineage of bacteria that was the precursor to mitochondria is not known, but they are likely related to alpha proteobacteria (Marín et al. 2018). During evolution, the genome of the mitochondrial genome was highly reduced, and the vast majority of mitochondrial proteins are encoded in the nuclear genome (Ku et al. 2015). This required the mitochondrial import machinery to evolve allowing proteins translated in the cytosol to be imported into the different compartments of the mitochondria, often with help of specific targeting peptides. Other originally bacterial functions were redirected to mitochondria and became operational elsewhere in the cell, for example, the cytosol and peroxisomes (Huynen et al. 2013), or were lost entirely.

Mitochondria usually contain >1,000 different proteins, for example, around 1800 proteins in mammals (Pihlström and Bross 2017), and perhaps >2000 in plant mitochondria (Rao et al. 2017). As the mitochondria-containing host cell was probably the ancestor for all eukaryotic lineages including plants, animals, and fungi, one would expect the majority of mitochondrial proteins to be of bacterial origin. However, of the ~800 human nuclear genes that bear clear resemblance to alpha proteobacteria genes, only about 200 are present in the human mitochondrial proteome (Sikorsky and Huyten 2010). The current view is that the mitochondrial proteome is a mixture of alpha proteobacteria related proteins, proteins from other (proteo-)bacteria obtained via lateral gene transfer, and viral proteins. Additionally, about 40% of the mitochondrial proteome has no clear viral or bacterial origin. These proteins are thought to be of perimitochondrial host cell origin.
or are of "lineage-specific" origin (e.g., plant specific) having originated after the last Eukaryotic Common Ancestor (Roger et al. 2017). The proteome of the plastid (derived from a photosynthetic cyanobacterial endosymbiont) appears to have a similarly complex origin (Leister 2016; Roger et al. 2017).

For the multitude of mitochondrial proteins that are related to bacterial, viral, or archaeal proteins, different mechanisms including endosymbiotic gene transfer (from the endosymbiont to the nucleus) or lateral gene transfer can be envisaged. It is, however, less evident how different eukaryotic lineages have obtained lineage-specific mitochondrial (or plastid) protein types. One possibility is via random creation of new open reading frames that encode completely novel proteins. Another mechanism may be gene duplication, whereby a new copy of a gene is created in the genome. In most cases, the encoded protein retains its original subcellular localization (intracompartmental duplication) (Szládek and Huyten 2009). However, in rare cases, the duplication results in one of the encoded proteins becoming targeted to another subcellular location (intracompartmental duplication).

Another consequence of the endosymbiotic nature of the eukaryotic cell is the need for more complex transcriptional regulation. As most of the genes encoding mitochondrial or plastid proteins are found in the nuclear genome, the individual mitochondrial or plastid genomes can no longer directly control all transcript levels. Instead, the organelles must provide feedback to the nucleus to steer gene expression, a process called retrograde signaling. Such retrograde signaling pathways have been described in fungi, animals, and plants. Especially, when the cellular or metabolic situation in the organelle changes (e.g., availability of substrates or circadian rhythms), the inhibition of important enzymes, and reactive oxygen species, adequate adjustments in transcript levels are needed to fine-tune the organelar proteomes. This further raises the question of how lineage-specific organelar proteins become regulated appropriately after their appearance. To be of optimal use to the cell, the new genes may become incorporated into existing transcriptional networks relevant to organelar functions. Alternatively, specific new needs may require new transcriptional modules to evolve. Indeed, the best-known retrograde signaling pathways in yeast, animals, or plants appear to be quite different between lineages and employ different (even lineage-specific) transcription factors (Ng et al. 2014; da Cunha et al. 2015).

In this study, we describe the phylogenetic history of lineage-specific Domain of Unknown Function 295 (DUF295) genes in plants. Despite its poorly understood function, the gene family is strongly expanded with 94 representatives in the Arabidopsis thaliana genome. Our findings show that relatively recent random gene duplications in the Brassicaceae family have led to neofunctionalization in plant mitochondria. Most likely through incomplete gene duplication, an ancestral DUF295 domain gene has lost its N-terminus and has instead obtained a functional mitochondrial targeting peptide. Furthermore, we show that several of these new mitochondrial proteins have been specifically integrated into pre-existing gene-expression networks containing "old" genes that regulate mitochondrial function.

**Results**

The DUF295 Proteins Form a Large Gene Family within Angiosperms

Despite many decades of intensive research, many conserved protein domains still remain unknown functions. The term Domain of Unknown Function (DUF) was originally coined to describe two bacterial domains (DUF1 and DUF2) found in bacterial signaling proteins (Schultz et al. 1998). Subsequent bioinformatics approaches identified thousands of additional uncharacterized domains that were assigned names in the PFAM database. The latest PFAM release 32 contains nearly 4,000 DUF families (up to DUF5652), representing around 172 of the known families (Bateman et al. 2010). The DUF295 domain was identified by Bateman et al. 2004 and currently contains 4,533 family members, with an average domain length of 578 amino acids. The A. thaliana Col-0 genome sequence was searched for proteins containing the DUF295 domain, based on the PFAM model PF01778 (Supplementary fig. 1, Supplementary Material online) and the TAIR 10 annotation (www.arabidopsis.org). Last accessed February 25, 2019. Using further homology searches, in total 94 unique loci encoding DUF295-related proteins were found (fig. 1A, table 1). To examine the evolutionary conservation and origin of the DUF295 protein family, homology searches were performed to identify representative families in other lineages. The DUF295 domain was not found in prokaryotes and animals. Interestingly, a single DUF295 domain protein was found by EBI (http://www.ebi.ac.uk/

Downloaded from https://academic.oup.com/molbev/advance-article-abstract/doi/10.1093/molbev/msab454/5446245 by UNIV OF WESTERN AUSTRALIA user on 12 January 2020
Most DUF295 Domain Proteins Also Contain an F-Box Domain

From the phylogenetic analysis, it was apparent that the DUF295 proteins can be divided in four major classes (Fig. 1A). One class of DUF295 proteins (indicated in green in fig. 1) was represented in the genomes of all studied plant species, with a clear subgroup of monocot and dicot representatives. This group is thus most likely the ancestral DUF295 protein class. Within the dicot subgroup, a clear expansion of Brassicaceae DUF295 homologs was observed. Analysis of the domain structure of the proteins in the group showed the presence of an N-terminal F-box domain and a C-terminal DUF295 domain (Fig. 1B). F-box domains are about 50 amino acids long and involved in protein-protein interactions. They are often found in Skp1-cullin-F-box (SCF) ubiquitin E3-ligases that mark proteins for degradation, with the F-box imparting specificity of the target proteins. Many key plant hormone receptors have been found to be SCF proteins, including SCFauxin (auxin receptor) and SCFjasmonic acid receptor (Kepinski and Leyser 2005; Karsir et al. 2008). The Arabidopsis genome contains 20 ancestral-type F-box/DUF295 proteins. The SKP1-interacting Protein SKIP23 (ADGR7031) is part of this group and was previously found to interact with ASK1 (Riesmeier et al. 2003), a component of, for example, the strigolactone SCFauxin receptor complex (Yao et al. 2016). In a more recent study, six ancestral-type F-box/DUF295 proteins were found to interact with ASK1 and related proteins by yeast two-hybrid screens (Kuroda et al. 2012). SKIP23 was also found to interact with Arabidopsis 14-3-3 proteins (Hong et al. 2017). Upward Curly
Table 1. Overview of DUF295-Related Genes in Arabidopsis thaliana.

<table>
<thead>
<tr>
<th>AGI</th>
<th>Gene Name</th>
<th>A. thaliana Mono/Dicert</th>
<th>Basic-Only F-Box</th>
<th>DUF295</th>
<th>SUBAcon</th>
<th>ASK1 Binding</th>
<th>MRR</th>
<th>Compressed Phylogenon</th>
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Note: A. thaliana conserved in Arabidopsis thaliana; N. benthamiana conserved in Nicotiana and D. cincta; Brachypodium conserved in Brachypodium; F-box protein contains an F-box domain; DUFE95 protein contains a DUFE95 domain; SUBACOS conserved in subcellular location as suggested by the SUBACOS algorithm; MS subcellular location as detected by MitoProt II; ASK1 binding experimentally protein-protein interaction with SLP1/ASK1 protein; MRR transcriptionally regulated by microtubule-associated signaling and proposed phylogenetic tree of the 100 most strongly conserved gene (Atg, angiosperm; Gm, Gossypium; V., v. d.; L., l.; B., Brassicaceae; T., Triticaceae; L., L.); Phylostomum leaf 1 [JCL, Atg57920] was found to be a nuclear protein interacting with Curly Leaf Polyclamb genes and ASK1 [Jeong et al. 2011]. This indicates that ASK1 binding is a common feature of this protein group. As most Arabidopsis DUFE95 proteins lack a systematic gene name, we named the genes in this group AtFDa1-10 (F-box/DUFE95 Ancestral) (Table 1 and Table 1). Interestingly, AtFDa9 (Atg15000X) is nearly identical to the adjacent gene AtFDa8 (Atg15000X), however a frame shift has occurred due to a single base deletion just before the start of the DUFE95 domain. This leads to a premature stop codon and truncated AtFDa9 protein of 322 amino acids, instead of around 415 amino acids as in AtFDa8 (where the DUFE95 domain is at position 319–360). If the AtFDa9 transcript sequence after the premature stop codon is translated in the +2 frame, the DUFE95 domain can be clearly identified, indicating that AtFDa9 was originally a DUFE95-containing gene. The structure of the dozy-day gene AtFDa10 (Atg16365X) appeared even more complex. In the current TAR10 annotation, Atg16365X is named photoperiodic control of hypocotyl1 (PC1), which is 778 amino acids long. The PC1“domain” (a phytochrome interaction domain) is located at the N-terminal, and the F-box is at amino acids 495–505. In the other AtFDa genes, the F-box is located right at the N-terminal (e.g., residues 3–49 in AtFDa8), suggesting it is a compound gene. Indeed, six splice forms have been annotated for Atg16365X, where Atg16365X.2 does not contain the F-box and downstream sequence. We checked a range of RNA-Seq data sets but could not find evidence for reads spanning the suggested third intron, which would connect the PCh1 region to the FDA region. There is also no proteomic support for the existence of proteins containing both PCh1 and FDA10 sequence, and the only short splice variant could be doned (Atg16365X.2) (Huang et al. 2016). Therefore, we propose that the currently annotated Atg16365X locus actually encodes two separate genes, PCh1 and FDA10. Additionally, FDA10 has a two-base insertion upstream of the DUFE95 domain, causing a premature stop codon and loss of the actual DUFE95 domain, as observed in FDA9. A second group of DUFE95 proteins was identified (indicated in red in fig. 1) which also contained an F-box/DUFE95 arrangement, but was clearly divergent from the ancestral FDA-type proteins. This type of protein was only represented...
have obtained functional mitochondrial and/or plastid targeting peptides in line with their strong organellar prediction.

**DUF295 Genes Show Remarkably Specific Expression Patterns**

The strong expansion of DUF295 genes does not necessarily indicate that the genes are functional and expressed. Therefore, the transcript levels of the 94 Arabidopsis genes were analyzed in a large set of available gene-expression experiments (Fig. S and supplementary Fig. S. Supplementary Material online). Starting from the transcript counts for 206 public RNA-Seq experiments, a gene-expression matrix was generated by summing transcript counts per locus (Vanechout et al. 2017). Out of 94 DUF295-related genes, 73 (78%) appeared to be expressed in one or more conditions (maximim transcript per million > 2). More than 50% (11) of the nonexpressed genes were of the AtHDA type, whereas only 1 AtFDA (AtFDA8) did not seem to be significantly expressed. Four AtDOA and five AtDOB genes also were not clearly expressed. Remarkably, most of the expressed genes were expressed under relatively specific conditions with only four DUF295 genes showing strong ubiquitous expression (AtFDA3, AtFDA15, AtKIP23, AtFDA14, and AtAFDR1). DUF295-related AtAFDR1 also appears to be expressed in most tissues and conditions. In contrast, most DUF295 genes were expressed under very specific tissues or conditions, often reproductive tissues such as young anthers, pollen, siliques, and young seeds. Others were specifically expressed during abiotic stress or biotic stress (Botrytis cinerea).

As many DUF295-containing genes are present in tandem duplications, often with up to six related genes in close proximity, we examined whether tandem duplicated genes are coexpressed. In many cases, tandem pairs of two genes were found to cluster together and showed very similar expression patterns (e.g., AtDOA1/2, AtDOA14/15, AtDOB4/5, and AtDOB16/17). Interestingly, such paired expression patterns were often observed for AtDOA and AtDOB genes, whereas F-box containing genes only rarely showed such clear coexpression between tandem repeated genes (AtEDE3) and AtEDE3, though these are interspersed by two non-coexpressed genes in the tandem repeat. We also noticed that in the larger tandem repeats like AtDOB-12 (six genes), only groups of maximum two genes were similarly expressed (AtDOB18/9 and AtDOB18/11), but these two pairs were very different from each other (Fig. 3). Several groups of genes showed remarkably similar expression patterns, such as eight mixed AtFDA/AtAFDR genes expressed in siliques, or eight genes induced by Botrytis cinerea infection (with members of AtFDADB/DOA/DOA groups). Clearly, the genes in these groups were not tandem repeats, so the mechanism behind their coexpression is most likely not tandem duplication of promoter regions.

**High-Impact Mutation Analysis across 1,135 A. thaliana Genomes**

To get more insight into which DUF295-related genes may be more active and/or functionally important, we assessed whether they are retained as intact open reading frames in the genome sequences of 1,135 A. thaliana accessions published by the 1001 Genomes Consortium (2016). For all 94 A. thaliana Col-0 DUF295-related genes, the occurrence of “high-impact mutations” (HIMs; e.g., gain or loss of start/stop codons and loss of splice acceptor sites) was searched in the other ecotypes. This varied widely, with some genes having accumulated no HIMs in other accesses, whereas others have accumulated many hundreds (supplementary
As stated above, this locus actually contains two separate genes (CCTI1 and ADIF2A), so it was excluded from the analysis.

Given the high rate of gene duplication, we postulated that a recently duplicated gene may develop into a functional gene ("consolidated": max tpm > 5, HIM < 50), gradually turn into a pseudogene and eventually disappear via mutations ("degenerating": HIM < 50) or temporarily remain in an intermediate stage ("undecided": max tpm < 5, HIM < 5) (Fig. 6B). More than 90% of the genes fell inside the intervals using cut-offs max tpm of 5 and HIMs of 50, suggesting they are relevant (Fig. 4A). When examining ancestral FDA genes, it appears that this selection is nearing completion, as nearly all ADIF2A genes are either "consolidated" or "degenerating" based on our cut-offs, with only one remaining "undecided" (ADIF2A16). This further supports the idea that the FDA genes are relatively ancient. Similarly, both ADIF4R genes show strong expression and very low HIMs (0.1), and thus seem completely "consolidated," supporting their premonocot/dicot divergence origin. For the probably more recent Brassicaceae-specific genes, the situation looks different. For the ADIF2B F-box genes an even distribution across the three groups can be seen, suggesting selection is still ongoing and balanced. More than 50% of the AtADO genes seem to be "degenerating," whereas fewer are being consolidated. Conversely, although most AtADO genes are still in a more "undecided" state, far more are being "consolidated" than are "degenerating." This suggests that there is higher selective pressure on ADIF4R, ADIF5B, and ADIF2B genes, whereas AtADO genes may be degenerating more often.

**DUF295 Organellar Genes Were Incorporated into the ANAC017 Retrograde Signaling Pathway**

Previously, we reported that eight DUF295 genes were constitutively induced in Arabidopsis mutants with mitochondrial defects (van Aken et al. 2016). Surprisingly, all eight of these are members of the DUF295 Organellar group (two AtADO and six AtADOB), whereas none of the F-box DUF295 proteins were represented (Table 1). To further examine the specificity of DUF295 Organellar proteins in responding to mitochondrial dysfunction, an antimonycin A treatment time course was set up. Gene expression levels were measured for the most highly induced AtADO representative (AtADO10, according to supplementary table 1, Supplementary Material online), and two highly induced AtADOB representatives (AtADO12 and AtADO65). Furthermore, AtFDI1/AtFDI3 and AtFDI2 were selected from the F-box DUF295 proteins, based on their relatively high expression in Col-0 seedlings of similar age in previous RNA-Seq data sets (van Aken et al. 2016) (supplementary table 3, Supplementary Material online). Figure 5 shows that only AtADO12, AtADO65, and AtADO10 were strongly induced by antimonycin A, whereas AtFDI1/AtFDI3 and AtFDI2 showed no induction.

As antimonycin A is known to induce gene expression via retrograde signaling, the response of the selected DUF295 genes was also monitored in mutants lacking ANAC017, a key transcription factor in plant mitochondrial and

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**Table 4, Supplementary Material online.** To clarify, if the same variant compared with Col-0 was found in multiple accessions, it was counted as the number of accessions it occurred in. In other words, if one mutation occurred in 300 accessions, this was counted as 300. Next, we plotted the number of HIMs against the transcript expression strength (maximal transcripts per million (max tpm) in the above 206 RNA-Seq data sets) (Fig. 4A). A clear trend could be observed that genes with high expression usually had a lower number of HIMs. Conversely, genes with low expression often had many mutations. The only clear exception was At2g15655 which had both the highest expression and the highest number of HIMs.
chloroplast regulation (De Clercq et al., 2013; Ng et al., 2013; Van Aken et al., 2016). The antimycin A-induced gene expression was almost completely suppressed during the first 8 h, which was where the peak expression occurs in wild-type plants (Fig. 5). Some delayed expression was observed toward 9–12 h, which was most likely due to contributions by ANAC017 homologs, such as ANAC013, ANAC053, and ANAC078 (De Clercq et al., 2013; Van Aken et al., 2016). No significant differences in gene expression for AFD4A11/SKIP23 or AFD4B2 were observed between Col-0 and the anac017 mutants. In summary, the tested DUF295 Organellar genes were strongly induced by mitochondrial dysfunction in an ANAC017-dependent way. The F-box DUF295 genes, however, seem to be largely unresponsive to mitochondrial stress signaling.

The promoters of the 8 DUF295 Organellar genes that were found as responsive to mitochondrial dysfunction based on RNA-Seq data (Van Aken et al., 2016) were searched for binding motifs of ANAC017 and/or its related NAC transcription factors (mitochondrial dysfunction motif) (De Clercq et al., 2013). The TF-Net tool was used and for seven out of eight genes an MDM-like motif (CTGimmtGGAAG or similar) was found (Kulkarni et al., 2018). Only for AtDOA3 (At1g30160) no MDM could be found, which is in line with its ANAC017-independent gene expression (supplementary table 2, Supplementary Material online). Furthermore, by using DNA affinity purification sequencing (DAP-seq, a variant of chromatin immunoprecipitation CHIP), we found that the promoters of these seven genes bind to ANAC017 and/or its homologs (supplementary table 2, Supplementary Material online) (O’Kearney et al., 2016).

It was surprising that only DUF295 gene variants with (predicted) mitochondrial targeting peptides have become incorporated into a mitochondrial signaling network. Additionally, this must have occurred relatively recently in evolutionary history, since the DUF295 Organellar proteins only evolved in Brassicaceae. As mitochondrial retrograde signaling occurs in all eukaryotic kingdoms (da Cunha et al., 2015), the plant-specific ANAC017-dependent mitochondrial retrograde pathway is most likely much more ancient than Brassicaceae (Kim et al., 2007). This would require that the recent DUF295 Organellar genes have been “adopted” by a much older, pre-existing coexpression set. To test this hypothesis, we performed a phylogenetic coexpression analysis of all DUF295 genes. The phylogenetic classification grouped A. thaliana genes in 13 clades based on their evolutionary conservation (Quin et al., 2012), ranging from genes universally conserved in cellular organisms (phytostratum 1), via Viridiplantae, to genes that are Brassicaceae (phytostratum 12) or even A. thaliana-specific (phytostratum 13). Next, a coexpression analysis was performed using publicly available gene-expression data, to identify the 30 most similarly expressed A. thaliana genes for each of the 92 DUF295 genes. Finally, these 300 coexpressed genes were searched for over-representation of genes from the different phytostrata (supplementary table 5, Supplementary Material online) (Ruppech et al., 2017). Based on this, coexpressed phytostrata were assigned to all DUF295 genes, giving an indication of the evolutionary age of their coexpression network.

For 82 DUF295 genes, one or more coexpressed phytostrata were identified (supplementary table 5, Supplementary Material online). When comparing the overall distribution of the A. thaliana genome (represented by 32,833 genes used in this analysis), the DUF295 gene family was particularly enriched in coexpression networks with genes from the Angiosperm and Eudicot phytostrata (Fig. 6). This is in line with the presumed age of the DUF295 domain, which appears to have originated early on in the Angiosperm lineage. The
specific group of eight
Brassicaceae-specific: DUF295
organellar genes that are responsive to mitochondrial dysfunction was
also enriched in coexpression networks consisting of
Angiosperm and Eudicot phyla. Interestingly, none of
these eight genes are coexpressed with Brassicaceae-specific
phyla, indicating they have been incorporated in coex-
pression network that is much older than the genes them-
sele. A similar analysis was performed for 21 "core"
mitochondrial retrograde target genes regulated by
ANAC017, based on previous data (supplementary table S,
Supplementary Material online). This indicated that the
strongest coexpression of core ANAC017-target genes is
also found with genes from Angiosperm phyla
(Fig. 6). Stronger coexpression of core ANAC017-target genes
was also found in the Landplants phyla. In summary,
this analysis further supports that the recent mitochondrial
stress responsive DUF295 genes have been adopted by
a much older coexpression network, which is of largely similar
age to the ANAC017 core regulon.

Characterization of DUF295 T-DNA Insertion
Mutants
To investigate the function of DUF295 genes in plants, we
isolated T-DNA insertion mutants for representatives of the
three main DUF295 groups (fig. 7A). For the ancestral F-box
DUF295 genes, AtFDB2/AtFDB3 was chosen, as it has been
picked up in several protein–protein interaction screens with
ASK1/SKIP1-type proteins and 14-3-3 proteins (Risseet al.
2003). Kuroda et al. 2012, Hong et al. 2017). It also had the
second highest gene-expression level in 2-week-old Col-0
based on RNA-Seq data (supplementary table S,
Supplementary Material online). For Brassicaceae-specific F-
Box DUF295 genes, AtFDB2 was selected as it was by far
the most strongly expressed gene in this group. For DUF295
organellar genes, AtDODA10 was selected as it showed the
highest fold-change induction to mitochondrial dysfunction
(supplementary table S, Supplementary Material online). Also
AtDOB5 and AtDOD12 were chosen because they were the
most highly induced representatives of two different AtDOB
tandem duplications (supplementary table 2, Supplementary Material online). They were also found to be targeted to mitochondria using GFP-fusions (fig. 2). Suitable T-DNA lines were selected from T-DNA express and homozygous lines were isolated using polymerase chain reaction (PCR)-based genotyping (supplementary table 6, Supplementary Material online).

First, the overall growth rate and phenotype of the mutant lines was compared with Col-0. The rosette surface area was monitored from 14 to 29 days after transfer to the growth room. However, no clear alterations in growth were observed compared with Col-0 for any of the lines tested (fig. 7B). Also, no obvious phenotypical differences in plant appearance were observed. As the selected DUF295 organellar genes clearly responded to mitochondrial dysfunction, root growth inhibition by antimycin A and methylviologen was tested (fig. 7C–F). Again, no obvious differences in root growth and resistance to inhibitors was observed for any of the lines compared with Col-0. Overall, no clear aberrant phenotypes were observed for any of the T-DNA lines analyzed, which is likely explained by the large extent of gene duplication leading to redundancy, for instance shown by often similar gene expression patterns of tandem duplications (fig. 3).

Discussion

Through extensive phylogenetic analysis of the DUF295 family, this study found that the F-box/DUF295 domain combination is the most prevalent and conserved configuration in Angiosperms (fig. 1). Most likely these types of proteins derived from F-box precursor proteins, and the DUF295 domain evolved gradually sometime after the Gymnosperm/Angiosperm divergence. A common factor in the limited functional information that is available on the FDA proteins is interaction with SKP1/ASK1-type proteins, which are part of SCF-type ubiquitin E3-ligases. ASK1 seems to mediate the interaction of the F-box protein with CUL1 (Jeong et al. 2011). The DUF295 domain is likely to be also a protein–protein interaction domain that may be bridging ASK1 and other proteins such as Curly Leaf (CLF), a polycomb SET-domain protein, thereby marking them for degradation. Overexpression of the DUF295 protein UCL1 resulted in similar phenotypes as a loss-of-function mutant in CLF, in line with the model that the interaction results in proteasome-mediated degradation of CLF (Jeong et al. 2011). Given the large number of FDA proteins within the same species, it is likely that a large range of proteins may be posttranslationally regulated by such a mechanism. The binding of at least six other AtFDA proteins with ASK1/SKP1 was shown using yeast two-hybrid assays, suggesting this is a common feature (Kuroda et al. 2012). AtFDA11/SKP23 was also found to interact with 14-3-3 proteins but could not be shown to be directly involved in ubiquitination (Hong et al. 2017). From the limited amount of information available, it thus seems that the DUF295 domain may be a protein–protein interaction domain. For the ancestral FDA proteins, it may help recruit target proteins to SCF E3-ligases for proteasomal degradation.

After several rounds of gene duplication in Brassicaceae, a variant to the F-box DUF295 configuration seems to have arisen (FDB proteins). The yeast two-hybrid screens could not identify an interaction with ASK1 (Kuroda et al. 2012) for any of the six tested FDB proteins, indicating that the F-box domain has diverged significantly. Whether these proteins have obtained a different function is currently unclear. At least a single loss-of-function mutation in AtFDA2 did not result in obvious phenotypic differences, but this may be due to the extensive redundancy.

The other group of Brassicaceae-specific DUF295 gene variants have led to more radical rearrangement, with the loss of the F-box domain, and the gain of a functional predicted organellar targeting peptide. Mitochondrial targeting of two AtDDB proteins was confirmed by GFP-fusions in this study (fig. 2), whereas proteomics identified at least one AtDDB protein in isolated mitochondria (Senker et al. 2017). From an evolutionary standpoint, this represents a clear example of how (partial) gene duplication can result in new organellar proteins. These new DUF295 Organellar proteins were not useful to plants, one would expect a fast accumulation of point mutations. However, at least for AtDDB proteins, there seems to be some selection pressure, indicating that the genes are being kept in a functional state. Also, most DUF295 genes are expressed at the mRNA level, often in very specific patterns, suggesting they are not pseudogenes. However, their function remains unclear for now. Assuming that the DUF295 domain is a protein–protein interaction domain, they may directly bind other proteins. Due to the loss of the F-box domain, this is unlikely to lead to ubiquitination and protein degradation of the potential binding partners. The DUF295 Organellar are generally only slightly shorter than FDA proteins (most between 350 and 400 amino acids), and the DUF295 domain is close to the C-terminal. Even without the F-box domain and the likely removal of the N-terminal organellar targeting peptide upon import, one would expect
at least 200–250 amino acids present in DUF295 proteins outside of the DUF295 domain itself. This would be more than sufficient for other (unknown) functions that are assisted by the DUF295 domain, or perhaps act as a flexible linker between the two domains. To some extent the organelar DUF295 proteins show similarities to microProteins, which are proteins that only contain a protein–protein interaction domain, but no other clear functional domains (Bharti et al. 2018). MicroProteins are thought to have regulatory effects for instance by preventing proteins from forming functional dimers, thus having dominant effects. MicroProteins have also been found in mitochondria, where they can bind mitochondrial elongation factors and stimulate micro-ribosome translation (Rachore et al. 2018). Further studies with gain/loss-of-function mutants and protein interaction screens may shed further light on the function of these evolving proteins.

A broad gene-expression analysis revealed that only very few DUF295 genes are ubiquitously expressed (Fig. 1), for example, AtFAD31/SKIP3 and AtFDR3, which were selected for further study. Most other genes had relatively specific expression patterns. Besides two groups of stress-responsive DUF295 genes, most patterns were strongly biased toward young reproductive tissues, such as siliques, anthers and pollen. Such a bias toward expression of recently evolved genes in the male germ line (“out of ezeis”) has been reported in animal systems. It has been proposed that male
garniters play an "innovation incubator" role in pushing species into new ecological niches and expanding their geographic range. This is particularly evident in the case of the non-ANAC017-regulated organelar DUF295 genes (fig 4) which have been shown to be involved in the transcriptional regulation of mitochondrial genes. Our study further demonstrates that such duplications can have a significant impact on the evolution of regulatory networks, which may in turn influence the creation of new and useful functions in plants.

Materials and Methods

Plant Materials and Growth Conditions

Arabidopsis thaliana (L.) Heynh. Col-0 was used in all experiments. Seeds were sown on soil mix or MS media with 2% sucrose and stratified for 2-3 days at 4°C, then grown under long-day conditions (16 h light/8 h dark) at 22°C and 100 μmol·m⁻²·s⁻¹. Previously published transgenic lines were obtained from Ng et al. (2013) ana017-ana017-1 SALK_022174, DUF295 mutant lines (fig 5) were generated using PCR on genomic DNA using primers shown in supplementary table 4, Supplementary Material online.

Stress Treatments of Plants

Seeds were sown on petri dishes containing MS medium (Duchefa Biotech) + 2% sucrose, stratified for 2–3 days in the cold room and then incubated in long-day growth conditions for 14 days. Plants were then collected before or after treatment and immediately placed in liquid nitrogen for storage and further processing. For transcript analysis, plants were stressed with 50 μM antamycin A. An in vitro stress assay was performed as previously described (De Clercq et al. 2013). For root growth assays, the different plant lines were incubated on vertically positioned plates supplemented with 50 μM antamycin A or 20 μM methylviologen. Plants were stratified in the cold room for 3 days and incubated for 7 days in long-day conditions. Primary root length was measured using ImageJ. Statistical analysis was performed using Student's t test.

Quantitative Reverse Transcription-PCR and Microarray Analysis

RNA isolation, cDNA generation, and quantitative reverse transcription-PCR (qRT-PCR) were performed as described in Van Aken et al. (2013) using Spectrum RNA Plant extraction kits (Sigma-Aldrich, Sydney, Australia). iScript cDNA synthesis kit (Bio-Rad), and a Roche LC480 Lightcycler using SYBRgreen detection assays. All primers for qRT-PCR are shown in supplementary table 4, Supplementary Material online. Relative expression values were normalized, with
Neofunctionalization of Mitochondrial Proteins - doi:10.1093/molbev/msv031

MBE

![Diagram](image)

183

Phylogenetic Analysis

Arabidopsis thaliana DUF295 genes were identified using a combination of searches for Pfam motif PF03478, TAIR10 annotation, and homology searches. Representative DUF295 genes from other plant species were obtained using homology searching. Protein sequences were aligned using MAFFT multiple sequence aligner (Katoh and Standley 2013) and edited in BioEdit. Phylogeny was inferred using the IQ-TREE webserver (http://iqtree.cibiv.ub.ac.at; last accessed February 25, 2019) using BIOLUX62 algorithm and 1000 bootstraps (Katoh and Standley 2013). Phylogenetic trees were visualized using FigTree v1.4.2.

Gene Duplication Analysis

Starting from the set of DUF295 genes reported in Table 1, the PLAZA 4.0 Dicots comparative genomics platform was used to retrieve information about gene duplications (Van Beert et al. 2018). Specifically, the PLAZA Workbench was used to define different gene sets and to determine the number of genes involved in a tandem gene duplication event. In the PLAZA database, tandem gene duplicates were identified using iADHoR v3.0.01 (gap_size 30, tandem_gap 30, cluster_gap 35, q_value 0.05, prob_cutoff 0.01, anchor_points 5, and multiple_hypothesis_correction FDR) (Prost et al. 2012).

1001 Genomes SNP Analysis

The 1001 Genomes polymorph tool (https://kitt.1001genomes.org/polymorph/; last accessed February 25,
2019) was searched for single nucleotide polymorphisms with high impact for all 94 A. diabolicus DUF295-related genes. The number of exons varies where specific polymorphisms compared with Col-0 occurred was added.

Phylostratum and Expression Analysis
Starting from the transcript counts reported by Vanechouette et al. (2017), a gene-expression matrix was generated by summing transcript counts per locus. Subsequently, for each gene, the top 300 coexpressed genes (denoted km300 cluster) were determined based on the Pearson Correlation Coefficient. Starting from phylostrata information derived from gene families defined in PLAZA 3.0 (Pico et al. 2015), significantly over-represented phylostrata per km300 cluster were identified using the hypergeometric distribution (incl. Benjamini-Hochberg correction for multiple hypothesis testing). All enrichments with corrected P values <0.05 were retained as significant. Expression patterns of DUF295 genes in 206 samples (from the Vanechouette et al. 2017 data set) were examined in an expression plot (fig. 3 and supplementary fig. 2, Supplementary Material online). For this, TPM expression values were first normalized for each gene by dividing them with the maximum TPM observed for that gene. Only genes with a maximum TPM larger than 2 were considered to be expressed and others were excluded from the heatmap. No expression data were available for Arabidopsis and so it was excluded from this analysis as well. Figure 3 shows a manually selected subset of 25 samples to highlight interesting expression behavior of the DUF295 genes.

Supplementary Material
Supplementary data are available at Molecular Biology and Evolution online.

Acknowledgments
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Author Contributions
The study and experiments were designed by O.V.A., K.V., and T.S. Experiments were performed by S.L., M.B., Z.A., K.B., D.V., K.V., and O.V.A. The manuscript was written by O.V.A. with contributions from the coauthors.

References
Appendix 3

A MYC2/MYC3/MYC4-dependent transcription factor network regulates water spray-responsive gene expression and jasmonate levels

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Mechanical stimuli, such as wind, rain, and touch affect plant development, growth, pest resistance, and ultimately reproductive success. Using water spray to simulate rain, we demonstrate that jasmonic acid (JA) signaling plays a key role in early gene expression changes, before it leads to developmental changes in flowering and plant architecture. The JA-activated transcription factors MYC2/MYC3/MYC4 mediate transiently induced expression of 266 genes, most of which peak within 30 min, and control 52% of genes induced >10-fold. Chromatin immunoprecipitation–sequencing analysis indicates that MYC2 dynamically binds >1,300 promoters and transcription assays show that MYC2 activates these promoters. By mining our multivariate data, we identified a core MYC2/MYC3/MYC4-dependent “regulation” of 82 genes containing many previously unknown MYC targets. Including transcription factors WRKY19 and WRKY29, both of which can in turn directly activate the OSH4/OSH5 promoter, indicating that MYC2/MYC3/MYC4 initiate a hierarchical network of downstream transcription factors. Finally, we also reveal that rapid water spray-induced accumulation of JA and JA-isoleucine is directly controlled by MYC2/MYC3/MYC4 through a positive modification loop that regulates JA biosynthesis genes.

significance

Plants are continuously exposed to mechanical manipulation by wind, rain, neighboring plants, animals, and human activities. These mechanical stimuli cause short-term molecular changes and long-term developmental effects, affecting flowering time, pathogen defense, and plant architecture. Using water spray to simulate rain, we show that jasmonic acid–signaling factors mediate rapid gene expression changes. Nearly 500 genes are regulated by MYC2/MYC3/MYC4 transcription factors, particularly affecting the most highly responsive genes. This is controlled by induced binding and activation of water spray–inducible promoters by MYC2. We have identified a core MYC2/MYC3/MYC4 regulon, including many secondary transcription factors that in turn activate downstream promoters, creating a hierarchical transcriptional network. Finally, we demonstrate that spray-induced jasmonate accumulation is transcriptionally regulated by a MYC2/MYC3/MYC4-controlled positive-feedback loop.

Significance

Plants are continuously exposed to mechanical manipulation by wind, rain, neighboring plants, animals, and human activities. These mechanical stimuli cause short-term molecular changes and long-term developmental effects, affecting flowering time, pathogen defense, and plant architecture. Using water spray to simulate rain, we show that jasmonic acid–signaling factors mediate rapid gene expression changes. Nearly 500 genes are regulated by MYC2/MYC3/MYC4 transcription factors, particularly affecting the most highly responsive genes. This is controlled by induced binding and activation of water spray–inducible promoters by MYC2. We have identified a core MYC2/MYC3/MYC4 regulon, including many secondary transcription factors that in turn activate downstream promoters, creating a hierarchical transcriptional network. Finally, we demonstrate that spray-induced jasmonate accumulation is transcriptionally regulated by a MYC2/MYC3/MYC4-controlled positive-feedback loop.
JA integrates environmental stresses and developmental signals to regulate plant growth and defense (26, 27). A key transcription factor (TF) of the JA-signaling pathway is the basic helix–loop–helix (bHLH) TF MYC2 (23), which is involved in many aspects of plant defense and development (2, 28–33). Importantly, in addition to CORONATINE-SENSITIVE 1 (COS1) (34) and the JAZ repressors (35), MYC2 and its paralogs MYC3 and MYC4 also regulate the JA-dependent delay of flowering time (60) and, whereas unmodified myc2 mutants show no flowering phenotype (35), the myc2, myc3, myc4 triple (myc2/3/4) mutant flowers early (56). Although many indirect targets of MYC2 have been identified through analyses of myc2 and myc2/3/4 mutants (26, 27, 31), few of its direct targets have been identified to date (31, 33, 39–41).

Besides JA, the volatile phytohormone ethylene has been linked to touch responses in the past, although it seems that for most touch responses ethylene is not directly involved (10). Both expression of the touch-responsive genes TCH2, TCH3, and TCH4, as well as the rhythmic nodal curling response, are not noticeably affected in the ethylene signaling mutants etr1 and etr2 (42). Similarly, touch-induced expression of the JA biosynthetic gene UPO3 appears independent from ETHYLENE RECEPTOR 1 (ETR1) (43). Nevertheless, some studies have reported ethylene as a coinducer and expression of the ethylene biosynthesis gene 2-3-5-OXYGENASE 2 (2-3-5-OX2) is induced after touch (10, 44, 45). Given the cross-talk between ethylene and other hormones like JA in regulation of growth and development (32, 33), a role for ethylene in some aspects of the touch response cannot be excluded (10). To what extent the genewide transcriptome is affected after touch in Arabidopsis is currently under study and the role of ethylene signaling mutants by extension, has however not yet been investigated.

A single touch can induce fast and widespread transcriptional changes (15, 17). Transcriptional, posttranscriptional, and translational mechanisms underlying the touch response have been identified. These include the identification of a regulatory region, the characterization of active mRNA degradation components, and posttranslational modifications that affect touch-induced transcript accumulation levels (46–50). Although transcription factors are central to such transcriptional reprogramming, a regulatory network underlying the touch response remains to be identified.

Using water sprays as a trigger (5), we have screened the responsiveness of hallmark mechanical stimulation-responsive genes in selected core signaling mutants to identify major pathways regulating their transcriptional response. To substantiate our findings, we have undertaken in-depth mulbinocys profiling of the early water spray-induced response in Arabidopsis in the context of JA-signaling components and have discovered a regulatory network governed by MYC2, MYC3, and MYC4.

Results

Identification of Key Regulatory Components of the Arabidopsis Touch Response. Touch-responses in Arabidopsis involve mechanosensitive channels (14, 51) as well as the accumulation of phytohormones, such as JA, GA, and ethylene (7). We have shown previously that the JA biosynthesis mutant con1 displays a normal transcriptional response for some of these genes (52). Here, we first assessed the expression of the touch marker genes WRY490, JAZ2, JAZ5, ETI1, and CALMODULIN-LIKE 39 (CML39), an independent of JA (7). Finally, whereas both touch and wounding cause a fast accumulation of calmodulin-like protein (CML39), the catalytically inactive CML39 mutant, in wounded leaves appears not to occur in touching leaves (25), hence further indicative of discriminating signaling cascades between touch and wounding responses.

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MYC2/MYC3/MYC4 for their full touch-inducibility (Fig. 1D). Notably, expression of the touch-induced genes TGA2/TGA3 was not affected in myc2a (Supplemental Fig. S4A). The most highly spray-induced TF genes appeared to be most affected by the myc2a mutations (Fig. 1E and Dataset S2). Of the 45 MYC2/MYC3/ MYC4-dependent touch-inducible TFs, 9 candidate TFs showed at least 40% reduction in their induction in myc2a compared to Col-0, and literature mining suggested their involvement in plant stress response (Dataset S4). To confirm the RNA-seq results, we performed quantitative RT-PCRs (qPCRs) on Col-0 and myc2a seedlings before (untreated) and after a 28-min spray treatment (Fig. 2). All selected TF genes were touch-inducible in Col-0 and significantly reduced touch-inducibility in myc2a was observed for OCT-DECAYANOIDE SYNSP1, AP2ERF-DOMAIN TF 47 (OR47), ERF5, ERF8, ERF11, ERF15, ERF16, and BHLH19. The relative effect of the myc2a mutations was most pronounced for transcript levels of ERF109 and ERF109 (Fig. 2A). To confirm the role of MYC2/MYC3/MYC4 in classic touch mechanical stimulation, Col-0 and myc2a seedlings were mechanically stimulated by slight gentle pinching with a soft paint brush (Fig. 2B). qPCR analysis clearly shows that representative transcripts ERF109, BHLH19, and JAZ28 are significantly less induced in myc2a than in Col-0 25 min after touching, as observed during water sprays mechanical stimulation. Together, these results show that MYC2/MYC3/MYC4 play an important role in regulating touch-responsive expression of particularly the most touch-induced transcripts.

Dynamic Transcript Profiling of the Water Spray-induced Response.

To investigate direct regulation by MYC2 of the TF genes indicated above, we performed a time-resolved water spray experiment in a myc2 mutant background expressing a MYC2 aromatic C-terminal FLAG-tagged MYC2 (myc2-FLAG). We sprayed 15-d-old myc2-FLAG seedlings and sampled after 0 (untouched), 10, 20, 40, 60, and 180 min (Dataset S5). This enabled profiling of the speed of responses at the transcript and protein level, as well as subsequent analysis of MYC2 DNA-binding by chromatin immunoprecipitation sequencing (ChIP-seq).

We found 2,612 DEGs in at least 1 time point after water spray (2-fold change; P < 0.05) (Dataset S6). Coexpression analyses were performed to quantify and visualize the dynamic of the response over time (Fig. 3A). The list of 2,612 contains many known touch-responsive genes, including TCH2, TCH3, and TCH4 (Fig. 3B), as well as different genes of the JA-signaling pathway like OPR3, JAZ10, and MYC2 (Fig. 3C); 1,504 transcripts (60%) were up-regulated, and overall, up-regulation of genes was much stronger than down-regulation (Fig. 3B).

To further capture the dynamic of the response, we grouped water sprayer-responsive genes based on significant differential expression by at least 2406 (P < 0.05) at each single time point. Using these parameters, 48% of the genes were exclusively induced at a single time point, of which the majority were expressed at 25 min (Fig. 3C). The other genes were differently expressed at multiple time points, including the aforementioned TCH and JA-signaling pathway genes, which nevertheless showed similar highly dynamic transcriptional patterns (Fig. 3B and C). Of the genes differentially regulated at 25 min, 50%, 70%, and 90% retain up-regulated transcriptional levels by the 40-min, 60-min, and 180 time points, respectively (Fig. 3A and B), illustrating the fast and transient nature of the water spray transcriptional responses. To further organize the 1,504 up-regulated genes, we used their peak expression level to assign them into a single “peak expression time point.” The majority of genes showed peak expression by 25 min after spray treatment (Fig. 3F), including the TCH genes, OPR3 and JAZ10, but not MYC2 that peaked 10 min after spray (Fig. 3B and C).

The 2,612 water spray-induced genes comprised 10% TFs, while genes that showed peak expression 10 min after spray were 25% TFs (Fig. 3E), representing strong enrichments compared to the 5% TF genes in the Arabidopsis genome (Plant Transcription Factor Database, http://planttfdb.cbi.pku.edu.cn/). In total, 55% more TFs were transcriptionally up-regulated at random sampling (P < 0.0001), demonstrating that fast reprogramming of a complex transcriptional network may be an important step in the activation of the defense response of plants.

MYC2/MYC3/MYC4 appear to mostly regulate early-responsive genes, with over 75% of the 206 MYC2/MYC3/MYC4-dependent genes peaking at the first 25 min after stimulation (Fig. 3F). Two of the most highly affected TF genes in myc2a were BHLH19 and ERF109 (Fig. 3 and Datasets S3 and S5). We assessed transcript accumulation levels in a time-course experiment spanning the first 3 h in Col-0 and myc2a using qPCR, showing peak expression at 25 min, which was severely impeded in the myc2a background (Fig. 3J).

Between our 2 RNA-seq experiments, 1,773 transcripts were water spray-responsive in both datasets. When combined, the 2 datasets comprise a total of 3,440 spray-responsive transcripts that we can define as water-spray-induced. At least ~10% of the 33,602 Arabidopsis genes (TAIR10), when combined
with 2 previously published touch-related transcriptome analyses (13, 15), a total of 2,944 mechanical stimulation-responsive transcripts can be obtained, with 1,671 transcripts (42.6%) represented in at least 2 of the 4 transcriptome datasets (SI Appendix, Fig. S5D). This so-called “core mechanical stimulation transcriptome” represents ~5% of the Arabidopsis genome and contained all 44 genes induced over 100-fold after water spray in Col-0 (SI Appendix, Fig. S5B and Dataset S7).

MYC2 Orchestrates a Hierarchical Regulatory Network. To gain genome-wide insight into the mechanical stimulation-regulated MYC2 signaling network, ChIP-seq was performed in the MYC2-KR MYC2-FLAG seedlings untreated and 25 min after water spray. Overall, 1,256 peaks were mapped to the vicinity of a coding sequence. Some peaks could be assigned to multiple adjacent genes, so in total 1,681 transcripts were represented (Dataset S8). Overlap of these 1,681 transcripts with all transcripts significantly altered by the myc2-24 mutation in the RNA-seq (P < 0.05, 899 transcripts) and our previously defined core mechanical stimulation transcriptome (1,671 transcripts) resulted in a total of 52 genes (SI Appendix, Fig. S5D) that we defined as the core MYC2 mechanical stimulation region. This region contained known direct MYC2-target genes such as JAZ2, LOX2, LOX3, OPR3, AOS1, AOC2, SULFOTRANSFERASE 16 (SOT16), and ORA47 (31, 41, 55, 56). Importantly, many previously unknown MYC2-targeted TF genes, as well as JA-signaling JA-hormesis, stress-related, and regulatory genes were identified (Dataset S9). About one-quarter (23 genes) of the region consisted of TFs (Fig. 4A), of which 17 are also JA-inducible, including bHLH19, ERF109, ZAT10, and ORA47. Overall, 74.3% (66 transcripts) were inducible by JA, including several JAZ proteins and JA biosynthesis genes (Fig. 4A and Table S2).

The effect of the myc2-24 mutation varied among the region genes and many of the previously identified MYC2-target genes were among the most affected transcripts (Fig. 4B). Of 26 genes that showed 50% or more reduced expression levels, 8 encoded TFs (Fig. 4B). Clustering the region TF genes with known MYC2-regulated genes using the water spray transcriptome analysis datasets bHLH19 with JAZ20, SOT16, and LOX3 and ERF109 in a clade with WOX18 (Fig. 4C). Of the 44 genes that are over 100-fold induced by spray after 25 min, 30 (68%) are MYC2-dependent based on the RNA-seq or ChIP-seq results (SI Appendix, Fig. S5D). In addition, 36 of the region genes were present in the 230 mechanical stimulation genes identified in all 4 RNA-seq experiments (SI Appendix, Fig. S5A).

To further establish a direct role for MYC2 in TF gene activation, we created fusion LUCIFERASE (LUC) reporter constructs with the promoter regions of the 9 selected MYC2/MYC2/MYC2-dependent touch TF genes (Dataset S8) for transient transactivation assays with MYC2 in tobacco protoplasts. A JAZ-inhibition derivative version of MYC2 (MYC2Δ359, 26) was used as well. The promoters of ORA47, ZAT10, ERF109, and bHLH19 could be directly activated by both MYC2 and MYC2Δ359 (Fig. 5A), supporting the myc2-24 RNA-seq results (Figs. 4A and S8B). qPCR results (Fig. 2E) and ChIP-seq results (Fig. 5C). Analysis of the upstream regions of bHLH19, ERF109, ZAT10, and ORA47 showed the presence of 1 or more G-boxes (CAGG) in their promoters (Fig. 5D and Dataset S10). None of the other tested promoters could be markedly activated by MYC2 (SI Appendix, Fig. S6). We then assessed if the selected promoters could be induced by bHLH19 or ERF109. A significant over 2-fold transactivation (P < 0.0005) could be observed for pORA47 by bHLH19 (SI Appendix, Fig. S7). These combined experiments delineated the core gene set directly regulated by MYC2 in response to mechanical stimulation and identified bHLH19 and ERF109 as direct target genes of MYC2, and ORA47 as a target gene of bHLH19.

Water Spray-Responsive Expression of ERF109 and bHLH19 Depends on JA. Despite an established link between touch and JA, the extent to which the mechanical stimulation transcriptome responds to JA is not known. Combining 2 published large-scale analyses on
Fig. 1. The dynamic profile of the water sprayer transcription. (A) Coexpression analysis of the 2,412 genes that are at least 2-fold and significantly (*P < 0.05) changed in at least 1 of 5 time points after spray treatment compared to the untreated seedlings. Average linkage clustering with Pearson correlation was used. The bar on top shows the log10 scale. Blue and yellow denote down- and up-regulation, respectively. B and C. Selection of genes from the RNA-seq dataset involved in the touch-response (B) and the JA signaling pathway (C). Asterisks indicate statistically significant differences (see Methods) compared to 0 min (P < 0.05 and 2-fold change). D. B shows clustering of the 2,412 water sprayer-regulated genes showing the number of dynamic genes that are up-regulated and down-regulated. The x-axis shows the different time points. The y-axis represents log10 transformed fold-induction. E. Venn diagram showing the dynamic of genes between the selected time points, for each time point, all genes that are at least 2-fold and significantly (*P < 0.05) changed were selected. F. The diagram showing the distribution of down-regulated genes (blue) and up-regulated genes (shades of yellow). For the letter, percentages of genes with maximum fold induction at each time point within the 2,412 water sprayer-regulated genes is given. G. Fraction of known TEs within defined collection of genes as indicated in F. Abbreviations: JAZ, Jasmonic acid 2M, OX1, 12-oxophytodienoic acid reductase; TCI, touch; (E) Coexpression analysis using the time course RNA-seq data of the 264 genes affected by myc234 (Fig. 1). The color scale shows log10-transformed values with blue and yellow representing down-regulation and up-regulation, respectively. (F) qPCR showing the effect of myc234 at different time points after spray for ERF019 (Upper) and ERF009 (Lower). The y-axis represents normalized fold induction compared to untreated (RT) Co0 (set to 1). Error bars are SEM (n = 5). Statistical significance was determined by the Student’s t test (**P < 0.05, ***P < 0.005, ****P < 0.0005).

(Me)JA-treated seedlings resulted in 4,309 unique (Me)JA-responsive transcripts (57, 58). Overlay of the combined 2,904 mechanical stimulation-responsive genes with the 2,904 (Me)JA transcription factors (3,859 transcripts) yielded 402 (1,356 transcripts) transcripts responding to both JA and mechanical stimulation. Similar, 20.9% of the JA-activated transcription factors were mechanical stimulation-responsive (Fig. 4d).

We tested the water sprayer-inducible expression of ERF009 and ERF019 in a range of JA-related mutants. In the JA-activated mutant coi1-1, spray-induced expression of both genes was nearly absent (Fig. 5a). In contrast, spray-induced transcript accumulation levels of TCI125/94 were CAO-independent (Fig. 5b). As was shown previously (29), the JA-related mutants opr1, stl1, and ERF009 expression was significantly affected (Fig. 5c) compared to Col-0. Analogously, in the opr1 mutant that cannot convert JA into its bioactive form jasmonate-3,5-diol (JA-3,5), water sprayer-induction of ERF019 was almost completely absent (Fig. 5b).

Finally, based on a literature search (30, 60) and screening patents DNA affinity purification cDNA library for ERF009 target genes, we have selected several mechanical stimulation-inducible genes and assayed their water sprayer-inducible transcript accumulation in a range of mutants (Fig. 4). Neither the basal nor sprayer-induced expression of the selected genes was significantly affected by the opr1 mutation (SI Appendix, Fig. 5d). This suggests that other additional factors acting redundantly to ERF009 must be active or that in the context of water spray, these genes are not targeted by ERF009.

Time-Resolved Proteomic of the Water Spray Response. To assess dynamic changes during touch responses in plants at the protein level an in-depth peptide mass spectrometry (MS) analysis was performed on the same sample sets used for the RNA-seq time-course analysis. For 12,413 proteins, 1 or more peptides were found in at least 1 biological replicate (n = 4) of at least 1 time point, with 4,243 proteins considered as reliably quantified. Of these, 347 proteins were significantly altered in abundance compared to the untreated samples (P < 0.05, 1.5-fold change) in at least 1 time point based on spectral counting (Fig. 5e and Dataset S1). Of the proteins, 159 were more abundant at their peak value, while 188 were less abundant. Different overall abundance patterns could be identified, ranging from rapid increase (cluster A) or decrease (clusters C and D), over initial increase with recovery at 40 to 60 min (clusters C and F), to later transient decrease (cluster B).

Protein levels of the JA-biosynthesis gene product ACO1 and the JA-signaling component ABABIDOPSIS SKP1 HOMOLOG1 (ASK1), which interacts with CO1 in part of the SCF-complex

Van Meerwater et al.
(62), were responsive to spray. Redox-related proteins are overrepresented in the proteins changing in abundance, compared to the proteins as a whole, including 6 flavonolins, 4 glutaredoxins, and 6 peroxidases. Glutathione peroxidase GPX2 increased 5-fold after 40 min, while GPX1 and GPX5 decreased in abundance after 10 min and recovered to pre-spray levels by 40 min. Ten protein kinases, including MITOGEN-ACTIVATED PROTEIN KINASE (MPK) 3, calcium-dependent protein kinases, and receptor-like kinases, and 11 protein phosphatases showed alterations in protein levels, indicating that phosphorylation cascades are likely to be important during early mechanical stimulation responses.

A targeted analysis of peptides derived from proteins whose transcripts were identified as being spray-responsive was conducted.
by manual curation of full-scan MS data (MSI analysis). Fig. 7B shows a comparison of protein and RNA levels of representative proteins that were identified as differentially during the water spray time course. JA-hyosynthetic enzyme (OPCL) and cold-responsive KIN2 showed a positive correlation between transcript induction and protein levels, while LIGHT HARVESTING COMPLEX PHOTOSYSTEM II (LHC-EL-2; ATFG80809) was transcriptionally down-regulated and the protein level fell. However, several proteins showed a more negative correlation, with transcript increasing while protein levels fell. For example, for ERP165, UDP-GLUCOSYL TRANSFERASE (UGT-74D1), and MAPK3.

MYC2/MYCC/MYC3 Regulate Water Spray-Induced JA Accumulation.

About 40% of the mechanical stimulation transcriptome is JA-responsive (Fig. 6D) and JA is reported to accumulate after touch (7). Further inspection of seed-removed hormone metabolism pathway genes showed that 10 of 26 JA pathway genes (38%) were water spray-responsive (Dataset S12). Furthermore, 4 of 14 ABA pathway genes (9%) and 9 of 17 indole-3-lactic acid (IAA) pathway genes (53%) were water spray-responsive (>2-fold up or down; P < 0.05) (Dataset S12). Half of the water spray-responsive JA pathway genes were dependent on MYC2, whereas none of the sprays-responsive IAA and ABA pathway genes were MYC2-dependent (Fig. 8A–C, SI Appendix, Fig. S9A–C and S10A–C, and Dataset S12). Thirteen of the JA biosynthesis and degradation genes were identified in the ChIP-seq using MYC2 (SI Appendix, Fig. S8).

Therefore, we performed hormone accumulation profiling on a time course spanning the first 3 h after water spray in Col-0 and myc2-24 seedlings. We also measured different active forms, precursors, conjugates, and degradation products of JA, auxin (IAA), cytokinin (CK), salicylic acid (SA), and ABA (Dataset S13). Levels of GA4 were below detection limit using our conditions, and ethylene was not included in the analysis. No difference in CKs between genotypes or in response to water spray could be observed (Dataset S13).

In untreated conditions (UT), there were no significant differences between Col-0 and myc2-24 for ABA (SI Appendix, Fig. S9J), JA, and its precursor cis-6-β-carotene-3-oxo-acid (c-OPDA) (Fig. S10D). In contrast, IAA levels were significantly reduced by 58% (P < 0.05; n = 5) (SI Appendix, Fig. S10D) and JA-lec levels could not be detected in myc2-24 compared to Col-0 (Fig. S10D). In response to water spray, IAA levels were unchanged but ABA levels dropped in Col-0 (SI Appendix, Figs. S9O and S10O) for the most highly up-regulated ABA pathway gene, CYP707A3, touch without spray would induce expression up to 56-fold, indicating the effect was not merely evoked by water for this gene as well (SI Appendix, Fig. S9E).

Interestingly, JA and its active conjugate IA-A-lec transiently peaked 25 min after touch in Col-0, restoring to near untreated levels after 3 h (Fig. S2D). The JA precursor cis-OPDA remained stable after water spray in Col-0. Significantly different patterns were found in the myc2-24 mutant. After water spray, cis-OPDA levels in myc2-24 were significantly lower than Col-0, pointing to transcribed MYC2/MYCC/MYC3-dependent activation of OPDA-2 oxidizing enzymes, such as LOX, AOC, and AOS. Whereas JA levels between Col-0 and myc2-24 were similar at UT and 10 min, the major peak of JA at 25 min in Col-0 in missing in myc2-24 (Fig. S2D). JA-lec levels are drastically lower at all times points measured in myc2-24 (Fig. S2D). Independently of the myc2-24 mutations, levels of the IAA-precursor tryptophan (Tyr) were reduced after water spray, although not significantly for the majority of time points. Interestingly, IAA and its conjugate IAA-asparate (IAA-Aip) were depleted in a water spray-independent manner: in myc2-24 compared to Col-0 (SI Appendix, Fig. S10D).
Overall, reduced levels of IAA and IAA precursor conjugates, and degradation products were measured in at least 1 time point of the touch time course, indicating that MYC2/3/4C MYC4 also modulate overall IAA metabolism. This is supported by subtle but significantly reduced levels for ANTHOCYANIN SYNTHESIS 1 (AS1) in myc2H compared to Col-0, independent of touch (Dataset SI 2) as previously reported (28). These analyses point to clear differences in hormone profiles when it comes to both water spray response and the contribution of MYC2/3/4C MYC4.

Discussion

Water Spray Invokes Major Dynamic Transcriptome and Proteome Changes through a Regulatory Network of Transcription Factors. Mechanical stimulation triggers a wide-spread transcriptional response. Our RNA-seq datasets were consistent with 2 published transcriptomic datasets (15, 16), with 230 genes in all 4 and 1,671 genes in 2 of 4 datasets differentially expressed. The increased time resolution of our dataset allowed for a more dynamic dissection. Over 700 genes respond to the water spray treatment within 10 min. Most of these genes continue to increase in expression, peaking at 25 min, returning to near unaltered levels within 1 h, including HLS19, ELF19, and TCH129. Only very few of the DEGs peak at 10 min with unclear representation of TFs such as MYC2 and ORA47, suggesting a transcriptional network is being initiated rapidly. Nearly half of the DEGs are differentially expressed at a single time point, illustrating the transient nature of this regulatory network and its implication for the transcriptional response. Accordingly, proteomic analysis revealed that the abundance of over 300 proteins was altered at least 1 time point after water spray. Several kinosomophasases were identified, confirming the importance of phosphorylation cascades in mechanical stimulation-generated signaling (50). In addition, involvement of PKs seems to play an important role with many peroxidases, thioredoxins, and glutaredoxins being altered in abundance after water spray. This is in line with previous reports of induced ROS bursts (12).

As a central JA, JA-1 response regulator, a critical role for MYC2 in jasmonate and wound response is well documented (53). Importantly, MYC2 and its paralogs MYC3 and MYC4 are also reported to be involved in the regulation of flowering time (17, 34), a hallmark feature of the touch response. However, their involvement in mechanistic stimulus-induced gene expression changes had been investigated to date. Here, we have assessed the genome-wide action of MYC2 in response to water spray through RNA-seq on myc2H, MYC2-targeted ChiP-seq, and promoter transactivation assays. The RNA-seq and ChiP-seq data combined showed that MYC2 (in addition to MYC3/4) directly controls the majority of the most water spray-responsive genes. MYC2/MYC3/MYC4 regulate in particular early-response genes, while MYC2 gene expression itself peaks 10 min after water spray. This further supports the concept that MYC2 activates a set of mRNAs in the first stimulus events following touch. Generally, our analyses support a preference for MYC2 to directly regulate other TFs, which is largely in agreement with a recent study in tomato (54). Overall, both JAs and MYC2 are necessary for coordinate responses.

MYC2 Regulates JA Biosynthesis and Hormone Levels. Previous reports have shown the importance of JA and the JA-signaling components JAR1, COII, OPP3, and AOS in thigmomorphogenesis (7, 26, 24, 35). However, it was unknown to what extent JA affects the transcriptional responses to mechanical stimulation. Our results show that ~30% of the water spray-induced genes are JA-responsive, whereas a clear non-JA-dependent circuit exists, exemplified by JA- and COII-independence of the FC31 genes (5, 57). Accordingly, at the protein level the JA biosynthesis enzymes OPL1 and AOC2 were found to be differentially abundant in response to water spray, underlining the importance of JA.

Some of the most striking effects of MYC2/MYC3/MYC4 were observed by hormone profiling. Although JA levels have previously been shown to be induced by touch and wounding in different species (7, 18, 19, 38, 39), the direct role of MYC2 on hormonal levels had not been described before. Our hormone analysis shows that the water spray-induced accumulation of JA and JA-Ele is largely dependent on MYC2/MYC3/MYC4.

Van Meerembe et al.
Whereas an initial increase in JA and JA-Ile levels is observed in both Col-0 and myc2-1, the large boost in JA and JA-Ile accumulation in Col-0 is completely absent in myc2-1. This correlates with the widespread MYC2/MYC3/MYC5-dependent up-regulation of JA biosynthetic genes after water sprays and is further supported by direct binding of lower than half of the JA metabolism genes promoter (12 of 26) by MYC2. JA and JA-Ile levels peak at 25 min and drop strongly by 40 to 60 min, which could be the result of enzymatic degradation of the active hormone, and thus attenuation of the JA signal. This is supported by earlier peak expression for JA-inducible genes like, for example, \textit{LOX}34 and \textit{OPCL1}, compared to JA-catabolism genes, such as \textit{JASMONIC ACID OXIDASE 2} (\textit{JA02}) and \textit{JA04} (70). Interestingly, \textit{JA02} and \textit{JA04} are also directly bound by MYC2 in our ChIP-seq analysis, indicating that in addition to JA biosynthesis, JA turnover appears transcriptionally regulated by MYC2 as well.

In conclusion, this study provides a high-resolution landscape of the transcriptional, hormonal, and proteomic effects of water spray in \textit{Arabidopsis}. It clearly shows the direct role of JA and the MYC2/MYC3/MYC5 TFs in the regulation of a large proportion of the transcriptional changes, both by directly setting up a second-order dependency of TFs in motion and directly controlling JA metabolism. Notably, however, this JA- and MYC2/MYC3/MYC5-dependent TF network does seem to modulate other classic touch marker genes, such as \textit{TCH3} and \textit{TCH4}, meaning that additional touch-induced signaling pathways await discovery.

\textbf{Methods}

\textbf{Plant Material and Treatment.} The \textit{myc2} myc3 \textit{myc2} myc3 \textit{myc2} \textit{myc3} and \textit{col-16} mutant lines have been described previously (30, 71) and were a kind gift from Roberto Solano, Consejo Superior de Investigaciones Científicas (CSIC), Madrid, Spain. \textit{apt-1} was kindly donated by Kirk Overmyer, University of Nebraska, Lincoln, Nebraska, and the red mutants were kindly provided by Elizabeth Kasschau, Washington University in St. Louis, St. Louis, MO. The mutant line \textit{erf102} (SALK, 156136C) originates from the Nottingham Arabidopsis Stock Centre and the \textit{myc2} MYC2/MYC3/FLAG line was described previously (72).

\textit{Arabidopsis} seed were dry-stabilized overnight in commercial bleach (1:3 dilution in water containing 1% H2O2), sown on 0.5x Murashige and Skoog media (including vitamins), 0.1% 2,4-D, 0.01% 2-methylbutanoic acid, pH 5.8, 0.7% Phytagel plates, and stratified for 2 to 3 d at 4 \degree C, after which the plates were transferred to standard growth conditions (16\,h, 16\,h light dark regim) for 10 to 14 d.

For transcript and metabolite analysis, seedlings were stimulated by spraying downward onto the plate from around 15 cm distance to tiles (depending on the size of the plate) with MilliQ water using a spraying bottle (Black/\textregistered). 100 mLs, multipurpose sprayer (cr. no. GLE6200), exact where specified. An example treatment is shown in Movie S1. The average droplet size of the spray was 2.15 \pm 1.2 \mu m as determined by microscopic sampling in silicon oil and measurement under a microscope. The water volume of 1 sprayer is around 625 \mu l, and we sprayed with enough force to allow the droplets to travel upward against gravity around 0.5 cm (implying an initial speed of about >3.0 m/s ignoring air frictions). Excess water was then drained.
Fig. 1. JAs-related metabolite and transcript profiling of the MYC2/MYCM14-dependent water spray response. (A) Venn diagram with selected JA biosynthesis genes (JA), MYC2/MYCM14-dependent genes (myc2/myc14), and water sprayer response genes (spray). The color code corresponds to the genes in B and C. In green are MYC2/MYCM14-dependent JA biosynthesis genes, in blue are water sprayer-responsive MYC2/MYCM14-dependent JA biosynthesis genes, and in pink are water sprayer-responsive MYC2/MYCM14-independent JA biosynthesis genes. (B) Pathways for JA biosynthesis and expression profiling of the genes avoiding the depicted enzymatic steps. Measured metabolites are boxed in green and the enzymatic steps are indicated. Visualization of the time-course expression analysis of the JA biosynthesis genes is depicted right-hand side of each enzymatic step. The size of the circle and the scale bar represents log10-transformed values. Red and blue denote up-regulation and down-regulation, respectively. (C) Hierarchical cluster analysis of the JA biosynthesis genes. Clustering was performed using transcript data of the time series shown in B as well as transcript data from Col-0 and myc2/myc14 seedlings untraced (ST) and 25 min after spray (25m). The scale bar represents log10-transformed values. Red and blue denote up-regulation and down-regulation, respectively. The color scale of the transcript is derived from the Venn diagram in A. Asterisks indicate genes identified by MYC2-ChIP (Dataset S2). (D) Accumulation of JA-OPDA, JA, and JA-Ile after water spray in Col-0 and myc2/myc14 seedlings. The y axis denotes (x+1)^{1/2} mean-centered. The x axis represents sampling time (0, 2, 5, 20, 40, 60, and 180 min after water spray). Black and colored asterisks indicate differences (Student’s t test, *P < 0.05, **P < 0.01, ***P < 0.001). ***P < 0.001)** with UT in Col-0 and myc2/myc14, respectively. Note that in myc2/myc14, JA-Ile cannot be detected. Grey asterisk indicates differences between Col-0 and myc2/myc14 at each time point (Student’s t test, P < 0.05). Some names can be found in Dataset S13.

Promoter Trans-Activation Assays in Tobacco Protoplasts. Transient promoter trans-activation assays in tobacco protoplasts were performed as described previously (7, 8).

ChIP-qPCR. Approximately 100 mg of myc2 MYCM14/FLAG or Col-0 seeds per plate were grown for 2 wk on Murashige and Skoog medium with 4% Suc and 0.8% Phytagel. Samples were spray-treated with 10 mM Brunt (a Tobacco ET77). In 10 mM NaOH, pH 7.4, and vacuum-infiltrated for 10 min. The vacuum was then released and re-infiltrated for 10 min. Next, formamide was replaced with 200 mM dithioerythritol and again vacuum-infiltrated for 10 min. Finally, the seeds were washed with distilled water, removed from the stock, and snap-frozen. ChIP experiments were performed as previously described (7, 14) with minor modifications. Approximately 100 mg of 2-wk-old myc2 MYCM14/FLAG and Col-0 seedling tissue was used. Experiments were conducted with antibodies against FLAG (F1804, Millipore Sigma). As a negative control, mouse IgG (545000000; Jackson ImmunoResearch) was used. Anti-FLAG antibody and IgG were coupled to 50 μl of protein G Dynabeads (100030; Thermo Fisher Scientific) in 50 μl and subsequently incubated overnight with equal amounts of sonicated chromatin. After overnight incubation, beads were washed twice with high salt buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100), low salt (50 mM Tris pH 7.4, 50 mM NaCl, 2 mM EDTA, 0.5% Triton X-100), and wash buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 2 mM EDTA). After elution, samples were cross-linked and digested with proteinase K digestion before the DNA was precipitated. ChIP-seq libraries were generated following the manufacturer’s instructions.