Running head: A two-phase mitochondrial stress response

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Research Area: Signaling and Response
Mitochondrial and chloroplast stress responses are modulated in distinct touch and chemical inhibition phases in Arabidopsis

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One-sentence summary:

Mitochondria and chloroplasts adapt to environmental changes via complex transcriptional regulation, with ANAC017 regulating chemical inhibition responses and WRKY factors modulating touch responses.
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Abstract

Previous studies have identified a range of transcription factors that modulate retrograde regulation of mitochondrial and chloroplast functions in Arabidopsis thaliana. However, the relative importance of these regulators and whether they act downstream of separate or overlapping signalling cascades is still unclear. Here we demonstrate that multiple stress-related signalling pathways, with distinct kinetic signatures, converge on overlapping gene sets involved in energy organelle function. The transcription factor ANAC017 is almost solely responsible for transcript induction of marker genes around 3-6 h after chemical inhibition of organelle function, and is a key regulator of mitochondrial and specific types of chloroplast retrograde signalling. However, an independent and highly transient gene expression phase, initiated within 10-30 minutes after treatment, also targets energy organelle functions, and is related to touch and wounding responses. Metabolite analysis demonstrates that this early response is concurrent with rapid changes in tricarboxylic acid cycle intermediates and large changes in transcript abundance of genes encoding mitochondrial dicarboxylate carrier proteins. It was further demonstrated that transcription factors AtWRKY15 and AtWRKY40 have repressive regulatory roles in this touch-responsive gene expression. Together, our results show that several regulatory systems can independently affect energy organelle function in response to stress, providing different means to exert operational control.
**Introduction**

As plants are sessile organisms, they must respond in an energy-efficient and effective way to cope with continuous change in the environment. Chloroplasts provide the energy equivalents to reduce CO₂ to carbohydrates via photosynthesis. Plant mitochondria are crucial to support energy metabolism, photosynthetic and photorespiratory functions, survival during dark periods and meristem activity (Ishizaki et al., 2006; Van Aken et al., 2007; Howell et al., 2009; Araujo et al., 2014). As mitochondria and chloroplasts are tightly involved in cellular metabolism, they are in a prime position to sense cellular dysfunction and are therefore thought to be one of the initial sites of stress recognition and can contribute to programmed cell death execution (Vanlerberghe, 2013). A significant body of evidence supports a crucial role for mitochondria and chloroplasts in perceiving and responding to biotic and abiotic stress conditions (Skirycz et al., 2010; Leister et al., 2011; Nomura et al., 2012; Zhang et al., 2014; Stael et al., 2015). This is further supported by several studies demonstrating that the transcriptomic response to mitochondrial or chloroplast dysfunction by chemical or genetic disruption appears to be part of broad biotic and abiotic stress responses (Schwarzländer et al., 2012; Van Aken and Whelan, 2012).

For mitochondria and chloroplasts to be involved in this cellular signalling network, communication from nucleus to organelle (anterograde) and from organelle to nucleus (retrograde) is essential (Ng et al., 2014). In terms of anterograde signals, it is known that nuclear genes encoding mitochondrial proteins respond to the diurnal cycle (Giraud et al., 2010), sugar content (Comelli et al., 2012), stress conditions (Van Aken et al., 2009) and germination cues (Law et al., 2012). On the other hand, retrograde signalling has been studied extensively by using chemical inhibition of organellar function (e.g. antimycin A, methyl viologen, norflurazon). Several studies showed that a subset of nuclear genes consistently responds to inhibition of mitochondrial function (Van Aken et al., 2007; Schwarzländer et al., 2012; De Clercq et al., 2013). This group of genes was termed the mitochondrial dysfunction stimulon (MDS) (De Clercq et al., 2013) and contains several genes that play important roles in stress tolerance in Arabidopsis thaliana (Arabidopsis). For example, the glycosyltransferase UGT74E2 targets auxins,
and upon overexpression triggers profuse shoot branching and drought tolerance (Tognetti et al., 2010). Also the classical mitochondrial stress marker gene alternative oxidase \( AOX1a \) was found to be important during osmotic stress (Dojcinovic et al., 2005; Dahal et al., 2014), and recently it was shown that the mitochondrial outer membrane ATPase AtOM66 (AtBCS1) is a regulator of cell death that controls pathogen resistance, senescence and drought tolerance (Zhang et al., 2014). Therefore, it is evident that energy organelle signalling is an important part of general stress responses, and understanding the way it is controlled could provide a means to enhance stress tolerance and thus productivity in crop species.

Over the past few years, regulatory proteins that are involved in retrograde responses in plants have been identified. Using a reverse genetic approach a number of transcription factors were identified, such as ABI4 (Koussevitzky et al., 2007; Giraud et al., 2009), AtWRKY15 and AtWRKY40 (Shang et al., 2010; Vanderauwera et al., 2012; Van Aken et al., 2013). In a forward genetic screen using the \( AOX1a \) promoter fused to luciferase, several genes that influenced plant retrograde signalling were identified. A cyclin dependent kinase CDKE;1 was found as a positive regulator of both mitochondrial retrograde signalling and chloroplast signalling (Ng et al., 2013a; Blanco et al., 2014). Additionally, a group of transmembrane NAC-type transcription factors was identified and shown to positively regulate mitochondrial retrograde signalling (De Clercq et al., 2013; Ng et al., 2013b), by binding a mitochondrial dysfunction DNA motif (MDM). Surprisingly, subcellular localisation studies showed that some of these NAC transcription factors are anchored in the ER by their transmembrane domain. This suggests that the NAC transcription factors are released from the ER upon stress perception and subsequently translocate to the nucleus. Furthermore, application of rhomboid protease inhibitors was able to partially suppress retrograde induction, indicating NAC transcription factors are released by proteolytic cleavage (Ng et al., 2013b). Inhibitors targeting ER and mitochondrial Ca\(^{2+}\) channels were demonstrated to enhance or reduce stress responsiveness of MDR genes (Vanderauwera et al., 2012), further pointing towards a role for the ER. It was also found that an antagonistic relationship occurs between retrograde and auxin signalling. Loss of transporters involved in auxin distribution such as PIN1 and BIG results in an enhanced activation of
mitochondrial retrograde response upon stimulation, while exogenous application of auxin suppresses retrograde signalling (Ivanova et al., 2014; Kerchev et al., 2014).

The relative importance of these regulators and whether they act downstream of separate or overlapping signalling cascades feeding into expression of common genes is still unclear. Here we demonstrate that multiple stress-related signalling pathways, with distinct time signatures, converge on overlapping gene sets involved in chloroplast and mitochondrial function. When plants are spray-treated with mitochondrial or chloroplast inhibitors, one expression phase related to inhibitory activity of the active compound peaks around 3-6 h post treatment, and is largely controlled by ANAC017.

Furthermore, a rapid phase of gene expression within 10 min of treatment also affects stress signalling target genes and correlates with rapid changes in metabolic substrate concentrations. We further demonstrate that this rapid phase is related to ‘touch’-signalling, and is modulated by WRKY transcription factors.
Results

NAC transcription factors involved in mitochondrial retrograde signalling show divergent expression patterns

Two previous studies identified five membrane-anchored NAC transcription factors as transcriptional regulators of plant mitochondrial retrograde signalling (De Clercq et al., 2013; Ng et al., 2013b), so the first aim was to clarify their relative contributions to retrograde signalling. To do this we assessed transcript abundance of NACs in 2-week-old seedlings treated with monofluoroacetate (MFA; a tricarboxylic acid (TCA) cycle inhibitor) for 6 hours (Figure 1). ANAC017 transcripts were most highly abundant in both untreated and stressed conditions, but showed no significant stress induction. Its closest homolog ANAC016 was almost 1300-times less abundant and was not further characterised in this study. ANAC013 was highly inducible, but even after induction transcript levels were still less abundant than ANAC017 in untreated conditions. Similar induction patterns were observed for ANAC053 and ANAC078: both were significantly induced by MFA, but did not reach the high transcript abundance of ANAC017.

Next, detailed time course experiments were performed with a wider range of stress-inducing factors: bacterial elicitor Flg22, UV and chemical inhibitors operating on complex III (antimycin A) and the TCA cycle (MFA) were applied or sprayed on 2-week-old seedlings, along with a mock spray treatment control. Samples were collected at a range of time points over 24 h, and mRNA abundance of the transcription factors and their previously identified target genes AOX1a and UGT74E2 were measured (Figure 1B). In accordance with the previous MFA experiment, ANAC016, ANAC017 and ANAC078 displayed no or very mild stress response. ANAC013, ANAC053 and the target genes AOX1a and UGT74E2 showed a clear induction after three hours of treatment. Transcript levels steadily decreased to almost basal levels 12 h and 24 h after treatment, except for the MFA treatment, which provoked longer-term induction. UV-treatment caused the most gradual rates of induction over time and in some cases expression increased at 1 h (Supplementary Figure 1).
To assess promoter activities, three-week-old GUS-based reporter lines were sprayed with antimycin A (Figure 1C). In untreated conditions the promoter of ANAC017 showed by far the strongest activity throughout the entire root and shoot of the plants.
but in accordance with its transcript levels no obvious changes after stress treatment were observed (a magnified image of ANAC017:GUS activity in root tips is shown in Supplementary Figure 1B). The promoter of ANAC013 showed low but discernible expression in young leaves and root tips. After stress treatment a clear induction in the leaves was observed, particularly in the youngest leaves, in line with a previous report (De Clercq et al., 2013). ANAC053 showed clear promoter activity throughout the root, but expression in leaf tissues was only observed after stress treatment. ANAC078 showed the weakest expression with some GUS staining in root tips, but no response to the stress treatment. Overall these experiments show that ANAC017 is the most strongly expressed, but was not induced by stress treatments.

**ANAC017 is a key regulator of responses to chemical mitochondrial inhibition**

To determine the effect of antimycin A treatment on the respiratory capacity of plants, dark respiration measurements were performed and showed no gross depletion of oxygen consumption after 4h of antimycin A treatment compared to untreated controls (Supplementary Figure 2B, p>0.50, n=4). This indicates that spray treatment with 50 µM antimycin A, while known to lead to cytochrome pathway inhibition and induction of AOX (Vanlerberghe and McIntosh, 1992; Ng et al., 2013b) is not a lethal chemical treatment, does not stop plant respiratory metabolism and thus represents a reasonable model for assessing signalling responses following a partial mitochondrial dysfunction.

Next, to assess the individual contributions of previously identified transcription factors in plant retrograde signalling (belonging to NAC and WRKY families, and ABI4), we collected a set of unpublished and previously published loss- and gain-of-function mutants (see Materials and Methods). This set of fifteen lines including wild type (Col-0) was subjected to antimycin A treatment. Samples were collected at 0, 0.5, 1, 3, 6 and 12 h post treatment in three independent pools of approximately 20 two-week-old seedlings per genotype to account for individual variations. Transcript abundance of target genes was measured using qRT-PCR (Figure 2). In untreated conditions overexpression lines of ANAC013, ANAC017, ANAC053 and ANAC078 already showed significantly elevated expression of AOX1a (p<0.05) compared to Col-0 (Figure 2).
Figure 2. ANAC017 is a key regulator of antimycin A-induced mitochondrial retrograde signalling. Two-week old in vitro grown seedlings were spray treated with antimycin A. Plants were collected in pools, relative mRNA levels were measured by qRT-PCR and normalised to Col-0 untreated samples (0h). For clarity, ANAC transcript levels in their respective overexpression and loss-of-function lines were not displayed. Coloured asterisks indicate statistically significant differential expression in a specific genotype vs Col-0 at the marked time point, white asterisks indicate statistically significant differential expression in Col-0 at the marked time point vs Col-0 0h (p<0.05; n=3).

AOX1a, left panel time 0). ANAC013 itself was significantly (p<0.05) induced in ANAC017, ANAC078 and ANAC013 overexpression lines (Figure 2, Supplementary Figure 2, ANAC013 panels). ANAC053 was significantly induced in ANAC017 and
ANAC053 overexpression lines (p<0.05) (Figure 2 ANAC053 panels, Supplementary Figure 2). Overall, these data suggest that all four NAC transcription factors upon overexpression have a basic capability of inducing mitochondrial retrograde signalling marker genes and some of its co-regulators.

By far the most drastic effect was observed in anac017 loss-of-function plants, where at the 3 h time point no significant induction of AOX1a (1.71-fold, p=0.47) was observed compared to Col-0 0h, as opposed to 10.4-fold induction in Col-0 (p<0.01) (Figure 2). anac017 loss-of-function lines also showed no induction of ANAC013 and ANAC053 at the 3 h time point, as opposed to 7.3-fold (ANAC013) and 2.9-fold (ANAC053) in Col-0 (p<0.05). This indicates that ANAC013 and ANAC053 are target genes downstream of ANAC017 in the signalling cascade, further supported by the observation that ANAC013 and ANAC053 are constitutively induced in ANAC017 overexpression plants (Figure 2). At 6 h in anac017 only a mild induction of AOX1a and ANAC013 was observed, which was significantly lower than in Col-0 (p<0.05). None of the loss- or gain-of-function lines for other NAC, WRKY or ABI4 transcription factors showed similarly strong reductions in antimycin A-induced target gene expression. In line with previous reports of ABI4 being a repressor of AOX1a expression, an increased expression of AOX1a was observed in abi4-1 at the 0 h time point (p<0.05) (Giraud et al., 2009).

To confirm the robust nature of the upstream signalling role of ANAC017 in antimycin A-induced gene expression, a repeat experiment was performed on plants grown in soil and Col-0 was also compared to two independent anac017 alleles (Supplementary Figure 2C). In agreement, both anac017 lines showed 4- to 6.5-fold reductions in AOX1a transcript induction at 3h (p<0.01, n=3). At 6h AOX1a transcript levels were already falling in the Col-0 plants, but showed a delayed increase in the anac017 lines that was well below the AOX1a levels reached in Col-0. Overall, these results indicate that ANAC017 is a key regulator of the transcriptional stress response to mitochondrial inhibition in seedlings grown in both MS media and soil, and it is also regulating induction of ANAC013 and ANAC053.

ANAC017 affects plant resistance to energy organelle inhibition
To consolidate that the role of ANAC017 extends beyond transcriptional responses, we performed a comprehensive phenotype analysis of all 15 NAC, WRKY and ABI4 lines for resistance to constitutive energy organelle function inhibition (Figure 3). To do this, the different plant lines were incubated on vertically positioned plates supplemented with antimycin A (complex III inhibitor), rotenone (complex I inhibitor) and methyl viologen (MV, superoxide generator that leads to damage in chloroplasts in lighted conditions). Plants were grown for 7 days and primary root length was measured (Figure 3). On control plates, a number of lines showed mild but significant growth reductions of between 13 and 30% compared to Col-0 plants. Antimycin A treatment resulted in significant growth reduction in all lines, and particularly anac017 plants were highly sensitive. In contrast, ANAC017 and ANAC013 overexpressing lines were significantly more tolerant to high concentrations of antimycin A. Rotenone had a milder effect on root length than antimycin A, but again plants overexpressing ANAC017 showed significantly higher resistance than Col-0, while anac017 plants were most susceptible. MV caused the most severe growth reductions (Figure 3). Again, plants overexpressing ANAC017 and ANAC013 showed a significant resistance to MV compared to Col-0, while the anac017 and anac013 loss-of-function lines showed increased susceptibility. On the other hand, AtWRKY15, 40, 63 and ABI4 have no discernible effects specific to any of the treatments. From these results, it is apparent that ANAC017 is crucial for optimal plant growth under conditions of organellar inhibition, and to a lesser extent also ANAC013 as reported previously (De Clercq et al., 2013).

**ANAC017 is also a regulator of chloroplast retrograde signalling**

Root growth characteristics showed a clear role for ANAC017 in resistance to antimycin A and rotenone. Interestingly, methyl viologen (MV) caused similar phenotypes in the gain- and loss-of-function lines of ANAC017 (Figure 3). MV is generally considered as a photosynthesis inhibitor that accepts electrons directly from thylakoid photosystem I (Fd subunit) and transfers them to molecular oxygen generating superoxide. In light conditions, MV is regarded as a chloroplast-specific ROS generator (Tsang et al., 1991). We sprayed 2-week-old Col-0 and anac017 seedlings with MV and assessed
the effects on gene expression (Figure 4). To confirm that MV did not cause a significant mitochondrial defect, the expression of mitochondrial dysfunction marker genes \textit{AOX1a} and \textit{AtOM66} was measured. The mock spray treatment caused mild inductions of both genes, however, no additional inductions were caused by the MV treatment (Figure 4). It was also evident that ANAC017 is required for the mock-induction at around 3 h. Based on an analysis of public microarray data, multiple marker genes were selected that respond more to MV treatment than mock (Kilian et al., 2007; Yoshida and Noguchi, 2009). qRT-PCR analysis revealed very high induction by MV of \textit{HSP17.6A} (approx. 80-fold) and \textit{HSP17.6CII} (approx. 250-fold) in Col-0, whereas no or minor inductions were caused in the mock treatment (Figure 4). Other genes such as the \textit{TINY} AP2 transcription factor, \textit{sHSP23.5}, \textit{AtWRKY30} and \textit{AtWRKY33} also showed several fold higher induction in MV treatment compared to mock in Col-0. In contrast, the MV-inducible genes were up to 10-fold less induced in \textit{anac017} plants compared to Col-0 (Figure 4). These experiments indicate that ANAC017 is also involved in the transcriptional response that is triggered by chloroplast-generated superoxide signalling in response to MV.
The further assess the role of ANAC017 in chloroplast retrograde signalling, Col-0 and anac017 plants were exposed to high light conditions (750 µmol s⁻¹ m⁻²). Samples were collected in biological triplicate and marker genes were tested using qRT-PCR (Supplementary Figure 3). HSP17.6 A and HSP17.6 CII were highly induced (> 1000 fold) by 1 h of high light in Col-0, while AtAPX2 was induced ~ 150-fold. To determine if the transcripts responded to temperature changes caused by the high light treatment or the light itself, an additional control experiment was performed where plants were incubated under normal light conditions but at a temperature matching that observed in the plates during high light treatment (28°C). From this it was clear that exposure to high light, rather than elevated temperature, was the main contributor to the gene expression increases observed. Mitochondrial stress marker gene AOX1a showed mild but significant high light induction (3-fold). Overall, anac017 plants showed no significant

Figure 4. ANAC017 is a regulator of chloroplast stress responses induced by methylviologen. Two-week old Col-0 and anac017 plants were spray treated with mock solution or 50 µM methylviologen. Pools of plants were collected in triplicate and mRNA levels were measured by qRT-PCR. Statistically significant changes in Col-0 caused by treatments are indicated (# p<0.05; ## p<0.01). Statistically significant changes of anac017 versus Col-0 within the same treatment are indicated (* p<0.05; ** p<0.01).
differences in high light-induced gene expression compared to Col-0 for any of the
marker genes tested, indicating that ANAC017 plays no significant role in high light-
induced signalling from the chloroplasts.

To clarify if ANAC017 plays a role in the previously described GUN (genomes
uncoupled) pathway of chloroplast retrograde signalling (Susek et al., 1993), Col-0 and
anac017 plants were treated with the carotenoid biosynthesis inhibitor norflurazon, and
the expression of GUN marker genes LHCB1.1 and LHCB2.4 was monitored
(Supplementary Figure 3B). As expected, transcript levels of LHCB2.4 and LHCB1.1
were strongly repressed within 3h of norflurazon application, but no genotype-specific
differences between Col-0 and anac017 plants were found.

Stress treatment by spray application induces a two-phase response in AtOM66
and WRKY transcription factors

The above experiments showed a clear role for NAC transcription factors –
especially ANAC017 – in retrograde responses to inhibitors around 3 h post-treatment.
Previous studies also identified AtOM66 (encoding an outer mitochondrial membrane
AAA ATPase) as a target for mitochondrial and chloroplast retrograde signalling, but
suggested it may be regulated differently to MDS genes such as AOX1a and UGT74E2
(Ho et al., 2008; Van Aken and Whelan, 2012). As AtOM66 was previously shown to be
regulated by WRKY transcription factors via direct binding of the AtOM66 promoter (Van
Aken et al., 2013), stress-related (antimycin A, MFA, Flg22, UV) WRKY expression
patterns were examined by qRT-PCR as described in Figure 1B (Figure 5,
Supplementary Figure 1). AtWRKY63 showed an expression pattern very similar to
AOX1a and ANAC013 with stress-inducible expression peaking around 3-6 h post
treatment for Flg22 and antimycin A, and 12 h for MFA. Surprisingly, a more complex
expression pattern was observed for AtWRKY40, AtWRKY15 and AtOM66, with a very
high level of induction by the 30 min time point (>380-fold for AtWRKY40, >100-fold for
AtOM66 and >10-fold for AtWRKY15) which rapidly declined by 1 h post treatment.
Following this initial transient peak, these genes showed a second phase of induction
similar in timing to AOX1a (Figure 1B, Figure 5). The mock treatment triggered a similar
induction of the first peak, but showed much weaker induction than the inhibitors around
This indicates that the spray treatment itself appears to be the main cause for the first peak, while the stress-inducing active compounds are mostly responsible for the second phase for both NAC and WRKY TFs. To determine the effect of TWEEN-20 on the early response, a repeat experiment using water without TWEEN-20 for the spray treatment was performed, and this showed similar highly-significant inductions within 25 minutes (e.g. AtWRKY40 was induced 226-fold, p<0.001) (Supplementary Figure 4). In conclusion, AtOM66 and its regulators AtWRK40 and AtWRKY15 display a two-phase
expression pattern, with a rapid peak at 30 min caused by the physical spray treatment, and a second peak around 3h or later caused by the sprayed compound.

*The rapid response peak is related to touch and wounding responses*

To investigate this 30 min peak in more detail, a mock treatment time course was performed and samples were collected every 10 minutes during the first hour, then at 3, 6 and 24 h in triplicate pools (Figure 5B). Significant induction of *AtOM66* (5.1-fold; p<0.02) and *AtWRKY40* (67-fold; p<0.005) was already observable by 10 min, with the highest expression level reached in the 20-30 min time points. *AOX1a* showed no significant induction at these early time points. Both *AtOM66* and *AOX1a* showed a mild but significant induction at the 3 h time point, possibly linked to the increased humidity and wetness caused by the spray treatment.

The rapid and highly transient nature of this early response could be caused by a number of different factors, including the mechanical stimulation of the spray treatment (similar to ‘touch’ responses) (Braam and Davis, 1990) or the changes in humidity and air composition by opening the petri dish plates. To investigate this, the effect of opening plates and mechanical stimulation by touching with blunt tweezers were each compared with the water spray treatment and a negative control. A similar control experiment was also performed on soil-grown plants. Samples of pooled plants were collected in triplicate after 25 minutes and mRNA expression levels of *AtOM66* and *AtWRKY40* were measured by qRT-PCR (Figure 5C). The touch and water spray treatments caused rapid and strong inductions both in soil and *in vitro*, while just transiently opening the lid that led to minor vibrations resulted in minor inductions. The water spray was the most uniform manipulation of the leaf surfaces of the plants and gave the highest fold changes overall.

A number of previous studies have looked at touch and early wounding responses using genome-wide expression studies. In Affymetrix ATH1 data set E-GEOD-48676, 5-week-old Arabidopsis leaves were wounded and collected 10 min after treatment. Although raw data files are publicly available on ArrayExpress, we could not find a published analysis. Therefore, we analysed the data in detail. In total, 1127 probe
sets of 22810 were significantly altered after wounding (>2-fold change, PPDE>0.95, Supplementary Table 1). 745 probe sets were up-regulated and 382 were down-regulated. In agreement with our observations in Figure 5A and 5B, *AtWRKY40* (131-fold), *AtOM66* (9.7-fold) and *AtWRKY15* (2.1-fold) were significantly induced within 10 minutes after wounding, with *AtWRKY40* being the second most induced gene across the genome. In contrast, *AOX1a* and *UGT74E2* were not induced significantly, which confirms the specificity of the response and similarity with our experiments. A study looking at changes in gene expression within 5 min of wounding also noted a significant induction of *AtWRKY40* using both Agilent microarrays and qRT-PCR (Walley et al., 2007).

In another study, 4-week-old plants were touched by gently folding back and forth the leaves, and not damaged (Lee et al., 2005). Samples were collected 30 minutes after treatment. The authors reported that expression of 589 genes was significantly touch-induced, while 171 were repressed. *AtWRKY40* (43-fold), *AtOM66* (10.7-fold) and *AtWRKY15* (4.3-fold) were also found to be significantly touch-induced, while *AOX1a* and *UGT74E2* were not. In conclusion, the rapid first peak observed in Figure 5 appears to reflect touch and wounding responses, which affects approx. 3-5% of the Arabidopsis transcriptome. This includes induction of several genes encoding mitochondrially targeted proteins, including *AtOM66*, as well as mitochondrial dicarboxylate substrate carriers (*DIC2*: 48-fold, *DIC1*: 21-fold, ppde >0.99), which are thought to provide the primary sources of reductant for mitochondrial function (Palmieri et al., 2008).

**Touch responses directly affect substrate levels and the expression of genes for transporters for energy metabolism**

Our initial interest in the early touch response was the strong induction of *AtOM66*, which encodes a mitochondrial protein. Touch responses caused by physical manipulation with water jets result in almost immediate mitochondrial and cytosolic Ca^{2+} spikes (Logan and Knight, 2003; Loro et al., 2012). This suggests mechanical manipulations may directly affect mitochondrial function. The touch- and wounding-related microarray expression data suggest that mitochondrial dicarboxylate carriers (*DICs*) are among the most highly induced genes (Lee et al., 2005). Therefore, we
tested if transcripts for the three Arabidopsis DIC genes are responding to the mock 403 spray treatment presented in Figure 5B. DIC2 was induced 91-fold, DIC1 was induced 17-fold and DIC3 was induced 25-fold (Figure 6). We also tested a chloroplast-located glucose-6-phosphate transporter GPT2, reported to be crucial for metabolic readjustment during dynamic acclimation (Athanasiou et al., 2010). GPT2 was 13-fold induced by touch treatment. Together, these findings suggest that transcriptional changes during touch responses are directed to modulate import/export of metabolic substrates from energy organelles.
To confirm if touch responses indeed affect mitochondrial metabolism, metabolite levels were analysed in the same samples used for the detailed mock spray time course used in Figure 5B (Figure 6). In line with the strong touch-induced expression of DIC transcripts, several of their major substrates including succinate and citrate decreased in abundance approximately twofold within 10 minutes (p<0.05). These metabolite levels started recovering after 30 minutes and returned to normal levels by 3-24 h. A number of amino acids such as L-alanine and L-threonine followed a similar trend, while other components such as glycerol showed increases towards the later time points. These results indicate that touch responses can affect mitochondria both through substrate availability and transcriptional signalling as part of a wider cellular response.

AtWRKY15 and AtWRKY40 are involved in regulation of touch-responses

To assess if the previously identified regulators could affect touch-related expression, AtOM66 expression was measured in the time course experiment with antimycin A treatment (Figure 2, Figure 7, n=3). At the 3 h time point, anac017 plants again showed a complete loss of AtOM66 induction, whereas none of the tested WRKY lines negatively affected AtOM66 induction. Similar reduced AtOM66 levels were observed during a treatment time course using two independent alleles of anac017 mutants grown in soil (Supplementary Figure 2C). However, loss of ANAC017 function had no effect on the touch-related peak at 30 minutes. This indicated that two separated signalling cascades were involved in these transcriptional responses. None of the transgenic lines tested above had a completely repressed touch-response peak, but our screen suggested that plants overexpressing AtWRKY15 and AtWRKY40 had an attenuated response (Figure 7A). Due to the highly transient nature of the touch response resulting in rapid changes in AtOM66 expression within minutes (see Figure 5), the inter-experiment variability caused these differences between AtWRKY15 OE or AtWRKY40 OE and Col-0 at the 30 min time point not to pass a standard unpaired t-test. However, within each of three experiments the AtOM66 expression values in AtWRKY15 OE and AtWRKY40 OE were consistently lower than in Col-0, and were found to show significantly reduced induction compared to Col-0 by a paired t-test (p<0.05).
To identify other regulators of the rapid touch responses, we carried out transcriptomic analysis focusing on the first 60 minutes following spray treatment. A range of mutants with previously described roles potentially relating to very early touch and wounding responses were included (see discussion for more details): touch-related plasma membrane Ca\(^{2+}\) channels *mca1 mca2*, extracellular ATP receptor *atdorn1-3*, *OXDORN1* (*AtDORN1* overexpressor) and *coi1* (coronatin-insensitive JA receptor). Two-week-old *in vitro* grown seedlings were mock spray treated and triplicate pools of plants were collected at 0, 30 and 60 min. mRNA abundance of touch-response marker genes was measured by qRT-PCR analysis (Figure 7B, Supplementary Figure 4). Overexpression lines of *AtWRKY15* and *AtWRKY40* (independent allele) were also included and again displayed a significant reduction of *AtOM66* expression at 30 min post-treatment (approx. 35% reduction; p<0.05). In contrast to our expectations, a significantly higher induction was found in *atdorn1-3* plants (approx. 50% increase; p=0.01), while the *OXDORN1* line did not show any significant differences (Figure 7B). Homozygosity of the T-DNA insertion in *atdorn1-3* was confirmed by PCR, and *AtDORN1* transcript levels were confirmed to be overexpressed in *OXDORN1* plants.
AtWRKY40 induction at 30 min was 50% reduced in AtWRKY15 OE plants (p<0.01), and 42% more induced in atdorn1-3 plants (Figure 7B). Also AtWRKY15 induction was significantly repressed in AtWRKY40 OE plants (approx. 55% reduction; p<0.05). The mca1 mca2 and coi1 plants showed no significant differences compared to wild type (Figure 7B). A mutant in gibberellin oxidase ga2ox7 that was recently reported to be involved in touch signalling (Joao Pimenta Lange and Lange, 2015) was also analysed, but no significant effect in transcriptional touch responses were observed compared to Col-0 at 25 min (Supplementary Figure 4). In conclusion, our results show that AtWRKY40 and AtWRKY15 are negative regulators of touch-induced transcriptional responses.
Discussion

Of several previously identified transcription factors involved in mitochondrial retrograde signalling in response to organelle inhibition, ANAC017 appears to be the main positive transcriptional regulator known to date. ANAC017 is most strongly expressed and appears to be epistatic over ANAC013 and ANAC053, as loss of ANAC017 abolishes their stress induction. This is in line with the direct binding of the ANAC013 promoter by ANAC017 as reported previously (De Clercq et al., 2013), and also the ANAC053 promoter contains a consensus ANAC017 MDM binding site. At 3 h post treatment the downstream target genes show an almost complete lack of induction in anac017 lines compared to WT plants. Some weak induction at 6 h is observed, which may be due to the partial redundancy with the other NAC family members, mainly ANAC013, ANAC053 and/or ANAC078. The later increase in signal may occur through a positive feedback loop by ANAC013 as it binds and stimulates its own promoter (De Clercq et al., 2013), which may take some hours to reach a noticeable threshold in the absence of ANAC017. As previously published, ABI4 appeared to have a significant repressive effect mainly on AOX1a in untreated conditions (Giraud et al., 2009). The role of WRKY transcription factors in response to mitochondrial inhibition is less clearly defined during the 3-6 h gene expression peak. This may be due at least in part to the large redundancy within the WRKY gene family, as previous studies have shown that more than 10 different WRKY proteins can bind the promoters of AOX1a and AtOM66 (Van Aken et al., 2013). For instance, AtWRKY40 is known to form a functional complex with related proteins AtWRKY18 and AtWRKY60 (Xu et al., 2006). Analysis of root growth also showed that ANAC017, and to a lesser extent ANAC013, provided the plants with enhanced resistance to inhibitors of organelle-function. Altered expression of a number of transcription factors resulted in reduced root growth (Figure 3), which likely reflected a perturbation of basal gene expression resulting in mild growth penalties to the plants.

Our previous analysis suggested that the role of ANAC017 in stress signalling is mostly confined to regulation of gene expression after mitochondrial function was inhibited at the level of Complex III (antimycin A), Complex IV (myxothiazol) and/or the TCA cycle (MFA) (Ng et al., 2013b). The experiments shown here suggest that the role of
ANAC017 may stretch further than just mitochondrial dysfunction, as shown by the very clear inhibition of stress signalling in response to chloroplast superoxide generator MV (Tsang et al., 1991). MV induces a transfer of electrons from photosystem I to oxygen in illuminated conditions, resulting in superoxide generation and oxidative stress that harms chloroplast function. The lack of mitochondrial stress marker gene induction (e.g. \textit{AOX1a} and \textit{AtOM66}) following treatment with MV compared to the mock treatment further supports that MV triggered signalling in the light is largely independent of mitochondria. Nevertheless, ANAC017 does not seem to mediate the signals for every type of stress that induces ANAC017 target genes. Stress-response triggers such as UV, salicylic acid, cold, ABA and Flg22 can also induce gene expression e.g. \textit{AOX1a} expression (Ng et al., 2013, this study). However, loss of ANAC017 did not significantly reduce the induction of \textit{AOX1a} to many of these triggers (Ng et al., 2013b). From our results ANAC017 is also not involved in every type of chloroplast retrograde response. High light stress causes a rapid induction within 1 h of many of the same target genes as MV (e.g. \textit{HSP17.6 A}, \textit{HSP17.6 CII}, \textit{TINY TF}, dual-targeted \textit{sHSP23.5}), however, loss of ANAC017 function did not significantly reduce these high light responses, nor did \textit{anac017} plants have a GUN-phenotype in our assays. Evidently, these genes are steered by separate pathways in the case of MV or high light, but have similar gene expression outcomes, providing an elegant example of convergent signalling. Previous reports also suggested a large overlap between target genes of ANAC017 and singlet oxygen-induced genes in the \textit{flu} mutant (Meskauskiene et al., 2001; Ng et al., 2013b). Our results indicate that both ANAC017 and \textit{flu}-mediated signalling may converge on multiple common target genes, but the signalling cascades that lead there appear to be distinct, shedding further light on the apparent cross-talk between different ROS stress signalling pathways (Laloi et al., 2007; Lee et al., 2007). Indeed, several of these genes contain consensus ANAC017 binding sites (CA(A/C)G) in their promoters, such as \textit{HSP17.6A} and \textit{sHSP23.5}.

Our results also raise the question of whether or not the ANAC017 pathway requires energy organelle dysfunction as part of the signalling event. Perhaps ANAC017 on the ER membrane receives inputs from many different upstream signalling pathways, of which organelar dysfunction, ER stress or unfolded protein responses may be some
examples (Senft and Ronai, 2015). To a large extent, the cell can distinguish the source of the stress despite the fact that ANAC017 is mediating multiple signals and overlapping target genes. This is evident from the observation that treatment with antimycin A or MV causes very different levels of induction of ANAC017 target genes: e.g. AOX1a and AtOM66 are 4-10 fold more induced by antimycin A than by MV, while HSP17.6A and HSP17.6 CII are 8-16 fold more induced by MV than antimycin A (Supplementary Figure 5, Figure 4). It appears that additional (currently unknown) factors may co-determine the specificity of ANAC017.

Unexpectedly, gene expression of mitochondrial and chloroplast proteins also responded to a much more immediate transient signalling cascade. This transient expression was initiated within 10 minutes of inhibitor application and appeared uncoupled from the organellar inhibition caused later on by the active compound. Again, overlapping genes with organelle inhibitor-induced expression were affected (e.g. AtOM66) but distinct pathways lead to activation, implying convergent signalling. This rapid response is very similar to mechanical manipulation signalling, such as touch and wounding responses, which induce Ca²⁺ spikes in mitochondria within seconds (Logan and Knight, 2003). Our control experiments showed that while water treatment induced the largest signalling responses, other mechanical processes also yielded significant responses (Figure 5). Overall the effects observed here appeared to be proportional to the degree of mechanical stimulation. Touch responsive gene expression was first described in the literature several decades ago (Braam and Davis, 1990), but only limited information on the genes that directly regulate these very rapid gene expression responses has been published. A number of studies suggested that plasma membrane located Ca²⁺ transporters MCA1 and MCA2 are involved in touch responses and loss-of-function mca1 mca2 mutants showed impaired mechanosensing (Nakagawa et al., 2007). An extracellular ATP (eATP) receptor AtDORN1, also on the plasma membrane, rapidly relays the presence of eATP (which is known to be released upon wounding) and triggers transcriptional responses (Choi et al., 2014) including induction of AtWRKY15, AtWRKY40 and AtOM66. Phytohormones are also thought to be involved in touch responsiveness and plants show altered morphology and pest resistance when touched on a regular basis, a process termed thigmomorphogenesis (Chehab et al.,
Jasmonic acid (JA) and gibberellic acid (GA) are thought to be main contributors to thigmomorphogenesis, but it is unclear whether JA or GA signalling is also involved in the very early transcriptional responses (Chehab et al., 2012; Joao Pimenta Lange and Lange, 2015).

Here we found that AtWRKY40 and AtWRKY15 may play repressive roles in the early touch response. Their transcripts are strongly induced by the touch treatment itself, suggesting that they may contribute to the rapid decline of mRNA expression between 30-60 min post-treatment. We selected 10 of the most strongly induced touch-responsive genes, as identified by Lee et al., 2004 (Supplementary Figure 6), for a more detailed promoter analysis, including AtOM66, DIC1, DIC2, AtWRKY40 and AtWRKY15. In line with a direct role for WRKY transcription factors in touch responses, all 10 genes contained W-boxes (TTGACY) in their 1 kb upstream promoter region (p<0.004) using the Athena tool (O'Connor et al., 2005). Accordingly, the promoter of AtOM66 was previously demonstrated to be directly bound by AtWRKY15 and AtWRKY40 (Van Aken et al., 2013). The presence of WRKY binding sites in stress responsive genes encoding energy organelle proteins has been described previously (Van Aken et al., 2009; Leister et al., 2011; Van Aken and Whelan, 2012). However, not all these genes exhibit touch-responsive expression patterns (e.g. AOX1a), suggesting that WRKY transcription factors play complex roles in multiple signalling cascades.

Although several of the genes induced by touch-responses play important roles in biotic and abiotic stress responses, the touch-response also targets energy organelle function and substrates for metabolism (Xu et al., 2006; Shang et al., 2010; Sheard et al., 2010; Zhang et al., 2014). All three DICs in Arabidopsis are highly induced by touch responses. DICs are the main transporters that allow substrates such as succinate, malate, oxaloacetate and 2-oxoglutarate to pass the inner mitochondrial membrane, in exchange for anions including phosphate, sulphate and arsenate (Palmieri et al., 2008). DICs can also function in exporting TCA intermediates as substrates for anaplerotic metabolism. For instance, DICs transport 2-oxoglutarate, which is important for anaplerotic pathways and nitrogen cycling, as well as malate for photorespiration and reductant exchange with chloroplasts. A metabolite analysis revealed that DICs...
substrates such as succinate and citrate are significantly reduced within 10 min of spray treatment, indicating that such rapid transcriptional responses occur to address a physiological change. Citrate is also known to trigger specific transcriptional response, but the target genes appear not to overlap with our findings, and the timeframe of 8h used in these experiments is different (Finkemeier et al., 2013).

Rapid (<1 h) transient changes in organic acid contents in Arabidopsis seedlings in response to CO₂ levels have also been reported, further showing that such dynamic responses in the metabolome are possible (Dutta et al., 2009). Recent metabolic studies have suggested a role for dicarboxylates in rapid respiratory responses to Pi starvation, and DICs are indeed induced within 1 h of transfer of plants to Pi depleted medium (Lin et al., 2011; Alexova et al., 2014). Rapid transcriptional responses within 10 minutes have also been found in response to high-light treatment, and involve chloroplast-located triose phosphate/phosphate translocator TPT (Vogel et al., 2014). Another gene related to energy metabolism and transport that is highly touch-responsive is the plastid glucose-6-phosphate translocator GPT2. GPT2 is involved in regulating transitions in metabolic states, with plants lacking GPT2 being unable to undergo dynamic photosynthetic acclimation under naturally viable conditions (Athanasiou et al., 2010). This further underlines the importance of short-term responses in maintaining plant energy organelle function.

Taken together, these findings suggest that rapid touch and wounding responses could prepare energy metabolism for a change in substrate availability, and potentially redirect nutrients and substrates from primary catabolic processes to secondary metabolism or biosynthesis. Touch-induced transcriptional responses are closely related to dark-shift responses (Lee et al., 2004) which also target AtWRKY40, DIC2 and AtOM66. Such a shift in light input could have dramatic effects on energy metabolism that need to be addressed quickly, perhaps by shifting to anaplerotic mitochondrial metabolism and usage of starch reserves, to allow sustained energy supply for the cell (Athanasiou et al., 2010). Plants thus seem to use overlapping signalling systems to respond to metabolic changes and biotic stress. Recent work showed that plants respond rapidly to even just the leaf vibrations caused by insect herbivores (Appel and Cocroft, 2014).
Such a mechanical signal can travel much faster through the plant than, for example, phytohormones. From this perspective, touch, wounding and dark-shift responses may all precede similar challenges, and thus initiating overlapping defence responses would have advantages for the plant.

**Conclusions**

In conclusion, we have found that multiple independent signalling pathways operate following a wide range of treatments that involve physical manipulations, changes in light intensity and inhibitor/elicitor treatments (Figure 8). The first gene expression peak within 10 minutes after physical manipulation or dark shifts is clearly related to pathogen defence signalling and appears to be regulated at least in part by WRKY transcription factors. Our study shows that this first expression phase may also facilitate rapid readjustments in energy metabolism and energy organelle functions, as plants anticipate or respond to changes in substrate or nutrient availability very soon after a manipulation. A second signalling system occurs around 3-6 h after chemical chloroplast or mitochondrial inhibition and is regulated to a large extent by ANAC017. This peak affects e.g. AOX1a, heat shock proteins, chaperones etc., and appears enriched in gene products primarily associated with repairing/maintaining the energy organelle machinery. This response may thereby prevent excessive ROS production during functional inhibition; rather than optimising substrate availability and usage. A third response triggered by excessive light, which causes damage to the photosystems, induces overlapping genes with the second pathway. However this high light pathway is ANAC017-independent and may at least partly be coordinated by the *flu/Executor and/or TPT* pathways (Meskauskiene et al., 2001; Lee et al., 2007; Vogel et al., 2014). Our results extend the idea that retrograde signals affect organelle biogenesis (biogenic control) or organelle performance (operational control) (Pogson et al., 2008), postulating that operational control involves separate signalling pathways for opportunity and risk (metabolic substrate availability and pathogen defence regulated e.g. by WRKYS) and for damage repair (organelle inhibitors, high light and ETC disruption regulated e.g. by NACs).
Figure 8. A working model for retrograde, high light- and touch-responsive operational control in plants. (A) High light treatment leads to chlorophyll over-excitation and reactive oxygen species (ROS) production. Signals are transduced to the nucleus possibly via flu/executer or triose-phosphate/phosphate translocator (TPT) signalling, leading to retrograde transcriptional responses. (B) Mechanical stress (e.g. by touch or spray application of an exogenous compound) triggers a transcriptional response within minutes. Studies suggest signalling molecules such as Ca²⁺, extracellular ATP (eATP), ROS, jasmonic acid (JA) and gibberellic acid (GA) are involved. This first 'touch' response is likely to be of key importance for stress recognition, successful pathogen resistance, morphological alterations and rapid readjustments of energy metabolism. A second, independent signalling network caused by the active inhibitor compound (e.g. affecting mitochondria and/or chloroplasts such as antimycin A (AA) and methylviologen (MV)) then launches a more general stress response, possibly dependent on ROS, via activation of NAC transcription factors present on the endoplasmic reticulum (ER). NAC target genes are at least partially overlapping with high light-induced genes, but different signalling pathways are involved.
Materials and Methods

Materials and growth conditions

Arabidopsis thaliana 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 (anac017: rao2.1), (anac017-2: SALK_022174) De Clercq et al., 2013 (anac013 amiRNA5.2, ANAC013 OE6, ANAC053 OE and ANAC078 OE), Van Aken et al., 2013 (AtWRKY15 OE1, atwrky40, AtWRKY40 OE2, atwrky63 and AtWRKY63 OE1), Giraud et al., 2009 (abi4-1), Choi et al., 2014 (atdorn1.3 and OXDORN1), Yamanaka et al., 2010 (mca1 mca2), coi1-1 was kindly provided by Dr Louise Thatcher (CSIRO, Floreat, Western Australia). We are grateful to Dr Gary Stacey (University of Missouri, USA), Dr Roberto Solano (Centro Nacional de Biotecnologia, Spain), Dr Kemal Kazan (CSIRO Agriculture Flagship, Australia) and Dr Hidetoshi Iida (Tokyo Gakugei University, Japan) to promptly share transgenic materials. Anac053 (SALK_009578), anac078 (SALK_025098) and ga2ox7 (SALK_055721C) were obtained from NASC. ANAC017 OE plants were generated by cloning the full length ANAC017 CDS into pB7WG2 (Karimi et al., 2002) using standard Gateway (Life Technologies) cloning strategies. For ANAC017, ANAC078 and ANAC053 expression levels were tested by qRT-PCR (supplementary Figure 2). The 1.5 kb promoter regions of ANAC013, ANAC017, ANAC053 and ANAC078 were amplified and cloned into pBGWFS7 (Karimi et al., 2002) via Gateway recombination, and transformed by floral dipping as described previously (Clough and Bent, 1998; Zhang et al., 2012). At least two independent lines per construct were analysed with comparable results. All primers used in this study are listed in Supplementary Table 2.

Stress treatments of plants

Seeds were sown on petri dishes containing MS media + 2% sucrose, stratified for two-three days in the cold room and then incubated in long day growth conditions for two weeks. Pools of plants were then collected before or after treatment and immediately placed in liquid nitrogen for storage and further processing. For transcript analysis...
plants were sprayed with mock (water + tween), 50 µM antimycin A, 50 µM monofluoroacetate, 1 µM Flg22, 50 µM methyl viologen or 20 µM norflurazon. For UV treatment, plants received 5 kJ/cm² UV radiation (254 nm) using a CX-2000 UV cross-linker (UVP Ltd). In vitro stress assays were performed as previously described using 50 µM antimycin A, 100 µM rotenone or 20 µM methyl viologen (De Clercq et al., 2013). Statistical analysis was performed using Student’s t-test. Additional control experiments for touch-related treatments were as follows: 1) unopened/untreated negative control, 2) plates opened for the same duration as spray treatment but otherwise untreated, 3) plates opened and gently mechanically stimulated by touching with blunt tweezers, and 4) spray treatment with water. A similar control experiment was also performed on soil-grown plants that were 1) untreated, 2) gently mechanically stimulated with blunt tweezers, and 3) spray treated with water. For high light treatment, in vitro-grown two-week-old plants were exposed to 750 µmol m⁻² s⁻¹ for 1h and compared to untreated control. Furthermore, to allow differentiation between high light treatment and associated increase in temperature, a heat control set was used that was exposed to normal light (100 µmol m⁻² s⁻¹) but incubated at 28°C (the temperature that was measured in the plates during the high light treatment). For root growth assays, the different plant lines were incubated on vertically positioned plates supplemented with 50 µM antimycin A, 10 µM rotenone and 50 µM methylviologen. Plants stratified in the cold room for 3 days and were for 7 days in long day conditions. Primary root length was measured using ImageJ. Statistical analysis was performed using student’s t-test.

**Promoter-GUS analysis**

The 1.5 kb promoter regions were cloned using standard Gateway (Life Technologies) procedures into pBGWFS7 (Karimi et al., 2002). 3 week in vitro grown plants were stained for GUS activity before and 6h after spray treatment with 50 µM antimycin A as described previously (Van Aken et al., 2007).

**Quantitative RT-PCR and Microarray analysis**

RNA isolation, cDNA generation and quantitative RT-PCR (qRT-PCR) was performed as described in (Van Aken et al., 2013) using Spectrum RNA Plant extraction kits.
(Sigma-Aldrich, Sydney), 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 2. Relative expression values were normalised, with untreated Col-0 samples set as 1. Statistical analyses were performed using student’s t-test throughout the manuscript, except where indicated. The E-GEOD-48676 microarray data set was obtained from ArrayExpress and analysed using Cyber-T as previously described (Zhang et al., 2014). Changes were considered significant at false discovery rate correction level of PPDE ($p >$0.95 and two-fold change.

**Metabolite Analysis**

Approx. 20-30 mg of frozen tissue from pooled two-week old plants grown in vitro were analysed in biological triplicate per time point after mock spray treatment using GC-MS. Full description of metabolite analysis is available in Supplementary Methods section. Data were imported and statistically analysed in Metabolome Express (Carroll et al., 2010).

**Acknowledgments**

We thank Dorothee Hahne of Metabolomics Australia for performing the GC-MS metabolite analysis and acknowledge the use of Metabolomics Australia facilities funded by National Collaborative Research Infrastructure Strategy (NCRIS) and the Government of Western Australia. Dr Sandy Vanderauwera for help with transgenic NAC lines.

**Author contributions**

OVA, JW, AHM, FVB and IDC designed experiments. OVA performed all experiments and data analysis, IDC isolated and genotyped *anac053* and *anac078* knockout lines, IDC and OVA cloned and created Promoter-GUS reporter lines for *ANAC017*, *ANAC053* and *ANAC078*, overexpression lines for *ANAC017*, and cloned ANAC053
and ANAC078 GFP vectors. Al optimised qRT-PCR assays for ANAC quantitation. OVA and AHM wrote the manuscript in collaboration with all co-authors.
Figure legends

Figure 1. Expression and of NAC Transmembrane transcription factors. (A) mRNA abundance of NAC transcription factors was quantified using qRT-PCR in 2 week old in vitro grown pooled Arabidopsis seedlings, before treatment and 6 h after treatment with 50 µM monofluoroacetate (MFA) (n=3; mean±SE). Expression levels were normalised to ANAC016 expression in untreated conditions (set to 1). * indicates significant induction of mRNA levels after MFA treatment compared to untreated control (p<0.05). (B) Two-week old in vitro grown seedlings were spray treated with Flg22, antimycin A, monofluoroacetate (MFA) or mock solution (water + tween). Pooled plants were collected, mRNA levels were measured by qRT-PCR (±SE) and normalised to untreated samples (0h). * p<0.05 vs 0h (n=3). (C) Promoter-GUS analysis of NAC expression patterns. The 1.5 kb promoter regions of selected NAC transcription factors fused to a GUS reporter gene was stably transformed into Arabidopsis Col-0 plants. 3 week old in vitro grown plants were stained for GUS activity before and after 6 h of treatment with 50 µM antimycin A.

Figure 2. ANAC017 is a key regulator of antimycin A-induced mitochondrial retrograde signalling. Two-week old in vitro grown seedlings were spray treated with antimycin A. Plants were collected in pools, relative mRNA levels were measured by qRT-PCR and normalised to Col-0 untreated samples (0h). For clarity, ANAC transcript levels in their respective overexpression and loss-of-function lines were not displayed. Coloured asterisks indicate statistically significant differential expression in a specific genotype vs Col-0 at the marked time point (p<0.05; n=3), white asterisks indicate statistically significant differential expression in Col-0 at the marked time point vs Col-0 0h (p<0.05; n=3).

Figure 3. ANAC017 is a determinant of organelle dysfunction tolerance. Seeds of different genotypes were grown vertically on MS media plates supplemented with antimycin A, rotenone or methylviologen. Primary root growth was measured after 7 days in the growth chamber (±SE). Significant differences with Col-0 within the same treatment are indicated (n>7, * p<0.05; **p<0.01; ***p<0.001).
Figure 4. ANAC017 is a regulator of chloroplast stress response induced by methylviologen. Two-week old Col-0 and anac017 plants were spray treated with mock solution or 50 µM methylviologen. Pools of plants (n=3) were collected in triplicate and mRNA levels were measured by qRT-PCR (±SE). Statistically significant changes in Col-0 caused by treatments are indicated (# p<0.05; ## p<0.01). Statistically significant changes of anac017 versus Col-0 within the same treatment are indicated (* p<0.05; ** p<0.01).

Figure 5. Spray treatment causes a two-phase stress response. (A) Two-week old in vitro grown seedlings were spray treated with Flg22, antimycin A, monofluoroacetate (MFA) or mock solution. Pooled plants were collected, mRNA levels were measured by qRT-PCR and normalised to untreated samples (0h). * p<0.05 vs 0h (n=3). (B) Two-week-old Col-0 plants were sprayed with water + tween (mock) and pools of plants were collected in triplicate. mRNA levels were measured using qRT-PCR (mean±SE) and normalised to time point 0h. (C) Two-week old in vitro or soil-grown seedlings were treated as indicated. Pools of plants were collected (n=3), mRNA levels were measured by qRT-PCR and normalised to untreated samples (0h). Statistically significant changes compared to untreated samples at time 0h are indicated (* p<0.05; ** p<0.01).

Figure 6. Touch responses are coupled to rapid changes in energy metabolism. Heat map of metabolite and transcript levels at indicated intervals (in minutes) after mock spray treatment, after hierarchical clustering (Pearson correlation and average linkage). Values represent fold changes normalised to 0 min time point. * indicate differential abundance during at least one of the time points (p<0.05). Transcript levels are indicated by red arrows to differentiate from metabolites (n=3).

Figure 7. AtWRKY15 and AtWRKY40 modulate touch-responsive gene expression. (A) Two-week-old in vitro grown seedlings were spray treated with antimycin A. Pooled plants were collected, mRNA levels were measured by qRT-PCR (n=3) and normalised to Col-0 untreated samples (0h). (B) Two-week-old seedlings of different genotypes were spray-treated and pools of plants were collected in triplicate before treatment and at the indicated times after treatment. mRNA levels were
calculated by qRT-PCR (±SE) and normalised to Col-0 untreated samples (0h).

Statistical significance is indicated by * (p<0.05), ** (p≤0.01), *** (p<0.001).

Figure 8. A working model for retrograde, high light- and touch-responsive operational control in plants. (A) High light treatment leads to chlorophyll over-excitation and reactive oxygen species (ROS) production. Signals are transduced to the nucleus possibly via flu/executer or triose-phosphate/phosphate translocator (TPT) signalling, leading to retrograde transcriptional responses. (B) Mechanical stress (e.g. by touch or spray application of an exogenous compound) triggers a transcriptional response within minutes. Studies suggest signalling molecules such as Ca²⁺, extracellular ATP (eATP), ROS, jasmonic acid (JA) and gibberellic acid (GA) are involved. This first ‘touch’ response is likely to be of key importance for stress recognition, successful pathogen resistance, morphological alterations and rapid readjustments of energy metabolism. A second, independent signalling network caused by the active inhibitor compound (e.g. affecting mitochondria and/or chloroplasts such as antimycin A (AA) and methylviologen (MV)) then launches a more general stress response, possibly dependent on ROS, via activation of NAC transcription factors present on the endoplasmic reticulum (ER). NAC target genes are at least partially overlapping with high light-induced genes, but different signalling pathways are involved.
Supplemental Data Files

Suppl. Table 1. Wounding microarray analysis

Suppl. Table 2. RT-PCR Primers

Suppl. Methods

Suppl. Figures 1-6

Suppl. Figure 1. UV-induced expression of retrograde-related genes.

Suppl. Figure 2. Confirmation of ANAC expression levels in transgenic lines, leaf respiration and antimycin A response.

Suppl. Figure 3. ANAC017 is not a regulator of high light- and norflurazon responsive gene expression.

Suppl. Figure 4. Touch responsive gene expression.

Suppl. Figure 5. Expression patterns of dicarboxylate carriers and HSP17.6 during antimycin A treatment.

Suppl. Figure 6. Promoter analysis of touch responsive genes.

Supplementary Figure Legends

Suppl. Figure 1. UV-induced expression of retrograde-related genes. (A) Two-week old in vitro grown seedlings were exposed to 5 kJ/cm² UV radiation or spray treated with antimycin A or mock solution (water + TWEEN-20). Plants were collected in pools at specified time points and relative mRNA levels of transcription factors and corresponding target genes were measured by qRT-PCR. * p<0.05 vs 0h (n=3). All expression values are fold changes normalised to untreated samples (0h). (B) Promoter-GUS analysis of ANAC017 expression patterns in root tips of 3 week old in vitro grown plants.
Suppl. Figure 2. Confirmation of ANAC expression levels in transgenic lines, leaf respiration and antimycin A response. (A) Expression levels of target NAC transcription factors in loss- or gain-of-function lines used in this study as determined by qRT-PCR. All values were normalised to Col-0. Error bars indicate standard error. (B) 2 week old soil-grown plants were left untreated (UT) or sprayed with 50 μM antimycin A. plants were collected after 4h and dark respiration was measured using a Clark-type oxygen electrode. No statistically significant differences were found between the two conditions (p>0.50, n=4). (C) 2 week old soil-grown plants were sprayed with 50 μM antimycin A. Pools of plants were collected in triplicate at the indicated time points and mRNA levels were measured using qRT-PCR and normalised to Col-0 untreated samples (0h). ** indicate statistically significant changes against Col-0 at 0h (** p<0.01). #, ## indicate statistically significant changes against Col-0 at the same time point. (## p<0.01; # p<0.05).

Suppl. Figure 3. ANAC017 is not a regulator of high light- and norflurazon responsive gene expression. Two-week-old in vitro grown seedlings of Col-0 and anac017 plants were treated with (A) 750 μmol s⁻¹ m⁻² of illumination and pooled plants were collected in triplicate (n=3) after 1 h or (B) sprayed with 20 μM norflurazon and pooled plants were collected in triplicate after 3h. Transcript abundance was measured using qRT-PCR and normalised to Col-0 in untreated conditions. Error bars indicate standard error. Statistical test vs Col-0 UT: Student’s t-test * p<0.08; ** p<0.05; ***p<0.01 (n=3); Mann-Whitney U rank-based test: # p<0.05.

Suppl. Figure 4. Touch responsive gene expression. (A) Pools of in vitro grown seedlings were spray-treated with water and collected in triplicate. mRNA levels were measured using qRT-PCR and normalised to Col-0 untreated. (B) Genotyping of AtDORN1 mutants with left (LP) and right (RP) genomic primers to check presence of WT AtDORN1 alleles, RP and SALK LBb1.3 T-DNA border primer to check presence of T-DNA insertion. (C) QRT-PCR measurement of AtDORN1 transcript levels in knock-out and overexpression lines. (D) Two-week-old seedlings of different genotypes were spray-treated and pools of plants were collected in triplicate. mRNA levels were...
measured by qRT-PCR and normalised to Col-0. Statistical significance is indicated by * (p<0.05), ** (p≤0.01), *** (p<0.001) (n=3).

**Suppl. Figure 5. Expression patterns of dicarboxylate carriers and HSP17.6 during antimycin A treatment.** Two-week old *in vitro* grown seedlings of various loss- and gain-of-function lines were spray treated with antimycin A. Plants were collected in pools at specified time points and relative mRNA levels were measured by qRT-PCR. All expression values are fold changes normalised to untreated Col-0 samples (0h). Black asterisks indicate significantly different expression in Col-0 vs Col-0 0h; green asterisks indicate significant differences between genotypes at the indicated time point: * p<0.10; ** p<0.05 (n=3).

**Suppl. Figure 6. Promoter analysis of touch responsive genes.** The 1 kb upstream promoter regions of 10 touch responsive genes were analysed by Athena (O’Connor et al., 2005). The top image represents locations in the promoter regions of statistically overrepresented W-box WRKYbinding sites. The table below shows the occurrence of all analysed promoter elements with respective p-values.


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