Running title: WRKY factors affect mitochondrial stress response

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AtWRKY40 and AtWRKY63 modulate the expression of stress responsive nuclear genes encoding mitochondrial and chloroplast proteins

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One-sentence summary:

Many nuclear genes encoding mitochondrial proteins are highly responsive to stress; WRKY transcription factors modulate expression of these genes via direct promoter binding and coordinate common stress responses caused by both mitochondrial and chloroplast dysfunction.
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Summary

The expression of a variety of nuclear genes encoding mitochondrial proteins is known to adapt to changes in environmental conditions and retrograde signalling. The presence of putative WRKY transcription factor binding sites (W-boxes) in the promoters of many of these genes prompted a screen of 72 annotated WRKY factors in the Arabidopsis thaliana genome for regulators of transcripts encoding mitochondrial proteins. A large-scale yeast one-hybrid screen was used to identify WRKY factors that bind the promoters of marker genes (Alternative oxidase 1a, NADH dehydrogenase NDB2 and AAA ATPase BCS1), and interactions were confirmed using electromobility shift assays. Transgenic overexpression and knockout lines for 12 binding WRKY factors were generated and tested for altered expression of the marker genes during normal and stress conditions. AtWRKY40 was found to be a repressor of antimycin A induced mitochondrial retrograde expression and high light induced signalling, while AtWRKY63 was identified as an activator. Genome-wide expression analysis following high light stress in transgenic lines with perturbed AtWRKY40 and AtWRKY63 function revealed that these factors are involved in regulating stress responsive genes encoding mitochondrial and chloroplast proteins, but have little effect on more constitutively expressed genes encoding organellar proteins. Furthermore, it appears that AtWRKY40 and AtWRKY63 are particularly involved in regulating the expression of genes responding commonly to both mitochondrial and chloroplast dysfunction, but not the genes responding to either mitochondrial or chloroplast perturbation. In conclusion, this study establishes the role of WRKY transcription factors in the coordination of stress-responsive genes encoding mitochondrial and chloroplast proteins.
Introduction

The expression of many nuclear genes encoding mitochondrial and chloroplast proteins is highly adaptive to changes in the environment. Furthermore, it is suspected that interplay exists between the regulatory mechanisms of both organelles. Nevertheless, very few transcription factors have been isolated that regulate transcripts of nuclear genes encoding organellar proteins, (for the remainder of the manuscript we refer to genes for organellar proteins that are encoded in the nucleus, unless otherwise stated). Examples of transcription factors that regulate genes encoding mitochondrial proteins are the B3 domain transcription factors ABI3, FUS3 and bZIP53, which are important for regulating expression of FeS proteins of Complex II (Roschztardtz et al., 2009). Also, a class of TCP transcription factors were found to regulate the expression of genes encoding mitochondrial proteins during the diurnal cycle (Giraud et al., 2010), while the CCA1 transcription factor was found to regulate diurnal control of chloroplastic genes such as thioredoxins (Barajas-Lopez et al., 2011). Also for chloroplasts, the GATA-type transcription factors GNC and CGA1 were shown to modulate the expression of genes encoding chloroplast proteins such as GUN4 and HEMA1 (Hudson et al., 2011). One example of a transcription factors that is known to regulate genes encoding both mitochondrial and chloroplast proteins is ABI4 (Abscisic Acid Insensitive 4; Koussevitzky et al., 2007; Giraud et al., 2009). ABI4 appears to be on the interface of external signals (such as ABA and sugars) and retrograde signals coming from chloroplasts and mitochondria.

A previous study identified a set of nuclear genes encoding mitochondrial proteins that are highly responsive to a wide range of stresses (Van Aken et al., 2009). These genes included alternative respiration pathway components such as alternative oxidase 1a (AOX1a) and NADH dehydrogenase B2 (NDB2), AAA ATPases (BCS1), mitochondrial substrate carriers, heat shock proteins and a variety of other functions. Past studies showed that a variety of signals can lead to activation of these genes (Ho et al., 2008; Giraud et al., 2012). This includes broad spectrum redox triggers such as hydrogen peroxide, but also more specific signals inhibiting mitochondrial function such as antimycin A and rotenone (Dojcinovic et al., 2005; Giraud et al., 2009). Also pathogen signals including hormones such as salicylic acid and abiotic stress signals such as ABA have been found to regulate genes encoding mitochondrial proteins (Van Aken et al., 2007; Ho et al., 2008). In silico analysis of the promoter regions of stress responsive genes encoding mitochondrial proteins revealed that most contain a TTGAC motif, the core binding site for WRKY-type transcription factors (W-box; Rushton et al., 1995; Van Aken et al., 2009). This observation suggests that WRKY...
transcription factors play a key role in the expression of genes encoding mitochondrial proteins.

WRKY transcription factors are a mostly plant-specific class of proteins that all contain at least one highly conserved Trp-Arg-Lys-Thr (WRKY) or related amino acid sequence and an additional zinc finger motif. Many WRKY factors have been reported to be involved in regulating stress responses (Eulgem and Somssich, 2007; Rushton et al., 2010) and in Arabidopsis 72 WRKY proteins have been annotated, varying in length from 109 (AtWRKY43) to potentially 1895 (AtWRKY19) amino acids. This diversity suggests a wide variety of upstream signalling factors and downstream target genes in plants, indicating a highly complex regulatory network with likely redundancies that controls gene expression.

WRKY transcription factors were found to be tightly correlated with biotic stresses (Rushton et al., 1996) and have been shown to be important regulators of microbe- or pathogen-associated molecular pattern-triggered immunity (MPI or PTI), and effector-triggered immunity (ETI) (Eulgem and Somssich, 2007; Rushton et al., 2010). In Hordeum vulgare HvWRKY1 and HvWRKY2 are involved in powdery mildew resistance, whereas NaWRKY3 and NaWRKY6 coordinate response to herbivory in the native tobacco species Nicotiana attenuata (Shen et al., 2007; Skibbe et al., 2008). In Arabidopsis, AtWRKY23 is involved in nematode infection (Grunewald et al., 2008) and a complex interaction between AtWRKY18, AtWRKY40 and AtWRKY60 in resistance to a variety of pathogens such as Botrytis cinerea (Xu et al., 2006) and Golovinomyces orontii (Shen et al., 2007) has been described extensively. It is therefore believed that WRKY TFs are closely involved in the signalling networks regulated by the phytohormones salicylic acid and jasmonic acid, often in opposite directions (Xu et al., 2006). Several reports have appeared linking WRKY TF function to abiotic stresses. For example overexpression of OsWRKY11 led to enhanced heat and drought tolerance, while in Arabidopsis AtWRKY25, AtWRKY26 and AtWRKY33 interact functionally in the regulation of salt and heat stress tolerance (Li et al., 2011; Jiang and Deyholos, 2009).

Recently, the involvement of WRKY factors in response to abscisic acid (ABA) has become apparent. A complex interaction of AtWRKY40, AtWRKY18 and AtWRKY60 with the C-terminal domain of the chloroplastic ABA receptor (ABAR, Genomes Uncoupled GUN5) was reported (Shang et al., 2010). In this model, AtWRKY40 is bound as a repressor to the promoters of ABA signalling factors and in the presence of ABA it re-localises to the cytosolic tail of ABAR, relieving repression of ABA signalling. This places AtWRKY40 upstream of several ABA signalling transcription factors such as ABI4, ABI5, DREB1/2A and MYB2. Another study identified AtWRKY63 in a screen for ABA hypersensitivity (Ren et al., 2010). AtWRKY63 would operate downstream of AtWRKY40 and ABI5, and stimulate the
expression of \textit{ABF2} and \textit{RD29A}. Therefore, it is clear that WRKY TFs play an important role in hormone-mediated responses triggered by environmental changes.

As putative WRKY binding sites are overrepresented in the promoters of genes encoding stress responsive mitochondrial proteins and WRKY transcription factors are known to contribute to responses that trigger expression of these genes, WRKY factors are plausible candidates to regulate expression of genes encoding mitochondrial proteins. Therefore, three widely stress responsive genes were selected as a starting point for identifying which WRKY transcription factors are specifically involved in regulating mitochondrial stress responses: \textit{AOX1a}, \textit{NDB2} and \textit{BCS1} (Van Aken et al., 2009). Our results indicate that W-boxes play a clear role in the expression of these genes, and furthermore WRKY transcription factors were identified that directly bind their promoters. Transgenic approaches show that AtWRKY40, AtWRKY57 and AtWRKY63 are regulators of stress responses of mitochondrially targeted proteins.

**Results**

\textit{W-boxes in the promoters of AOX1a, NDB2 and BCS1 are required for normal promoter activity}

The function of the putative TTGAC W-boxes in the promoters of \textit{BCS1}, \textit{NDB2} and \textit{AOX1a} was tested to establish if the putative W-boxes play a role in regulating transcriptional activity. The 2 kb promoter of \textit{AOX1a} contains 3 TTGAC sequences, and the 1.5 kb promoters of \textit{NDB2} and \textit{BCS1} promoters contain 2 TTGAC motifs, respectively (Figure 1a). An additional putative W-box was present 3.3 kb upstream of the \textit{BCS1} start codon, but given the long distance this was not further analysed experimentally. To assess promoter activity, the 1.5-2 kb promoter sequences were cloned in front of a β-glucuronidase (GUS) reporter gene using the pLUS reporter vector (Ho et al., 2008). The putative W-boxes were mutated using site-directed mutagenesis to repeats of A (see Supplementary Table 2 for details) either singly or in the different combinations. Cell cultures were mock treated to assess basal promoter activity and treated with H$_2$O$_2$ to assess general stress responsiveness of the promoters. GUS activity in mock and H$_2$O$_2$ treated cells was measured and normalised for transformation efficiency using a luciferase reporter gene (Figure 1b). The levels of promoter activity induction by H$_2$O$_2$ were comparable to previous reports using this system (Ho et al., 2008). The observation that increases in promoter activity after H$_2$O$_2$ treatment are lower than the maximal fold-change induction of the
respective transcript levels (Ho et al., 2008), may be due to the fact that the transient transformation is in itself a stress, and thus even ‘mock’ treatments will have an elevated promoter activity. The results show that in the \( AOX1a \) promoter mutation of each W-box significantly reduced promoter activity under mock conditions by up to 50 %, and mutation of W-box W1 also reduced inducibility by \( \text{H}_2\text{O}_2 \). This is in agreement with a previous study showing that deletion of an 18 bp region overlapping with \( AOX1a \) W1 resulted in strongly reduced promoter induction by antimycin A and monofluoracetate (Dojcinovic et al., 2005). In this study specific mutation of the \( AOX1a \) W1 TTGAC sequence also resulted in a more than two-fold decrease in promoter induction by monofluoroacetate and a mild decrease in antimycin A induction (Dojcinovic et al., 2005). \( AOX1a \) W1 was also identified as putative regulatory element in another study but was not functionally characterised (Ho et al., 2008).

In the \( BCS1 \) promoter mutation of W2 abolished induction by hydrogen peroxide, and mutation of both W1 and W2 almost halved normal promoter activity compared to the wild type promoter. \( \text{H}_2\text{O}_2 \) induction could be observed after mutation of both W1 and W2, but the resulting induced promoter activity was significantly lower than in the WT promoter and similar to that after deletion of \( BCS1 \) W2. Similarly, in the \( NDB2 \) promoter mutation of W1 boxes resulted in no observable changes and mutation of W2 abolished induction by \( \text{H}_2\text{O}_2 \). Simultaneous deletion of W1 and W2 resulted in a significant reduction of basal promoter activity and no induction by \( \text{H}_2\text{O}_2 \). Mutations in multiple W-boxes resulted in reduced or unchanged promoter activity compared to WT, indicating a complex interplay of these elements. In conclusion, these experiments indicate a complex interplay between the putative W-boxes in the promoters of stress responsive mitochondrial genes, pointing at both positive and negative roles in regulating promoter activity.

**WRKY transcription factors bind the promoters of mitochondrial stress responsive genes**

To identify which of the 72 \( \text{AtWRKY} \) transcription factors present in Arabidopsis are responsible for binding and potentially regulating the promoters of mitochondrial stress responsive genes, the WRKY transcription factors were cloned into the pGADT7-rec2 yeast one-hybrid prey vector. \( \text{AtWRKY16, AtWRKY19, AtWRKY24, AtWRKY52 and AtWRKY64} \) were not cloned despite several attempts. The genes for \( \text{AtWRKY16, AtWRKY19 and AtWRKY52} \) are exceptionally long (more than 3.8 kb), which may explain difficulty in amplification. The 50 bp promoter regions surrounding selected W-boxes of \( AOX1a, NDB2 \) and \( BCS1 \) were cloned into the pHIS2 yeast one-hybrid bait vector (Supplementary table 1).
For *AOX1a* W1 and W3 were selected, for *NDB2* W2 and for *BCS1* W1 and W2 were selected as their mutation had the strongest effects on promoter activity (Figure 1).

Given the large number of combinations between the 67 transcription factors and selected bait promoter sequences, a 96-well plate assay was optimised and adapted to the yeast one-hybrid transformation and screening procedure (Supplementary Figure 1). As self-activation and false positive interactions form a potential difficulty in yeast one-hybrid interactions screens, background binding of all cloned WRKY factors against an active promoter sequence that does not contain the TTGAC W-box sequence (pHIS2-p53) was tested. No significant binding of any of the WRKY factors could be observed with the pHIS2-p53 negative control, while the p53 protein showed a strong positive interaction, allowing to proceed the screen against the W-boxes (Supplementary Figure 1). Additionally, some WRKY transcription factors were recalcitrant towards transformation, likely because their expression causes lethal defects within the yeast host cells (Supplementary Figure 1). Interaction assays were performed using selected W-boxes of *AOX1a*, *NDB2* and *BCS1* as bait (Supplemental Table 1). Several WRKY transcription factors displayed clear growth on the selective interaction media as compared to the pHIS2-p53 negative control. Based on the Y1H screen results a selection of 12 WRKY transcription factors was made for further confirmation using spotting assays in a dilution series (Figure 2). Positive interactions of specific WRKY factors were observed with *AOX1a* W1 and W3, *NDB2* W2 and *BCS1* W1, though none of the WRKY factors showed a positive interaction with *BCS1* W2 W-box (Figure 2, Supplemental table 1). As an additional negative control, the transcription factor ABF4 (Abscisic acid response-element Binding Factor 4) that is known to bind the AGCTC motif (Choi et al., 2000) was included to demonstrate that binding of WRKY transcription factors to the TTGAC motif is specific. These experiments revealed that overall *AOX1a* W1, *NDB2* W2 and *BCS1* W1 are bound most strongly. This may be explained by the observation of previous studies that WRKY transcription factors appear to bind most strongly to TTGAC(C/T) motifs. The strongest binding sites based on our Y1H screen *AOX1a* W1, *NDB2* W2 and *BCS1* W1 are effectively followed by a C or T, whereas the weakest binding sites *AOX1a* W3 and *BCS1* W2 are followed by an A or G respectively. The *BCS1* W2 shows similarity to the AS-1 element found in the Cauliflower Mosaic Virus 35S promoter and may potentially bind bZIP transcription factors (Hara et al., 2000). Most of the 12 selected WRKY factors show binding to these three elements, but the relative strength of the interactions varies for each specific protein.

To further confirm binding of WRKY factors electromobility shift assays were performed (EMSAs, Figure 3). *AtWRKY57*, *AtWRKY63* and *AtWRKY75* were found to
express most efficiently in *E. coli* BL12 DE3 Rosetta 2 cells, whereas the other WRKY proteins showed little or no expression of soluble protein. Purified proteins were incubated with radiolabelled dsDNA probes containing wild type or mutated W-boxes. In an additional reaction a surplus of unlabelled wild type competitor probe was added to verify the specificity of binding. The W1 W-box of the *AOX1a* promoter was used as a representative for binding to confirm the yeast one-hybrid results. Figure 3 demonstrates that *AtWRKY57*, *AtWRKY63* and *AtWRKY75* are able to bind the *AOX1a* promoter, and that their binding is abolished when the W-box is mutated, indicating the binding is specific for the W-box. Binding was also abolished when an excess of unlabelled competitor probe was added. In conclusion, our results show that the promoters of mitochondrial stress response genes can effectively be bound by multiple WRKY transcription factors, indicating a complex regulatory interplay between different transcription factors.

*Genes encoding mitochondrial genes respond differentially in transgenic lines with altered WRKY expression*

In the previous sections we demonstrated that the promoters of stress responsive genes encoding mitochondrial proteins contain active W-boxes and are bound by WRKY transcription factors. It is therefore plausible that WRKY transcription factors play a part in controlling expression of these genes. To study the effects of WRKY transcription factors we generated transgenic *Arabidopsis thaliana* lines with reduced and increased expression for the selection of 12 *WRKY* genes in Figure 2 (Table I, supplementary figure 2). For all 12 genes overexpression was confirmed by QRT-PCR, and a clear reduction in expression was confirmed in the insertion or RNAi mutant lines with the exception of *AtWRKY30*, for which no T-DNA mutant lines are available, and *AtWRKY15* for which the T-DNA is present in the 5' UTR.

Expression profiles for the genes encoding mitochondrial proteins (*AOX1a*, *NDB2* and *BCS1*) were investigated in the mutant lines for the 12 selected WRKY transcription factors under both normal and stress conditions. To cover the wide spectrum of signalling pathways that affect the expression of stress responsive mitochondrial genes we selected antimycin A for mitochondrial retrograde pathways (Dojcinovic et al., 2005), Flg22 for biotic signalling (Ho et al., 2008) and ABA for abiotic stress signalling (Giraud et al., 2009). Untreated plants and randomised plants treated with AA, Flg22 or ABA were collected in biological duplicates for all 24 transgenic lines including Col-0. The expression of *AOX1a*, *NDB2*, and *BCS1* were analysed by QRT-PCR. Furthermore, the ABA response marker *RD29A* and the universal ROS marker gene *UPOX*, which encodes a mitochondrial protein...
of unknown function, were also included (Gadjev et al., 2006). The results of this large scale expression analysis are shown in supplementary figure 3. AOX1a and NDB2 expression was strongly induced by AA, and responded more moderately to Flg22 and ABA. BCS1 responded strongly to Flg22 and AA, but not ABA, whereas UPOX was highly induced by AA and slightly by ABA. RD29A expression only responded to ABA treatment. Detailed transcript analysis revealed that the expression of the marker genes was different in several WRKY transgenic lines compared to wild type plants (Supplementary Figure 3). In brief, AtWRKY13 and AtWRKY40 appeared to affect response to AA, AtWRKY63 appeared to affect basal expression of BCS1, while AtWRKY40, AtWRKY57 and AtWRKY63 affected response to Flg22 (Supplementary Figure 3).

Based on the initial screening assay for altered responses to stress treatments (Supplementary Figure 3) AtWRKY13, AtWRKY40, AtWRKY57 and AtWRKY63, were retained for confirmation in a repeat experiment under selected conditions using two independent overexpression lines and where available two independent knockout alleles (Figures 4 to 6). Strikingly, under untreated conditions BCS1 transcript levels were significantly higher in both AtWRKY63 overexpression lines, whereas AOX1a and NDB2 levels were unchanged. This confirms that BCS1 induction can be uncoupled from AOX1a induction and can respond to different signals at least under these conditions (Ho et al., 2008). Upon Flg22 treatment the induction of BCS1 and AOX1a expression was approximately four- and two-fold repressed in atwrky63 knockout plants (Figure 4). Expression levels of BCS1 after Flg22 treatment remained similar to the already elevated levels in untreated conditions, while no change in the induction of AOX1a was observed in AtWRKY63 overexpression lines. These observations imply that AtWRKY63 is an activator of biotic responses and BCS1 responds specifically to these signals. For AtWRKY57, up to 7.5 fold reduction (p<0.05) of BCS1 expression upon Flg22 stimulation was observed in the overexpression lines. Atwrky57 knockout lines showed no or little change in induction, indicating that AtWRKY57 can act as a repressor of biotic responses. In atwrky40 knockout plants a more than two-fold reduction in Flg22 response of BCS1 and AOX1a was observed. Interestingly, there was also a strongly reduced induction of BCS1 in AtWRKY40 overexpression lines, suggesting reprogramming has resulted in an upset balance of repression and activation in these mutants.

Based on the initial screening (Supplementary Figure 3) transgenic lines AtWRKY40 and AtWRKY13 were treated with AA. As expected AA increased marker gene expression in WT, but strikingly a significantly higher induction was observed in atwrky40 plants for BCS1, AOX1a and UPOX. Conversely, less induction of BCS1 and AOX1a was observed in both
AtWRKY40 OE lines (Figure 5A). These experiments indicate that AtWRKY40 can act as a repressor during stress responses to AA. The effect of AtWRKY13 on AA responses did not appear to be consistent in the repeat experiments (Supplementary Table 4).

Whereas AA was used for inducing mitochondrial retrograde signalling, the transgenic lines for AtWRKY13, AtWRKY40, AtWRKY57 and AtWRKY63 were also treated with high light (HL, 1h at 1000 µE m\(^{-2}\) s\(^{-1}\)) to induce chloroplast retrograde signalling (Gonzalez et al., 2011). Single replicates were initially tested for changes in marker gene expression (Supplementary Table 3), and based on these results transgenic lines for AtWRKY40 and AtWRKY63 were selected for further analysis in biological triplicate (Figure 5B). Significant changes in expression of AOX1a, BCS1, NDB2 and UPOX were observed in the transgenic lines for AtWRKY40 and AtWRKY63. As control marker genes for the HL treatment ascorbate peroxidase 2 (APX2) and early light-inducible protein 2 (ELIP2) were used (Gonzalez et al., 2011). As observed for the AA treatment, again atwrky40 plants showed a significantly stronger induction (> 2 fold) of the mitochondrial stress marker genes by HL. This further supports the role of AtWRKY40 as a repressor of mitochondrial stress responsive genes. Overexpression of AtWRKY40 did not consistently change marker gene expression under the used conditions for the mitochondrial marker genes, suggesting AtWRKY40 activity is limited by other factors and may be part of a multiprotein complex as described previously (Xu et al., 2006). Interestingly, the two marker genes for HL were also significantly affected by perturbation of AtWRKY40 levels: APX2 behaved similarly to the mitochondrial stress response marker genes, i.e. more strongly induced in atwrky40 plants, while ELIP2 that encodes a chloroplast protein behaved in an opposite fashion, i.e. more strongly induced in the AtWRKY40 overexpression lines.

For AtWRKY63, both overexpression lines again showed a significant induction of BCS1 expression in untreated conditions, whereas the basal expression of BCS1 was downregulated in atwrky63 lines (Figure 5B). Moreover, after the HL treatment a significantly higher induction of AOX1a, BCS1, NDB2, UPOX and also APX2 were observed in the AtWRKY63 overexpression lines. These expression patterns are consistent with a role of AtWRKY63 as a positive regulator of mitochondrial stress responses. In conclusion, the identified WRKY transcription factors are capable of modulating gene expression patterns of stress responsive genes encoding mitochondrial proteins, with AtWRKY40 acting as a repressor and AtWRKY63 as an activator.

AtWRKY13 and AtWRKY57 affect basal expression levels of ABA-related genes
Previous reports have shown the involvement of several WRKY transcription factors in the regulation of ABA responses (Ren et al., 2010; Shang et al., 2010; Rushton et al., 2011). Therefore, the expression of ABA-signalling related genes was analysed in transgenic lines for selected WRKY transcription factors (Figure 6A, Supplementary Figure 3). In the large scale transcript analysis AtWRKY13 and AtWRKY57 overexpression lines showed increased basal expression of the ABA response marker gene RD29A (Supplementary Figure 3). While AtWRKY40 and AtWRKY63 showed no significant effects (Supplementary Figure 3, Supplementary Table 4). In a repeat experiments with independent transgenic lines AtWRKY13 overexpression lines consistently showed nearly threefold higher expression levels of RD29A and a minor induction of ABI5 (Abscisic Acid Insensitive 5, a bZIP transcription factor; Finkelstein et al., 2000). ABF2 (Abscisic acid-response element binding factor 2) was unaffected. AtWRKY57 overexpression lines showed a similar 3 to 4 fold induction of RD29A and about twofold induction of ABI5 (Figure 6). In agreement with a role of AtWRKY57 as an activator of ABA related gene expression, RD29A was 4 to 5 fold downregulated in both atwrky57 mutant lines.

AtWRKY13, AtWRKY40 and AtWRKY57 affect early expression of genes encoding chloroplast proteins.

The four WRKY transcription factors identified here as affecting transcripts encoding mitochondrial proteins were investigated for a role in the expression of genes encoding chloroplast proteins. Wild type, AtWRKY13, AtWRKY40, AtWRKY57 and AtWRKY63 overexpression and knockout seedlings were grown under continuous light conditions for 5 days on MS and MS + 5 μM norflurazon (a chlorophyll synthesis inhibitor) in an assay identical to the GUN (Genomes Uncoupled) plastid-to-nucleus signalling screen (Koussevitzky et al., 2007). LHCb2.4 (Light-Harvesting Chlorophyll B binding protein 2.4, At3g27690) and HEMA1 (glutamyl-tRNA reductase involved in chlorophyll synthesis) were selected as marker genes for chloroplast function and stress responses (Koussevitzky et al., 2007; McCormac and Terry, 2004) (Figure 6). For AtWRKY63 transgenic lines no consistent changes were observed under untreated conditions, however both AtWRKY40 and AtWRKY57 were found to be basal repressors of the chloroplast function reporter genes, with knockout lines showing induced expression and overexpression lines showing strong repression. Conversely, atwrky13 knockout lines showed strong repression of the chloroplast reporter genes indicating that AtWRKY13 is an activator of chloroplast reporter genes. Interestingly, AtWRKY13 overexpression lines also showed reduced expression of LHCb2.4 and HEMA1, suggesting a perturbed steady state of expression in these mutants. Treatment with norflurazon triggered the previously reported reduction in expression of LHCb2.4 and
HEMA1 in wild type plants, which was alleviated in the GUN5 mutant that was included as control (Koussevitzky et al., 2007). In this study, none of the WRKY transgenic lines showed a classical GUN phenotype (Figure 6).

**WRKY transcription factors directly bind the promoters of genes encoding chloroplast proteins**

Being transcription factors, the subcellular localisation of AtWRKY factors is expected to be nuclear. Previous reports indicated the presence of AtWRKY40 in the nucleus as well as associated with chloroplast membranes, whereas AtWRKY63 was found to be exclusively nuclear (Shang et al., 2010, Ren et al., 2010). As the subcellular localisation of AtWRKY13 and AtWRKY57 was not determined previously, GFP fusion protein assays confirmed their nuclear localisation (Figure 7A). We showed that a range of AtWRKY transcription factors regulate expression of genes encoding chloroplast proteins (Figure 6). Therefore, the occurrence of putative W-boxes was investigated in the promoters of chloroplast protein marker genes *LHCB2.4* and *HEMA1*. The promoter of *LHCB2.4* contained one (1465 bp upstream of ATG) and the promoter of *HEMA1* contained two putative W-boxes (774 (W-box 2) and 164 (W-box 1) bp upstream of ATG) in the 1.5 kb promoter regions, respectively. To confirm if the regulation of genes encoding chloroplast proteins by AtWRKY13, AtWRKY40 and AtWRKY57 is through direct promoter interactions, the *LHCB2.4* and *HEMA1* W-box promoter regions were cloned into the yeast one-hybrid reporter system and interactions with AtWRKY13, AtWRKY40 and AtWRKY57 were assessed. Figure 7B shows that AtWRKY13, AtWRKY40 and AtWRKY57 clearly bind the *LHCB2.4* W-box and *HEMA1* W-box 1. Only AtWRKY40 showed a weak interaction with *HEMA1* W-box 2. Given the previous reports of the involvement of ABI4 in the regulation of genes both encoding mitochondrial and chloroplast proteins (Koussevitzky et al., 2007; Giraud et al., 2009) it was assessed whether direct interactions of WRKY transcription factors occurred with ABI4 using the yeast two-hybrid protein-protein interaction method. ABI4, AtWRKY13, AtWRKY40, AtWRKY57 and AtWRKY63 were cloned into the yeast two–hybrid reporter vectors and screened for binding (Figure 7C). No binding of WRKY proteins with ABI4 was observed, however direct binding of AtWRKY13 with itself, AtWRKY40 and AtWRKY57 was demonstrated.

**Genome-wide expression analysis reveals role of AtWRKY40 and AtWRKY63 in Arabidopsis stress responses**


The starting point of this study was to assess whether the putative W-boxes in the promoters of highly stress-responsive genes encoding mitochondrial proteins were functionally involved in the regulation of gene expression. In the previous sections we identified AtWRKY40 and AtWRKY63 as regulators of marker genes such as AOX1a. To obtain a clear picture of which other genes and stress response pathways are modulated by the identified WRKY transcription factors we performed a genome-wide microarray expression analysis. The transgenic lines atwrky40, AtWRKY40 OE1, atwrky63 and AtWRKY63 OE1 were compared to WT plants under untreated and HL conditions in biological triplicate using the Affymetrix ATH1 GeneChip platform. The HL treatment itself caused profound changes in gene expression in WT plants (Table II) and the changes in the selected marker genes (Figure 5B) due to perturbed levels of AtWRKY40 or AtWRKY63 were confirmed by the microarray analysis (Supplementary Table 4-5). Compared to WT plants all four mutant lines displayed significantly altered expression profiles (PPDE(<p) > 0.95) both under untreated and HL treated conditions (Table II), demonstrating the role of AtWRKY40 and AtWRKY63 in modulating the expression of more than just the initially selected marker genes. Comparison of the expression profiles revealed a several fold higher number of overlapping significantly changed probe sets than randomly expected, indicating a functional relatedness between AtWRKY40 and AtWRKY63 (Table III). 18 probe sets were more than two-fold significantly changed in all four mutants compared to WT under untreated conditions, with the expected number of overlapping probe sets by random selection being close to 0 (Table III).

A survey of the direction of change (i.e. up- or down regulation) in the four mutant lines demonstrated that significantly more genes than expected were upregulated in atwrky40 plants and downregulated in AtWRKY40 OE1 in untreated conditions (p<0.001), consistent with the suggested role of AtWRKY40 being mainly a repressor (Supplementary Table 6). In AtWRKY63 OE1 plants significantly more genes were upregulated compared to WT, consistent with a role as activator. The same trends were observed in response to HL treatment for atwrky40 and AtWRKY63 OE (Supplementary Table 6). In general, the atwrky63 mutation caused the least changes compared to WT and no bias could be detected towards up or downregulation, indicating other redundant factors may partially complement the loss of AtWRKY63. Of the 18 probe sets that were commonly changed in the four mutant lines, 10 followed the expected pattern consistent with a role of AtWRKY63 as activator (downregulated in atwrky63 and upregulated in AtWRKY63 OE), and of these seven followed the expected pattern of AtWRKY40 being a repressor (upregulated in atwrky40 and downregulated in AtWRKY40, Supplementary Figure 4). AtWRKY40 and AtWRKY63 have
previously been described to affect ABA signalling (Ren et al., 2010; Shang et al., 2010). To estimate the effect of perturbing AtWRKY40 and AtWRKY63 on ABA responsive genes, the expression characteristics of the approximately 3000 genes that are thought to be affected by ABA were analysed in the microarray dataset (Nemhauser et al., 2006). In agreement with previous findings, a significantly higher proportion of these genes were affected in the four analysed lines compared to random expectation (Supplementary Table 6) both under untreated and HL treated conditions. Again, the basal expression of ABI5 and RD29A was not different in AtWRKY40 and AtWRKY63 mutant lines compared to WT plants (Supplementary Table 3, Supplementary Figure 3), suggesting ABA responses are controlled by multiple pathways. Interestingly, the mild but significant induction of RD29A by HL treatment was completely repressed in atwrky40 plants (Supplementary Table 3).

**AtWRKY40 and AtWRKY63 modulate the mitochondrial stress response**

Subsequently, we analysed the expression profiles of the 26 genes encoding mitochondrial proteins that were defined previously as the core mitochondrial stress response and display an overrepresentation of W-boxes (Van Aken et al., 2009). Of these 26 genes, 23 indeed responded significantly to the HL treatment in WT plants (Figure 8A). Moreover, the responsiveness to HL of 20 of these 23 genes was significantly affected in the WRKY mutant lines: 16 genes responded differently to HL compared to WT in atwrky40, 12 in AtWRKY63 OE1, 6 in AtWRKY40 OE1 and 1 in atwrky63 plants, respectively (Figure 8A, Table IV). Furthermore, 11 genes were commonly affected in atwrky40 and AtWRKY63 OE1. To rule out that these observations could be the result of random distribution, chi-squared statistical tests indicated up to 5.7 times more changes (p<0.01) as compared to random sampling for all lines (Table IV). Next, it was assessed whether the changes caused by WRKY perturbations were biased towards genes encoding energy organelle proteins in general. Therefore, we assembled lists of all the genes that putatively encode energy organelle proteins and tested if there was a significant overrepresentation of these genes in the gene sets that were altered by AtWRKY40 or AtWRKY63 perturbation (Supplementary Table 6). Interestingly, this analysis revealed that 1.7 to 3.8 times less (p<0.01) genes encoding chloroplast or mitochondrial proteins were significantly changed in the WRKY mutant lines compared to WT plants than expected. This was the case for all four mutant lines under untreated conditions and all except atwrky63 under high light conditions. Changes in expression of genes encoding peroxisome proteins were also significantly underrepresented in the AtWRKY63 OE1 plants.
A previous study examined the gene expression patterns of genes encoding chloroplast and mitochondrial proteins in a set of nearly 1300 ATH1 microarray chips, encompassing experiments related to light signalling, hormones, sugars, chloroplast function, reactive oxygen species and general stresses (Leister et al., 2011). In this study, the genes encoding chloroplast and mitochondrial proteins were subdivided into functional subclasses. For chloroplast proteins the subclasses photosynthesis, organellar gene expression and tetrapyrrole metabolism were annotated; genes encoding mitochondrial proteins were subdivided into respiration and organellar gene expression. The AtWRKY40 and AtWRKY63 microarray data set after HL treatment was analysed to determine if any of these categories were specifically affected by WRKY perturbations (Supplementary Table 6). For the purpose of this study only nuclear encoded genes were analysed. Interestingly the three chloroplast categories were represented either as randomly expected or even significantly underrepresented in the case of photosynthesis and tetrapyrrole metabolism in *atwrky40* and *AtWRKY63 OE1* plants (Supplementary Table 6). Similarly, the mitochondrial organellar gene expression category was represented either as randomly expected or significantly underrepresented in *atwrky40* and *AtWRKY63 OE1* plants. The mitochondrial respiration category was represented as randomly expected for all lines, except *atwrky63* where an overrepresentation could be observed. This discrepancy could be caused by reduced statistical power due to the low number of statistically changed genes in general (Table II).

The study by Leister and colleagues (2011) also ranked the genes encoding mitochondrial and chloroplast proteins by the number of treatments or mutations to which the genes were responsive. The categories “very highly responsive” (VHR, 127 genes) and “very weakly responsive” (VWR, 120 genes) were analysed and indeed significantly more of the VHR genes were HL responsive than randomly expected, while significantly less of the NSR genes were HL responsive (p<0.001, Supplementary Table 6). Furthermore, it was noted that a highly significant proportion of the VHR genes were altered in WRKY mutants compared to WT after HL treatment, whereas VWR genes were significantly less affected by WRKY perturbations (except *atwrky63*) than expected. These analyses suggest that AtWRKY40 and AtWRKY63 are specifically involved in regulation of the highly environment-responsive genes encoding mitochondrial and chloroplast proteins, but are apparently less involved in more constitutively expressed genes encoding energy organellar proteins.
AtWRKY40 and AtWRKY63 modulate common gene expression responses to organellar dysfunction

A recent study performed a meta-analysis on a wide range of microarray data sets that were specifically related to chemical or genetic inhibition of chloroplast or mitochondrial functions (Van Aken and Whelan, 2012). One of the main conclusions of this study was that perturbation of mitochondrial or chloroplast function triggered distinct transcriptional responses directed towards the impaired organelle. However, it was also found that a higher than expected overlap exists between the two organelle dysfunction responses and that most of the common genes are part of the general stress response. Three sets of marker genes were identified that respond specifically to either chloroplast or mitochondrial dysfunction, or that responded to both organelar dysfunctions. Therefore, we analysed the expression changes for these three marker gene sets in the AtWRKY40 and AtWRKY63 microarray datasets (Figure 8B). For genes responding specifically to chloroplast dysfunction no more significant changes could be observed in the WRKY mutant lines than what is randomly expected by HL treatment in WT plants. Likewise, no more significant changes could be observed for genes responding specifically to mitochondrial dysfunction in all WRKY mutant lines, except in atwrky63. However, 3 to 10 times more of the marker genes which respond to both chloroplast and mitochondrial perturbation than randomly expected were affected in all four WRKY mutant lines (p<0.001, Table IV). The fact that WRKY factors specifically affect the genes responding in common to but not specific to chloroplast or mitochondrial dysfunction is further supported by the observation that W-boxes are present in nearly all of the promoters of these genes (Van Aken and Whelan, 2012). In contrast, no overrepresentation of W-boxes is found in the promoters of the genes responding specifically to either chloroplast or mitochondrial dysfunction.

Discussion

In this study a series of WRKY transcription factors were identified that are involved in regulating the expression of stress responsive genes encoding both mitochondrial and chloroplast proteins. The presence of over 70 different WRKY transcription factors in the Arabidopsis genome and the apparent lack of abnormal visual phenotypes in most WRKY knockout lines, suggest a high level of redundancy between the different WRKY proteins.
However, the clear changes in expression in single WRKY mutants indicate that individual proteins have discrete functions in the regulatory network (Figures 4 to 6).

The initial goal of this study was to investigate if the overrepresentation of putative W-boxes in the promoters of the most strongly stress responsive genes encoding mitochondrial proteins was functionally relevant to the expression patterns of these genes. Several lines of evidence now support this hypothesis. First, mutation of specific W-boxes significantly affects the promoter activity of the selected marker genes AOX1a, BCS1 and NDB2. Secondly, a number of WRKY factors are capable of directly binding to these promoter regions, and thirdly, perturbing the activity of AtWRKY40 or AtWRKY63 significantly altered the expression pattern and stress responsiveness of a very large proportion of the 26 mitochondrial stress response marker genes after stress treatment (Van Aken et al., 2009). From the observed expression patterns it is apparent that AtWRKY40 acts mainly as a repressor of these genes, whereas AtWRKY63 acts mostly as an activator. Interestingly, some genes respond in an opposite manner such as ELIP2 encoding a chloroplast protein, indicating a complex network of direct and possibly indirect regulation.

Using genome wide expression analysis, we have further expanded understanding of the role of these transcription factors. The results indicate that WRKY transcription factors are not significantly involved in the regulation of constitutive functions carried out by energy organelles such as photosynthesis, respiration and organellar gene expression. In contrast, it appears that WRKY transcription factors are more involved in modulating gene expression of genes that are specifically responsive when organellar function is inhibited either by a genetic defect or by external factors. A proportion of the genes responsive to such cellular dysfunctions encode organellar proteins, as exemplified by AOX1a, BCS1 and other mitochondrial stress response genes, as well as the “Very highly responsive” gene set encoding chloroplast and mitochondrial proteins as identified by Leister et al., 2011. The study by Leister and colleagues did not report an overrepresentation of W-boxes in the promoters of genes encoding organellar proteins. This may be explained by the fact that WRKY factors appear to regulate mainly stress responsive genes encoding organellar proteins, but not the large group of genes encoding organellar proteins that are less environment responsive. Furthermore, many stress-responsive genes not encoding energy organellar proteins (Figure 8) appear to be modulated by WRKY transcription factors.

When specifically looking at how AtWRKY40 and AtWRKY63 regulate the expression of marker genes directly responsive to chloroplast or mitochondrial dysfunction (Van Aken and Whelan, 2012), a number of interesting patterns arose. The analysis showed that the WRKY factors are not significantly involved in the regulation of genes that specifically
respond to either chloroplast or mitochondrial dysfunction. However, it was evident that the identified WRKY transcription factors are important regulators of the genes that are commonly affected by both chloroplast and mitochondrial perturbation. Moreover, these marker genes are widely affected during more general stresses (Figure 8). The promoters of these genes (which include BCS1) almost all contain W-boxes, suggesting the regulation is due to direct binding of WRKY transcription factors

Most of the identified WRKY factors seem to play multiple roles in seemingly disparate functions ranging from response to ABA and biotic stress to regulation of organellar stress responsive proteins. One explanation may be the formation of multimeric protein complexes, where different combinations of interaction partners bind to different sets of promoters and have different effects. The formation of these multimeric WRKY protein complexes has been reported multiple times in present literature, with AtWRKY18, AtWRKY40 and AtWRKY60 being a well-studied example with roles in biotic stress and ABA signalling (Xu et al., 2006; Shang et al., 2010). Also AtWRKY25 and AtWRKY33 are known to interact with roles in heat and osmotic stresses (Li et al., 2011). A genome wide study using the yeast two-hybrid screening method identified additional homomultimeric interactions of AtWRKY18, WRKY36, and AtWRKY60, and heteromultimers AtWRKY17-18 and AtWRKY38-40 (Arabidopsis Interactome Consortium, 2011). Here, multimeric complexes were identified of AtWRKY13 with itself, AtWRKY40 and AtWRKY57 (Figure 7). The precise make-up and stoichiometry of these complexes needs further investigation. Given the complex interactions of repression and activation by different transcription factors, it is reasonable to speculate that they accomplish this in a coordinated or competitive manner. It is thus conceivable that AtWRKY40 and AtWRKY63 may achieve their opposing effects by sequestration or competition for binding sites.

Another explanation for the role of WRKY transcription factors in the regulation of divergent processes may be found in the upstream signalling events leading to their activation or inactivation. WRKY transcription factors have been found to be regulated by interactions with different types of proteins such as MAP kinases (Kim and Zhang, 2004; Mao et al., 2011) and calmodulin (Park et al., 2005; Arabidopsis Interactome Consortium, 2011). AtWRKY40 was shown to bind the cytosolic tail of the ABA receptor (ABAR, GUN5) on the chloroplast surface upon ABA treatment (Shang et al., 2010). The differential binding with WRKY or non-WRKY interaction partners seems to be the most logical explanation for
the observation that some WRKY factors are found to be both repressors or activators depending on the conditions and which downstream gene is being examined.

For AtWRKY40 a significant amount of functional information is available (Xu et al., 2006; Shen et al., 2007). Here AtWRKY40 was found to be a novel repressor of retrograde expression in response to AA. AOX1a, BCS1 and UPOX expression in response to AA treatment was up to 80% more induced in atwrky40 plants than in wild type plants. This is only the second report of transcription factors involved in mitochondrial retrograde signalling in plants, besides ABI4 (Giraud et al., 2009). ABI4 was found to keep the AOX1a promoter repressed under normal conditions, whereas AtWRKY40 appears to limit AOX1a expression in its induced state (Figure 5). Also here AtWRKY40 was found to be a regulator of responses to Flg22. Atwrky40 knockout plants show a reduced induction by Flg22 of BCS1, and interestingly both AtWRKY40 OE lines show an even stronger repression of BCS1. Looking at transcripts in response to HL, reduced expression of AtWRKY40 triggers overall an induction while AtWRKY40 overexpression results in an overall reduction (Figure 5 and Supplementary table 6), although inverse correlations are also found as exemplified by ELIP2 (Figure 5). The fact that AtWRKY40 seems to have bi-directional roles in responses to biotic stimuli has been observed earlier in that AtWRKY40 mutant plants have induced expression of PR1, but reduced expression of PDF1.2 (Pandey and Somssich, 2009). During early seedling establishment AtWRKY40 was found to repress expression of GUN marker genes LHCBC2.4 and HEMA1. Interestingly, once plants are more established (3 weeks old), AtWRKY40 appears to have no significant control over basal expression of these genes (Supplementary Table 3), while instead modulating how plants respond to stress stimuli.

An atwrky63 mutant was found in a screen for mutants in response to ABA (Ren et al., 2010) and in agreement with this report a reduced induction of RD29A by ABA in atwrky63 plants was also seen (Supplementary figure 3). Interestingly, a role for AtWRKY63 as an activator in response to Flg22 treatment was also observed. Atwrky63 mutant plants showed a reduced induction of BCS1 and AOX1a (Figure 4) while AtWRKY63 OE plants showed several fold induction of BCS1 already in untreated conditions. AtWRKY63 was also identified to play a significant role in response to HL stress. AtWRKY63 appears to act as an activator for many of the stress responsive genes encoding mitochondrial proteins as evidenced by the often strong super-induction following HL stress. Some redundancy for this role as an activator for AtWRKY63 appears to be in place as the atwrky63 knockout plants only showed little changes compared to wild type plants. The identity of these redundant
factors, whether belonging to the WRKY family or not, still needs to be determined in future work.

For AtWRKY57 a role as a potential repressor of Flg22 signalling could be observed as the AtWRKY57 OE lines consistently showed a reduced induction of for instance BCS1 and AOX1α upon stimulation. No consistent changes were observed in the atwrky57 knockout lines pointing at potential functional redundancy with other proteins. AtWRKY57 also appears to stimulate basal expression of ABA marker genes RD29A and ABI5, and represses the expression of LHC2.4 and HEMA1 during the first days of plant development.

The data presented in this study and the results shown by other groups expands the current network of cellular responses influenced by WRKY and other transcription factors. One of the findings that became apparent in this study is the coordination of transcripts responding to both chloroplast and mitochondrial dysfunction. Previously ABI4 has been found be a regulator of both organelles (Koussevitzky et al., 2007; Giraud et al., 2009). This study suggests that signalling events through ABI4 and WRKY transcription factors are not directly linked. First of all, no direct protein-protein interactions of ABI4 and WRKY transcription factors could be detected. Furthermore, abi4 mutant lines were reported to have a GUN phenotype, namely that expression of chloroplast marker genes is not repressed as in wild type plants by treatment with norflurazon (Koussevitzky et al., 2007). Such a GUN phenotype was not observed in wrky mutant lines, rather an effect on the constitutive expression during seedling establishment. Finally, ABI4 was found to be a repressor of AOX1α and needs to be relieved for AOX1α induction after inhibition of mitochondrial function (Giraud et al., 2009), whereas AtWRKY40 keeps AOX1α induction levels in check once stress is occurring.

Conclusion

Though chloroplasts and mitochondria are physically separated from each other, their essential roles in energy metabolism necessitate a regulatory system that optimises cross-talk and interactions between the two organelles both under normal and stress conditions. Therefore it makes sense that there are common transcription factors able to coordinate gene expression when these organelles are functionally compromised.
Materials and Methods

Plant growth conditions

Arabidopsis Col-0 seeds were sterilized using with chlorine gas (100 ml 12% NaOCl and 3 ml 37% HCl). In vitro plants were grown on MS media (4.3 g/L Murashige-Skoog (Duchefa, Haarlem, The Netherlands), 0.5 g/L 2-(N-morpholino)ethanesulfonic acid, 20 g/L sucrose, 8 g/L agar (LabM, Bury, UK), 1 mL/L Gamborg B5 vitamins (Duchefa) at 22°C and 100 μE m⁻²s⁻¹ radiation in a 16-h light/8-h dark photoperiod. For stress treatments plants were grown in soil for 3 weeks and sprayed with 50 μM AA (Sigma-Aldrich), 10 μM Flg22 (Biomatik, Ontario, Canada) or 200 μM ABA (Sigma-Aldrich). Plants treated with AA were snap frozen after 4h, with Flg22 after 45 min, and with ABA after 6 h. For the HL treatment, plants were exposed to 1000 μE m⁻²s⁻¹ for 1h and snap frozen Arabidopsis thaliana Ler cell cultures were grown under continuous light conditions with shaking and treated with 100 mM H₂O₂.

T-DNA insertion lines and overexpression lines.

T-DNA insertion lines as shown in table I were obtained from the ABRC stock centre. Plants were genotyped by PCR, and insertion positions were confirmed by sequencing. Primers sequences are listed in Supplemental table II. Atwrky40 and atwrky18 atwrky40 atwrky60 mutant lines were kindly obtained from Prof. Da-Peng Zhang (Tsinghua University, Beijing). AtWRKY75 RNAi lines were kindly provided by Prof. Kashchandra Raghothama (Purdue University). WRKY overexpression lines were produced by cloning the coding sequences into CaMV 35S expression vector pK7WG2 (Karimi et al., 2002) using the Gateway cloning system (Invitrogen, California, USA) and dipped into Arabidopsis thaliana Col-0 plants according to (Clough and Bent, 1998). Homozygous plants with single T-DNA locus were selected on MS media containing 35 mg/L kanamycin. Overexpression was confirmed using QRT-PCR (for primer sequences see supplementary table II).

Quantitative RT-PCR

Quantitative RT-PCR was performed on snap-frozen Arabidopsis tissue. Total RNA isolation and cDNA synthesis was carried out as described previously (Lister et al., 2004). Transcript levels were assayed using the LightCycler 480 (Roche, Sydney, Australia). From each cDNA preparation, transcripts were analysed in duplicate and normalised to UBC as housekeeping gene. QRT-PCR primers used for the genes AOX1a, BCS1, NDB2, UPOX and UBC have
been previously described (Clifton et al., 2005), additional primers are shown in supplementary table II. Statistical analysis was performed using student t-test.

**Cloning of Arabidopsis promoter regions**

Promoter regions were cloned using standard protocols into pDRIVE (Qiagen, Sydney, Australia) and subcloned into pLUS as described by (Ho et al., 2008). The constructs were made as translational fusions with GUS, with the first ATG of the gene used as start codon for GUS. The elements to be tested (Figure 1) were mutated in the promoter via site-directed mutagenesis using the Stratagene Quikchange II Site-directed mutagenesis kit (La Jolla, California, USA).

**Biolistic transformation and assays for Luc and GUS**

Transformation was performed using the PDS-1000 system using the Hepta adaptor according to the manufacturer’s instructions (Biorad, Sydney Australia), as previously described using Arabidopsis suspension cell cultures (Ho et al., 2008). Standard errors are shown and to determine statistical significance, a student's t-test was performed assuming unequal variances. For comparison of GUS activities of the motif deletions with that of the un-mutated promoter, a t-test was also performed. Significance was defined as $p \leq 0.05$.

Following comparisons were carried out to determine the activity of each element:

1) Comparison of normalised GUS activity between wild-type promoter and mutated promoter. Significance is indicated with a blue asterisk.

2) Comparison between mock treated and stress treated GUS values. Significance is indicated with a red asterisk.

**Construction of yeast 1-hybrid vectors**

Coding regions of WRKY transcription factors were cloned from *Arabidopsis thaliana* Col-0 cDNA into pDRIVE (Qiagen) or by recombination directly into pGADT7-rec2 (according to Clontech handbook) with the Roche Expand High Fidelity PCR System (Roche, Sydney, Australia; primers in Supplemental table II). The PCR products in pDRIVE were then subcloned into the pGADT7-rec2 prey vector (Clontech, California, USA). As a control, the binding capacity of the p53 transcription factor to a DNA sequence containing (p53 +) or not
containing (p53 -) a p53-binding motif was used. For construction of the pHIS2 bait vectors, forward and reverse oligonucleotides (Supplementary Table 2) were annealed and subcloned into EcoRI–SacI linearised pHIS2 vector. The 50 bp sequence surrounding the W-box elements were cloned into pHIS2 upstream of the HIS3 promoter region and HIS3 reporter gene.

Yeast one hybrid screen

Yeast one hybrid transformation screens were performed in strain Y187 using the Clontech Matchmaker One Hybrid kit (Clontech, California, USA) and optimised for transformation in 96-well deep well sterile plates (Axygen, Australia). For each yeast one hybrid transformation, 50 μL of competent yeast cells were incubated with 100 ng pHIS2 bait vector and 100 ng pGADT7-Rec2 prey vector, 100 μg Herring Testes Carrier DNA (Clontech, California, USA) and 0.3 mL PEG/LiAc solution. Cells were transformed with 35 μL dimethylsulfoxide at 42°C for 15 minutes, then cooled on ice. 10 μL transformation was spotted onto SD media –leu -trp (double dropout DDO) to select co-transformed cells and SD media –his –leu –trp containing 150 mM 3-amino-1,2,4-triazole (3AT; triple dropout TDO; Sigma-Aldrich, Sydney, Australia). Plates were incubated four days at 28 °C. The pGADT7-rec2-p53 prey vector in combination with p53HIS2 was used as positive control, and pGADT7-rec2-p53 with pHIS2 or pGADT7-rec2-ABF4 with p53pHIS2 as negative controls. For specific confirmations in Figure 3, transformed yeast cells were grown overnight in YPD liquid media to OD_{600} of 0.1 and diluted in a 10x dilution series. From each dilution 10 μL was spotted on DDO and on TDO plates. Plates were incubated for three days at 28 °C.

Electromobility shift assays

30 bp oligonucleotide probes (Supplemental table II) with W-box or mutated W-box were annealed by heating to 99°C and gradual cooling. Annealed probes were radiolabelled using $^{32}$P γ-ATP (Perkin Elmer, USA) and polynucleotide kinase (Roche), and purified using Sephadex G-25 radiolabelled DNA Quick Spin columns (Roche). WRKY proteins were cloned into GST-tag expression vector pDEST15 (Invitrogen) and transformed into E. coli Rosetta 2 (DE3) pLysS competent expression cells. Proteins were expressed in 500 mL culture for 4h with 0.2 mM isopropyl-β-D-thio-galactoside at 18°C and 250 rpm. Cells were harvested, resuspended in extraction buffer (5x extraction buffer: 250 mM Tris-Cl pH 8.5, 500 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol) and lysed by sonication. Lysate was centrifuged at 16000 g for 20 min to clarify. Filtered lysate was then incubated with
glutathione-agarose beads (Scientifix, Australia) and washed with 10 volumes of extraction buffer containing Complete no-EDTA protease inhibitor cocktail (Roche). Purified proteins were eluted using extraction buffer containing 10 mM reduced glutathione. For gel shift assays, 20 μL reactions were setup with 4 μL 5x binding buffer (100 mM HEPES pH 7.8, 0.5 M KCl, 5 mM MgCl₂, 2.5 mM DTT, 5 mM EDTA, 0.25 mg/mL poly dI-dC, 50 % glycerol), 1 fmol radiolabelled probe, (500 fmol unlabelled probe for competitor reactions), 1.5 μg purified protein extract. Reactions were incubated for 20 min and separated on polyacrylamide gels (0.5x TBE, 2.5% glycerol, 6 % acrylamide) for 2 h at 200V on a 16 x 20 cm² Bio-Rad Protean II gel system. Gels were then dried on Whatman paper in a gel dryer and exposed overnight or longer and visualised using phosphorimager detection plates.

**Yeast two-hybrid analysis**

Full length coding sequences were subcloned into the pGBK7T and pGADT7-rec yeast two-hybrid vectors (Clontech). pGBK7T vectors were transformed into Y187 yeast cells and pGADT7-rec vectors into AH109. Transformed cells were mated overnight in YPD with gentle shaking at 28°C and spotted onto DDO and QDO (quadruple dropout SD –leu –Trp –His –adenine) with or without 10 mM X-α-gal. Plates were incubated at 28°C for 1 week until interactions were clearly visible.

**Subcellular localisation**

Coding sequences were cloned into GFP fusion vectors as described in (Carrie et al., 2008). AtWRKY13 was cloned into pDEST-NGFP and AtWRKY57 was cloned into pDEST-CGFP. As a mitochondrial marker the alternative oxidase (AOX) targeting signal of 42 amino acids fused to RFP was used (Carrie et al., 2008). Biolistic co-transformation of the GFP and RFP fusion vectors was performed on Arabidopsis cell culture and onion epidermal cells as previously reported (Carrie et al., 2008). GFP and RFP expression and targeting were visualized using a BX61 Olympus microscope using excitation wavelengths of 460/480 nm (GFP) and 535/555 nm (RFP), and emission wavelengths of 495–540 nm (GFP) and 570–625 nm (RFP). Images were captured using CellR imaging software.

**Microarray analysis**

Analysis of the changes in transcript abundance between Col-0, and double mutants was performed using Affymetrix GeneChipTM Arabidopsis ATH1 Genome Arrays (Affymetrix, Santa Clara, CA), along with preliminary data quality assessment, as described previously (Zhang et al., 2012). In total 16993 probe sets were deemed present by the MAS5.0 algorithm. Once processed, GC Robust Multigarray Average normalized gene expression
values were analyzed to identify differentially expressed genes by a regularized t test based on a Bayesian statistical framework using the software program Cyber-T (Kayala and Baldi, 2012). Changes were considered significant at a false discovery rate correction level of PPDE (>P) >0.95. Representations of gene categories were calculated using chi-square statistical tests. Lists of energy organelle proteins and expression values of the selected marker genes in publicly available microarray experiments were collected as described in Van Aken and Whelan, 2012.

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**Figure legends**

**Figure 1.** Location and activity of W-boxes in the promoters of stress responsive genes encoding mitochondrial protein. (a) The location of W-boxes with TTGAC core sequence in the promoters of three widely stress responsive genes encoding mitochondrial proteins. The red line indicates 5’ UTR. Numbers indicate base positions upstream of the start codon. (b) The relative activity of the promoter sequences fused to the GUS reporter gene compared the promoter sequence with single or multiple W-boxes mutated. The mock treated wild type (WT) promoter activity was normalised to one. Blue asterisks indicate a significant difference (p<0.05) compared to the WT promoter for the mock treatments, red asterisks indicate significant difference (p<0.05, n=9) of the H₂O₂ treatments compared to the mock treatment of the same construct. Error bars indicate standard error.

**Figure 2.** Yeast one-hybrid analysis of selected WRKY transcription factors against W-boxes of AOX1a, NDB2 and BCS1 promoters and negative control (promoter region without W-box). Non-WRKY transcription factor ABF4 was also included as a negative control for bait self-activation. Three 10-fold dilutions were spotted vertically for each assay on DDO (transformation control) and TDO (interaction) media, including p53 positive and negative controls. DDO: double dropout; TDO: triple dropout.

**Figure 3.** Electromobility shift assays of purified WRKY proteins against radiolabelled probes with wild type and mutated W1 W-box for the AOX1a promoter sequence. Radiolabelled 30 bp probes were incubated with purified WRKY proteins in the presence or absence of unlabelled promoter probe. As a negative control the promoter probe with mutated W-box was included. WT: wild type; comp: competitor. Arrows indicate specific shifts that are abolished by adding competitor or using mutated probes.

**Figure 4.** QRT-PCR analysis of transcripts encoding mitochondrial proteins in transgenic WRKY overexpression or knockout/knockdown Arabidopsis lines. Wild type and mutant plants with increased or reduced expression of WRKY transcription factors were grown in soil for three weeks, then treated with Flg22 for 45 min in biological duplicate. Samples were harvested and mRNA levels were measured using QRT-PCR. Expression values are shown and colour-coded for visual representation, yellow: unchanged, green: reduced, red:
induced. Underlined values indicate a statistically significant difference (t-test, p<0.05) compared with the expression in Col-0 following the same treatment.

**Figure 5.** QRT-PCR analysis of transcripts encoding mitochondrial proteins in transgenic WRKY overexpression or knockout/knockdown lines. Wild type and mutant plants with increased or reduced expression of WRKY transcription factors were grown in soil for three weeks, then treated with antimycin A for 4h in biological duplicate, or high light for 1h in biological triplicate. Samples were harvested and mRNA levels were measured using QRT-PCR. Asterisks indicate statistically significant difference (p<0.05) compared with the expression in Col-0 within the same treatment.

**Figure 6.** QRT-PCR analysis of transcripts encoding chloroplast proteins in transgenic WRKY overexpression or knockout/knockdown lines. (A) Expression of ABA-related genes in three week old AtWRKY13 and AtWRKY57 mutant lines. (B) Wild type and mutant plants were grown on MS plates with or without norflurazon (NF) for five days. Samples were harvested and analysed using QRT-PCR in duplicate. Expression values are shown and colour-coded for visual representation, yellow: unchanged, green: reduced, red: induced. Underlined values indicate statistically significant difference (p<0.05) compared with the expression in Col-0 within the same treatment.

**Figure 7.** Subcellular localisation and yeast one-/two-hybrid assays for WRKY proteins (A). Full length coding sequences were fused to a GFP coding sequence and transformed into Arabidopsis suspension cell and onion epidermal cells. AOX-RFP (red fluorescent protein) was included as a mitochondrial marker. Scale bare indicates 20 μm. (B) Binding of WRKY transcription factors to W-boxes present in the promoters of genes encoding chloroplast proteins was examined using yeast one-hybrid screening. A p53 promoter sequence not containing a W-box was included as negative control. Transformation is indicated by growth on double dropout media (DDO), interaction is indicated by growth on triple dropout media (TDO). Cultures were diluted 10-fold in a vertical pattern. (C) Screening of protein-protein interactions between WRKY transcription factors and ABI4 using the yeast two-hybrid system. pGADT7-rec and pGBK7 are included as empty vector controls. Growth on double dropout media (DDO) indicates presence of bait and prey vectors, interaction is indicated by growth on quadruple dropout media (QDO) with and without X-α-gal.
Figure 8. Heat maps representing the expression of marker genes of mitochondrial stress responses (a) and chloroplast or mitochondrial dysfunction (b). The relative expression in a range of previously published microarray experiments involving abiotic stress, biotic stress, chemical or genetic inhibition of chloroplast or mitochondrial functions, and from this study the expression in AtWRKY40 and AtWRKY63 knockout/overexpression in untreated (UT) or high light (HL) conditions.
Table I. WRKY transgenic lines. Overview of transgenic overexpression (OE) and loss-of-function lines for selected WRKY genes. For loss-of-function lines the respective line names and the position of the insertion is indicated. N/a: not available.

| Gene      | OE line | T-DNA line 1 | insertion site | T-DNA line 2 | Insertion site |
|-----------|---------|--------------|----------------|--------------|----------------|
| AtWRKY9   | yes     | SALK_067122  | intron 4 of 4   |              |                |
| AtWRKY13  | yes     | SALK_064346  | exon 2 of 3     | SALK_032911  | exon 1 of 3    |
| AtWRKY15  | yes     | GABI_097A12  | 5'-UTR          |              |                |
| AtWRKY27  | yes     | SALK_048952  | exon 2 of 3     |              |                |
| AtWRKY33  | yes     | SALK_006602  | exon 3 of 5     |              |                |
| AtWRKY40  | yes     | CSHL_ET5883  | exon 2 of 4     |              |                |
| AtWRKY42  | yes     | SALK_121674  | exon 3 of 6     |              |                |
| AtWRKY45  | yes     | RATM11-0634-1_H | intron 1 of 1   |              |                |
| AtWRKY57  | yes     | SALK_076716  | intron 1 of 2   | GABI_078H12  | intron 1 of 2  |
| AtWRKY63  | yes     | SALK_007496  | exon 3 of 3     | n/a          |                |
| AtWRKY75  | yes     | amiRNA line  |                |              |                |
| AtWRKY30  | yes     | n/a          |                |              |                |
Table II. Microarray analysis of WRKY mutants during high light stress.
Number of significantly differentially expressed probe sets compared to WT plants (PPDE(<p) > 0.95) with a range of foldchange cut-offs. Numbers between brackets indicate up- or downregulated genes compared to WT. Abbreviations: WT: wild type; UT: untreated; HL: high light.

Number of significantly changed genes compared to WT

| Compared to WT untreated (UT) | PPDE(<p) > 0.95 | FC > 1.5   | FC > 2   |
|------------------------------|-----------------|------------|------------|
| wrky40 UT                    | 2585 (1446, 1139) | 2309 (1286, 1023) | 1336 (723, 613) |
| WRKY40 OE1 UT                | 953 (358, 595)   | 897 (324, 573)   | 583 (180, 403)   |
| wrky63 UT                    | 512 (267, 245)   | 490 (257, 233)   | 315 (165, 150)   |
| WRKY63 OE1 UT                | 1499 (975, 524)  | 1353 (914, 439)  | 744 (580, 164)   |

| Compared to WT high light (HL) | PPDE(<p) > 0.95 | FC > 1.5   | FC > 2   |
|------------------------------|-----------------|------------|------------|
| wrky40 HL                    | 4239 (2408, 1831) | 2690 (1535, 1155) | 1271 (681, 590) |
| WRKY40 OE1 HL                | 805 (391, 414)   | 739 (366, 373)   | 470 (250, 220)   |
| wrky63 HL                    | 253 (124, 129)   | 194 (92, 102)    | 71 (34, 37)      |
| WRKY63 OE1 HL                | 1698 (1007, 691) | 1544 (965, 579)  | 960 (700, 260)   |
| Col-0 HL vs Col-0 UT         | 9105 (3919, 5186) | 6313 (2662, 3651) | 3611 (1687, 1924) |
Table III. Comparison of overlap between WRKY mutant lines. Chi-square analysis of representation compared to random distribution of overlapping two-fold significantly changed genes compared to wild type in untreated or high light treated conditions. Abbreviations: diff.: differential; WT: wild type; UT: untreated; HL: high light.

| Twofold UT vs WT UT         | Line 1 | Line 2 | Line 3 | Line 4 | Observed overlap | Expected overlap | p-value |
|-----------------------------|--------|--------|--------|--------|------------------|------------------|---------|
| wrky40 vs WRKY40 OE1       | 1336   | 583    |        |        | 175              | 46               | <0.001  |
| wrky40 vs WRKY63 OE1       | 1336   | 744    |        |        | 153              | 58               | <0.001  |
| WRKY40 OE1 vs wrky63       | 583    | 315    |        |        | 107              | 11               | <0.001  |
| wrky63 vs WRKY63 OE1       | 315    | 744    |        |        | 109              | 14               | <0.001  |
| All four lines             | 1336   | 583    | 315    | 744    | 18               | 0                | <0.001  |

| Twofold HL vs WT HL        | Line 1 | Line 2 | Line 3 | Line 4 | Observed overlap | Expected overlap | p-value |
|-----------------------------|--------|--------|--------|--------|------------------|------------------|---------|
| wrky40 vs WRKY40 OE1       | 1271   | 470    |        |        | 164              | 35               | <0.001  |
| wrky40 vs WRKY63 OE1       | 1271   | 960    |        |        | 327              | 72               | <0.001  |
| WRKY40 OE1 vs wrky63       | 470    | 71     |        |        | 21               | 2                | <0.001  |
| wrky63 vs WRKY63 OE1       | 71     | 960    |        |        | 29               | 4                | <0.001  |
| All four lines             | 1271   | 470    | 71     | 960    | 9                | 0                | <0.001  |
Table IV. Representation of marker genes for mitochondrial stress response and organellar dysfunction. Chi-square analysis of representation compared to random distribution. Abbreviations: diff.: differential; WT: wild type; HL: high light; obs: observed; exp: expected; dysf: dysfunction.

| Mitochondrial stress response genes | Changes vs WT HL | present in category | Observed diff. to WT HL | Expected diff. to WT HL | p-value | ratio obs/exp |
|------------------------------------|------------------|---------------------|-------------------------|-------------------------|---------|---------------|
| wrky40 HL                          | 4239             | 26                  | 17                      | 6.5                     | <0.001  | 2.6           |
| WRKY40 OE1 HL                      | 805              | 26                  | 7                       | 1.2                     | <0.001  | 5.7           |
| wrky63 HL                          | 253              | 26                  | 2                       | 0.4                     | <0.01   | 5.2           |
| WRKY63 OE1 HL                      | 1688             | 26                  | 13                      | 2.6                     | <0.001  | 5.0           |

| Mitochondrial and chloroplast dysf. | Changes vs WT HL | present in category | Observed diff. to WT HL | Expected diff. to WT HL | p-value | Ratio obs/exp |
|-------------------------------------|------------------|---------------------|-------------------------|-------------------------|---------|---------------|
| wrky40 HL                           | 4239             | 13                  | 10                      | 3.2                     | <0.001  | 3.1           |
| WRKY40 OE1 HL                       | 805              | 13                  | 3                       | 0.6                     | <0.001  | 4.9           |
| wrky63 HL                           | 253              | 13                  | 2                       | 0.2                     | <0.001  | 10.3          |
| WRKY63 OE1 HL                       | 1688             | 13                  | 7                       | 1.3                     | <0.001  | 5.4           |

| Mitochondrial dysf. only            | Changes vs WT HL | present in category | Observed diff. to WT HL | Expected diff. to WT HL | p-value | ratio obs/exp |
|-------------------------------------|------------------|---------------------|-------------------------|-------------------------|---------|---------------|
| wrky40 HL                           | 4239             | 12                  | 3                       | 3.0                     | >0.05   | 1.0           |
| WRKY40 OE1 HL                       | 805              | 12                  | 1                       | 0.6                     | >0.05   | 1.8           |
| wrky63 HL                           | 253              | 12                  | 3                       | 0.2                     | <0.001  | 16.8          |
| WRKY63 OE1 HL                       | 1688             | 12                  | 1                       | 1.2                     | >0.05   | 0.8           |

| Chloroplast dysf. only              | Changes vs WT HL | present in category | Observed diff. to WT HL | Expected diff. to WT HL | p-value | ratio obs/exp |
|-------------------------------------|------------------|---------------------|-------------------------|-------------------------|---------|---------------|
| wrky40 HL                           | 4239             | 14                  | 3                       | 3.5                     | >0.05   | 0.9           |
| WRKY40 OE1 HL                       | 805              | 14                  | 2                       | 0.7                     | >0.05   | 3.0           |
| wrky63 HL                           | 253              | 14                  | 0                       | 0.2                     | >0.05   | 0.0           |
| WRKY63 OE1 HL                       | 1688             | 14                  | 1                       | 1.4                     | >0.05   | 0.7           |
Supplementary figure legends

Supplementary Figure 1. Self-activation assay and example of large scale yeast one-hybrid screen of WRKY transcription factors against non-W-box containing sequence (p53 binding site) and AOX1a W-box 1. Yeast cells were co-transformed with bait (pHIS2 vector containing 50 bp promoter region with W-box) and prey vectors (pGADT7-rec2 containing WRKY transcription factor fused to GAL4 activation domain). Growth on double dropout (-leu/-trp) media indicates transformation of both plasmids, growth on triple dropout (-leu/-trp/-his) indicates interaction between promoter element and transcription factor. The bottom left diagram indicates grid position of WRKY transcription factors. P53 binding to p53 DNA binding site or empty pHIS2 vector were included as positive and negative controls, respectively. DDO: double dropout, TDO: triple drop-out media.

Supplementary Figure 2. RT-PCR expression analysis of AtWRKY transgenic lines. (a) Relative mRNA analysis of CaMV 35S overexpressor plants normalised to expression levels in Col-0 wild type plants. (b) Relative mRNA expression analysis of insertional knockout and RNAi knockdown lines normalised to expression levels in Col-0 wild type plants. (c) RT-PCR using full-length AtWRKY9 primers on Col-0 and atwrky9 cDNA. Primers for At5g08670 were used as control for cDNA quality. Error bars indicate standard error.

Supplementary Figure 3. RT-PCR expression analysis of AtWRKY transgenic lines. qRT-PCR analysis for BCS1, AOX1a, NDB2, UPOX and RD29A on WRKY transgenic knockout/knockdown and overexpression lines untreated and treated with Flg22 (45 min), Antimycin A (4h) and ABA (6h). For simplicity knockout/knock-down lines were abbreviated as wxx (for atwrkyxx) and OExx (for AtWRKYxx OE). Error bars indicate standard error.

Supplementary Figure 4. Heat map representing expression of 18 probe sets commonly affected by AtWRKY40 and AtWRKY63 knockout/overexpression in untreated (UT) or high light (HL) conditions.
Figure 1. Location and activity of W-boxes in the promoters of stress responsive genes encoding mitochondrial protein. (a) The location of W-boxes with TTGAC core sequence in the promoters of three widely stress responsive genes encoding mitochondrial proteins. The red line indicates 5’ UTR. Numbers indicate base positions upstream of the start codon. (b) The relative activity of the promoter sequences fused to the GUS reporter gene compared the promoter sequence with single or multiple W-boxes mutated. The mock treated wild type (WT) promoter activity was normalised to one. Blue asterisks indicate a significant difference (p<0.05) compared to the WT promoter for the mock treatments, red asterisks indicate significant difference (p<0.05, n=9) of the H2O2 treatments compared to the mock treatment of the same construct. Error bars indicate standard error.
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|                     | Untreated       | Fig22 (a)       |
|---------------------|-----------------|-----------------|
|                     | BCST | AOX1a | NDB2 | BCST | AOX1a | NDB2 |
| Col-0               | 1.00 | 1.00 | 1.00 | 37.86 | 4.11 | 1.36 |
| atwrky13-1          | 1.07 | 1.27 | 1.18 | 37.90 | 4.50 | 1.38 |
| atwrky13-2          | 0.27 | 0.63 | 0.52 | 29.43 | 4.30 | 1.37 |
| AtWRKY13 OE1        | 0.49 | 0.93 | 0.69 | 28.78 | 4.34 | 1.50 |
| AtWRKY13 OE2        | 0.41 | 0.81 | 0.56 | 16.12 | 2.73 | 0.70 |
| atwrky40            | 0.49 | 0.75 | 0.61 | 16.79 | 1.96 | 1.07 |
| AtWRKY40 OE1        | 0.32 | 0.93 | 0.95 | 11.70 | 3.74 | 1.31 |
| AtWRKY40 OE2        | 0.46 | 1.00 | 0.59 | 6.33  | 2.08 | 1.07 |
| atwrky57-1          | 0.69 | 0.55 | 1.03 | 23.85 | 3.27 | 1.02 |
| atwrky57-2          | 1.14 | 0.84 | 0.59 | 36.40 | 4.30 | 1.30 |
| AtWRKY57 OE1        | 0.65 | 1.37 | 0.67 | 11.83 | 3.19 | 0.95 |
| AtWRKY57 OE2        | 0.82 | 1.54 | 0.84 | 4.99  | 2.12 | 0.41 |
| atwrky63            | 0.77 | 0.85 | 0.60 | 10.80 | 2.11 | 0.54 |
| AtWRKY63 OE1        | 15.13 | 1.45 | 1.10 | 11.41 | 3.90 | 2.08 |
| AtWRKY63 OE2        | 21.02 | 0.90 | 1.21 | 19.51 | 3.69 | 0.51 |
Figure 5. QRT-PCR analysis of transcripts encoding mitochondrial proteins in transgenic WRKY overexpression or knockout/knockdown lines. Wild type and mutant plants with increased or reduced expression of WRKY transcription factors were grown in soil for three weeks, then treated with antimycin A for 4h in biological duplicate, or high light for 1h in biological triplicate. Samples were harvested and mRNA levels were measured using QRT-PCR. Asterisks indicate statistically significant difference (p<0.05) compared with the expression in Col-0 following the same treatment.
Figure 6. QRT-PCR analysis of transcripts encoding chloroplast proteins in transgenic WRKY overexpression or knockout/knockdown lines. (A) Expression of ABA-related genes in three week old AtWRKY13 and AtWRKY57 mutant lines. (B) Wild type and mutant plants were grown on MS plates with or without norflurazon (NF) for five days. Samples were harvested and analysed using QRT-PCR in duplicate. Expression values are shown and colour-coded for visual representation, yellow: unchanged, green: reduced, red: induced. Underlined values indicate statistically significant difference (p<0.05) compared with the expression in Col-0 following the same treatment.
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