A Membrane-Bound NAC Transcription Factor, ANAC017, Mediates Mitochondrial Retrograde Signaling in Arabidopsis

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Plants require daily coordinated regulation of energy metabolism for optimal growth and survival and therefore need to integrate cellular responses with both mitochondrial and plastid retrograde signaling. Using a forward genetic screen to characterize regulators of alternative oxidase1a (AOX1a) mutants, we identified RAO2/Arabidopsis NAC domain-containing protein17 (ANAC017) as a direct positive regulator of AOX1a. RAO2/ANAC017 is targeted to connections and junctions in the endoplasmic reticulum (ER) and F-actin via a C-terminal transmembrane (TM) domain. A consensus rhomboid protease cleavage site is present in ANAC017 just prior to the predicted TM domain. Furthermore, addition of the rhomboid protease inhibitor N-p-Tosyl-L-Phe chloromethyl abolishes the induction of AOX1a upon antimycin A treatment. Simultaneous fluorescent tagging of ANAC017 with N-terminal red fluorescent protein (RFP) and C-terminal green fluorescent protein (GFP) revealed that the N-terminal RFP domain migrated into the nucleus, while the C-terminal GFP tag remained in the ER. Genome-wide analysis of the transcriptional network regulated by RAO2/ANAC017 under stress treatment revealed that RAO2/ANAC017 function was necessary for >85% of the changes observed as a primary response to cytosolic hydrogen peroxide (H₂O₂), but only ~33% of transcriptional changes observed in response to antimycin A treatment. Plants with mutated rao2/anac017 were more stress sensitive, whereas a gain-of-function mutation resulted in plants that had lower cellular levels of H₂O₂ under untreated conditions.

INTRODUCTION

Mitochondria and plastids (chloroplasts) are composed of ~1500 and ~3000 proteins, respectively, with >95% of these proteins encoded by nuclear-located genes (Woodson and Chory, 2008). It has been shown that two-way communication pathways exist between the nucleus and mitochondria and chloroplasts, called anterograde and retrograde signaling pathways (Rhoads and Subbaiah, 2007; Woodson and Chory, 2008). Anterograde regulation refers to a top-down regulatory pathway, where signals have a direct impact on gene expression in the nucleus. Conversely, nuclear gene expression is also influenced by signals that originate from within the organelles, mitochondria, or chloroplasts and is referred to as retrograde regulation.

Several components involved in plastid retrograde signaling have been identified, with at least five different pathways characterized: reactive oxygen species (ROS), redox signals, plastidial gene expression, pigment biosynthesis, and specific signaling metabolites (Pfannschmidt, 2010). The most intensively studied retrograde signaling pathway is in the genomes uncoupled (gun) mutants that uncouple the expression of nuclear-encoded chloroplastic proteins from the functional state of chloroplasts (Susek et al., 1993). A recently identified plastid-bound transcription factor, PTM (plant homeodomain–type transcription factor with transmembrane [TM] domains), was also identified as a regulator for plastid retrograde signaling and acts downstream of GUNs (Sun et al., 2011). Additionally, plastid retrograde signals that have been identified more recently include 3′-phosphoadenosine 5′-phosphate (Estavillo et al., 2011), β-cyclophilin that is produced in plastids during high-light stress (Ramel et al., 2012), methylerythritol cyclo-diphosphate, a precursor of isoprenoids (Xiao et al., 2012), and pathogen-associated molecular pathogen signals that are

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relayed via the chloroplasts through a Ca\textsuperscript{2+}-mediated signal transduction pathway (Nomura et al., 2012).

At least some of the components involved in mitochondrial retrograde signaling pathways have been identified in yeast (Butow and Avadhani, 2004). ROS have been implicated in mitochondrial retrograde signaling in both plants and animals (Murphy, 2009; Kim et al., 2010; Petrov and Van Breusegem, 2012), but the means by which a ROS signal can be transmitted to activate gene expression in the nucleus is still unknown, and ROS is produced in a variety of locations in the cell (Möller and Sweetlove, 2010). In plants, several mitochondrial retrograde regulation deficient (mrrd) mutants have been isolated using the response of ALTERNATIVE OXIDASE1a (AOX1a) to mitochondrial dysfunction as the marker for mitochondrial retrograde regulation (Dojcinovic et al., 2005; Zarkovic et al., 2005). However, the identities of these mrrd mutants are yet to be characterized. To date, three components mediating the mitochondrial retrograde response in plants have been identified: ABSCISIC ACID INSSENSITIVE4 (ABI4) (Giraud et al., 2009), CYCLIN-DEPENDENT KINASE E1 (Ng et al., 2013), and WRKY40 (Van Aken et al., 2013).

In this work, we employed a forward genetic approach using AOX1a as marker gene for mitochondrial retrograde regulation to identify regulators of AOX1a (rao) mutants. Here, we report a membrane-bound NAC (for NO APICAL MERISTEM/ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR/CUP-SHAPED COTYLEDON) transcription factor, RAO2/Arabidopsis NAC domain-containing protein17 (ANAC017), that is a transcriptional activator of AOX1a during mitochondrial dysfunction. Furthermore, we show that this transcription factor mediates hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced changes in transcript abundance, thus integrating anterograde and retrograde regulatory pathways.

RESULTS

RAO2 Encodes the NAC Domain Transcription Factor ANAC017

To identify the molecular components necessary for mitochondrial retrograde signaling under stress in plant cells, a forward genetic screen was conducted using AOX1a as a model, which is a stress-responsive nuclear-encoded mitochondrial component, to identify rao mutants (Rhoads and Subbaiah, 2007). A construct with the AOX1a 2-kb upstream promoter region driving a firefly luciferase gene (AOX1a-LUC) was used as a reporter gene system and permanently transformed into Arabidopsis thaliana Columbia-0 (Col-0) to generate Col:LUC germ lines (Figure 1A) (Ng et al., 2013). Two-week-old Col:LUC seedlings, when treated with 50 \mu M antimycin A (AA), showed high expression of LUC driven by the AOX1a promoter (Figures 1A to 1C). Col:LUC plants were subjected to ethyl methanesulfonate (EMS) mutagenesis, and two independent lines, rao2-1 and rao2-2, were identified as loss-of-function alleles that showed little or no induction of AOX1a-LUC under AA treatment (Figure 1A). Consistent with the results from AA treatment, AOX1a-LUC was expressed in mycothiazol-treated Col:LUC seedlings but not in the rao2 lines (Figure 1B). For both AA and mycothiazol treatments, LUC was fivefold less abundant (P < 0.001, Student’s t test) in the rao2 background compared with Col:LUC seedlings (Figure 1C). A number of other treatments, including monofluorooacetate (Dojcinovic et al., 2005; Zarkovic et al., 2005), and to a lesser extent H\textsubscript{2}O\textsubscript{2}, revealed a significant reduction in the induction of LUC in the rao2 background (see Supplemental Figure 1 online). By contrast, exposure to more general cellular stresses, such as UV light, 4°C cold, abscisic acid (ABA), and salicylic acid, did not result in compromised induction of LUC, suggesting that RAO2 is likely to mediate a mitochondrial-dependent signaling pathway (see Supplemental Figure 1 online).

A combination of classical positional cloning techniques with next-generation sequencing technology was used to identify the mutations in rao2 mutants. Both rao2-1 and rao2-2 are recessive mutations and mapped to the same 2.47-Mb region on chromosome 1 between In/Del CEREON Genomics markers CER449719 and CER450671 (see Supplemental Data Set 1 online) and were therefore crossed to test whether these were allelic mutations within the same gene product. Plants from the first filial generation (F1) were unable to induce AOX1a-LUC expression in response to AA treatment (Figure 1D), confirming that rao2-1 and rao2-2 were in fact allelic mutations. To identify candidate point mutations within this 2.47-Mb region in the rao2 mutant lines, whole-genome next-generation resequencing was used (see Supplemental Data Set 1 online). A candidate gene at the locus At1g34190, encoding a NAC domain transcription factor, ANAC017, was identified with nonsynonymous mutations in each of the mutant alleles (see Supplemental Data Set 2 online; Figure 1F). Wild-type coding sequence for At1g34190, expressed under the control of the cauliflower mosaic virus 35S promoter, was transformed into rao2-1 mutants and restored the ability of transgenic plants to induce AOX1a-LUC under AA treatment (Figure 1E), thus confirming that the rao2 phenotype was the result of a specific mutation in ANAC017. Furthermore, point mutations in both rao2-1 and rao2-2 were confirmed by Sanger sequencing (Figure 1G). A single nucleotide change in rao2-1 introduced a stop codon replacing Trp at amino acid position 99 (Trp-99), whereas the change in rao2-2 produced an amino acid substitution from a Pro into a Leu at amino acid 180 (P180L; Figures 1G and 1H). The RAO2/ANAC017 protein is predicted to be a type II membrane protein with a single TM motif at the C-terminal end and an N terminus containing the NAC domain (PROSITE accession number PS51005) (Figure 1G) (Kim et al., 2010). RAO2/ANAC017 belongs to NAC2 subfamily according to the classification of Ooka et al. (2003) (see Supplemental Figure 2 and Supplemental Data Set 3 online). The point mutations in rao2-1 and rao2-2 are in highly conserved protein regions between members of the NAC2 subfamily (Figure 1H). The rao2-1 allele harbors a mutation in the NAC domain, while the rao2-2 mutation lies near the boundary of the NAC domain (Figures 1G and 1H).

RAO2 Is Required for Induction of AOX1a Transcript and Protein

To further confirm that any deficiency in mitochondrial retrograde signaling in the rao2 mutant lines was a result of specific inactivation of ANAC017, two T-DNA lines (SALK_022174 and
Figure 1. Identification of RAO2/ANAC017 as a Regulator of AOX1a.

(A) Two-week-old seedlings (top panel) and luminescence of ColLUC and rao2 mutants after treatment with AA. ColLUC plants generated from Col-0 transformed with a firefly LUC reporter gene driven by the AOX1a promoter (AOX1a-LUC). LUC activity was then visualized in a NightOWL bioluminescence imager.

(B) Luminescence of rao2-1 and rao2-2 after 6 h of myxothiazol treatment.

(C) Quantified luminescence of rao2 mutants 6 h following AA or myxothiazol treatment. Bars indicate se, and asterisks indicate a significant difference (P < 0.001, Student’s t test) of LUC activity between ColLUC and rao backgrounds.

(D) Allelic test of rao2-1 and rao2-2. F1 seedlings were the first generation from a cross between rao2-1 and rao2-2 mutants.

(E) Complementation test of rao2-1 transformed with full-length coding sequences of ANAC017. The F2 generation of transformants (Complement) was able to induce LUC following AA treatment.

(F) Next-generation sequence analysis identifying a candidate mutation in ANAC017 (At1g34190).

(G) ANAC017 gene model. ANAC017 contains a predicted NAC domain at its N terminus and a predicted TM domain at the C terminus. Sanger sequencer analysis of rao2-1 and rao2-2 confirmed the point mutations that caused amino acid changes.

(H) Alignment of NAC proteins from rice (ONAC) and Arabidopsis (ANAC). Identical amino acids are colored in black, and amino acids that shared high similarity are colored in gray. Asterisk indicates the position of the point mutation in rao2 mutants.
SALK_044777) in the Col-0 background were also used in subsequent analyses (Figure 2A; see Supplemental Figure 3A online). The two lines were screened and found to have T-DNA insertions in the second and fourth exons of the ANAC017 coding sequence, respectively (see Supplemental Figures 3B to 3D online). One SALK line (SALK_022174, referred to as anac017-1) contained the insertion in the same exon as the EMS mutants and was shown to be a true knockout line, as the full-length transcript could not be amplified in this background (see Supplemental Figure 3F online, using primers F1 and R1) and the NAC domain was disrupted. Interestingly, the T-DNA insertion in the second line (SALK_044777, referred to as anac017-2) was in exon 4, just upstream of the predicted TM region (Figure 2A), but did not disrupt the N-terminal NAC domain at all. While no transcript could be detected using the F1 and R1 primers (see Supplemental Figure 3F online), a truncated transcript could be detected with primers F1 and R2 (see Supplemental Figure 3F online), with a mobility on an agarose gel indicating a size of ~1500 bp, close in size to the predicted size of 1424 bp, compared with the size of a genomic DNA product that has a predicted size of 1936 bp (see Supplemental Figure 3F online). Based on this, a new set of primers for quantitative RT-PCR (qRT-PCR) analysis of the ANAC017 transcript abundance in anac017-2 (see Supplemental Figure 3G online) revealed that transcript abundance was similar to that in the wild-type line (see Supplemental Figures 4C and 4D online). Sequencing revealed that the T-DNA was inserted after codon 438, adding 10 amino acids from the T-DNA sequence followed by a stop codon (see Supplemental Figure 3E online). Thus, the predicted TM region from amino acids 523 to 548 could not be translated in this line.

AOX1a transcript and protein abundance were examined by qRT-PCR and immunoblot analyses in Col:LUC and independent rao2/anac017 mutant lines: rao2-1 representing an EMS mutant, the SALK T-DNA insertion line, anac017-1 (SALK_022174) and the SALK T-DNA insertion line, and anac017-2 (SALK_044777) over a period of 6 h of treatment with AA. After 3 h of treatment, >40-fold significant induction (P < 0.001, Student’s t test) of AOX1a transcript level was observed in Col:LUC as a primary response, and after 6 h of treatment, this response had already decreased (Figure 2Bi). The magnitude of induction was significantly lower (P < 0.001, Student’s t test) in rao2-1 and anac017-1, with only 10- to 20-fold AOX1a induction (P < 0.001, Student’s t test) (Figure 2Bi). This result was consistent with a higher level of the LUC transcript abundance in Col:LUC under AA treatment (12-fold induction, P < 0.001, Student’s t test) compared with that of rao2-1 (threefold induction, P < 0.01, Student’s t test) at 3 h after AA treatment (see Supplemental Figure 4A online). In the case of anac017-2, AOX1a transcript abundance was significantly higher (P < 0.01, Student’s t test) than Col:LUC under untreated conditions at all time points examined (Figure 2B). Thus, it appears that the anac017-2 allele may produce a constitutively functional protein that is able to induce AOX1a expression even without AA treatment and is likely to be a gain-of-function mutant. However, induction of AOX1a in anac017-2 under stress conditions, like both rao2-1 and ana017-1 mutants, was significantly lower compared with Col:LUC.

To confirm that the aberrations in transcriptional regulation of AOX1a were carried through at a protein level, immunoblot analysis was performed (Figure 2Bii). Consistent with the transcript analysis, AOX1a protein abundance was fourfold higher in Col:LUC after AA treatment compared with rao2-1 and anac017-1 (Figure 2Bii).

Next, we examined the role of RAO2/ANAC017 in response to general cellular stresses. Excessive ROS is toxic to cells, and it has been shown that AOX1a expression increases under conditions of oxidative stress (Vanlerberghe and McIntosh, 1997; Rhooids and Subbaiah, 2007; Millar et al., 2011). Seedlings were treated with 20 mM H2O2 for 6 h. Three hours after treatment, a primary stress response was observed, with a greater than sixfold AOX1a induction (P < 0.001, Student’s t test) in Col:LUC plants (Figure 2Ci). Induction of AOX1a in response to H2O2 was significantly reduced (P < 0.001, Student’s t test) in rao2-1 and anac017-1 at both a transcript level and a protein level (Figures 2Ci and 2Cii; see Supplemental Figure 4B online). In the case of anac017-2, as observed previously, the constitutive level of AOX1a was higher than in Col:LUC (Figure 2Ci). Two points to note are as follows: (1) Induction with AA produced a greater induction in AOX1a than treatment with H2O2 (Figures 2B and 2C; see Supplemental Figures 4A and 4B online); in fact, the response to AA in the rao mutant and T-DNA lines was still greater than the response in the wild-type control, Col:LUC, to H2O2. (2) At 6 h, the response was decreasing and was returning to untreated levels in Col:LUC, and in mutants, with H2O2 treatment. This is consistent with the LUC imaging results for the rao mutants, where imaging takes place 6 h after application of AA or H2O2. In the case of AA (and myxothiazol and monofluoracetate), a large reduction in luminescence was still evident, but for H2O2, the difference after 6 h, while significant (P < 0.05, Student’s t test), was smaller in magnitude (see Supplemental Figure 1 online).

**RAO2/ANAC017 Binds to Specific NAC Binding Sites in the AOX1a Promoter**

Validation of the binding ability of ANAC017 to the AOX1a promoter to influence stress responsive induction was performed to confirm direct regulation of AOX1a by ANAC017. Analysis of the AOX1a promoter sequence revealed three consensus NAC protein binding site sequences, (T/G)CGTGT, identified in two independent studies (Tran et al., 2004; Olsen et al., 2005) (Figure 3A). NAC binding sites 1, 2, and 3 are at positions −311, −264, and −94 bp upstream from the transcriptional start site, respectively (Figure 3A). Transient biolistic transformation assays using β-glucuronidase (GUS) activity driven by the AOX1a promoter in Arabidopsis suspension cells revealed that the AOX1a promoter activity increases significantly in response to AA treatment (P < 0.05, Student’s t test) (Figure 3Bi). Mutation of any single NAC binding site within the promoter led to limited or no significant changes in basal or induced promoter activity. However, when a combination of elements was deleted, basal promoter activity and stress induction were severely compromised (P < 0.01, Student’s t test) (Figure 3Bi). Analysis of the AOX1a promoter in the anac017-1 T-DNA mutant showed that treatment with AA did not induce the promoter as it did.
in Col-0 (Figure 3Bi). *ana017-2* also failed to induce the GUS activity (Figure 3Bi), which was consistent with the induction of the \textit{AOX1a} transcript abundance in this mutant background as shown in Figure 2Bi, in that the induction of \textit{AOX1a} with AA was much reduced compared with Col: \textit{LUC}. The level of \textit{ANAC017} transcript in the *ana017-2* background was similar to that of Col: \textit{LUC} (see Supplemental Figures 4C and 4D online).

The activity of the proposed NAC binding sites was also tested in stably transformed lines. Treatment of plants transformed with the wild-type \textit{AOX1a} promoter with AA resulted in a clear induction of GUS activity (Figure 3C; see Supplemental Figure 5 online). Deletion of NAC binding site 2 resulted in a complete loss of induction of GUS activity (Figure 3C). Deletion of NAC binding site 1 resulted in very little loss of staining, and deletion of NAC binding site 3 resulted in a loss of staining.

\textbf{Figure 2.} Characterization of \textit{AOX1a} Transcript and Protein Abundance in \textit{rao2} and \textit{ana017} T-DNA Insertion Lines.

\textbf{(A)} A schematic gene model of \textit{ANAC017}. Numbers indicate the nucleotide position of the mutation or the T-DNA insertion. \textit{rao2} mutants are marked in red asterisks, and positions of the T-DNA inserts (\textit{ana017-1} and \textit{ana017-2}) are indicated by inverted triangles. TSS, transcriptional start site; NLS, putative nuclear localization signal.

\textbf{(B) \textit{AOX1a} transcript (i), \textit{UBQUITIN} (\textit{UBC}) transcript (ii), and \textit{AOX1a} protein (iii) abundance under AA treatment.} (i) Two-week-old seedlings were exposed to 50 μM AA or deionized water (Mock, −AA) and were harvested at 1, 3, and 6 h and transcript abundance analyzed by qRT-PCR. (ii) \textit{UBC} was used as transcript abundance control. (iii) Protein abundance was quantified and \textit{AOX1a} abundance was normalized against \textit{TOM40-1} to give a loading corrected quantification. Relative protein abundance, below each blot, was expressed as a percentage of the highest value of the set (i.e., for \textit{AOX1a}, that for Col: \textit{LUC} treated with AA is set to 100). Apparent molecular mass is indicated in kilodaltons. Bars indicate SE, and asterisks indicate a significant difference (P < 0.001, Student’s t test) of \textit{AOX1a} transcript abundance in Col: \textit{LUC} compared with \textit{rao2} backgrounds with AA treatment. Pound signs indicate a significantly difference (P < 0.001, Student’s t test) of \textit{AOX1a} transcript abundance in untreated versus AA treated, both in Col: \textit{LUC} and \textit{rao2} backgrounds.

\textbf{(C) As in (B) except that induction was performed with 20 mM H}_2\textit{O}_2\textsubscript{2}.
Figure 3. RAO2/ANAC017 Binds to Predicted NAC Binding Sites in the AOX1α Promoter.

(A) A schematic diagram of AOX1α promoter region with NAC binding sites 1, 2, and 3. TSS, transcriptional start site.

(B) Analysis of AOX1α promoter activity using GUS reporter assay. (i) Wild-type plants (Col-0) were transiently transformed with constructs expressing wild-type AOX1α promoters fused with GUS or with constructs with NAC binding sites mutated in the AOX1α promoter. Bars indicate SE, asterisks indicate a significant difference of GUS activity between wild-type promoter and mutated promoter constructs (P < 0.05, Student’s t test), and pound signs indicate significant induction of GUS activity between mock-treated (deionized water) control and AA treatment (P < 0.05, Student’s t test). (ii) Analysis of AOX1α promoter activity using GUS reporter assay in wild-type plants (Col-0) and anac017-1 and anac017-2 genetic backgrounds. Bars indicate SE, asterisks indicate a significantly difference of GUS activity between wild-type and mutant plants, and pound signs indicate significant induction of GUS activity between untreated control and AA treatment (P < 0.05, Student’s t test). All assays in (i) and (ii) were performed using three biological replicates.

(C) GUS histochemical staining of 2-week-old transgenic Arabidopsis seedlings carrying either the wild-type (WT) AOX1α 2 kb promoter driving GUS expression (top panel) or constructs with the NAC binding sites deleted (bottom panel) 6 h after AA treatment. Data from three independent transformed lines are shown in Supplemental Figure 5 online.

(D) Yeast one-hybrid binding assay of RAO2/ANAC017 to AOX1α promoter. Bait vector contained a 50-bp region surrounding NAC binding sites. Prey vector contained the full-length coding sequence of RAO2/ANAC017. Serial dilutions of cotransformed yeast were spotted on synthetic dropout (SD) –Trp/Leu to select for cotransformants and SD-Trp/Leu/His to select for positive interactions. Numbers 1, 2, and 3 in bold represent predicted NAC binding sites.
in the blade of the leaves, but not in the veins (Figure 3C; see Supplemental Figure 5 online).

Further evidence of positive binding of RAO2/ANAC017 to the AOX1a promoter was detected by yeast one-hybrid analyses, with RAO2/ANAC017 and AOX1a promoter fragments containing NAC binding sites 1, 2, and 3 (Figure 3D). Binding was greatly reduced, as evidenced by little or no yeast colony growth, when NAC binding sites 2 and 3 were mutated, indicating that the binding of RAO2/ANAC017 to binding site 2 and 3 was specific (Figure 3D), consistent with the GUS staining results (Figure 3C). Finally, the binding of RAO2/ANAC017 to the AOX1a promoter was also validated by electrophoresis mobility shift assays (EMSAs). Whereas NAC binding sites 1 and 3 did not show a specific interaction with ANAC017 protein (Figure 3E), a clear band shift of radiolabeled probes containing the NAC binding site 2 was detected when RAO2/ANAC017 protein was added (Figure 3E, NAC binding site 2), due to binding to the protein. Unlabeled NAC binding site 2 probes could effectively compete out binding (Figure 3E, NAC binding site 2) and mutation of the NAC binding site 2 resulted in a loss of that binding (Figure 3E, NAC binding site 2).

**RAO2/ANAC017 Is Colocalized to Actin and the Endoplasmic Reticulum and Released to the Nucleus upon Proteolytic Cleavage of the C-Terminal TM Region**

The subcellular localization of RAO2/ANAC017 was examined using green fluorescent protein (GFP) targeting assays. Full-length RAO2/ANAC017 fused to GFP was seen dispersed in the cytoplasm and formed a web-like structure that did not colocalize with mitochondria (Figure 4A), unlike other transcription factors involved in plastid retrograde signaling that have been recently characterized to be bound within the organelle envelope (Sun et al., 2011). When the predicted TM region was deleted from the RAO2/ANAC017-GFP fusion (as would be predicted to be produced by anac017-2; see Supplemental Figure 3E online), GFP fluorescence was detected only in the nucleus (Figure 4Ai). A GFP fusion containing only the predicted TM region of RAO2/ANAC017 displayed a similar pattern as the full-length construct (Figure 4Aiii). Thus, it was concluded that the predicted TM region was responsible for the localization of RAO2/ANAC017 outside the nucleus.

Furthermore, the full-length RAO2/ANAC017-GFP showed overlapping fluorescent signal with F-actin with two independent fluorescent signals with F-actin with two independent markers for actin. First, with a rhodamine-labeled phalloidin control (Figure 4Bi) (Olyslaegers and Verbelien, 1998), and second in cotransformations with an AtFimbrin-1-RFP (for red fluorescent protein) control (Figure 4Bii) (Wang et al., 2004). Together these analyses confirm that RAO2/ANAC017 is co-localized with F-actin filaments within the cell. The endoplasmic reticulum (ER) is intimately connected with F-actin and forms a cellular transportation network (Boevink et al., 1998). Colocalization with ER was also tested and revealed consistent overlapping fluorescent signal between the full-length RAO2/ANAC017-GFP and ER-RFP (Figure 4C). Thus, we concluded that RAO2/ANAC017 is targeted to connections and junctions in the ER and F-actin via a C-terminal TM domain and upon release of this domain is exclusively located in the nucleus.

To characterize the mechanism by which an ER-located transcription factor can activate transcription in the nucleus, the two known mechanisms described for activation of ER-bound transcription factors were investigated: alternative splicing and proteolytic activation (Howell, 2013). For the ER-bound transcription factor bZIP60, initially believed to be released from the ER membrane by an unknown proteolytic cleavage (Iwata and Koizumi, 2005; Iwata et al., 2008), it has been shown that under stress, alternative splicing of the mRNA transcript is performed by an ER-bound RNA splicing enzyme called inositol-requiring enzyme 1 (IRE1), a dual-functioning protein kinase (Nagashima et al., 2011). Two genes encode IRE1 in Arabidopsis, called IRE1a and IRE1b (Nagashima et al., 2011). In order to test if alternative splicing was involved in the induction of AOX1a under AA treatment, the increase in transcript abundance for AOX1a was measured in ire1a, ire1b, and ire1a ire1b double mutants (Figure 5A) (Nagashima et al., 2011). It was evident that in each single mutant, there was no difference in the induction of the AOX1a transcript, while in the double mutants, the induction of AOX1a transcript was double that observed in the wild-type or single mutants (Figure 5A). Additionally, amplification and sequencing of the ANAC017 transcript revealed no difference between normal and stress conditions in terms of alternative splicing or insertion of a stop codon before the predicted TM domain (data not shown). Thus, it was concluded that alternative splicing of the ANAC017 transcript was not the mechanism of activation.

A variety of treatments were performed that have been previously used to inhibit the proteolytic release of membrane-bound transcription factors in plants (Seo et al., 2010). Col:Luc plants were treated with inhibitors targeting various proteases, MG132 (26S proteasome inhibitor), ALLN (calpain protease inhibitor), a plant protease inhibitor cocktail, and 1,10-phenanthroline (a metalloprotease inhibitor). All these treatments failed to reduce the induction of LUC activity in Col::Luc or inhibit the induction of AOX1a transcript abundance under AA treatment (see Supplemental Figure 6 online). The ER stress response is characterized in plant and mammalian cells to be involved in protein release and modification events; therefore, chemicals known to induce the ER stress response were also tested for the ability to activate the AA-responsive induction of AOX1a. Tunicamycin, a mixture of homologous compounds that prevent the first committed step of N-linked glycosylation of proteins in the ER, causes extensive protein misfolding and induces
a slow-motion ER stress response (Li et al., 2011). Treatment with tunicamycin resulted in normal AOX1a induction profiles in response to AA (see Supplemental Figures 6C and 6D online). Interestingly, treatment with DTT, which induces a fast-motion ER stress response by inhibiting disulphide bond formation in proteins (Deng et al., 2011), resulted in no increase in LUC activity or AOX1a transcript abundance in response to mitochondrial stress (see Supplemental Figures 6C and 6D online). Finally, using a T-DNA mutant of the site 2 protease (S2P), which previously had been shown to be required for the release of the membrane tethered bZIP28 and bZIP17 transcription factors (Seo et al., 2010; Howell 2013), did not compromise the induction of AOX1a (see Supplemental Figure 6E online).

A group of proteases that are capable of proteolysis in a membrane environment are rhomboid proteases. The first rhomboid substrate identified from Drosophila melanogaster was an epidermal growth factor ligand called Spitz, characterized by the seven amino acids highlighted in gray (Figure 5B).

Figure 4. In Vivo Targeting of Full-Length and Truncated Forms of RAO2/ANAC017 Linked to Green Fluorescence Protein.

(A) Subcellular targeting of full-length RAO2/ANAC017 (i), full-length RAO2/ANAC017 minus the predicted TM region (ii), and the predicted TM region of RAO2/ANAC017 (iii) were tagged with GFP to assess targeting ability. These three constructs were transiently transformed into both Arabidopsis suspension cells and onion epidermal cells, in addition to a mitochondrial RFP (Mito-RFP) control (see Methods).

(B) Colocalization of RAO2/ANAC017 to actin filaments. Based on the localizations observed for the full-length construct, colocalization of RAO2/ANAC017 was confirmed with actin stained with rhodamine-labeled phalloidin control (i) and AtFim1-RFP control (ii). A magnified view of this colocalization has been provided.

(C) Colocalization of RAO2/ANAC017 to the ER with an ER-RFP control.
Figure 5. Identification of the Activation Mechanism of ANAC017.

(A) Characterization of AOX1a transcript abundance in splicing mutants. Relative AOX1a and UBIQUITIN (UBC) transcript abundance after 3 h of AA treatment. Two-week-old seedlings of Col-0, ire1a (At2g17520 T-DNA SALK_018112), ire1b (At5g24360 T-DNA GABI_638B07), and ire1a ire1b double mutants were treated with 50 µM AA and harvested 3 h later or before the treatment. Transcript abundance was measured by qRT-PCR. Bars indicate SE, asterisks indicate a significant difference of AOX1a transcript abundance between the wild type and mutant in AA-treated samples (P < 0.001, Student’s t test), and pound signs indicate a significant difference between untreated (DMSO and ethanol control) and AA-treated samples (P < 0.001, Student’s t test).

(B) Rhomboid protease cleavage site in the C-terminal region of ANAC017. Potential rhomboid substrates have a concentration of helix-breaking residues (S, Q, P, and G) around the cleavage site (underlined); the presence of a GA (helix-breaking) pair (indicated in red) and the presence of an ASI motif (LSI present in ANAC017) (as both L and S are hydrophobic, they have similar properties, indicated in red). The seven amino acids previously shown to be sufficient for cleavage by the Spitz rhomboid from D. melanogaster is shaded in gray (Strisovsky et al., 2009). The arrow indicates the cleavage site in the Spitz TM domain. Dm, Drosophila melanogaster.

(C) Inhibition of the induction of AOX1a transcript abundance by the rhomboid protease inhibitor TPCK. Four-day-old suspension cell culture was treated with 100 µM TPCK or DMSO for 3 min followed by 50 µM AA treatment for 1 and 2 h. Ethanol and DMSO were used as a solvent-only control. Samples were then collected for AOX1a transcript abundance measurement using qRT-PCR. UBIQUITIN (UBC) was used as transcript abundance control. Bars indicate SE, asterisks indicate a significant difference of AOX1a transcript abundance between untreated (DMSO and ethanol control) and AA-treated samples (P < 0.001, Student’s t test), and pound signs indicate a significant difference between samples treated with and without TPCK (i.e., AA plus DMSO versus AA plus TPCK treatments) (P < 0.001, Student’s t test).
that are necessary and sufficient for cleavage (Strisovsky et al., 2009). Analyses of the sequence of ANAC017 in the region of the TM domain revealed that it contains a similar motif, except that Ala, a hydrophobic residue, is replaced by Leu, another hydrophobic residue (Figure 5B). It has been determined that structural features rather than sequence motifs define cleavage for rhomboid proteases (Strisovsky, 2013), and it appears that ANAC017 contains all the features required for processing by a rhomboid protease (Figure 5B). In order to test if a rhomboid protease was involved in activation of ANAC017, cell cultures were treated with 100 μM N-p-Tosyl-L-Phe chloromethyl (TPCK), a rhomboid protease inhibitor (Urban et al., 2001), and the induction of AOX1a with AA treatment was assessed (Figure 5C). The induction was reduced approximately fourfold by pretreatment with TPCK, indicating that cleavage by a rhomboid protease was the most likely mechanism of activation of ANAC017.

To obtain additional evidence for proteolytic activation of ANAC017, a construct was designed that was tagged with RFP at the N terminus and GFP at the C terminus (Figure 6A). The hypothesis tested with this construct is that since the GFP is at the C terminus, green fluorescence will be observed only in the ER, as it will remain in the ER when the N-terminal portion of ANAC017 is released by proteolysis. Furthermore, since RFP is at the N terminus, it is proposed that in addition to being located in the ER, this fragment will also be relocated to the nucleus. Finally, upon treatment with AA, the amount of RFP in the nucleus compared with the ER should increase. Visualization of GFP and RFP in onion cells revealed that, indeed, green fluorescence was restricted to the ER network (Figure 6B), as observed in Figure 4. However, red fluorescence could be distinctly detected in the nucleus as well as the ER (Figure 6B). No green fluorescence was detected in the nucleus, although it could be seen as strands overlaying the nucleus (Figure 6B, 60x). Carrying out a similar experiment in the presence of AA revealed that without treatment, red fluorescence was detected in both the ER and nucleus, and with treatment it could be exclusively detected in the nucleus (Figure 6C). Again no green fluorescence was detected in the nucleus. Additionally, using this dual-tagged construct with fluorescent proteins labeling actin and ER confirmed that while GFP coincides with the ER and actin, the RFP was also located inside the nucleus, with ER and/or actin on the outside of the nucleus (see Supplemental Figure 7 online). Thus, the combined evidence of the above approaches strongly indicates that activation of ANAC017 occurs via proteolytic activation by a rhomboid protease releasing ANAC017 from the ER membrane to migrate to the nucleus.

**Global Transcriptional Analysis Reveals RAO2/ANAC017 as a Primary Response Regulator in H2O2-Mediated Stress Signaling**

In order to investigate the extent of the transcriptional network regulated by this transcription factor under the two model stress treatments (i.e., AA and H2O2), changes in the transcriptome were examined using microarray analysis. While AA acts as an inhibitor of mitochondrial electron transport, H2O2 in addition to being produced in mitochondria by manganese superoxide dismutase from the more reactive superoxide radical (O2−) (Murphy, 2009), can be produced by a variety of other cellular sources (Petrov and Van Breusegem, 2012). Analysis was performed on Col::LUC, as a wild-type control, the rao2-1 EMS mutant, and the anac017-1 T-DNA insertion knockout line. Additionally, transcriptomic profiles were examined in anac017-2, which is predicted to be missing the TM region and thus is likely to produce a truncated protein targeted to the nucleus based on the localization studies (Figures 2A to 2C).

For both AA and H2O2 stress treatments, transcripts were cataloged into one of five gene list sets based on changes in transcript abundance in response to stress in wild-type versus the mutant lines (see Supplemental Figure 8, AA, and Supplemental Figure 9, H2O2, online for summary of classifications). For both treatments, genes classified as Group 1 did not change in transcript abundance in response to stress in any lines tested (i.e., are constitutively expressed; 7767 genes for AA and 14,153 genes for H2O2). Group 2 consists of genes that were stress responsive in Col::LUC but for which changes in transcript abundance were not altered by rao2-1 and anac017-1 mutation (i.e., stress-responsive genes that are regulated independently from RAO2/ANAC017 function; 3288 genes for AA and 229 genes for H2O2) (Figures 7A and 7B). Genes from Groups 3 and 4 were stress responsive, and this transcriptional response to stress was mediated either positively or negatively through ANAC017 functionality, respectively (Figures 7A and 7B; see Supplemental Figures 8 and 9 online). These genes represented in Groups 3 and 4 make up the transcriptomic network regulated through ANAC017 as the primary stress response in plant cells in response to general oxidative stress and specific mitochondrial dysfunction (see Supplemental Data Set 4 online). The transcriptomic network that appears to be regulated through the functionality of ANAC017 for the primary stress response at 3 h comprises 1569 transcripts for AA and 1413 transcripts for H2O2 (see Supplemental Figures 8 and 9 online, respectively, Group 3 plus Group 4). An additional group was also cataloged, as it was noticed that, particularly in response to AA treatment, there were a number of transcripts that were nonresponsive in Col::LUC plants at the time point tested but significantly responded to the stress treatment at this time point in the rao2 background (639 transcripts for AA treatment and 60 transcripts for H2O2 treatment). These groups of transcript could represent stress-responsive transcripts that under normal conditions are regulated under a different response kinetic, with the response timing affected in the rao2 background, or they may represent downstream effects of the lack of functional ANAC017 in the plants that results in an altered stress response in these plants. Notably, the proportion of these transcripts is relatively small, compared with the defined stress response in Col::LUC plants, indicating minimal perturbation of the mutants under normal conditions (between 23 and 34% of the stress response for AA and between 3 and 4% for H2O2; see Supplemental Figures 8 and 9 online).

The proportions of the total stress-responsive transcriptomic changes that are mediated through ANAC017 function were examined for the two stress treatments, compared with the percentage of stress response changes mediated independently of ANAC017 function. Strikingly, ANAC017 function was necessary
and sufficient for the correct induction and repression of 87% of transcripts genome-wide that are involved in a primary response to H$_2$O$_2$, and this cannot be compensated for by other transcription factors in plants lacking a functional ANAC017 (Figure 7C). Furthermore, this phenomenon was observed in the stress responses of two independent lines; the rao2-1 EMS line and the anac017-1 T-DNA knockout line showed remarkable overlap in transcriptomic profiles in response to H$_2$O$_2$, with >91% of transcripts (15,855 out of 17,301) responding in a similar manner (see Supplemental Figure 9 online). By contrast, only 32.3% of the AA response could be attributed to RAO2/ANAC017 function (Figure 7C), while the remaining 67.7% of AA-responsive transcripts maintained the correct induction and repression in the absence of a functional ANAC017. This indicates that AA treatment may recruit multiple signaling pathways from the mitochondria, using numerous transcription factors or signal transduction mechanisms that allow redundancy and other factors to compensate for the absence of ANAC017 on a genomic scale.

To further investigate the molecular pathways and functional significance of the involvement of ANAC017 particularly in the

**Figure 6.** Localization of N- and C-Terminal Tagged ANAC017 Using Fluorescence Microscopy.

(A) In order to test whether ANAC017 is initially targeted to the ER, and subsequently relocated to the nucleus, a fusion protein was constructed, consisting of full-length ANAC017 with RFP fused to the N-terminal region and GFP fused to the C-terminal region. This construct allows the location of the N and C termini of ANAC017 to be determined independently.

(B) The construct was transiently transformed into onion epidermal cells using biolistic transformation and observed using a fluorescence microscope at ×20 and ×60 magnification. It was observed that GFP colocalized only with the ER, while RFP was observed to colocalize partially with the ER, but was also found in the nucleus; note GFP was always conspicuously absent from the nucleus.

(C) To influence the proportion of each fluorophore’s localization, onion cells transformed with this construct were treated with AA and incubated for 120 min prior to examination under a fluorescence microscope. AA treatment results in all RFP being detected in the nucleus. Also, GFP was not detected in the nucleus.
primary response to \( \text{H}_2\text{O}_2 \), the transcripts significantly responding to \( \text{H}_2\text{O}_2 \) in \( \text{Col}:\text{LUC} \) plants that were affected by ANAC017 function were visualized in MapMan regulation overview pathways (Figure 7D). The transcript and responses plotted here represent the stress network regulated through ANAC017 function (in conjunction with other transcription factors) under the conditions tested in this study. Numerous transcription factors were alternately regulated, as were protein modification and degradation pathways (Figure 7D). Interestingly, numerous transcripts involved with ABA that are ubiquitously upregulated under \( \text{H}_2\text{O}_2 \) stress were regulated through ANAC017 function as were a number of mitogen-activated protein kinases and many transcripts involved in calcium signaling (Figure 7D).

ANAC017 is a Regulator Mediating Primary and Specific Responses to Stress via the Induction of Transcription Factor Cascades

Analysis of the 728 genes positively regulated through the activity of RAO2/ANAC017 under AA treatment revealed 62 annotated as transcription factors (8.5% versus 4.9% of the whole set of transcripts present on the array throughout this analysis) and five other NAC transcription factors, ANAC042, ANAC087, ANAC019, ANAC04, and ANAC096 (arranged by order of fold change in \( \text{Col}:\text{LUC} \)) were in this group (see Supplemental Data Set 5 online). None of these NAC transcription factors were predicted to contain a TM region (see Supplemental Figure 2 online). Notably, bZIP60 is also within this list of stress-responsive transcription factors that are putatively positively regulated by ANAC017 (see Supplemental Data Sets 4B, 4F, and 4I online). Under \( \text{H}_2\text{O}_2 \) treatment, of the 804 genes that were defined as being positively regulated through the functionality of RAO2/ANAC017, 86 were annotated as transcription factors (10.5% versus 4.9% of the whole set of transcripts present on the array throughout this analysis), ANAC019, ANAC042, ANAC102, ANAC055, ANAC087, ANAC046, ANAC003, and ANAC032 were in this group, and again none are predicted to contain a TM region (see Supplemental Data Set 5 and Supplemental Figure 2 online).

Furthermore, analysis of the second T-DNA insertion line, anac017-2 (see Supplemental Figure 3 online), that likely contains a constitutively nuclear-localized ANAC017 and has elevated levels of \( \text{AOX1a} \) transcript under untreated conditions (Figures 2B and 2C; four- to sixfold elevated), provided insight into the extent of secondary regulatory networks provided by the transcription factors that are directly regulated via ANAC017. Transcripts that are altered in abundance in this background may represent components that are directly regulated via ANAC017 due to the constitutive nuclear localization in this line. Analysis of the 1000-bp promoter regions of the 533 transcripts with altered abundance in the anac017-2 line under normal conditions showed that >85% contain a consensus NAC binding site in their promoters (350/533 genes; see Supplemental Data Set 4I online). Among Gene Ontology annotations for transcripts with altered abundance in this line, components involved in responses to biotic and abiotic stress were overrepresented and those in functional categories or cell organization, biogenesis, protein metabolism, and development were underrepresented (see Supplemental Figure 10 and Supplemental Data Set 4I online). Additionally, 50 out of these 533 transcripts encoded transcription factors (see Supplemental Data Set 5ii online), many of which have well-established roles in the literature in regards to integrating stress and energy metabolism, and of these 50 transcription factors, 84% (42/50) contain one or more consensus NAC binding sites within their 1000-bp promoter regions (see Supplemental Data Set 5 online). Within this list of transcription factors, in order of magnitude of fold change, are ANAC013, ANAC102, ANAC019, ANAC002/ATAF1, and ANAC032. Furthermore, all five of these NAC transcription factors contain one or more NAC consensus binding sites within the promoter regions and are all likely to be involved in regulating downstream secondary responses to cellular stress with metabolic signals and other stress signals (see Supplemental Data Set 5 online). ANAC002/ATAF1 was also significantly upregulated in the anac017-2 background under normal conditions. This transcription factor has been linked with mediating and integrating cellular responses to abiotic and biotic stresses, ABA, drought, and cellular ROS as a high level regulator (Wu et al., 2009).

In order to gain a better understanding of the RAO2/ANAC017-dependent and -independent stress-responsive changes, changes observed were compared with (1) common changes that occur under oxidative stress as defined previously (Gadjev et al., 2006) and (2) genes encoding mitochondrial proteins defined as stress responsive (Van Aken et al., 2009). In terms of stress-responsive mitochondrial components, RAO2/ANAC017 accounts for 11/26 and 10/26 of these changes under AA and \( \text{H}_2\text{O}_2 \) treatment (see Supplemental Data Set 6 online). From 66 genes reported to change in the \( \text{flu} \) mutant, ozone, and methyl viologen (Gadjev et al., 2006), 34 of these transcripts were defined in this study as stress responsive and mediated via ANAC017 function (see Supplemental Data Set 6 online). From these analyses, it appears that RAO2/ANAC017 not only accounts for a large proportion of the \( \text{H}_2\text{O}_2 \)-driven changes in transcript abundance observed in this study, but can also be seen to account for ~50% of the changes observed under various oxidative stress treatments from diverse cellular signals.

Whereas the above analysis suggests that ANAC017 may act upstream of a variety of other transcription factors to regulate stress responses, it is unclear if this is due to ANAC017 directly regulating these factors or via intermediate pathways. We examined whether ANAC017 could bind to the promoter regions of several transcription factors that were found to be upregulated in transcript abundance in the anac017-2 mutant line (see Supplemental Data Set 5iii online). Specifically, we tested CYTOKININ RESPONSE FACTORS5 and 6 (At2g46310 and At3g61630), belonging to the AP2 family of transcription factors and proposed to negatively regulate senescence during stress in Arabidopsis (Zwack et al., 2013) and rice (Oryza sativa; Jung et al., 2010), an AP2 domain transcription factor (At2g47520), defined as a hypoxia-induced transcription factor (Licau et al., 2010), and NFXY1 (At1g10170) defined as having a heat acclimation phenotype (Larkindale and Vierling, 2008). All four promoters contained one or more CACG or CAAAG putative core NAC binding site (Yabuta et al., 2010). Direct binding of ANAC017
**Figure 7.** Characterization of the Genome-Wide Transcriptional Response to Stress That Is Regulated through RAO2/ANAC017 and Cannot Be Compensated for by Other Cellular Components.

(A) and (B) Hierarchical cluster of gene set lists categorized as either positively or negatively regulated through ANAC017 activity under AA (A) or H$_2$O$_2$ (B) treatment, as described in Supplemental Figures 8 and 9 online. Blue color represents transcripts that are downregulated in response to treatment and red/yellow color represents transcripts that are upregulated in response to treatment as shown by the color scale, log$_2$ fold changes in untreated versus treated conditions. Fold changes in response to stress were calculated for Col:LLUC (wild type), rao2-1, and anac017-1 T-DNA insertional knockout lines.

(C) Proportional breakdown of the degree that the response to AA or H$_2$O$_2$ is mediated through RAO2/ANAC017 function genome wide.

(D) MapMan visualization of changes in transcript abundance for Col:LLUC under treatment with H$_2$O$_2$ for transcripts that are regulated through ANAC017 function (i.e., the ANAC017 H$_2$O$_2$ stress-regulated pathway).
to putative NAC binding motifs in all these promoters could be demonstrated (Figure 8), suggesting that regulation by ANAC017 is direct. Mutation of the CA(C/A)G sequence was sufficient to completely abolish binding (Figure 8). Furthermore, direct binding of ANAC017 to the promoter of ANAC013 could also be demonstrated (De Clercq et al., 2013).

Physiological and Phenotypic Consequences of Altered RAO2/ANAC017 Expression

It has been previously shown that aox1a mutant plants are more sensitive to drought stress combined with moderate light treatment compared with the wild type and do not recover after rewatering (Giraud et al., 2008). As we have shown that RAO2/ANAC017 is a regulator of AOX1a, the effect of drought stress combined with moderate light on rao2/anac017 mutants was also investigated. Exposure to moderate light (300 µmol m⁻² s⁻¹) during drought stress produced a notable difference in leaf color between Col:LUC and rao2, anac017-1, and aox1a plants, in that the wild type remained largely green, in contrast with the other lines that were purple in color (Figure 9A, top panel). Previously under similar conditions, it was shown that this was due to a 10-fold increase in anthocyanins in aox1a compared with wild-type plants (Giraud et al., 2008). Notably, anac017-2 appeared to behave as the wild type, likely because of the constitutive nuclear localization of the ANAC017 in this line. The purple pigmentation resulting from increased anthocyanin content (Giraud et al., 2008) was much greater in aox1a and even greater in rao2 and anac017-1 plants. Both Col:LUC and anac017-2 survived the stress treatment and recovered after being returned to normal conditions, while rao2, anac017-1, and aox1a plants died (Figure 9A, bottom panel). Analysis of cellular

<table>
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<th>Promoter site</th>
<th>Sequence</th>
<th>Binding</th>
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<tr>
<td>At2g47520 NAC1</td>
<td>GACATAATTGGGTT CACG TTAAAAATTTTA</td>
<td>-</td>
</tr>
<tr>
<td>At2g47520 NAC2</td>
<td>TAGAAGAACTTGGCGTG CAAAG CAAAACAGCAAC</td>
<td>+</td>
</tr>
<tr>
<td>At2g47520 NAC3</td>
<td>CTTTAACCTTGCGCCCCCTGT CAAAG TACATCAC</td>
<td>-</td>
</tr>
<tr>
<td>At3g61630 NAC1</td>
<td>CCCCTTTTGGGCGGC CACG AAACGCCCTTA</td>
<td>+</td>
</tr>
<tr>
<td>At2g46310 NAC1</td>
<td>TACCGAAATAATC CACG TGCTAAAAATCG</td>
<td>-</td>
</tr>
<tr>
<td>At2g46310 NAC2</td>
<td>TGGATCTGACTCGCTC CAAAG CAAACAGATCATA</td>
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<td>At2g46310 NAC3</td>
<td>CGCGCATGACCTT CACG AACACATAGGCCCG</td>
<td>-</td>
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<tr>
<td>At1g10170 NAC1</td>
<td>TGAAGTGGGGATGTCG CACG TGTTGCAATTGA</td>
<td>+</td>
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Figure 8. EMSAs Show Binding of ANAC017 to Promoters of Downstream Transcription Factor Genes.
(A) The promoters of four selected transcription factors proposed to be regulated by ANAC017 contain one or more putative NAC transcription factor binding sites, CA(C/A)G, indicated in the central column and underlined. Binding of ANAC017 to these promoters was tested by EMSA. For some promoters, up to three sites were predicted and tested. The underlined sequences represent bases that were changed in the mutated probes (see Supplemental Data Set 1 online for sequences).
(B) EMSA confirmed binding of ANAC017 to the four target promoters. Only putative NAC binding sites that displayed binding are shown. In all cases, binding could be abolished by unlabeled competitor probe or was not observed when the predicted NAC binding site was mutated.
content of H$_2$O$_2$ by staining with 3,3'-diaminobenzidine (Förster et al., 2005; Giraud et al., 2008) showed that there were no visible differences between nonstressed leaves of Col:LUC and rao2/anac017 mutants; however, for anaco17-2, there were substantially lower levels of 3,3'-diaminobenzidine precipitate, indicating the response within these plants to a nuclear-localized ANAC017 protein results in lower ROS production under normal conditions (Figure 9B, left panel). No significant difference was observed when visualizing superoxide levels by staining with nitroblue tetrazolium (Figure 9B). Thus, dysfunction of RAO2/ANAC017 protein resulted in significantly reduced drought/moderate light stress tolerance possibly due to failed induction of AOX1a protein itself. By contrast, a gain-of-function allele, anac017-2, with elevated AOX1a transcript levels did not exhibit a stress phenotype and had less cellular H$_2$O$_2$ under normal conditions.

**DISCUSSION**

Whereas the induction of AOX under a variety of treatments from several plant species has been documented (Millar et al., 2011), the results presented here identify a positive regulator required for mitochondrial retrograde signaling. Our results support a role for RAO2/ANAC017 in directly binding the promoter region of AOX1a to regulate transcription and shed light on the mechanism of signal transduction from signals perceived in the cytosol that release ANAC017 from its bound state within the ER/F-actin to the nucleus to affect a response to stress. This subcellular

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**Figure 9.** Characterization of the Physiological Sensitivity to Environmental Stress and H$_2$O$_2$ in rao2/anac017.

**(A)** Thirty-four-day-old plants growth for 16 h at 120 µmol m$^{-2}$ s$^{-1}$ light/8 h dark, 22°C, were transferred to 16 h at 300 µmol m$^{-2}$ s$^{-1}$ light/8 h dark, 22°C, without watering, and responses were observed after 9 d (top panel), after which water was resupplied at 120 µmol m$^{-2}$ s$^{-1}$ light for 3 d (bottom panel).

**(B)** Thirty-four-day-old plants grown for 16 h at 120 µmol m$^{-2}$ s$^{-1}$ light/8 h dark, 22°C, were stained for H$_2$O$_2$ (3,3'-diaminobenzidine [DAB]) and superoxide radical O$_2^*$ (nitroblue tetrazolium [NBT]).
relocalization provides a mechanism for the activation of RAO2/ANAC017 by mitochondrial-generated ROS as follows (Figure 10): Actin, and F-actin in particular, have been established to play a role in mitochondrial movement in several independent studies (Van Gestel et al., 2002; Sheahan et al., 2004, 2005; Wang et al., 2004; Boldogh and Pon, 2006; Logan, 2010), and F-actin has also been shown to match the pattern of the ER network (Boevink et al., 1998). Thus, mitochondria and ER can be positioned together via interaction with F-actin. Intimate mitochondrial–ER interactions have been described in yeast and mammalian systems that play an important role in stress signaling (Pizzo and Pozzan, 2007; Kornmann and Walter, 2010). Thus, mitochondrial ROS production, specifically H$_2$O$_2$, may directly signal RAO2/ANAC017 activation in the ER by close intimate contacts (this may also explain why ANAC017 activity seems so central to the transcriptomic response to H$_2$O$_2$). This signaling also means that ROS-mediated mitochondrial signaling could be specific (Møller and Sweetlove, 2010). A recent study by Maruta et al. (2012) defined transcripts that respond specifically to plastid-generated H$_2$O$_2$ signaling; interestingly, there is little overlap between this list and the list of transcripts that respond to exogenous H$_2$O$_2$ application in this study (only 15 transcripts show common responses out of more than 1000 transcripts that are altered in abundance in the two studies) (Maruta et al., 2012). This would indicate that there is a distinct signal for plastid-generated H$_2$O$_2$ separate from ANAC017 function or mitochondrial/cytosolic H$_2$O$_2$ production. It has been suggested that plastid-generated H$_2$O$_2$ is involved in antagonistic signaling to balance pathways initiated via plastid generated superoxide, and there appears to be little or no overlap with plastidial H$_2$O$_2$ production and the hallmarks of oxidative stress (Gadjev et al., 2006; Laloi et al., 2007; Maruta et al., 2012). Here, we have shown that ANAC017 is necessary for correct cellular responses to exogenous H$_2$O$_2$ signaling molecules, these stress responses are intimately linked with mitochondrial dysfunction, and there is a significant overlap in these responses and those defined as hallmarks of general oxidative stress and superoxide stress (see Supplemental Data Set 6 online). Thus, ANAC017 appears to induce a primary response to H$_2$O$_2$ at a point of convergence that is distinct from plastid-specific H$_2$O$_2$. This also appears to be a primary response, due to the fact that it is most significantly compromised at the 3-h time point; by 6 h, secondary transcriptional networks were induced to result in increased AOX1a transcript abundance (Figure 2). At 6 h and later,
it is likely that downstream stress-responsive transcriptional networks are activated involving other transcription factors (see below).

Two distinct mechanisms have been described for activation of ER tethered transcription factors that appear universal in plant and animals (Deng et al., 2013; Howell, 2013). One mechanism uses two Golgi-located proteases, S1P and S2P, in combination with the ER chaperone binding protein (Srivastava et al., 2013). A second mechanism involves alternative splicing of mRNA to produce a transcript that does not contain a predicted TM region. It is proposed that release and activation of ANAC017 from the ER employs a novel mechanism: proteolytic cleavage by a rhomboid protease. In fact in the splicing mutants ire1a ire1b, which are disrupted in the activation of bZIP60 by alternative splicing, the response of AOX1a to AA treatment was greater than in the wild type (Figure 5A), suggesting that these pathways may overlap in the stress response. Also notable is that bZIP60 transcript abundance was positively regulated by ANAC017 (see Supplemental Data Set 5 online). Whereas induction of AOX1a transcript in the s2p mutant under AA treatment did not display any significant increase in magnitude compared with the wild type, transcript abundance of AOX1a with AA treatment was higher (see Supplemental Figure 6E online).

Overall, the data also suggest that ANAC017 acts as a primary response and that it is involved in regulating secondary responses. This is based on the fact that induction of AOX1a by ANAC017 appears to peak at 3 h after treatment and declined afterwards. Based on the analysis of transcript abundance changes in the anac017 loss-of-function mutants with AA or H₂O₂ treatment and analysis of the transcriptome of anac017-2, which likely produces a constitutive activated ANAC017 protein, a variety of transcription factors increase in transcript abundance. This increase in transcript abundance may be due to direct or indirect consequences of altered ANAC017 function, but the presence of predicted NAC transcription factor binding sites in many of the promoters for these genes, combined with the fact that direct binding of ANAC017 to these predicted NAC transcription sites can be demonstrated (Figure 8), suggests that at least in part, ANAC017 is responsible for the induction of a variety of other stress-responsive transcription factors. In addition to the factors for which direct binding of ANAC017 was demonstrated, many of the genes have previously described roles in stress responses, such as ANAC013 (De Clercq et al., 2013), WRKY transcription factors (Dojcinovic et al., 2005; Giraud et al., 2009), several transcription factors in the AP2 family, including DRE2B and RAP2 involved in resistance to abiotic stresses (Zhu, 2002; Mizo et al., 2012), and other NAC transcription factors, ATAF1/ANAC002 (Wu et al., 2009), ANAC042 (JUNGBRUNNEN1) (Wu et al., 2012), and ANAC019 (Bu et al., 2008). Many NAC transcription factors are known to play a role in mediating stress, development, and growth responses (Chen et al., 2008; Nakashima et al., 2012), and NAC transcription factors represent one of the largest family of transcription factors in plants (Riechmann and Ratcliffe, 2000). However, no function has been previously ascribed to RAO2/ANAC017 in Arabidopsis.

Given the central role of ANAC017 in mediating ROS responses in the cell, it is somewhat surprising that prior studies have not identified this factor. RAO2/ANAC017 is not induced by stress or light treatment in Arabidopsis and the closest orthologues are not stress induced in rice (Nuruzzaman et al., 2010); thus, it could be overlooked in profiling experiments that look for stress-responsive targets. Indeed, an analysis of ANAC017 alterations in transcript abundance across a number of aberrations and developmental and anatomical conditions in the public database Genevestigator (https://www.genevestigator.com/gvl/) revealed that ANAC017 is constitutively expressed and shows fold changes >2.5-fold in only nine conditions out of several hundred experimental conditions (Hruz et al., 2008) (see Supplemental Figure 9B online). Furthermore, inactivation of RAO2/ANAC017 did not cause a severely altered phenotype under normal/standard growth conditions (see Supplemental Figure 11 online), so it would be overlooked if directed screening was performed. Thus, the forward screen we performed can identify important regulatory factors that would go unnoticed by other discovery-based screening methods.

In conclusion, we identified a biological role for ANAC017 as an integral cellular component in mitochondrial retrograde signaling and a high-level transcriptional regulator that is necessary for H₂O₂-mediated primary stress responses in plants. The location-based regulation of ANAC017 functionality provides an elegant mechanism for signal transduction and integration within the plant cell under stress; together, these results significantly contribute to our understanding of retrograde regulation pathways.

METHODS

Col:LUC Construction, Mutagenesis, Mutant Screen, Stress Treatments, and Gene Identification

The cloning of the 2-kb AOX1a upstream promoter region and generation of Col:LUC line with single T-DNA insert, EMS mutagenesis, stress treatments and screening, genetic mapping, and gene identification and verifications were performed as previously described (Ng et al., 2013). All plant materials were grown in growth chambers at 22°C, 16 h 120 μmol m⁻² s⁻¹ light/8 h dark. Seeds were sterilized and sown onto Gamborg’s B5 agar medium supplemented with 3% (w/v) Suc or sown directly onto soil and stratified for 48 h before being moved to growth chambers.

T-DNA Insertion Mutants

Two T-DNA insertion lines for anac017 (SALK_022174 and SALK_044777) were obtained from the European Arabidopsis Stock Centre. T-DNA insertion homozygous lines were confirmed by PCR using the gene-specific primers (LP and RP) and T-DNA-specific primer (LB) (see Supplemental Data Set 1 online). The location of the T-DNA insert was confirmed by sequencing. Additionally, knockout lines of the s2p plants in Arabidopsis thaliana encoded by At4g20310 were obtained (NASC N444004), and AOX1a induction was measured in these lines in response to AA treatment (outlined below) by qRT-PCR. T-DNA lines for ire1a, ire1b, and ire1a ire1b were a kind gift from Nozomu Koizumi (Osaka Prefecture University), as previously described (Nagashima et al., 2011).

Phylogenetic Analysis

Members of the rice (Oryza sativa) and Arabidopsis NAC protein families belonging to the NAC-b group according to Shen et al. (2009) were downloaded from The Arabidopsis Information Resource (www.Arabidopsis.org) or
from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/). ANAC075 does not belong to the NAC-b group but was also included in the analysis because it has a TM domain. The phylogenetic tree was calculated with MEGA5.2.2 (Tamura et al., 2011), aligned using Clustal (Larkin et al., 2007) and analyzed using the maximum likelihood tree method (Jones et al. 1992) and the Jones-Thornton-Taylor model of amino acid substitution. Numbers represent the bootstrapping values of maximum likelihood and maximum parsimony after 1000 replications (Felsenstein, 1985).

qRT-PCR

Rosette leaf tissue from Col:LUC, rao-2,1, anac017-1, and anac017-2 2-week-old seedlings treated with 50 μM AA or 20 mM H2O2, as described above, were harvested in biological triplicate at 1, 3, and 6 h after treatment. RNA isolation, cDNA generation, and qRT-PCR were performed as described previously (Giraud et al., 2008). For mock control, plants were treated with deionized water. Primers and assay details for AOX1a have been described previously (Giraud et al., 2008). LUC primers are listed in Supplemental Data Set 1 online. Plants for mutants analyzed, namely, s2p, ire1a, ire1b, and ire1a ire1b, were treated in a similar manner and AOX1a transcript abundance measured.

Protease Inhibitor Treatments

For MG132 (40 μM; Sigma-Aldrich), ALLN (2 μM; Sigma-Aldrich), 1,10-phenanthroline (100 μM; Sigma-Aldrich), and Protease Inhibitor Cocktail for plant cell and tissue extracts (Sigma-Aldrich P9599) treatment, 2-week-old Col:LUC seedlings were treated with the protease inhibitors or with DMSO (mock) for 90 min followed by 50 μM AA treatment for 6 h before sprayed with luciferin for imaging under a bioluminescent imager. For transcript abundance analysis, 4-d-old Arabidopsis suspension cell culture grown under continuous light conditions was treated with the protease inhibitors for 90 min before treated with or without AA (50 μM) for 3 h before samples were collected for RNA isolation. For tunicamycin (5 μg/mL; Sigma-Aldrich) and DTT (4 mM; Sigma-Aldrich) treatment, 2-week-old Col:LUC seedlings were first treated with the inhibitors or water (mock) for 30 min, then sprayed with 50 μM AA. Plants were then collected for RNA isolation after 3 h of AA treatment or treated with luciferin for imaging after 6 h of AA treatment.

Rhomboid Protease Inhibitor Assay

Four-day-old Col-0 suspension cell culture grown under continuous light conditions were treated with 100 μM TPCK (Sigma-Aldrich) for 3 min before AA was added to a final concentration of 50 μM. Absolute ethanol and DMSO were added as a solvent only control. Tissue samples were then collected 1 and 2 h after AA was added.

Immunoblots

Mitochondria were isolated from 2-week-old plants treated with 50 μM AA and 20 mM H2O2, as described above. Immunodetections were performed as described previously (Wang et al., 2012) using antibodies to AOX (Elthon et al., 1989) and TOM40 (Carrie et al., 2009). To ensure linearity of detection, three dilutions of mitochondria were loaded. The intensity of cross-reacting bands was quantified with Quantity One software (Bio-Rad). The pixel densities were expressed relative to Col:LUC, where the highest value was adjusted to 100 (i.e., mitochondria isolated from Col:LUC treated with AA). Three biological replicates were performed and the average determined. The intensity of the cross-reacting bands probed with the antibody to AOX was adjusted to the intensity of TOM40 that was used as a loading control.

**Transgenic Transformation Constructs**

A pPLUS transformation vector containing 2-kb upstream sequences of AOX1a promoter driving a GUS gene, previously used by Ho et al. (2008), was used as a template for the mutagenesis of NAC binding sites using the Quik Change II XL site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Site-directed mutagenesis primers are listed in Supplemental Data Set 1 online.

**Biolistic Transient Transformation**

To measure the activity of AOX1a promoter with NAC binding site(s) deleted, transformation was performed on suspension cell culture. Col-0 suspension cell culture was grown on half-strength Murashige and Skoog growth medium (PhytoTechnology), supplemented with 3% (w/v) Suc and 0.75% (w/v) agar, under continuous 120 μmol m−2 s−1 light for 5 d before transformations. Transformation was performed with PDS-1000 system with the hepta adaptor according to the manufacturer’s instructions (Bio-Rad), as described previously (Ho et al., 2008). Cell culture was treated with AA to a final concentration of 12 μM or with equal amount of absolute ethanol (control) for half an hour before transferring to osmoticum plates 2 h before transformation was performed. Tissue was collected 4 h after transformation. AOX1a promoter activity was also examined in Col-0 and two T-DNA insertion lines (anac017-1 and anac017-2). Transformation was performed on 2-week-old seedlings grown under 16-h-light/8-h-dark conditions. Plants were left for 18 h after transformation for recovery before treating with 50 μM AA for 6 h. The promoter activities were measured and analyzed as described previously (Ho et al., 2008).

**Yeast One-Hybrid Screen**

The coding sequence of RA02/ANAC017 was cloned from Arabidopsis Col-0 cDNA into pDRIVE (Qiagen) using standard protocols (see Supplemental Data Set 1 online for primer sequences). The RA02/ANAC017 coding sequence was then subcloned into the pGADT7-rec2 prey vector (Clontech) using NdeI and Smal restriction sites. The pGADT7-rec2-p53 prey vector in combination with p3SHIS2 was used as a positive control as outlined in the manufacturer’s instructions (Clontech). Approximately 50 bp surrounding the putative NAC binding sites were cloned into the pHi52 vector using EcoRI and SalI restriction sites (see Supplemental Data Set 1 online for primer sequences), upstream of the HIS3 promoter region and HIS3 reporter gene. Yeast one-hybrid screens were performed in strain Y187 according to the Clontech Matchmaker One-Hybrid kit (Clontech). Transformed cells were grown on SD media –Leu/Trp to select for cotransformed cells and SD media –His/Leu/Trp containing 150 mM 3-amino-1,2,4-triazole (Sigma-Aldrich).

**EMSAs**

The 30- to 40-bp oligonucleotide probes (see Supplemental Data Set 1 online) with wild-type or deleted NAC binding sites were annealed by heating to 99°C and cooled gradually. Annealed probes were radiolabeled using [γ-32P]ATP (Perkin-Elmer) and polynucleotide kinase (Roche) and purified using Sephadex G-25 radiolabeled DNA Quick Spin columns (Roche). The coding sequence of ANAC017 lacking the C-terminal TM domain (i.e., amino acids 1 to 523) was cloned into glutathione S-transferase-tag expression vector pDEST15 (Invitrogen) and C-terminal TM domain (i.e., amino acids 1 to 523) was cloned into the pHIS2 vector using EcoRI and SalI restriction sites (see Supplemental Data Set 1 online for primer sequences), upstream of the HIS3 promoter region and HIS3 reporter gene. Yeast one-hybrid screens were performed in strain Y187 according to the Clontech Matchmaker One-Hybrid kit (Clontech). Transformed cells were grown on SD media –Leu/Trp to select for cotransformed cells and SD media –His/Leu/Trp containing 150 mM 3-amino-1,2,4-triazole (Sigma-Aldrich).
20 min and separated on polyacrylamide gels (0.5× Tris/borate/EDTA, 2.5% glycerol, and 6% acrylamide) for 2 h at 200 V on a 16 × 20-cm² Bio-Rad Protein II gel system.

**Subcellular Localization of ANAC017**

A number of fusion proteins were constructed for this study. The cDNAs of full-length ANAC017 (amino acids 1 to 557), ANAC017 minus the TM domain (amino acids 1 to 523), and the isolated TM of ANAC017 (amino acids 458 to 557) were cloned in frame with GFP using Gateway cloning (Invitrogen), as previously described (Carrie et al., 2009). To track the dynamic relocation of ANAC017 following AA treatment, Gibson Assembly (New England Biolabs) was used to fuse RFP to the N terminus and GFP to the C terminus of full-length ANAC017. The resulting chimeric insert was subsequently cloned into a destination vector using Gateway cloning (Invitrogen). Constructs were transiently transformed into both Arabidopsis suspension cell and onion (Allium cepa) epidermal cells using biolistic transformation, as reported previously (Carrie et al., 2007). In addition to the GFP constructs, a series of organelle markers were employed, including an ER-targeted RFP (Nelson et al., 2007), a mitochondrial-targeted RFP (Carrie et al., 2007), and the isolated actin binding domain of the FIM1 protein (At4g26700) fused to RFP. Additionally, actin was stained using the glycerol method for actin staining outlined by Olszewski and Verbel (1998) with a rhodamine-labeled phalloidin conjugate (Invitrogen, Molecular Probes). Visualization of the fluorescent proteins was performed using an Olympus BX61 fluorescence microscope with excitation wavelengths of 460/480 nm for GFP and 535/555 nm for RFP, while emission wavelengths were measured at 495 to 540 nm for GFP and 570 to 625 nm for RFP. Micrographs were captured and processed using Cell Imaging software as previously described (Carrie et al., 2007).

**Global Transcript Analyses**

Analysis of the global changes in transcript abundance in response AA treatment in the rao2 mutant was performed using Affymetrix ATH1 microarray gene chips. Arrays were performed using RNA isolated 3 h after treatment in biological triplicate from Col:Luc, rao2-1, anac017-1, and anac017-2. Two-week-old seedlings were treated with 50 μM AA or 20 mM H₂O₂. Amplified RNA generation, hybridization, washing, and scanning of gene chips were performed according to manufacturer’s instructions. Data quality was assessed using GeneChip Operating Software 1.4 prior to CEL files being exported into AVADIS Prophetic version 4.3; Strand Genomics) and Partek Genomics Suite software, version 6.3, for further analysis. CEL files are available at the Gene Expression Omnibus under the accession number GSE41136. MASS normalization algorithms were performed only to generate present/absent calls across the arrays. Probe sets that recorded absent calls in two or more of the three biological replicates for genotypes and treatment conditions for a particular analysis were removed. CEL files were also subjected to GC content background robust multiarray normalization for computing fluorescence intensity values used in differential expression analyses. Correlation plots were examined between all arrays using the scatterplot function in the Partek Genomics Suite, and in all cases r > 0.98 (data not shown). Analysis of differential expression was performed using a regularized t test based on a Bayesian statistical framework using the software program Cyber-T (Long et al., 2001) (http://cybert.microarray.ics.uci.edu/). Cyber-T employs a mixture model-based method described by Allison et al. (2006) for the computation of the global false-positive and false-negative levels inherent in a DNA microarray experiment. To accurately control for false discovery rate and minimize false positives within the differential expression analysis, posterior probability of differential expression PPDE(P) values and Posterior Probability of Differential Expression (>P) values were calculated, as a means to measure the true discovery rate (1 - false discovery rate). Changes in transcript abundance were considered significant with a Posterior Probability of Differential Expression (>P) > 0.95 and a log₂ fold change > 1.5-fold. Details for the classification of transcripts into one of five gene lists based on the nature of the stress response in mutant backgrounds can be found in Supplemental Figures 8 and 9 online for AA and H₂O₂, respectively. Hierarchical clusters were generated using Euclidean distance and average linkage measures in Partek Genomics suite V6.3.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g34190 (RAO2/ANAC017) and At3g22370 (AOX1a). All microarray data have been deposited at the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under the accession data set GSE41136.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Characterization and Quantification of AOX1a Promoter Inductions in rao2-1 and rao2-2 Following Different Stress Treatments.

**Supplemental Figure 2.** Phylogenetic Tree of Arabidopsis and Rice NAC Domain–Containing Proteins.

**Supplemental Figure 3.** Confirmation of T-DNA Insertion Lines, anac017-1 and anac017-2.

**Supplemental Figure 4.** Transcript Abundance of Luciferase and ANAC017 in 2-Week-Old Seedlings under Antimycin A or H₂O₂ Treatment.

**Supplemental Figure 5.** Histochemical Staining of AOX1a promoter: GUS Transgenic Plants Treated with or without Antimycin A (+AA and −AA).

**Supplemental Figure 6.** The Ability of Various Inhibitors to Prevent the Induction of AOX1a under Antimycin A Treatment.

**Supplemental Figure 7.** Onion Epidermal Cells Transiently Expressing the nRFP-ANAC017-cGFP Construct in Isolation, with an Actin Control, or with an Endoplasmic Reticulum Control.

**Supplemental Figure 8.** Overview of the logic and criteria for global transcript classification into one of four lists of genes depending on stress responses to Antimycin A and regulation through ANAC017 function.

**Supplemental Figure 9.** Overview of the Logic and Criteria for Global Transcript Classification into One of Four Lists of Genes Depending on Stress Responses to H₂O₂ and Regulation through ANAC017 Function.

**Supplemental Figure 10.** ANAC017 Is Constitutively Expressed and Regulates Stress-Responsive Transcripts Genome-Wide.

**Supplemental Figure 11.** Phenotypic Analysis of rao2 Mutants.

**Supplemental Data Set 1.** List of Primers.

**Supplemental Data Set 2.** Next-Generation Sequencing Candidate SNP Calls.

**Supplemental Data Set 3.** Text File of the Alignment Used for the Phylogenetic Analysis Shown in Supplemental Figure 2.

**Supplemental Data Set 4.** RAO2/ANAC017-Regulated Stress-Responsive Transcripts Based on Microarray Analyses.
Supplemental Data Set 5. Transcripts Regulated by ANAC017 Function That Encode Transcription Factors.

Supplemental Data Set 6. Overlap between RAO2/ANAC017-Regulated Stress-Responsive Transcript and Hallmarks of Oxidative Stress.

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AUTHOR CONTRIBUTIONS

J.W. and A.I. conceived and designed the screen. A.I. carried out the screen. A.I., S.N., and E.G. carried out the genetic mapping, complementation, qRT-PCR transcript measurements, and microarray analysis. S.N. carried out the site-directed mutagenesis and biolistic transient transformation. O.D., Y.W., and B.K. carried out biochemical analyses. S.R.L., L.X., and C.C. carried out GFP localization. E.G. and H.W. carried out bioinformatic analyses of next-generation sequencing data. O.V.A. carried out yeast one-hybrid assays and EMSA. E.G., S.N., A.I., F.V.B., I.D.C., and J.W. designed and interpreted results with regards to integration with cellular stress and energy pathways. All authors contributed to writing the article.

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