Plant, Cell and Environment (2015) doi: 10.1111/pce.12647

SPi | P C E | 1 2 6 4 7 | Journal Code | Article ID | Dispatch: 16.10.15 | No. of Pages: 17 | ME: | CE: Greena Cesa Pesalbon

Original Article

The cytochrome c oxidase biogenesis factor AtCOX17 modulates stress responses in Arabidopsis

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ABSTRACT

COX17 is a soluble protein from the mitochondrial intermembrane space that participates in the transfer of copper for cytochrome *c* oxidase (COX) assembly in eukaryotic organisms. In this work, we studied the function of both *Arabidopsis thaliana AtCOX17* genes using plants with altered expression levels of these genes. Silencing of *AtCOX17-1* in a *cox17-2* knockout background generates plants with smaller rosettes and decreased expression of genes involved in the response of plants to different stress conditions, including several genes that are induced by mitochondrial dysfunctions. Silencing of either of the *AtCOX17* genes does not affect plant development or COX activity but causes a decrease in the response of genes to salt stress. In addition, these plants contain higher reactive oxygen and lipid peroxidation levels after irrigation with high NaCl concentrations and are less sensitive to abscisic acid. In agreement with a role of AtCOX17 in stress and abscisic acid responses, both *AtCOX17* genes are induced by several stress conditions, abscisic acid and mutation of the transcription factor ABI4. The results indicate that AtCOX17 is required for optimal expression of a group of stress-responsive genes, probably as a component of signalling pathways that link stress conditions to gene expression responses.

Key-words: abscisic acid; mitochondrion; salt stress.

INTRODUCTION

The mitochondrial respiratory chain is composed of several complexes that catalyse the oxidation of reduced substrates by O_2 and the subsequent production of ATP (Millar *et al.* 2011). These complexes are formed by subunits encoded either in the nuclear or in the mitochondrial genome, and their expression must be finely coordinated to ensure the proper assembly and function of the respiratory chain (Giege *et al.* 2005; Gonzalez *et al.* 2007; Welchen *et al.* 2014). In plants, the respiratory chain is also composed of the so-called alternative components [i.e. alternative NAD(P)H dehydrogenases and the alternative oxidase]. Particularly, the alternative oxidase (AOX) bypasses the transport of electrons through complexes III and IV, thus participating in a cyanide-insensitive respiratory pathway that drives electrons directly from ubiquinone to oxygen without the concomitant synthesis of ATP (Moore *et al.* 2013). Several components of the alternative respiratory pathways are induced in response to stress (Van Aken *et al.* 2009a; Millar *et al.* 2011), and current evidence supports the hypothesis that they function as safety valves to avoid overreduction of respiratory chain components and the subsequent increase in reactive oxygen species (ROS) production (Van Aken *et al.* 2009b). This function may be particularly important during stress conditions that block electron transport through the cyanide-sensitive pathway (Giraud *et al.* 2008; Wang & Vanlerberghe 2013). Accordingly, *AOX1a*, one of the genes that encode the alternative oxidase, is induced by several stresses and after inhibition of the cyanide-sensitive pathway (Van Aken & Whelan 2012; Vanlerberghe 2013).

In addition to their polypeptidic components, respiratory complexes need the presence of several redox co-factors that directly participate in the transport of electrons and thus are important for respiratory function. An example of the requirement of co-factors for the catalytic function is complex IV or cytochrome *c* oxidase (COX), which needs the presence of two heme *a* groups and two copper centres (Fontanesi *et al.* 2008; Winge 2012). Studies in yeast revealed the requirement of several accessory proteins, which are not part of the complex, for correct copper insertion into COX (Barrientos *et al.* 2009). One of these proteins, COX17, is a small protein from the intermembrane space that receives copper from an unknown source (possibly located in the matrix; Cobine *et al.* 2004) and delivers it to COX11 and synthesis of cytochrome *c* oxidase1 (SCO1), which are involved in copper insertion into COX (Cobine *et al.* 2006; Leary *et al.* 2009). COX17 contains four cysteines arranged in two CX_9C motifs that form a couple of intramolecular disulfide bonds and two additional cysteines involved in copper coordination (Khalimonchuk & Winge, 2008). At least *in vitro*, COX17 participates in several redox reactions, forming species with different amounts of bound copper and different numbers of disulfide bonds, including an oligomeric form with a polycopper cluster bound to reduced thiolates (Heaton *et al.* 2001; Abajian *et al.* 2004; Palumaa *et al.* 2004; Voronova *et al.* 2007; Palumaa 2013).

Proteins similar to yeast COX assembly factors are encoded in plant genomes. In the case of COX17, two *Arabidopsis thaliana* (Arabidopsis) genes, *AtCOX17-1* and *AtCOX17-2*, are able to complement the respiratory deficiency of a yeast

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cox17 null mutant (Balandin & Castresana 2002; Wintz & Vulpe 2002), suggesting that they encode functional proteins that participate in COX assembly. It has also been reported that the expression of *AtCOX17* genes is induced under biotic and abiotic stress conditions (Balandin & Castresana 2002; Attallah *et al.* 2007). In this work, we studied the role of AtCOX17 in stress responses by using Arabidopsis plants with altered expression of *AtCOX17* genes. Our results suggest that AtCOX17 is required for an efficient response of plants to stress.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana

Heyhn. ecotype Columbia (Col-0) was purchased from Lehle Seeds (Tucson, AZ). T-DNA insertion (Alonso *et al.* 2003) and other mutant lines were obtained from the Arabidopsis Biological Resource Center, Ohio State University, or The Q4 European Arabidopsis Stock Centre (Scholl *et al.* 2000). The stock numbers of the lines are CS8104 (*abi4-1*), N24 (*abi3-1*), N6131 (*abi3-5*), N16263 [*mt-gk*; marker line overexpressing green fluorescent protein (GFP) targeted to mitochondria] and SALK_062021C (*cox17-2*). The location of the T-DNA insertion in the *cox17-2* knockout mutant was determined by PCR, using gene-specific primers on genomic DNA prepared according to Edwards *et al.* (1991). The presence of the wild-type (WT) allele was assessed using gene-specific primers flanking the insertion site (Supporting Information Table S1). To determine the exact location of the insertion, the products Q5 were cloned into the vector pCR 2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and sequenced.

Plants were grown on soil in 7 cm height pots at 22 to 24 °C under long-day (16 h light/8 h darkness) or short-day (8 h light/16 h darkness) photoperiod at an intensity of approximately 100 μmol m^{-2} s^{-1}, unless indicated otherwise. Alternatively, Arabidopsis seeds were surface-sterilized in a solution containing 70% (v/v) ethanol and 0.1% (w/v) SDS for 5 min, washed in distilled water and sown in Petri dishes containing 0.5× Murashige and Skoog (MS) medium and 1% (w/v) agar. Plates were cold-stratified at 4 °C for 2 d and transferred to a growth chamber under long-day conditions.

Gene cloning and plant transformation

To obtain plants that express AtCOX17-1 fused to monomeric red fluorescent protein (mRFP), a 990 bp *Bgl*II/*Xho*I fragment comprising *AtCOX17-1* genomic sequences located upstream of the translation stop codon was amplified from Arabidopsis genomic DNA using specific oligonucleotides (Supporting Information Table S1) and cloned into pENTR 3C (Invitrogen). This fragment, together with its flanking recombination sites, was amplified by PCR and transferred into the destination binary vector pGWB554 (kindly provided by Dr Tsuyoshi Nakagawa, Shimane University, Japan; Nakagawa *et al.* 2007), using the Gateway cloning system (Invitrogen). This vector allows C-terminal fusions of proteins to mRFP/mCherry under the control of the enhanced CaMV *35S* promoter. The construct was introduced into the line *mt-gk* (Nelson *et al.* 2007).

To obtain plants that overexpress *AtCOX17-1*, the *Bgl*II/*Xho*I fragment described in the previous text was used, except that a stop codon was added with the reverse primer at the end of the *AtCOX17-1* coding sequence. The fragment was transferred into the destination vector pEarleyGate100 (Earley *et al.* 2006), using the Gateway cloning system (Invitrogen). This binary vector allows protein expression under the control of the CaMV *35S* promoter.

Plants silenced in *AtCOX17* genes were obtained using amiRNAs designed with the WMD3 tool (Web MicroRNA Designer; wmd3.weigelworld.org). Two different amiRNAs for each gene ranked top by the server were used to silence *AtCOX17-1* or *AtCOX17-2*. Once the amiRNA sequence was selected, overlap extension PCR with specific primers (Supporting Information Table S1) was used to obtain the corresponding amiRNA precursors, as described in WMD3. Vector pNB47 (kindly provided by Dr Javier Palatnik, IBR, Rosario, Argentina) was used as template. This binary vector includes the sequence of the precursor for miR319a as backbone for amiRNA expression under the CaMV *35S* promoter. PCR products were cloned into pNB47 replacing the miR319a precursor sequence. Because both amiRNAs used for each gene led to similar silencing levels, only plants expressing one of them were used for most experiments. The expression analysis of stress-responsive genes by RT-qPCR was confirmed with both amiRNAs.

For plant transformation, the *Agrobacterium tumefaciens* strain LBA4404 transformed with the respective constructs was used to obtain transgenic Arabidopsis plants by the floral dip procedure (Clough & Bent 1998).

Confocal laser scanning microscopy

Roots of transgenic seedlings (7 to 10 d old) containing the *35S*::*AtCOX17-1-mRFP* construct in the *mt-gk* background selected on hygromycin were analysed using the Zeiss confocal Q6 laser scanning microscope 780. The imaging procedure and settings are described in Steinebrunner *et al.* (2014). For co-localization analyses, intensity-scatter plots were generated with the 'Coloc 2' and 'Colocalization Threshold' plug-ins of the Fiji software (Schindelin *et al.*, 2012).

High-salt treatments

To measure changes in respiration as a consequence of growth under high-salt conditions, seeds of WT and transgenic plants were sown in Petri dishes containing 0.5× MS medium, 150 mM NaCl and 1% (w/v) agar. Plates were cold-stratified at 4 °C for 2 d and then transferred to a growth chamber under 16 h light/8 h darkness photoperiod for 10 d. Finally, seedlings were harvested and used to measure oxygen consumption (see succeeding text). Samples for gene expression studies were obtained from 15-day-old plants grown in

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0.5× MS medium transferred to fresh MS medium supplemented with 250 mM NaCl for the time periods indicated in the figure legends. Seedlings were harvested and immediately frozen in liquid nitrogen before proceeding with the RNA isolation. To analyse long-term physiological responses to salt, plants were grown on soil under 8 h light/16 h darkness photoperiod. Twenty-day-old plants were acclimated to salt by irrigation with a solution containing 50 mM NaCl for 7 d. Then, plants were irrigated with a solution containing 150 mM NaCl every 7 d, and growth parameters were measured periodically.

RNA isolation and analysis

RNA samples were prepared with TRIzol reagent (Invitrogen). RT-qPCR analysis was performed according to O'Connell (2002). First-strand cDNA synthesis was performed using the oligo$(dT)_{18}$ primer and MMLV reverse transcriptase (Promega, Madison, WI, USA) under standard conditions. Quantitative PCR (RT-qPCR) was performed on an aliquot of the cDNA synthesis reaction with specific primers (Supporting Information Table S2). RT-qPCR was carried out using either an MJ Research Chromo4 or an Applied Biosystems StepOne apparatus in a 20 μL final volume containing 1 μL SYBR Green, 10 pmol of each primer, 3 mM $MgCl_2$, dilutions of the reverse transcription reaction and 0.2 μL PhireII polymerase (Thermo Scientific, Waltham, MA, USA). Fluorescence was measured at 72 °C during 40 cycles. Relative transcript levels were calculated by a comparative C_t method. Expression values were normalized using *PP2AA3* or *ACT2* and *ACT8* transcript levels as standards (Charrier *et al.* 2002; Czechowski *et al.* 2005).

For microarray analysis, duplicate biological replicates obtained from WT plants and plants with reduced expression of *AtCOX17-1* and *AtCOX17-2* were hybridized in Agilent Arabidopsis (V4) 4 × 44K arrays using two-colour reciprocal labelling. Labelling, hybridization, scanning and feature extraction were performed in the Ontario Cancer Institute Genomic Centre (http://www.ocigc.ca/ocigc/) according to the manufacturer's protocols. Further processing of probe intensities was performed with the R statistical programming environment (R Core Team 2013) and the limma package from the Bioconductor project (Gentleman *et al.* 2004). Firstly, probes were filtered to exclude those with log2-background intensities above 7.2. Intensities were then background corrected employing the 'normexp' method with an offset of 16 (Silver *et al.* 2009). Next, arrays were normalized using the locally weighted scatter plot smoothing (LOESS) procedure. Finally, genes differentially expressed between *AtCOX17* and WT plants were identified applying the empirical Bayes method (Gentleman *et al.* 2004), a false discovery rate (FDR) adjusted probability value of 0.05 and a threshold of 1 for the absolute value of the log2 fold change. Analyses of enriched biological process gene ontology (GO) terms and of the overlaps between different gene sets were performed using the VirtualPlant 1.3 site (Katari *et al.* 2010; http://virtualplant.bio.nyu.edu/cgi-bin/vpweb/).

Blue native-polyacrylamide gel electrophoresis, in-gel activity staining and western blot analysis

Extracts enriched in mitochondrial proteins were prepared from seedlings grown for 10 d under 16 h light/8 h darkness photoperiod in liquid MS medium as described in Steinebrunner *et al.* (2014). Proteins from crude mitochondrial fractions were separated by blue native-polyacrylamide gel electrophoresis (BN-PAGE) according to the protocol described by Wittig *et al.* (2006). Digitonin (detergent:protein ratio 4:1) was used for membrane protein solubilization. Equal amounts of mitochondrial proteins, as deduced from citrate synthase activity measurements, were loaded in each lane. Proteins were visualized by Coomassie Brilliant Blue colloidal staining (Neuhoff *et al.* 1988). COX was detected by in-gel activity assay (Jung *et al.* 2000; Wittig *et al.* 2006).

For western blot analysis, proteins separated on SDS-PAGE were transferred to Hybond-ECL (GE Healthcare, Little Chalfont, UK). Blots were incubated with polyclonal rabbit antibodies against AtCOX2 at a dilution of 1:2000, AOX1/2 or VDAC1 (Agrisera) at a dilution of 1:1000 or CA (gamma-carbonic anhydrase; kindly provided by Dr Eduardo Zabaleta, IIB, Mar del Plata, Argentina) at a dilution of 1:500 and developed with anti-rabbit immunoglobulin conjugated with horseradish peroxidase using the SuperSignal West Pico chemiluminescent Substrate (PIERCE).

Oxygen consumption measurements

Oxygen consumption was measured according to Welchen *et al.* (2012). Plants were kept in darkness for 40 min and then 100–150 mg of aerial parts was transferred to 800 μL reaction buffer [300 mM mannitol, 1% (w/v) bovine serum albumin, 10 mM potassium phosphate pH 7.2, 10 mM KCl, 5 mM $MgCl_2$]. Measurements were made at 25 °C using a Clark-type oxygen electrode (Hansatech, Norfolk, England). The cyanide-sensitive pathway was inhibited by addition of 10 mM KCN.

Cytochrome *c* oxidase activity was measured in crude mitochondrial extracts using a Clark-type oxygen electrode as described by Sweetlove *et al.* (2007). Briefly, 500 μL of reaction buffer [0.3 mM sucrose, 10 mM TES/KOH pH 7.5, 10 mM KH_2PO_4,3 mM $MgSO_4$, 10 mM NaCl, 0.1% (w/v) bovine serum albumin] were supplemented with 200 μg of extract, 10 mM sodium ascorbate and 50 μM reduced cytochrome *c* in a final volume of 500 μL. The reaction was started after lysing mitochondrial membranes with 10 μL of 10% Triton X-100.

Chlorophyll content

Chlorophyll content was spectrophotometrically measured as described by Porra (2002). Rosette leaves (100 mg) were placed in a tube containing 2 mL of 80% acetone and incubated overnight at 4 °C. After incubation, samples were centrifuged at 5000 *g* for 3 min, and absorbance was measured in the supernatant. Results were expressed as percentage of the chlorophyll content of WT leaves.

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Determination of lipid peroxidation

Malondialdehyde content was measured according to Hodges *et al.* (1999). Plant tissue (100 to 150 mg) samples were homogenized with a mortar and pestle in 25 mL of 6% (w/v) trichloroacetic acid per gramme of fresh weight (FW) followed by centrifugation at 3000 *g* for 10 min. A 1 mL aliquot of appropriately diluted sample was added to a test tube with 1 mL of either (1) a solution comprised of 20% (w/v) trichloroacetic acid and 0.01% butylated hydroxytoluene or (2) a solution containing the previous plus 0.65% thiobarbituric acid (TBA). Samples were then mixed vigorously, heated at 95 °C for 25 min, cooled and centrifuged at 3000 *g* for 10 min. Absorbance was measured at 440, 532 and 600 nm. Malondialdehyde equivalents were calculated according to Hodges *et al.* (1999).

Determination of superoxide by nitroblue tetrazolium staining

Histochemical analysis of superoxide levels was performed by incubating plants with 0.6 mM nitroblue tetrazolium (NBT) in 1 mM HEPES (pH 10.0). After incubation, plants were cleared with ethanol and analysed.

Cluster analysis

The microarray experiments for the cluster analysis were downloaded using the GEOquery R package (Davis & Meltzer 2007) with the corresponding accession codes (Rotenone and OligomycinLo/Hi, GSE3709; ndufa1, E-MEXP-2178; ndufs4, E-MEXP-1967; aox1a.1 and aox1a.2, E-ATMX-32; asAOX1a.1 and AOX1aOE, E-GEOD-4113; asAOX1a.2, GSE2406; dsr1 and dsr1.SA, GSE22942; and AntimycinA, GSE41136). The normalized datasets were analysed with the limma R package (Smyth 2005), and the log2 ratios with respect to the corresponding control samples were extracted for the genes termed MRR by De Clercq *et al.* (2013). A hierarchical clustering of the genes and experiments was performed first calculating Euclidean distances between the expression vectors and then performing a complete-linkage clustering. The heat map was produced using the corresponding function from the g plots R package.

Statistical analysis

Data are quoted as statistical mean +/− standard deviation (SD). Data were analysed by one-way ANOVA, and the means were compared by Tukey test. Statistical analysis was performed using InfoStat Version 2013 for Windows (http://www.infostat.com.ar).

RESULTS

AtCOX17 is localized to mitochondria *in vivo*

Dual subcellular localization in the cytosol and mitochondria was reported for yeast (Beers *et al.* 1997) and human Cox17 (Oswald *et al.* 2009). However, Cox17 is functional when confined to mitochondria in yeast (Maxfield *et al.* 2004). AtCOX17 is predicted to be localized in mitochondria (e.g. SUBA3 database; Tanz *et al.* 2013). Recently, mitochondrial localization was suggested for AtCOX17-1 by LC-MS/MS (König *et al.* 2014). We initially confirmed the subcellular localization of AtCOX17 *in vivo* by confocal microscopy. For this purpose, we expressed a gene construct encoding a fusion of AtCOX17-1 to the mRFP under the control of the CaMV*35S* promoter in the background of the *mt-gk* line, which expresses a green fluorescent protein-labelled mitochondrial marker (mt-GFP) (Nelson *et al.* 2007). Seedlings from lines co-expressing *AtCOX17-1-mRFP* and *mt-GFP* were assessed by confocal microscopy. The mt-GFP signal appeared in numerous spots of 0.5 μm in diameter, as described by Nelson *et al.* (2007). The fluorescence corresponding to the AtCOX17-1-mRFP fusion displayed a similar pattern, indicating that AtCOX17-1-mRFP was localized in mitochondria (Fig. 1a). In fact, merged images of the mt-GFP and AtCOX17-1-mRFP signal showed overlap of the two signals, which was confirmed by intensity-scatter plot analysis (Fig. 1b). These co-localization results suggest that AtCOX17-1 is restricted to mitochondria *in vivo*. We have not performed a similar analysis for AtCOX17-2. However, considering the high degree of redundancy that seems to exist in the function of these proteins and the fact that both proteins are able to complement a yeast *cox17* null mutant (Balandin & Castresana 2002; Wintz & Vulpe 2002), it can be assumed that AtCOX17-2 also performs its functions inside mitochondria.

Plants with reduced expression of both *AtCOX17* genes show a decrease in the expression of stress-responsive genes

To analyse the function of *AtCOX17* in Arabidopsis, we generated plant lines with post-transcriptional silencing of *AtCOX17-1* or *AtCOX17-2*. For this purpose, constructs expressing specific artificial microRNAs (amiRNAs; Ossowski *et al.* 2008) under the control of the CaMV *35S* promoter were stably introduced in plants (Supporting Information Fig. S6). Lines with reduced *AtCOX17* transcript levels, ranging from 21 to 80% of WT levels, were obtained. As shown in Supporting Information Table S3, silencing was specific for each of the *AtCOX17* genes. Because silencing effects tended to decrease after two or three generations, independent lines with silencing levels over 50% were periodically obtained and used in this study. For *AtCOX17-2*, a homozygous line with a T-DNA insertion located within the coding region of the gene was also used (Supporting Information Fig. S6). *AtCOX17-2* transcripts spanning the T-DNA insertion site were not detected in this *cox17-2* mutant.

Plants with decreased expression of either *AtCOX17* gene were used to obtain plants in which both genes show reduced expression. While attempts to simultaneously silence *AtCOX17-1* and *AtCOX17-2* using amiRNAs were unsuccessful, introduction of a construct that expresses an amiRNA for *AtCOX17-1* into the *cox17-2* mutant line resulted in a relatively low number of independent lines with significantly reduced transcript levels of both genes. These plants (amiCOX17-1/

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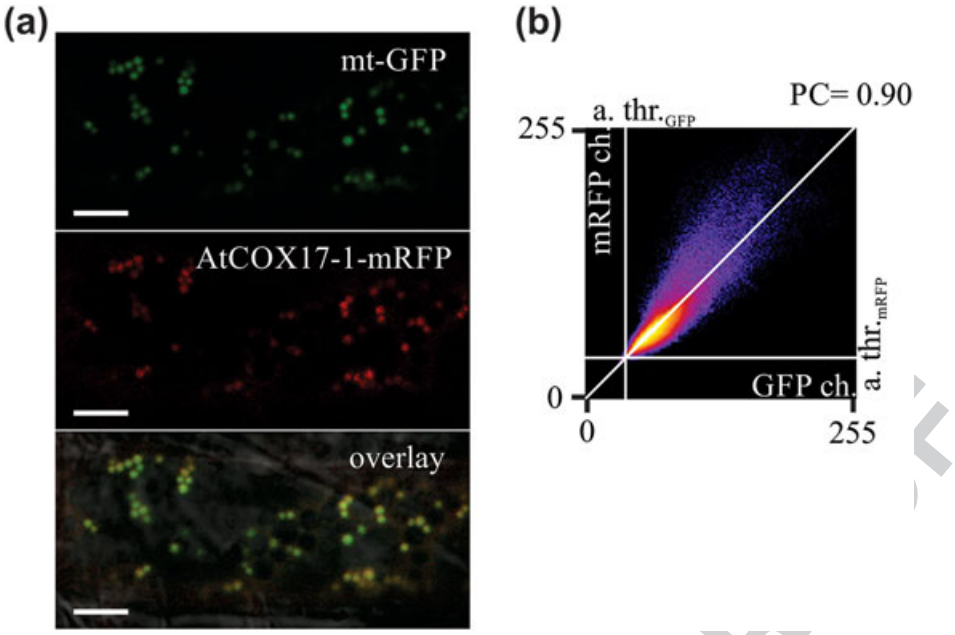

Figure 1. AtCOX17-1 is localized in mitochondria. (a) Roots of 7-day-old transgenic seedlings overexpressing GFP with a mitochondrial targeting signal (mt-GFP) and AtCOX17-1-mRFP were imaged by confocal microscopy. A representative image is shown. The overlay represents the merged images of the GFP and mRFP fluorescence detection channels and the transmitted light. Yellow signals indicate co-localization of equally intense green and red fluorescent signals. Scale bars = 5 μm. (b) The mt-GFP and AtCOX17-1-mRFP images presented in (a) were analysed for co-localization of the GFP (*x*-axis) and mRFP (*y*-axis) detection channel (ch.) intensities by performing a scatter plot, displaying the frequencies of the red–green signal overlays. The automatic thresholds (a. thr.) were set by the Fiji software. Co-localization is assumed when the pixels from both channels cluster along a straight line with a PC greater than 0.5. A PC of 1.0 indicates perfect co-localization. GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; PC, Pearson's coefficient.

cox17-2 plants) were largely affected in development, showing retarded growth and smaller rosettes than WT plants (Fig. 2). It is possible that the growth defect observed in amiCOX17-1/*cox17-2* plants is due to impaired COX assembly caused by the lack of AtCOX17 function. Previous studies indicated that the knockout of proteins involved in COX assembly causes arrest in embryo development or severely affects plant growth (Attallah *et al.* 2011; Steinebrunner *et al.* 2011, 2014; Dahan *et al.* 2014). However, respiration measurements indicated that oxygen consumption in these plants was similar to WT in both the absence and presence of KCN (Supporting Information Fig. S7), suggesting that the capacity of the COX pathway is not severely affected.

To analyse the effect of silencing both *AtCOX17* genes on global gene expression, we performed a microarray experiment using RNA extracted from amiCOX17-1/*cox17-2* plants. We observed that 1531 genes changed their expression more than twofold in these plants compared with WT of the same developmental stage. Among these, 536 genes were up-regulated and 995 were down-regulated. An analysis of the biological processes in which up-regulated and down-regulated genes participate according to The Arabidopsis Information Resource gene ontology (TAIR GO) classifications is shown in Supporting Information Tables S4 and S5, respectively. The set of up-regulated genes is enriched in genes that participate in several metabolic processes, mainly the biosynthesis of organic acids, nitrogen compounds and lipids. Processes such as translation and regulation of the cell cycle are also enriched (Supporting Information Table S4). The set of down-regulated genes shows an enrichment of genes involved in the response of plants to a variety of stress conditions (Supporting Information Table S5).

Among the genes that were down-regulated as a result of *AtCOX17* silencing, there are several genes encoding transcription factors from families typically involved in stress responses: WRKY, NAC [no apical meristem, ATAF (Arabidopsis transcription activation factor) and cup-shaped cotyledon] and AP2/ERF (APETALA2/ethylene response factor) (Chen *et al.* 2012; Mizoi *et al.* 2012; Nakashima *et al.* 2012; Table 1). To validate these results, we selected members of these families (*WRKY53*, *ANAC019*, *CBF2* and *ERF1*) and analysed their expression by RT-qPCR. All genes showed a significant decrease in expression as a result of *AtCOX17* silencing, in agreement with the result of the microarray experiment (Supporting Information Fig. S8).

Genes induced by mitochondrial perturbations show decreased expression in amiCOX17-1/*cox17-2* plants

We also found that the expression of several genes that encode mitochondrial components, such as *AOX1a*, *AOX1d*, *NAD(P)H dehydrogenase b2* (*NDB2*) and *cytochrome bc1 synthesis 1* (*BCS1*), was significantly down-regulated in amiCOX17-1/*cox17-2* plants (Table 1; validation by RT-qPCR for *AOX1d*

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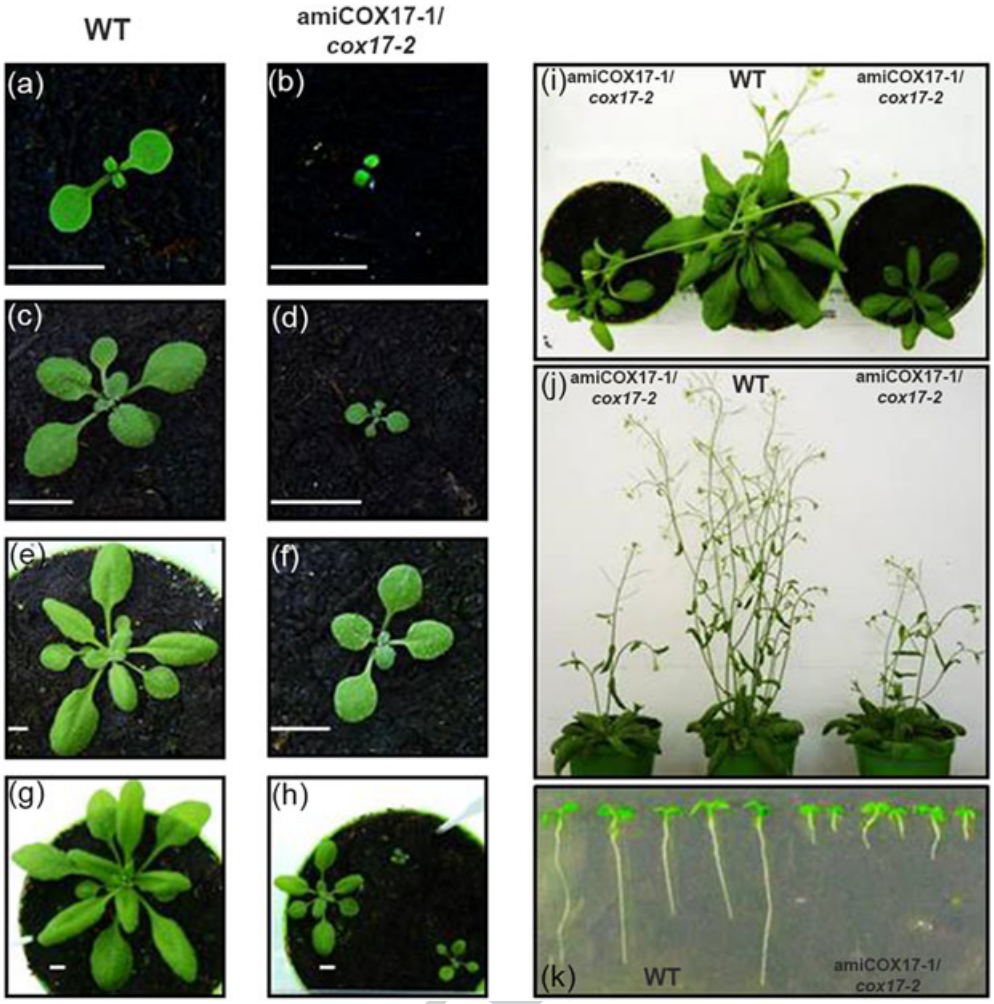

Figure 2. Silencing of both *AtCOX17* genes impairs plant growth. (a–k) Phenotype of amiCOX17-1/*cox17-2* and WT plants at different growth stages. Pictures were taken at days 5 (a,b), 10 (c,d), 17 (e,f), 21 (g,h), 28 (i) and 35 (j) after sowing. In (k), plants grown during 5 d in Petri dishes are shown. Panel (h) shows plants of different size obtained after sowing amiCOX17-1/*cox17-2* plants. Bigger plants, like the one shown to the left, were used for expression analysis and oxygen consumption measurements. Scale bars represent 0.5 cm. WT, wild type.

is shown in Supporting Information Fig. S8). These are stress-responsive genes (Clifton *et al.* 2005; Ho *et al.* 2008) and are also components of a set of genes induced as a result of mitochondrial perturbations (called MRR genes by De Clercq *et al.* 2013). Considering this, we performed a comparative cluster analysis of the expression of MRR genes in amiCOX17-1/*cox17-2* plants and plants with mitochondrial perturbations, due to either mutation of mitochondrial components or treatment with inhibitors of the respiratory chain. The results indicate that a group of MRR genes shows opposite expression characteristics in amiCOX17-1/*cox17-2* plants and plants with mitochondrial dysfunction, mainly those treated with the inhibitors antimycin A, rotenone and oligomycin (Fig. 3). It has been reported that a block in electron transport from ubiquinone to O_2 through complexes III and IV, as observed after inhibition of complex III with antimycin A, induces a set of stress-responsive genes, among them genes that participate in alternative respiratory pathways (Schwarzländer *et al.* 2012; Umbach *et al.* 2012; Ng *et al.* 2013). More recently, a similar behaviour was observed in plants defective in COX activity (Dahan *et al.* 2014). The fact that amiCOX17-1/*cox17-2* plants show opposite changes in expression of these genes suggests that these changes are not a consequence of a decrease in COX activity. In addition, the expression of MRR genes in amiCOX17-1/*cox17-2* plants has similarities with that observed in *AOX1a* antisense plants, defective in the alternative oxidase (Fig. 3).

General oxidative stress-responsive genes also show decreased expression in amiCOX17-1/*cox17-2* plants

Because several MRR genes induced by antimycin A are repressed in amiCOX17-1/*cox17-2* plants, we also compared the global expression profiles of amiCOX17-1/*cox17-2* plants and wild-type and *anac017* mutant plants treated with antimycin A or H_2O_2 (results from Ng *et al.* 2013). ANAC017 is a transcription factor involved in the response of *AOX1a* and other genes to antimycin A and H_2O_2. Cluster analysis indicated that

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Table 1. Selected stress-responsive genes with decreased expression in plants in which both *AtCOX17* genes were silenced

Gene name	Locus	Expression (logFC)	*P* value
WRKY53	AT4G23810	−1.29	3.74E-03
WRKY75	AT5G13080	−1.92	2.69E-05
WRKY40	AT1G80840	−1.90	2.67E-05
WRKY50	AT5G26170	−1.53	6.54E-05
WRKY51	AT5G64810	−1.44	2.83E-03
WRKY26	AT5G07100	−1.25	3.87E-03
WRKY46	AT2G46400	−1.24	1.92E-04
WRKY6	AT1G62300	−1.22	1.99E-04
NAC3	AT3G15500	−1.61	2.15E-03
NAC6	AT5G39610	−2.26	1.11E-03
NAC042	AT2G43000	−2.15	1.51E-03
NAC046	AT3G04060	−1.91	1.33E-03
NAC019	AT1G52890	−1.29	3.58E-03
NAC036	AT2G17040	−1.23	5.40E-03
NAC053	AT3G10500	−1.14	2.83E-04
ERF1	AT3G23240	−2.10	1.13E-03
CBF2	AT4G25470	−2.03	1.27E-03
CBF1	AT4G25490	−1.86	1.49E-03
ERF5	AT5G47230	−1.82	1.53E-03
ERF104	AT5G61600	−1.40	2.78E-03
ERF-1	AT4G17500	−1.33	2.19E-04
ERF4	AT3G15210	−1.23	3.75E-03
ERF6	AT4G17490	−1.19	4.10E-03
ERF13	AT2G44840	−1.16	4.47E-03
ERF11	AT1G28370	−1.11	7.21E-03
AOX1A	AT3G22370	−1.29	4.87E-03
AOX1D	AT1G32350	−2.21	2.73E-05
BCS1	AT3G50930	−1.08	6.72E-03
NDB2	AT4G05020	−1.62	1.96E-03

Genes from the WRKY, NAC and AP2/ERF transcription factor families that belong to the gene ontology category 0006950 (stress response process) and show at least twofold reduced expression after *AtCOX17* gene silencing. Stress-responsive genes encoding mitochondrial proteins are also shown. Expression values are log2 relative to the wild type. Genes in bold were validated by RT-qPCR (Supporting Information Fig. S8).

many genes induced in amiCOX17-1/*cox17-2* plants are down-regulated by antimycin A and H_2O_2 treatments in WT and *anac017* plants, and vice versa (Supporting Information Fig. S1). A detailed analysis indicated that 303 of the 2036 genes induced by antimycin A (15%) are down-regulated in amiCOX17-1/*cox17-2* plants, while 190 of the 2640 genes repressed by antimycin A (7%) are up-regulated in amiCOX17-1/*cox17-2* plants (Fig. 4a). For H_2O_2 treatment, these numbers are 195 out of 871 (22%) and 114 out of 695 (16%), respectively (Fig. 4b). Essentially no differences were observed when the subsets of genes regulated and not regulated by ANAC017 were considered, suggesting that the expression changes in amiCOX17-1/*cox17-2* plants are not particularly related to ANAC017 function.

We also analysed sets of genes that were previously described by Gadjev *et al.* (2006) as markers of general oxidative stress responses (27 genes) and of specific oxidative stress conditions [methyl viologen (MV), O_3 and *flu* mutant; 66 genes]. Figure 4c shows that 17 (63%) and 30 (45%) genes from these sets, respectively, are down-regulated in amiCOX17-1/*cox17-2* plants. Conversely, among the up-regulated genes, only one gene induced by general stress conditions is present. The results suggest that AtCOX17 affects the expression of general stress-responsive genes.

AtCOX17 genes are induced by abiotic stress conditions

The results obtained with amiCOX17-1/*cox17-2* plants suggest that *AtCOX17* genes may have a function related to stress responses. Accordingly, we measured *AtCOX17-1* and *AtCOX17-2* transcript levels after salt, ultraviolet B (UV-B) and high-light intensity treatments (Fig. 5a). These treatments caused a twofold to fourfold increase in transcript levels of both genes. Induction was also observed after incubation with MV and high $CuSO_4$ concentrations (Fig. 5b), which cause an increase in ROS (Fujii *et al.* 1990; Drazkiewicz *et al.* 2004). The results agree with previous studies that showed that *AtCOX17* genes are induced by wounding, treatment of leaves with the bacterial pathogen *Pseudomonas syringae* and incubation with agents that cause oxidative stress (Balandin & Castresana 2002; Attallah *et al.* 2007). The significant induction of *AtCOX17* genes observed in Arabidopsis plants exposed to several stress conditions reinforce the idea that AtCOX17 may have a role in the response to general stress situations.

Silencing of either *AtCOX17* gene impairs the response to salt stress

The severe growth phenotype of amiCOX17-1/*cox17-2* plants precluded further phenotypic analysis of these plants. Plants silenced in either of the two *AtCOX17* genes, on the contrary, developed normally and had no obvious phenotypic differences compared with WT plants (Supporting Information Fig. S2). Considering the role of COX17 in COX assembly, we measured several parameters related with COX function in plants with reduced expression levels of either of the *AtCOX17* genes. COX2 protein levels and total and cyanide-insensitive respiration were not significantly different from those of WT in these plants (Supporting Information Fig. S2). COX activity levels of mitochondrial extracts, analysed by BN-PAGE, were similar in WT, amiCOX17-1 and amiCOX17-2 plants (Supporting Information Fig. S2). For *cox17-2* mutant plants, a slight decrease in COX activity was apparent in some cases (Supporting Information Fig. S2), but this was not consistently observed. To obtain a more quantitative measure of COX activity levels, we analysed oxygen consumption in mitochondrial extracts using reduced cytochrome *c* as substrate. Using this method, COX activity levels were not significantly different from WT in any of the lines analysed (Supporting Information Fig. S2). Again, a tendency to reduced activity levels was evident in *cox17-2* mutant plants. The results indicate that decreasing the expression of single *AtCOX17* genes does not significantly affect COX assembly or respiratory activity.

Considering the expression changes observed in amiCOX17-1/*cox17-2* plants, implying that *AtCOX17* genes influence the

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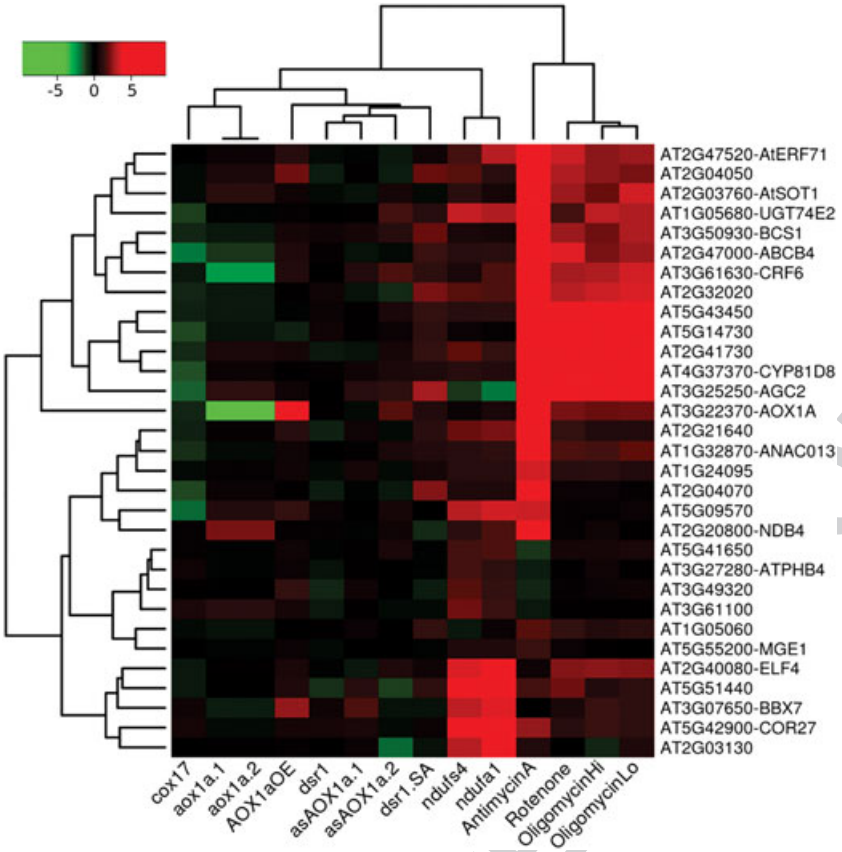

Figure 3. Cluster analysis of the expression of MRR genes in amiCOX17-1/*cox17-2* plants and plants with mitochondrial dysfunctions. The expression of genes that are induced by mitochondrial perturbations (termed MRR genes by De Clercq *et al.* 2013) was analysed in amiCOX17-1/*cox17-2* plants (cox17) and plants with mitochondrial dysfunctions: aox1a.1 and aox1a.2, two lines with mutations in *AOX1a* (Giraud *et al.* 2008); AOX1aOE, an AOX1a overexpressor line (Edgar *et al.* 2002); dsr1 and dsr1.SA, a line with a mutation in the complex II subunit gene *SDH1-1*, either under control conditions or treated with salicylic acid (Gleason *et al.* 2011); asAOX1a.1 and asAOX1a.2, two lines that express an antisense construct for *AOX1a* (Edgar *et al.* 2002; Umbach *et al.* 2005); ndufs4 and ndufa1, lines with mutations in the corresponding complex I subunit genes (Meyer *et al.* 2009); AntimycinA, plants treated with 50 μM antimycin A for 180 min (Ng *et al.* 2013); Rotenone, plants treated with 40 μM rotenone for 180 min (Clifton *et al.* 2005); and OligomycinHi and OligomycinLo, plants treated with 1.25 or 0.125 μM oligomycin, respectively, for 180 min (Clifton *et al.* 2005). The results are shown as a heat map; red and green indicate induction and repression relative to controls, respectively.

expression of stress-responsive genes, and the fact that *AtCOX17* genes are induced by stress, we decided to analyse the behaviour of plants silenced in either of the *AtCOX17* genes under stress conditions. Particularly, we focused on salt stress, because an analysis of genes with decreased expression in amiCOX17-1/*cox17-2* plants showed the presence of many salt stress-responsive genes. In fact, 27% (169/634) of the genes induced by salt stress described by Ma *et al.* (2006) are down-regulated in amiCOX17-1/*cox17-2* plants (Supporting Information Fig. S3). To analyse the effect of salt stress, we incubated plants in 250 mM NaCl for 1 h and measured the expression of stress-responsive genes by RT-qPCR after the treatment. We focused on two sets of genes: (1) genes that are known from the literature to be induced by NaCl treatment and (2) genes that encode mitochondrial components whose expression is induced under salt stress and reduced in amiCOX17-1/*cox17-2* plants. From the first group (Fig. 6a), we observed that *catalase 3* (*CAT3*), which was induced about sixfold in WT plants, did not change its expression after salt treatment in plants silenced in either *AtCOX17-1* or *AtCOX17-2*. In addition, two other genes, *L-ascorbate oxidase* (*ASO*) and *responsive to dehydration 29b* (*RD29b*), showed a decreased response (Fig. 6a). No differences were observed for *peroxiredoxin IIF* (*PRXIIF*), *ferritin 3* (*FER3*) and *ascorbate peroxidase 1* (*APX1*) (Fig. 6a). In the second set, the four tested genes (*AOX1a*, *AOX1d*, *NDB2* and *BCS1*) were induced after salt treatment in WT plants but showed no significant induction in plants silenced in *AtCOX17-1* or *AtCOX17-2* (Fig. 6b). The results indicate that silencing of either *AtCOX17-1* or *AtCOX17-2* originates a decreased response of several genes to salt stress. These genes encode stress-responsive proteins located in different cell compartments, including mitochondria.

The decreased response to salt stress observed after silencing either *AtCOX17* gene may indicate that these plants are not experiencing stress (i.e. that they are more stress-tolerant) or that they are unable to activate stress responses even after experiencing stress conditions. To analyse the physiological impact of growth under high-salt conditions in the plants under study, plants were grown on soil for 20 d with normal water irrigation, acclimated to salt by irrigation with 50 mM NaCl for

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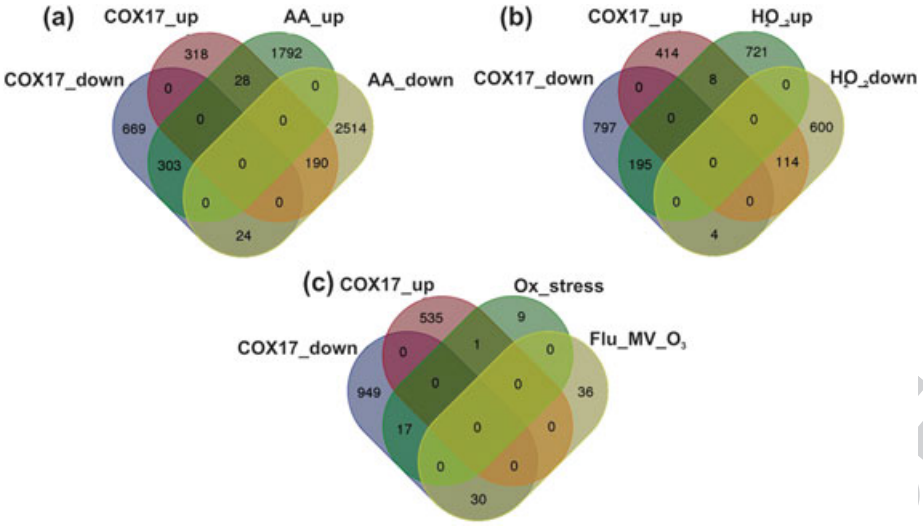

Figure 4. Sets of genes induced by several oxidative stress conditions are preferentially down-regulated in amiCOX17-1/*cox17-2* plants. Venn diagrams of the genes with increased or decreased expression in amiCOX17-1/cox17-2 plants (COX17_up and COX17_down, respectively) with groups of genes whose expression is modified under oxidative stress conditions. (a) Genes induced or repressed by antimycin A (AA_up and AA_down, respectively) according to Ng *et al.* (2013). (b) Genes induced or repressed by H_2O_2 (H_2O_2_up and H_2O_2_down, respectively) according to Ng *et al.* (2013). (c) Sets of genes induced by general oxidative stress (Ox_stress) or by specific oxidative stress conditions (Flu_MV_O_3) according to Gadjev *et al.* (2006).

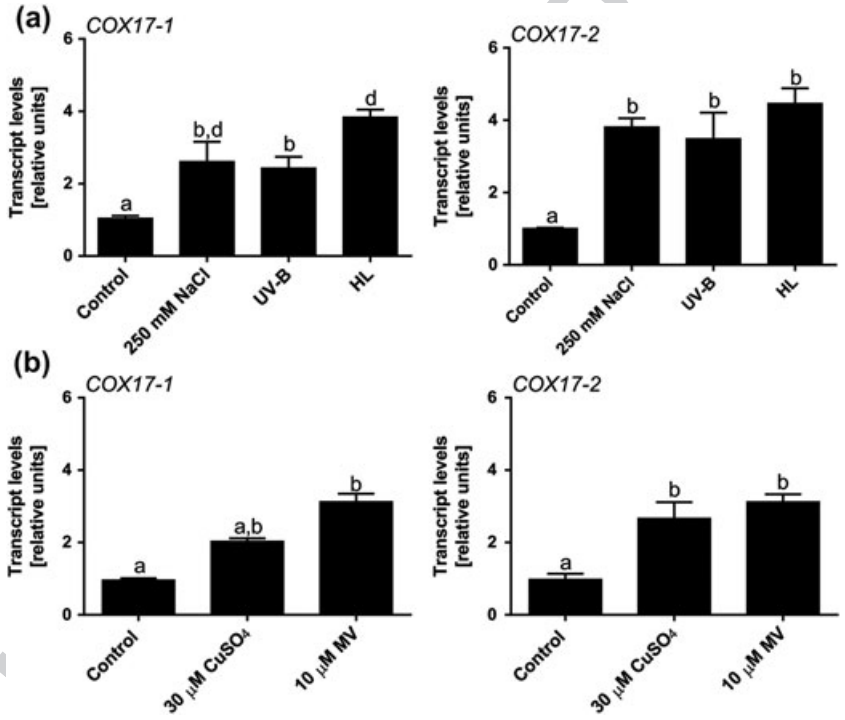

Figure 5. *AtCOX17-1* and *AtCOX17-2* are induced under different stress conditions. Transcript levels were determined by RT-qPCR and referred to the value obtained under control conditions (no treatment). Experiments were carried out with 10 to 15 plants for each treatment and repeated twice with similar results. Results are expressed as mean ± standard deviation of three biological replicates. Columns with different letters are significantly different at $P < 0.05$ (ANOVA; Tukey test). (a) Arabidopsis (Col-0) plants grown on soil for 15 d were treated with either 250 mM NaCl, ultraviolet B (UV-B) light or high-light intensity (HL; 600 μmol m^{-2} s^{-1}) for 3.0, 0.5 and 4.0 h, respectively. (b) Plants grown on 0.5× Murashige and Skoog medium for 15 d were treated with 30 μM $CuSO_4$ or 10 μM methyl viologen (MV) for 0.5 h.

7 d and then treated with 150 mM NaCl. Measurements of rosette growth rates during the stress treatment indicated that the growth of WT plants and plants silenced in one *AtCOX17* gene was not differentially affected by salt (Fig. 7a). In addition, no significant differences in chlorophyll levels (Fig. 7b) and plant survival rate could be observed. However, plants **F7**

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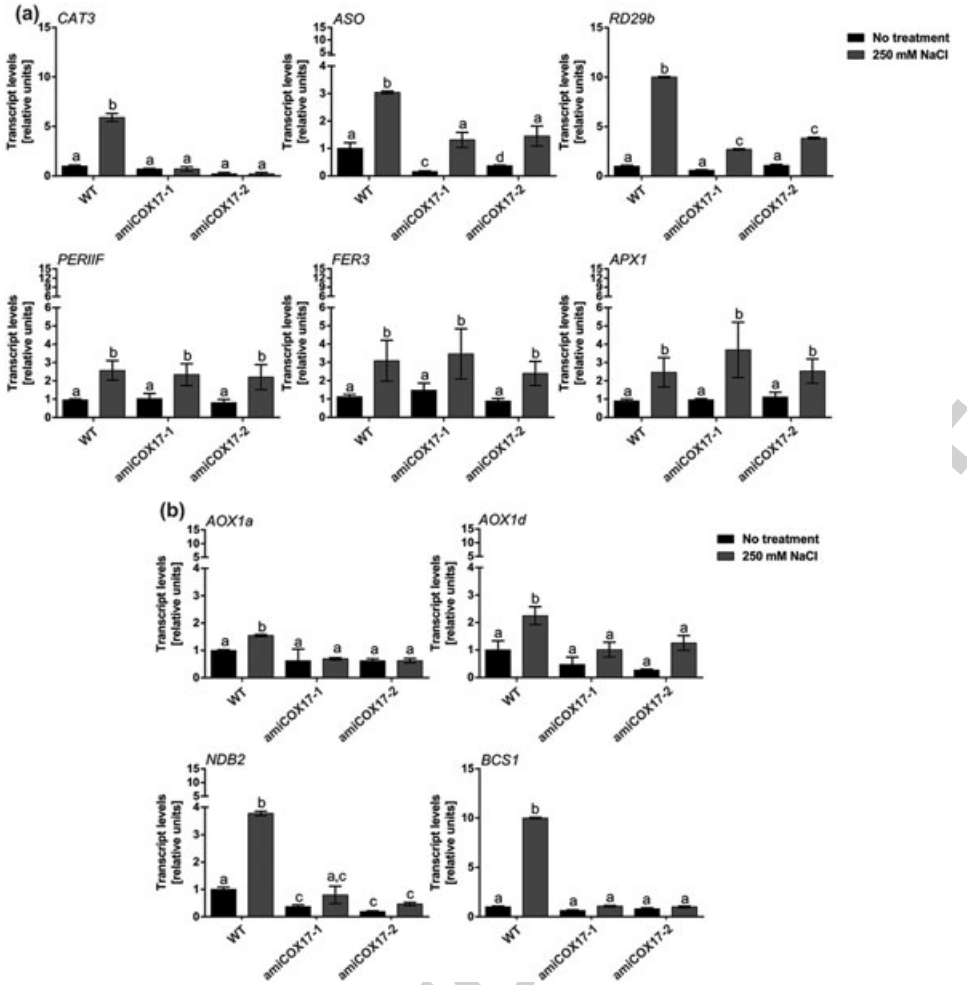

Figure 6. Silencing of *AtCOX17-1* or *AtCOX17-2* causes a decreased response of a group of genes to salt stress. Transcript levels of genes induced by salt stress were measured in WT, amiCOX17-1 and amiCOX17-2 plants before and after incubation with 250 mM NaCl for 1 h. For each assay, two different lines of each genotype were used [amiCOX17-1 lines 1 ($n = 5$) and 10 ($n = 5$) and amiCOX17-2 lines 8 ($n = 5$) and 23 ($n = 5$)]. All values were normalized relative to the value measured in WT plants under normal conditions (no treatment), arbitrarily assigning a value of 1. Results are expressed as mean ± standard deviation of three independent assays. Columns with different letters are significantly different at $P < 0.05$ (ANOVA; Tukey test). (a) A set of genes known from the literature to be induced by salt stress. (b) Genes encoding stress-induced mitochondrial proteins whose expression is decreased in amiCOX17-1/*cox17-2* plants. WT, wild type.

silenced in *AtCOX17* contained higher ROS levels than WT after salt treatment, as deduced from NBT staining (Fig. 7c) and lipid peroxidation measurements (Fig. 7d). This is probably due to the decreased response of a group of genes that influence ROS production and detoxification under stress conditions, like several of those described in Fig. 6. The results indicate that silencing of either *AtCOX17* gene causes an increased sensitivity to high-salt conditions, reflected in increased ROS levels. This suggests that the observed decrease in the induction of stress-responsive genes by high NaCl concentrations is most likely due to a defective response to stress conditions.

We also measured O_2 consumption in plants before and after salt treatment. As reported in the literature (Jacoby *et al.* 2011), the rate of O_2 consumption increased after salt stress in WT plants, mainly because of an increase in alternative respiration as deduced from the effect of KCN (Supporting Information Fig. S4). The increase in alternative respiration is presumed to prevent inhibition of the respiratory chain and to reduce the subsequent increase in ROS levels under stress. A similar increase in respiration was observed after salt treatment in plants silenced in *AtCOX17* genes (Supporting Information Fig. S4). However, the increase in alternative respiration was less pronounced in this case. This is in agreement with the fact that *AOX1a* and *AOX1d* induction by salt is impaired in these plants. Apparently, increased respiration results from an increase in cyanide-sensitive respiration, further suggesting that COX function is not compromised in these plants.

Overexpression of *AtCOX17-1* increases tolerance to salt stress

We also analysed the possible effect of increasing *AtCOX17* expression levels on the tolerance of Arabidopsis plants to salt stress. For this purpose, we cloned the *AtCOX17-1* transcribed

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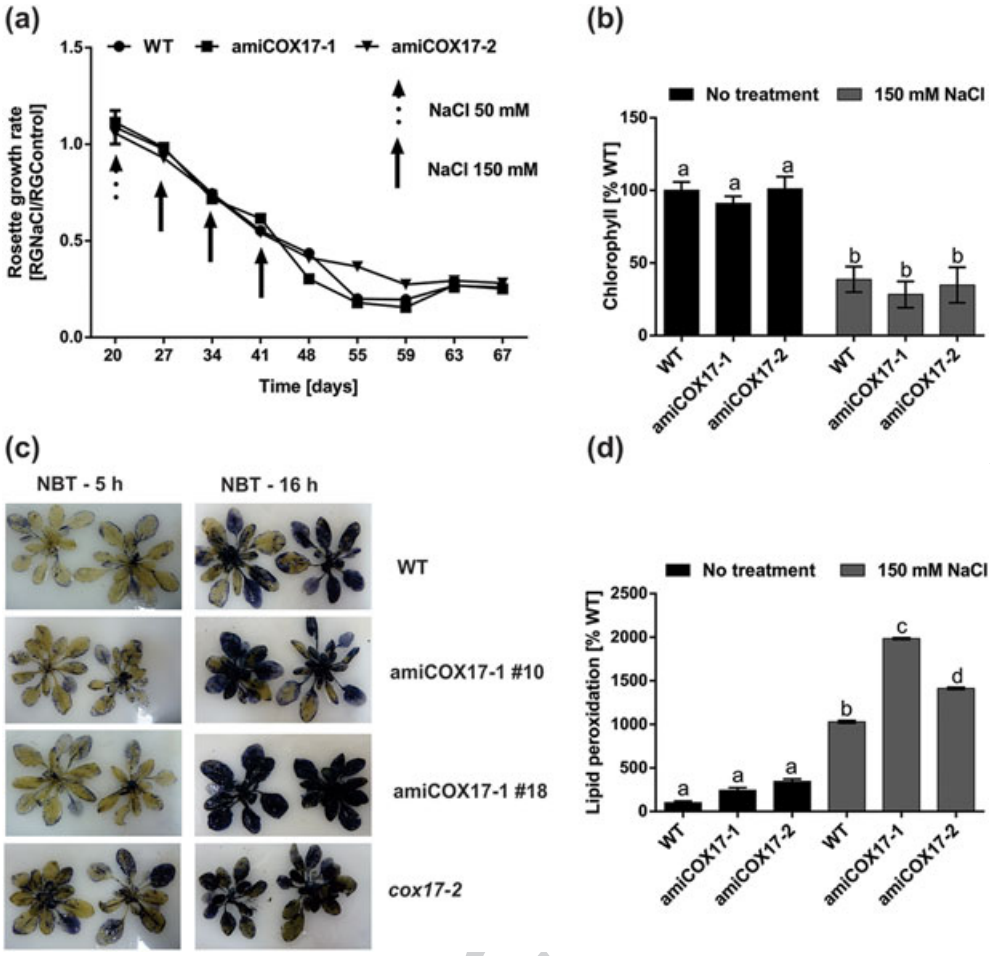

Figure 7. Plants silenced in *AtCOX17-1* or *AtCOX17-2* show increased ROS and lipid peroxidation levels after prolonged salt stress. Plants were grown under normal conditions for 20 d, after which one acclimation treatment with 50 mM NaCl plus three irrigation treatments with 150 mM NaCl (at days 27, 34 and 41) were performed. (a) Rosette growth rate was measured relative to untreated plants. The growth rate of untreated plants was set to 1 at every time point measured. Arrows indicate the time of NaCl treatments. Between 23 and 32 plants of each genotype were used for this assay. Results are expressed as mean ± standard deviation of two lines for each genotype (amiCOX17-1 lines 2 ($n = 20$) and 10 ($n = 23$) and amiCOX17-2 lines 8 ($n = 29$) and 23 ($n = 32$) were used). (b) Chlorophyll content at day 45 of growth. (c) Nitroblue tetrazolium (NBT) staining at day 45 of growth. (d) Lipid peroxidation levels at day 45 of growth. Results are expressed as mean ± standard deviation of two lines for each genotype. This assay was repeated three times. Columns with different letters are significantly different at $P < 0.05$ (ANOVA; Tukey test). WT, wild type.

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region located upstream of the translation stop codon, which includes a leader intron, under the control of the CaMV *35S* promoter and introduced this construct into plants. When growing on soil under basal conditions, plants that overexpress *AtCOX17-1* (oeCOX17-1) showed no obvious phenotypic differences compared with the WT. However, rosette growth after irrigation with 150 mM NaCl was less affected in oeCOX17-1 F8 plants than in WT plants (Fig. 8a). In addition, oeCOX17-1 plants showed slightly increased chlorophyll content and lower lipid peroxidation levels than WT after salt treatment (Fig. 8b, c). The results suggest that an increase in AtCOX17-1 levels increases tolerance to salt stress. Similar experiments using plants that overexpress *AtCOX17-2* were not conclusive.

Measurements of the expression of stress-responsive genes in oeCOX17-1 plants compared with WT showed increased transcript levels of *AOX1d* and *NDB2* under basal growth conditions (Supporting Information Fig. S5). Increased expression of these genes was also observed after 1 (for *NDB2*) or 2 h (for *AOX1d*) of salt treatment (Supporting Information Fig. S5). *AOX1a* transcript levels were also higher after 1 h of treatment in one of the lines analysed. For other genes, like *RD29a* and *ASO*, the response to salt stress was similar to WT after 1 h of treatment, but transcript levels were lower than those of WT plants after 2 h (Supporting Information Fig. S5). The results indicate that the expression of some stress-responsive genes is altered in oeCOX17-1 plants.

AtCOX17 genes are ABA-responsive and are regulated by the transcription factor ABI4 Q11

Considering the observed induction of *AtCOX17* genes by abiotic stress treatments, we analysed the effect on *AtCOX17* gene expression of a treatment with ABA, known to be involved in many responses to abiotic stress (Raghavendra *et al.* 2010). Both *AtCOX17* genes were induced in Arabidopsis

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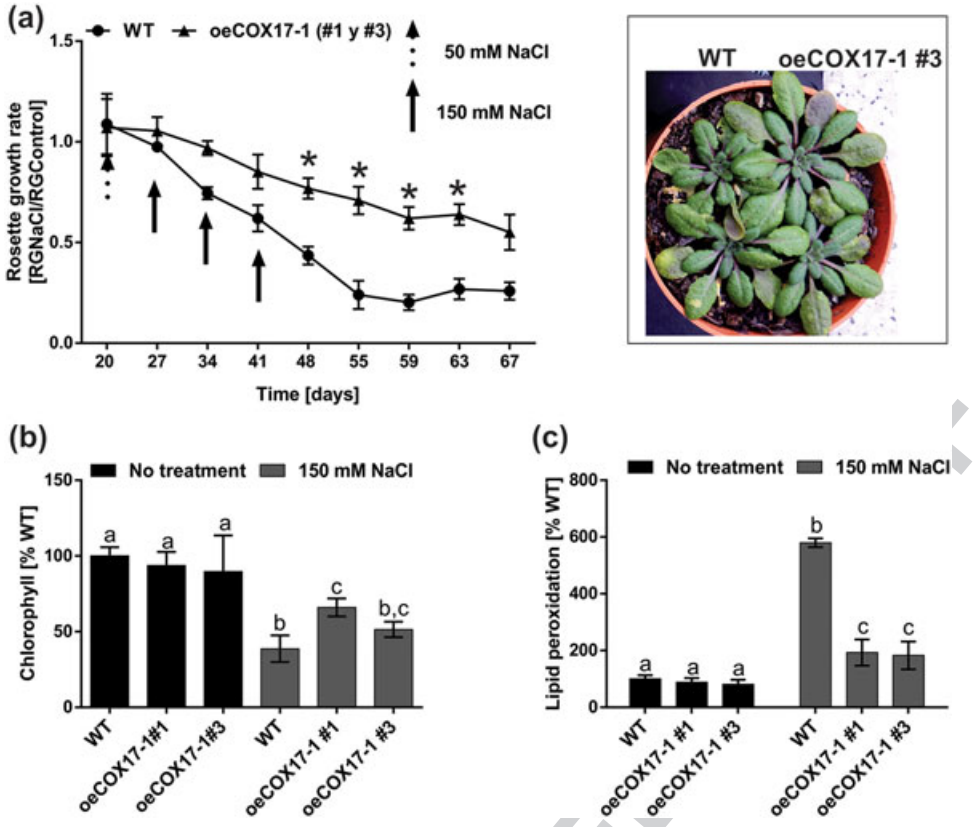

Figure 8. Plants that overexpress *AtCOX17-1* are more tolerant to salt stress. Wild-type (WT) and oeCOX17-1 plants were treated with 150 mM NaCl (three applications on days 27, 34 and 41). (a) Rosette growth rate of WT and oeCOX17-1 plants subjected to NaCl treatment. Between 17 and 25 plants of each line were used for this assay. Results are expressed as mean ± standard deviation of two oeCOX17-1 lines (line 1, $n = 21$; line 3, $n = 17$). Asterisks indicate significant difference relative to WT ($P < 0.05$, Student's *t*-test). A representative image of WT and oeCOX17-1 plants at day 45 of treatment is shown on the right. (b) Chlorophyll content of treated and untreated WT and oeCOX17-1 plants at day 45 of growth. (c) Lipid peroxidation levels of treated and untreated WT and oeCOX17-1 plants at day 45 of growth. Results in (b) and (c) are expressed as mean ± standard deviation of four biological replicates. This assay was repeated three times. Columns with different letters are significantly different at $P < 0.05$ (ANOVA; Tukey test).

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seedlings after 180 min of incubation with the hormone (Fig. 9a). To analyse if components involved in the ABA signal transduction pathways influence the expression of *AtCOX17* genes, we determined *AtCOX17* transcript levels in *aba-insensitive* (*abi*) mutants. Interestingly, transcript levels of both genes were increased in *abi4-1* mutants (Fig. 8b), which are impaired in the function of the AP2/ERF transcription factor ABI4 (Yamaguchi-Shinozaki & Shinozaki 2006). Conversely, expression was not affected by mutations in *ABI3* (Fig. 9b), which encodes a B3-domain transcription factor that mediates responses to ABA through a different signalling element (Yamaguchi-Shinozaki & Shinozaki 2006). This behaviour is similar to the one reported earlier for the stress-responsive *AOX1a* gene (Giraud *et al.* 2009). The result suggests that *AtCOX17* genes may be targets of the same signal transduction pathway involved in the induction of alternative respiratory chain components. Notably, mutation of *ABI4* abolished the response to ABA (Fig. 9c), suggesting that induction by ABA operates through ABI4, which acts as a repressor of *AtCOX17* gene expression.

We also analysed the sensitivity to ABA of plants with decreased expression of *AtCOX17-1* or *AtCOX17-2*. For this purpose, we sowed seeds in medium containing 0.3 μM ABA and analysed the percentage of germinated seeds 18 h later. While in WT plants ABA inhibited germination of about 40% of seeds, no effect of ABA was observed in plants with reduced expression of *AtCOX17-1* or *AtCOX17-2* (Fig. 10). This result suggests that silencing of either *AtCOX17-1* or *AtCOX17-2* decreases the sensitivity to ABA.

DISCUSSION

In the present study, we analysed the function of *A. thaliana COX17* genes using an insertion mutant and silencing with amiRNAs. In yeast, COX17 is a small soluble protein from the mitochondrial intermembrane space that is involved in the delivery of copper for COX assembly (Cobine *et al.* 2006; Khalimonchuk & Winge 2008; Leary *et al.* 2009). The conservation of the protein structure and the fact that AtCOX17 proteins are able to replace yeast COX17 as COX assembly factors (Balandin & Castresana 2002; Wintz & Vulpe 2002) suggest that these proteins fulfil a similar role in plants. The role of AtCOX17 in COX assembly may explain the fact that

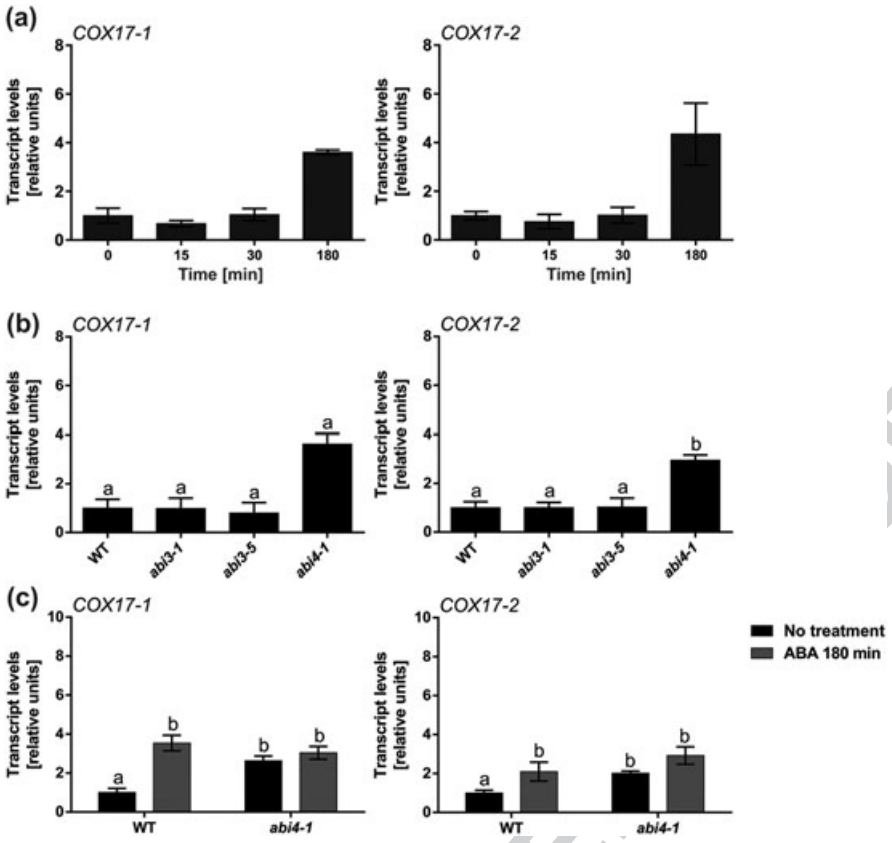

Figure 9. *AtCOX17* gene expression is induced by ABA and modulated by ABI4. (a) Transcript levels of *AtCOX17-1* and *AtCOX17-2* at different times after treatment with 10 μM ABA. (b) Transcript levels of *AtCOX17-1* and *AtCOX17-2* under basal growth conditions (no treatment) in mutants in *ABI3* (*abi3-1* and *abi3-2*) and *ABI4* (*abi4-1*). (c) Effect of ABA treatment on *AtCOX17* gene expression in the wild type (WT) and the *abi4*yyy*1* mutant. Results are expressed as mean ± standard deviation of three different experiments. Columns with different letters are significantly different at $P < 0.05$ (ANOVA; Tukey test).

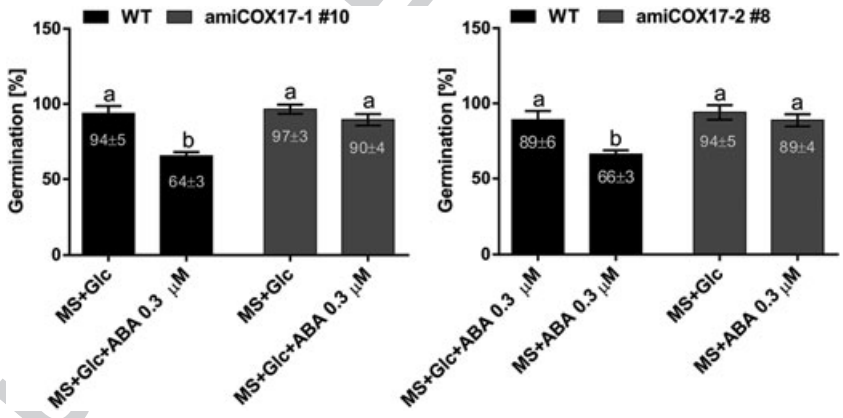

Figure 10. Silencing of *AtCOX17* decreases sensitivity to ABA. Germination of wild type (WT), amiCOX17-1 and amiCOX17-2 seeds in Murashige and Skoog (MS) 0.5× medium containing 2% glucose and either 0 or 0.3 μM ABA. Percentage of germination was determined 18 h after the plates were transferred to growth chamber conditions. Results are expressed as mean ± standard deviation of three different plates, each one with 300 to 350 seeds from each genotype.

silencing of both *AtCOX17* genes originated only a limited number of lines that were affected in growth to different levels. Notably, oxygen consumption rates in the plants analysed were similar to WT in both the absence and presence of KCN, suggesting that COX pathway capacity is not severely affected. However, we also observed the presence of very small plants that arrested their development at the rosette stage and eventually died. These plants were not further analysed, but we speculate that their phenotype may arise from a defect in COX activity.

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Analysis of gene expression in amiCOX17-1/*cox17-2* plants indicated that these plants show a general decrease in the expression of genes that participate in the response to many different types of stress. In fact, genes involved in the responses to general oxidative stress, to salt stress and to mitochondrial dysfunctions are all overrepresented in the set of genes down-regulated in amiCOX17-1/*cox17-2* plants. Even though it can be argued that the results observed with these plants may be due to a secondary effect of their impairment in growth, we strongly favour the idea that they are a more direct reflection of AtCOX17 action. This is suggested by the observation that altered stress responses also occurred in plants with only one *AtCOX17* silenced gene, which are not affected in development. It is also unlikely that the decreased expression of stress-responsive genes observed in amiCOX17-1/*cox17-2* plants is due to defects in COX activity. In fact, current evidence indicates that inhibition of electron transport through complexes III and IV, due to either treatment with inhibitors or a deficiency in complex IV, induces the expression of stress-responsive genes (Schwarzländer *et al.* 2012; Umbach *et al*. 2012; Ng *et al*. 2013; Dahan *et al*. 2014), among them those encoding alternative respiratory components that show reduced expression or responses after *AtCOX17* silencing. Among the genes with down-regulated expression due to *AtCOX17* silencing, many encode transcription factors from families typically known to be involved in stress responses. Whether these are true and direct intermediates in the signalling process originated by decreased *AtCOX17* expression will require further investigations.

Silencing of single *AtCOX17* genes did not significantly affect plant growth. These plants showed normal COX activity levels and a reduced or delayed response to stress. These results also suggest that the reduced response to stress is not related to COX deficiency and that *AtCOX17* genes have acquired functions related to stress responses in addition to their role in COX assembly. The fact that silencing of single *AtCOX17* genes produced plants that were unable to properly induce a set of stress-responsive genes supports a role of AtCOX17 in optimizing or amplifying stress responses.

Plants respond to stress through a variety of mechanisms. The sessile nature of plants is probably one of the reasons why plants have developed such sophisticated responses. Even though each type of stress triggers specific defense programmes, features common to many different stress conditions can be recognized, one of them being the generation of ROS (Mittler 2002; Gill & Tuteja 2010). ROS damage cell structures and are also intermediates in signalling pathways that induce the expression of stress-responsive genes (Suzuki *et al*. 2012; Baxter *et al.* 2014). The finding that plants with decreased expression of *AtCOX17* genes show increased ROS levels and lipid peroxidation after salt treatment but are unable to properly induce stress-responsive genes suggests that stress signalling is altered in these plants. Besides specific mechanisms that generate ROS at the plasma membrane (i.e. during biotic stress; Torres 2010), ROS are also generated in energy-producing organelles (chloroplasts and mitochondria) because of inhibition of the respective electron transport chains followed by overreduction of their components (Apel & Hirt 2004; Navrot *et al*. 2007). As a consequence, these organelles are also major targets of ROS damage, thus generating a positive feedback cycle that causes further inhibition of electron transport chains. In recent years, it has become evident that these organelles also participate in signal transduction pathways involved in responses to increased stress and ROS production (Noctor *et al*. 2007; Vanlerberghe *et al*. 2009; Pfalz *et al*. 2012; Kleine & Leister 2013). In the case of mitochondria, several reports have shown that mutations or changes in the expression of genes that encode mitochondrial proteins modify the response of plants to biotic and abiotic stress or the sensitivity to the hormone ABA (Zsigmond *et al*. 2008; Gleason *et al*. 2011; Laluk *et al*. 2011; Cvetkovska & Vanlerberghe 2013). Our results suggest that AtCOX17 is another mitochondrial protein that participates in the modulation of plant stress responses.

It is noteworthy that some expression characteristics of *AtCOX17* genes resemble those of components of the alternative respiratory pathways (Ho *et al*. 2008; Giraud *et al*. 2009), because they are induced by stress, ABA and mutation of the transcription factor ABI4. Because ABI4 is involved in diverse signalling processes, it can be speculated that *AtCOX17* genes are sensitive to signals that impact in the nucleus through an ABI4-dependent pathway (Wind *et al.* 2013), probably the same pathway that modulates the expression of *AOX1a* (Giraud *et al*. 2009).

Considering that *AtCOX17* expression levels influence the expression of a set of stress-responsive genes and that *AtCOX17* genes themselves are induced by stress, it can be proposed that AtCOX17 reinforces regulatory pathways involved in the induction of a group of stress-related genes. It can be speculated that the amount or redox state of AtCOX17 influences signals involved in these regulatory pathways. COX17 proteins can exist in multiple forms according to the amount of copper present in the medium and the redox state of the environment (Heaton *et al.* 2001; Abajian *et al*. 2004; Palumaa *et al*. 2004; Voronova *et al*. 2007; Palumaa 2013). In addition, it was observed that COX17 species with different oligomeric states can form depending on protein concentration (Heaton *et al*. 2001). Because redox signals are thought to be important components of stress-related signal transduction pathways, it can be envisaged that AtCOX17 may act as a transducer of these signals. We cannot completely rule out that the action of AtCOX17 on stress responses is also related to its role in COX assembly. Even if strong inhibition of COX-dependent respiration triggers the induction of stress-responsive genes (Schwarzländer *et al.* 2012; Umbach *et al*. 2012; Ng *et al*. 2013; Dahan *et al*. 2014), it is possible that small changes in COX activity, which went unnoticed in our studies, may produce a different effect. Vanlerberghe *et al.* (2009) hypothesized that AOX may participate in the modulation of stress signal transduction pathways, helping to keep the balance between ROS production by the respiratory chain and ROS scavenging through the induction of antioxidant systems. Additionally, a role of AOX in signalling was demonstrated at least during pathogen infection (Cvetkovska & Vanlerberghe 2013). According to our results, AtCOX17 would be another component of this or a related signal transduction pathway,

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possibly acting to modulate the magnitude and timeliness of the response to stress. A future challenge will be to identify the signals and components that integrate this pathway.

ACKNOWLEDGMENTS

We thank the Light Microscopy Facility of the Biotechnology Center Technische Universität Dresden for their excellent training and professional assistance with the CSLM imaging. We also thank several researchers, listed in the text, who provided vectors and antibodies used in this study. This work was supported by grants from ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica, Argentina), CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina) and Universidad Nacional del Litoral. The stays of L.G. and E.W. in Dresden and of U.G. in Santa Fe were supported by grant 1455/5-1 from the German Research Foundation (DFG) awarded to I.S. The stays of E.W. and U.G. were also supported by a collaborative research grant from CONICET. E.W. and D.H.G. are members of CONICET. L. G. and A.L.A. are CONICET fellows.

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Received 12 June 2015; accepted for publication 22 September 2015

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Schematic representation of the strategies used to analyse *AtCOX17* gene function.
Figure S2. Respiratory rates of wild-type and amiCOX17-1/*cox17-2* plants.
Figure S3. Transcript levels of stress-responsive genes in amiCOX17-1/*cox17-2* plants.
Figure S4. Hierarchical clustering of gene expression changes in wild-type and *anac017* mutant plants (rao2, anac017-1) after treatment with either antimycin A (AA) or H_2O_2 (Ng *et al.* 2013) and those observed in amiCOX17-1/*cox17-2* plants (cox17).
Figure S5. Plants with reduced *AtCOX17-1* or *AtCOX17-2* transcript levels show normal growth and respiration.
Figure S6. Venn diagram of the genes with increased or decreased expression in amiCOX17-1/*cox17-2* plants (COX17_up and COX17_down, respectively) with genes whose expression is modified under salt stress conditions (Ma *et al.* 2006).
Figure S7. Plants silenced in *AtCOX17* genes are not able to induce the alternative respiratory pathway after salt treatment.
Figure S8. Expression of stress-responsive genes in oeCOX17-1 plants.
Table S1. Oligonucleotides used for cloning and genomic analysis.
Table S2. Oligonucleotides used in RT-qPCR.
Table S3. *AtCOX17-1* and *AtCOX17-2* transcript levels in different lines of plants transformed with constructs expressing amiRNAs specific for each of these genes.
Table S4. Enriched biological process gene ontology terms in genes that are up-regulated in plants with decreased expression of both *AtCOX17* genes.
Table S5. Enriched biological process gene ontology terms in genes that are down-regulated in plants with decreased expression of both *AtCOX17* genes.

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Original Article

The cytochrome c oxidase biogenesis factor AtCOX17 modulates stress responses in Arabidopsis

Lucila Garcia, Elina Welchen, Uta Gey, Agustín L. Arce, Iris Steinebrunner and Daniel H. Gonzalez

COX17 is a mitochondrial intermembrane space protein that participates in the transfer of copper for cytochrome *c* oxidase biogenesis. This study shows that silencing *COX17* genes in *Arabidopsis thaliana* affects the response of plants to stress and the sensitivity to abscisic acid. The results indicate that COX17 is a component of signalling pathways that link stress conditions to gene expression responses in plants.

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Q8	AUTHOR: Please give manufacturer information for this product: company name, town, state (if USA), and country.	
Q9	AUTHOR: If HEPES is an abbreviation, please define.	
Q10	AUTHOR: If LC-MS/MS is an abbreviation, please define.	
Q11	AUTHOR: If ABA is an abbreviation, please define.	
Q12	AUTHOR: Please provide the page ranges for references Pfalz et al. (2012). in the Reference List – not just the first page.	
Q13	AUTHOR: Please provide the page ranges for references Steinebrunner et al. (2014). in the Reference List – not just the first page.	
Q14	AUTHOR: Please provide accessed date for reference Team R. (2013).	
Q15	AUTHOR: Please provide the page ranges for references Umbach et al. (2012). in the Reference List – not just the first page.	
Q16	AUTHOR: Please provide the page ranges for references Van Aken O. & Whelan J. (2012). in the Reference List – not just the first page.	

Query No.	Query	Remark
Q17	AUTHOR: Reference “Vanlerberghe & McIntosh (1997)” is not cited in the text. Please indicate where it should be cited; or delete from the reference list.	

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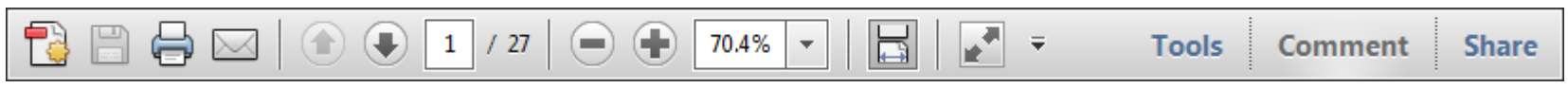
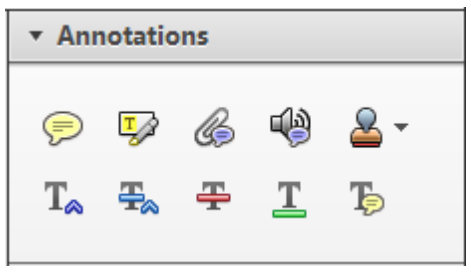
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

Required software to e-Annotate PDFs: Adobe Acrobat Professional or Adobe Reader (version 7.0 or above). (Note that this document uses screenshots from Adobe Reader X)
The latest version of Acrobat Reader can be downloaded for free at: http://get.adobe.com/uk/reader/

Once you have Acrobat Reader open on your computer, click on the Comment tab at the right of the toolbar:



This will open up a panel down the right side of the document. The majority of tools you will use for annotating your proof will be in the Annotations section, pictured opposite. We've picked out some of these tools below:

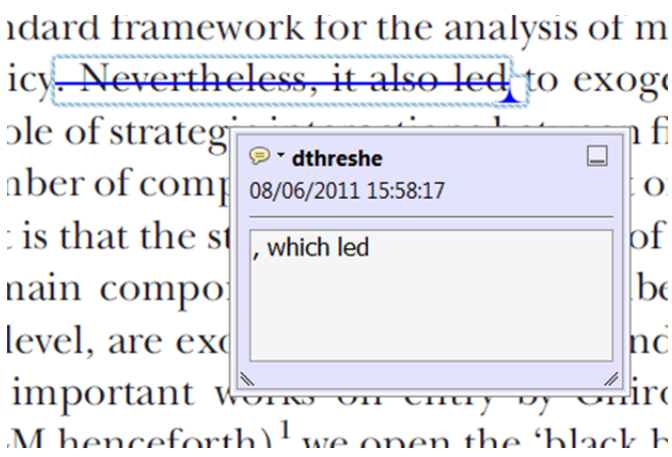


1. Replace (Ins) Tool – for replacing text.

Strikes a line through text and opens up a text box where replacement text can be entered.

How to use it

- Highlight a word or sentence.
- Click on the Replace (Ins) icon in the Annotations section.
- Type the replacement text into the blue box that appears.

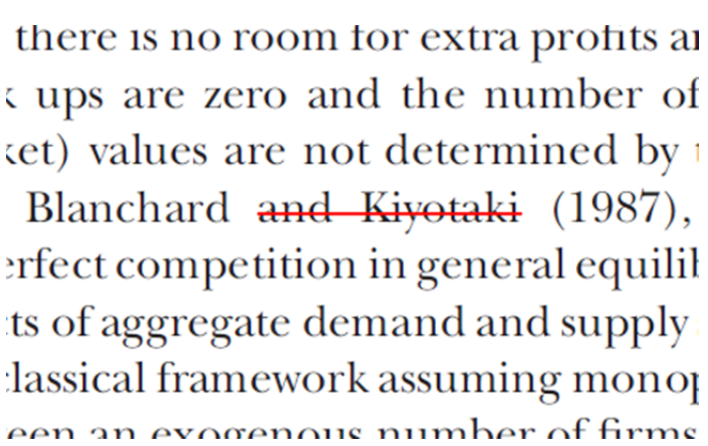


2. Strikethrough (Del) Tool – for deleting text.

Strikes a red line through text that is to be deleted.

How to use it

- Highlight a word or sentence.
- Click on the Strikethrough (Del) icon in the Annotations section.

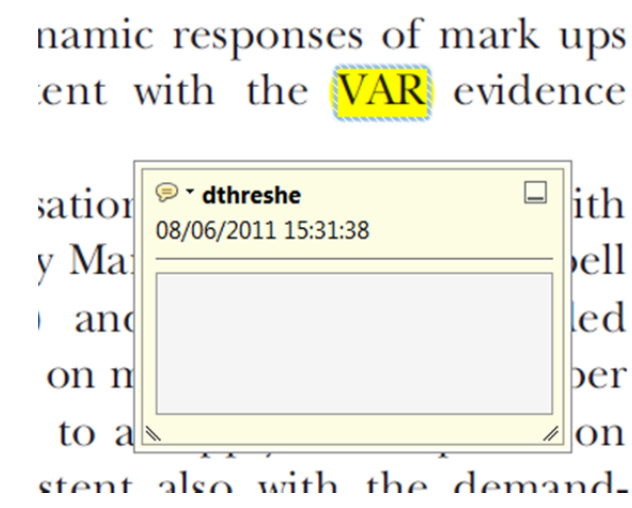


3. Add note to text Tool – for highlighting a section to be changed to bold or italic.

Highlights text in yellow and opens up a text box where comments can be entered.

How to use it

- Highlight the relevant section of text.
- Click on the Add note to text icon in the Annotations section.
- Type instruction on what should be changed regarding the text into the yellow box that appears.

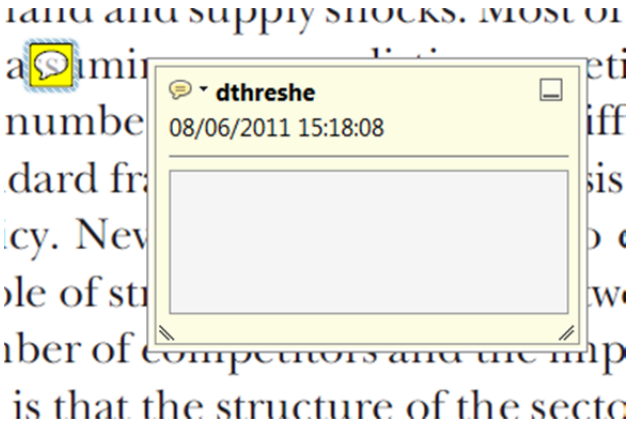


4. Add sticky note Tool – for making notes at specific points in the text.

Marks a point in the proof where a comment needs to be highlighted.

How to use it

- Click on the Add sticky note icon in the Annotations section.
- Click at the point in the proof where the comment should be inserted.
- Type the comment into the yellow box that appears.



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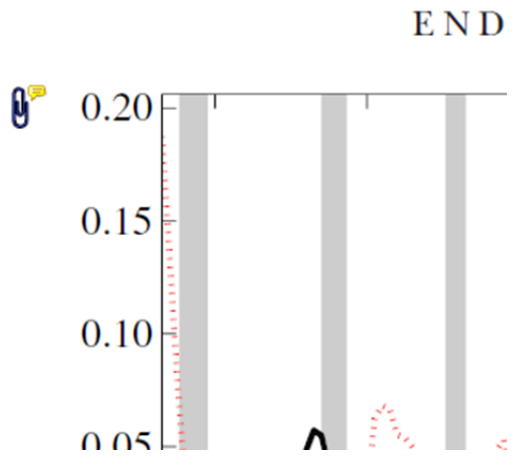
USING e-ANNOTATION TOOLS FOR ELECTRONIC PROOF CORRECTION

5. Attach File Tool – for inserting large amounts of text or replacement figures.

Inserts an icon linking to the attached file in the appropriate pace in the text.

How to use it

- Click on the Attach File icon in the Annotations section.
- Click on the proof to where you'd like the attached file to be linked.
- Select the file to be attached from your computer or network.
- Select the colour and type of icon that will appear in the proof. Click OK.

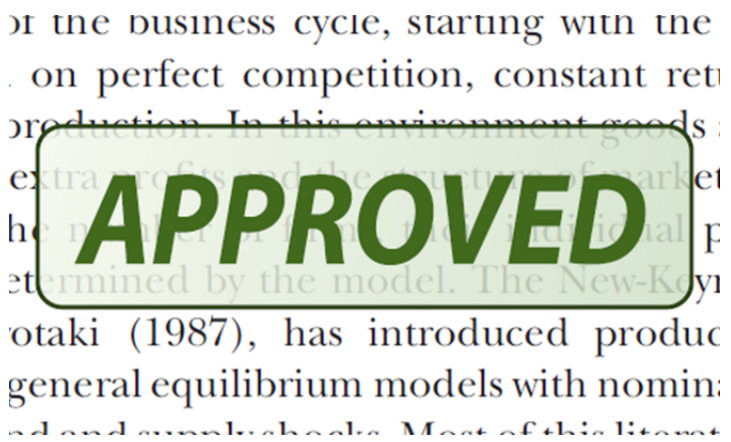


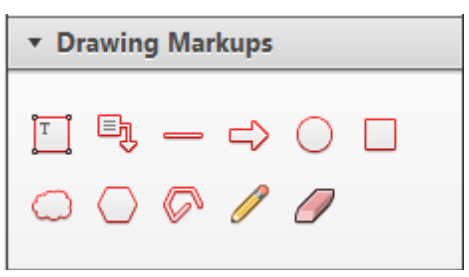
6. Add stamp Tool – for approving a proof if no corrections are required.

Inserts a selected stamp onto an appropriate place in the proof.

How to use it

- Click on the Add stamp icon in the Annotations section.
- Select the stamp you want to use. (The Approved stamp is usually available directly in the menu that appears).
- Click on the proof where you'd like the stamp to appear. (Where a proof is to be approved as it is, this would normally be on the first page).



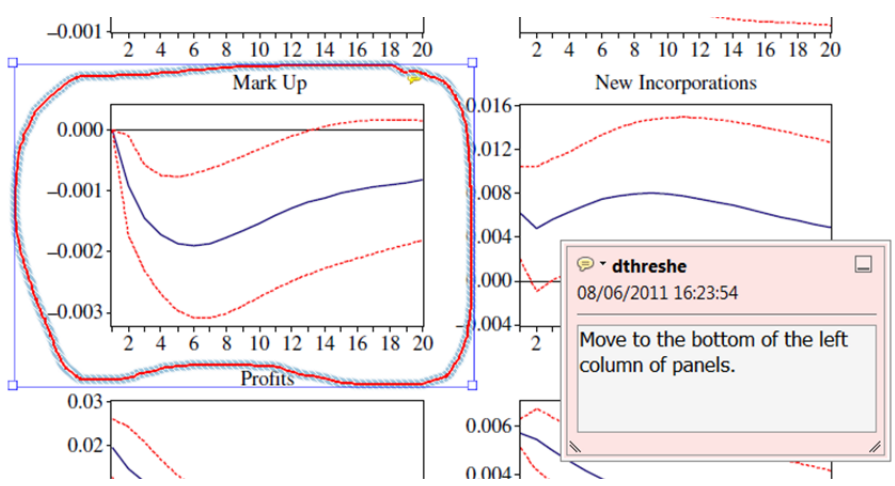


7. Drawing Markups Tools – for drawing shapes, lines and freeform annotations on proofs and commenting on these marks.

Allows shapes, lines and freeform annotations to be drawn on proofs and for comment to be made on these marks..

How to use it

- Click on one of the shapes in the Drawing Markups section.
- Click on the proof at the relevant point and draw the selected shape with the cursor.
- To add a comment to the drawn shape, move the cursor over the shape until an arrowhead appears.
- Double click on the shape and type any text in the red box that appears.



For further information on how to annotate proofs, click on the Help menu to reveal a list of further options: