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The effects of induced production of reactive oxygen species in organelles on endoplasmic reticulum stress and on the unfolded protein response in arabidopsis

Rengin Ozgur, Baris Uzilday, A. Hediye Sekmen and Ismail Turkan*

Department of Biology, Faculty of Science, Ege University, Bornova, Izmir, 35100, Turkey

*For correspondence. E-mail ismail.turkan@ege.edu.tr

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• **Background and Aims** Accumulation of unfolded proteins caused by inefficient chaperone activity in the endoplasmic reticulum (ER) is termed 'ER stress', and it is perceived by a complex gene network. Induction of these genes triggers a response termed the 'unfolded protein response' (UPR). If a cell cannot overcome the accumulation of unfolded proteins, the ER-associated degradation (ERAD) system is induced to degrade those proteins. In addition to other factors, reactive oxygen species (ROS) are also produced during oxidative protein-folding in the ER. It has been shown in animal systems that there is a tight association between mitochondrial ROS and ER stress. However, in plants there are no reports concerning how induced ROS production in mitochondria and chloroplasts affects ER stress and if there is a possible role of organelle-originated ROS as a messenger molecule in the unfolded protein response. To address this issue, electron transport in chloroplasts and mitochondria and carnitine acetyl transferase (CAT) activity in peroxisomes were inhibited in wild-type *Arabidopsis thaliana* to induce ROS production. Expression of UPR genes was then investigated.

• **Methods** Plants of *A. thaliana* ecotype Col-0 were treated with various H₂O₂- and ROS-producing agents specific to different organelles, including the mitochondria, chloroplasts and peroxisomes. The expression of ER stress sensor/transducer genes (*bZIP28*, *bZIP17*, *IRE1A*, *IRE1B*, *BiP1*, *BiP3*), genes related to protein folding (*CNX*, *ERO1*) and ERAD genes (*HRD1*, *SEL1*, *DER1*, *UBC32*) were evaluated by qRT-PCR analysis.

• **Key Results** Relatively low concentrations of ROS were more effective for induction of the ER stress response. Mitochondrial and chloroplastic ROS production had different induction mechanisms for the UPR and ER stress responses.

• **Conclusions** Chloroplast- and mitochondria-originated ROS have distinct roles in triggering the ER stress response. In general, low concentrations of ROS induced the transcription of ER stress-related genes, which can be attributed to the roles of ROS as secondary messengers. This is the first time that ROS production in organelles has been shown to affect the ER stress response in a plant system.

Key words: Oxidative stress, endoplasmic reticulum stress, unfolded protein response, UPR, endoplasmic reticulum-associated degradation, ERAD, reactive oxygen species, ROS, signalling, *Arabidopsis thaliana*.

INTRODUCTION

The endoplasmic reticulum (ER) acts as a hub in the production of secretory proteins in eukaryotic cells. Secretory proteins are modified and folded in the ER, before transfer to their final destination via the secretory pathway (Iwata and Kouzumi, 2012). A series of well-coordinated processes are required for synthesis and secretion of proteins from the ER. First, nascent proteins, synthesized by the ER membrane-bound ribosomes, are moved into ER by a Sec61-like translocon during translation. Then, these proteins are stabilized by HSP40 and HSP70-like proteins such as ERdj3 and binding protein (BiP) in the ER before they are properly modified and fold (Howell, 2013). The interaction of these chaperones with nascent polypeptides prevents aggregation and helps their proper folding. Glycosylated proteins are modified by oligosaccharide transferase in the ER and are folded by a folding apparatus that constitutes the lectin protein calreticulin/calnexin complex and protein disulfide isomerase (PDI). PDI acts as a mediator during formation of

disulfide bonds (Kamauchi *et al.*, 2005; Lu and Christopher, 2008). ER oxidoreductase 1 (ERO1) is a glycosylated flavoenzyme that is tightly associated with the luminal face of the ER and catalyses formation of disulfide bonds by oxidizing PDI. In parallel with its role in oxidative protein folding, ERO1 is a significant source of oxidizing equivalents and is responsible for the regulation of ER oxidation state (Dixon *et al.*, 2003; Onda *et al.*, 2009).

Protein load and folding capacity in ER is meticulously controlled. Accumulation of unfolded proteins due to insufficient folding capacity in ER triggers a response called the unfolded protein response (UPR), which induces genes and pathways that facilitate protein folding and degradation of misfolded proteins (Martinez and Chrispeels, 2003; Schröder and Kaufman, 2005; Deng *et al.*, 2011). More detailed information on this subject can be found in a recent excellent review by Howell (2013).

ER stress is sensed by sensors/transducers that are located on the ER membrane. The ER stress response signalling pathway

comprises two arms. One arm involves bZIP28, a membrane-associated transcription factor, and the other involves inositol requiring enzyme 1 (IRE1), a membrane-associated dual functioning protein kinase/ribonuclease (Koizumi *et al.*, 2001; Liu and Howell, 2010a, b; Iwata and Koizumi, 2012). Upon accumulation of unfolded proteins in the ER lumen, bZIP28 is mobilized from ER to Golgi bodies, where it is processed by S1P and S2P proteases to release the N-terminal portion of bZIP28 to the cytosol (Liu *et al.*, 2007a, b; Che *et al.*, 2010). Cleaved bZIP28 moves to the nucleus and induces the ER stress response (Tajima *et al.*, 2008). By contrast, upon ER stress, IRE1 splices bZIP60 mRNA and the translated product activates the ER stress response. In addition, bZIP28 and bZIP60 can heterodimerize and these two arms of the pathway may overlap to induce a stress response (Gao *et al.*, 2008; Liu and Howell, 2010a, b).

Another important mechanism in response to ER stress is the removal of unfolded or misfolded proteins from the ER lumen to prevent their aggregation (Su *et al.*, 2011). ER-associated protein degradation (ERAD) effectively removes proteins from the ER and these proteins are degraded by 26S proteasome. Briefly, unfolded glycosylated proteins are recognized by a luminal lectin OS9 which works together with SEL1/HRD3 (a membrane spanning protein) and HRD1 (an E3 ligase). These unfolded proteins are then transferred to cytosol by this complex. After removal from ER lumen, ubiquitinated proteins are degraded by 26S proteasome in the cytoplasm (Su *et al.*, 2012).

In a previous study, we investigated the relationship among ER stress, reactive oxygen species (ROS) signalling and antioxidant defence in arabidopsis and showed that treatment with the ER stress agent tunicamycin (Tm) can induce expression of NADPH oxidase encoding genes *RBOHD* and *RBOHF* and can induce the activities of ROS scavenging enzymes leading to a change in redox status of the cell (Ozgur *et al.*, 2014). A need for an oxidizing environment in ER for proper disulfide bond formation and results from our previous study clearly imply that ROS signalling and cellular redox status are important players during response to ER stress. Chloroplasts and mitochondria are the major sources of ROS in the plant cell. In animals, mitochondrial ROS production is tightly associated with ER stress (Berridge, 2002; Hotamisligil, 2010). It has been shown that ER stress and mitochondrial dysfunction can induce each other and there is a cross-talk between them via ROS. However, despite the significance of this issue, it is still not known how ROS produced in different compartments of the plant cell such as mitochondria, chloroplasts and peroxisomes affect ER stress response and ER folding machinery.

The aim of this study was to investigate the effects of organellar ROS on different components of the ER stress response such as ER quality control (ERQC), UPR and ERAD at the transcriptional level. For this, different chemicals were used to induce ROS production/accumulation in different cellular compartments: (i) rotenone in mitochondria, (ii) methyl viologen (MV) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in chloroplasts, and (iii) 3-amino-1,2,4-triazole (3-AT) in peroxisomes, in addition to exogenous H₂O₂ treatments.

Expressions of binding protein 1 (*BiP1*), *BiP3*, *bZIP28*, *bZIP17*, inositol requiring enzyme 1A (*IRE1A*), *IRE1B*, calnexin (*CNX*), endoplasmic reticulum oxidoreductase (*ERO1*), *HRD1*,

SEL1, *UBC32* and *DER1* were investigated to elucidate the effects of organellar ROS on ER stress response.

MATERIAL AND METHODS

Plant material, growth conditions and stress treatments

In this study, *Arabidopsis thaliana* ecotype Col-0 was used as plant material. Plants were grown in a plant growth chamber using a hydroponic system under controlled conditions (23/21 °C day and night temperatures, 60 % relative humidity, 12/12-h light/dark period, 200 μmol photons m⁻² s⁻¹ light intensity) with half-strength Hoagland's solution. Fully expanded rosette leaves of 21-d-old plants were used for the experiments. Leaves were detached and floated on solutions containing H₂O₂ (10 μM, 100 μM, 1 mM, 10 mM), rotenone (Rot; 1, 10, 25, 50 μM), MV (1, 10, 25, 50 μM), DCMU (1, 10, 25, 50 μM), 3-AT (1 mM) and tunicamycin (0.5 μg mL⁻¹). First, leaves were floated in the dark for 2 h and then lights were turned on for an additional 2 h. Properties of the chemicals used are given in Table 1. ROS staining was done using fresh leaves. For gene expression studies leaves were immediately frozen in liquid nitrogen and stored at -80 °C.

All experiments were done in detached leaves and we tested whether a control detached leaf shows a similar pattern of gene expression compared with an attached leaf (Supplementary Data Fig. S1).

Staining of O₂⁻ and H₂O₂ with NBT and DAB

In situ staining of O₂⁻ and H₂O₂ was done according to Dutilleul *et al.* (2003). For detection of O₂⁻, after treatments, leaves were vacuum infiltrated (three cycles) in 0.5 mg mL⁻¹ nitro blue tetrazolium (NBT) prepared in 10 mM potassium phosphate buffer (pH 7.8). Samples were incubated for 1 h in the dark at room temperature, and then were cleared in 90 % ethanol at 70 °C until chlorophyll was completely removed. As a control, superoxide dismutase (10 u mL⁻¹) and 10 mM MnCl₂ were added to the staining medium before infiltration. For detection of H₂O₂, leaves were vacuum infiltrated (three cycles) with 1 mg mL⁻¹ 3,3'-diaminobenzidine (DAB) in 10 mM sodium acetate buffer (pH 3.8). Samples were incubated in the dark at room temperature for 12 h. After incubation samples were transferred to 3:1:1 (v/v) ethanol/acetic acid/glycerol and were cleared at 70 °C until complete removal of chlorophyll. Both NBT- and DAB-stained leaves were examined in 70 % glycerol.

Determination of H₂O₂ content

H₂O₂ was determined according to Cheeseman (2006) using eFOX reagent. In this assay, 1 % ethanol is added to the reagent, which increases its sensitivity to H₂O₂ by 50 % (i.e. eFOX). Extraction was carried out using ice-cold acetone containing 25 mM H₂SO₄. Samples were then centrifuged for 5 min at 3000 g at 4 °C. eFOX reagent [950 μL of 250 μM ferrous ammonium sulfate, 100 μM xylene orange, 100 μM sorbitol, 1 % ethanol (v/v)] was used for 50 μL of supernatant. Reaction mixtures were incubated at room temperature for 30 min and then absorbance at 550 and 800 nm was measured.

TABLE 1. Summary of agents used to induce ROS production/accumulation in specific cellular compartments

Treatment	Concentration range	Cellular compartment	Function	ROS accumulation
H ₂ O ₂	10 μM – 10 mM	–	–	–
Rotenone (Rot)	1–50 μM	Mitochondria	Inhibits NAD(P)H dehydrogenase	O ₂ ^{•-}
Methyl viologen (MV)	1–50 μM	Chloroplast	Accepts electrons from PSI and transfers them to O ₂	O ₂ ^{•-}
DCMU	1–50 μM	Chloroplast	Blocks the plastoquinone binding site of photosystem II	¹ O ₂
3-Amino-triazole (3-AT)	1 mM	Peroxisome	Inhibits catalase	H ₂ O ₂

H₂O₂ concentrations were calculated using a standard curve prepared with known concentrations of H₂O₂.

Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was isolated from 0.1 g of leaf tissue using the Qiagen RNeasy kit according to the manufacturer's recommendations. Total RNA was treated with DNase I (Fermentas) to remove residual genomic DNA. Then, reverse transcription was performed (1 μg total RNA for each treatment group) using M-MuLV reverse transcriptase (New England Biolabs). These cDNAs were used as templates for qRT-PCR. The amount of RNA in each reaction was normalized to the *Arabidopsis thaliana* *ACTIN8* gene. Power SYBR Green Master Mix was used (Applied Biosystems) to perform the qRT-PCR. Three independent experiments were performed for qRT-PCR assays with an Applied Biosystems StepOne Plus System. The conditions for PCR amplification were as follows: 95 °C for 5 min, and 40 cycles at 94 °C for 15 s, 60 °C for 15 s and 72 °C for 30 s. qRT-PCR data analyses were performed with StepOne Plus software. Non-treated *A. thaliana* plants were used as a reference point and relative expression levels were calculated with respect to this reference value (set to 1) for genes that were studied.

Expression of *bZIP28* (AT3G10800), *bZIP17* (AT2G40950), *IRE1A* (AT2G17520), *IRE1B* (AT5G24360), *BiP1* (AT5G28540), *BiP3* (AT1G09080), *CNX* (AT5G61790), *ERO1* (AT1G72280), *HRD1* (AT1G65040), *SEL1* (AT1G18260), *DER1* (AT4G29330) and *UBC32* (AT3G17000) were identified by qRT-PCR. The primers were synthesized by Sentromer DNA Technologies.

Primers used in this study are: *bZIP28* forward 5'-ATCCTA AGCCTGTCTCGAGTTGTA-3', reverse 5'-CGCCGACCAT TAAAACCCCTC-3'; *bZIP17* forward 5'-CAAGCTTGTGAAG ATAGATGGGA-3', reverse 5'-TAGAGGCAGTGCAGGGGT AT-3'; *IRE1A* forward 5'-GCGCTACAGGCGTTACAAATA-3', reverse 5'-TCGTCAATCCTTCTGGAAC-3'; *IRE1B* forward 5'-AGTGGGAAAAACCAGTTCC-3', reverse 5'-AACCAAGTCTCGGAAACAGTG-3'; *BiP1* forward 5'-TCA GTCCTGAGGAGATTAGTGCT-3', reverse 5'-TGCCTTTG AGCATATTGAA-3'; *BiP3* forward 5'-CGAAACGTCTGAT TGGAAGAA-3', reverse 5'-GGCTTCCCATCTTTGTTTCCAC-3'; *CNX* forward 5'-ATGAGACAACGGCAACTATT-3', reverse 5'-TTCCTGAGGACGGAGGTACT-3'; *ERO1* forward 5'-TGGCGATGGCCTTTAGCGACT-3', reverse 5'-GGCCAG AATGGGCAGTCACACC-3'; *HRD1* forward 5'-TCTCTGTT GGGTTTATCTCTTTGGTT-3', reverse 5'-CGGACATGAGA GAGCAAAGTCA-3'; *SEL1* forward 5'-TGATGGAAGAAGC AGTGGATGA-3', reverse 5'-CAGCTGCAAATTATGGTGA AG-3'; *DER1* forward 5'-CGTAGAAGAGTGGTACAAGC AGATG-3', reverse 5'-ACCCGACGGTGGTACTACA-3';

UBC32 forward 5'-CGAGGGCGGGATTTATCATGGG-3', reverse 5'-GTTGCCAATGCTCAGGGTGGTAG-3'; *ACTIN8* forward 5'-TCAGCACTTTCCAGCAGATG-3', reverse 5'-AT GCCTGGACCTGCTTCAT-3'.

Statistical analysis

The experiments were repeated twice, and each data point was the mean of three replicates ($n=6$). The results are expressed as mean with error bars indicating standard error of mean (\pm SEM). Groups were compared using Student's *t*-test.

RESULTS

Quantification of ROS (H₂O₂ and O₂^{•-}) under inhibitor treatments

To confirm the effects of different treatments, ROS production in the leaves was evaluated by NBT and DAB staining. Among treatment groups, the highest H₂O₂ level was observed in H₂O₂- and MV-treated plants, which can also be seen in H₂O₂ contents measured spectrophotometrically (Fig. 1).

O₂^{•-} production was induced by H₂O₂, rotenone, MV, DCMU and 3-AT treatments (Fig. 1).

Expression levels of ER stress sensors and transducer genes: bZIP28, bZIP17, IRE1A and IRE1B

H₂O₂ and rotenone treatments increased the transcription of *bZIP28* except in 10 mM H₂O₂. While 1 μM MV treatment enhanced *bZIP28* expression 5.5-fold, enhanced concentrations of MV (10, 25, 50 μM) gradually decreased its transcription levels to the control levels under 50 μM MV. By contrast, 1 μM DCMU did not have any effect on expression levels of *bZIP28*, while 10, 25 and 50 μM DCMU decreased the *bZIP28* transcript abundance. 3-AT and Tm treatments enhanced expression of this gene by 3- and 18-fold, respectively (Fig. 2).

Low concentrations of H₂O₂ (10 μM) and rotenone (1 and 10 μM) enhanced the *bZIP17* transcripts, while other concentrations of these two agents did not affect expression of this gene. MV, DCMU and 3-AT treatments decreased *bZIP17* expression. *bZIP17* transcripts were highly induced by Tm treatments approx. by 100-fold as compared with control levels (Fig. 2).

Expression of *IRE1A* and *IRE1B* were slightly increased by H₂O₂ and rotenone treatments as compared with control. By contrast, MV treatments increased the expression of *IRE1A* but decreased *IRE1B* expression levels. In the DCMU treatments, neither *IRE1A* nor *IRE1B* expression was changed by 1 μM DCMU, while it was decreased by higher concentrations. 3-AT and Tm did not affect the transcription levels of *IRE1A* and *IRE1B* (Fig. 3).

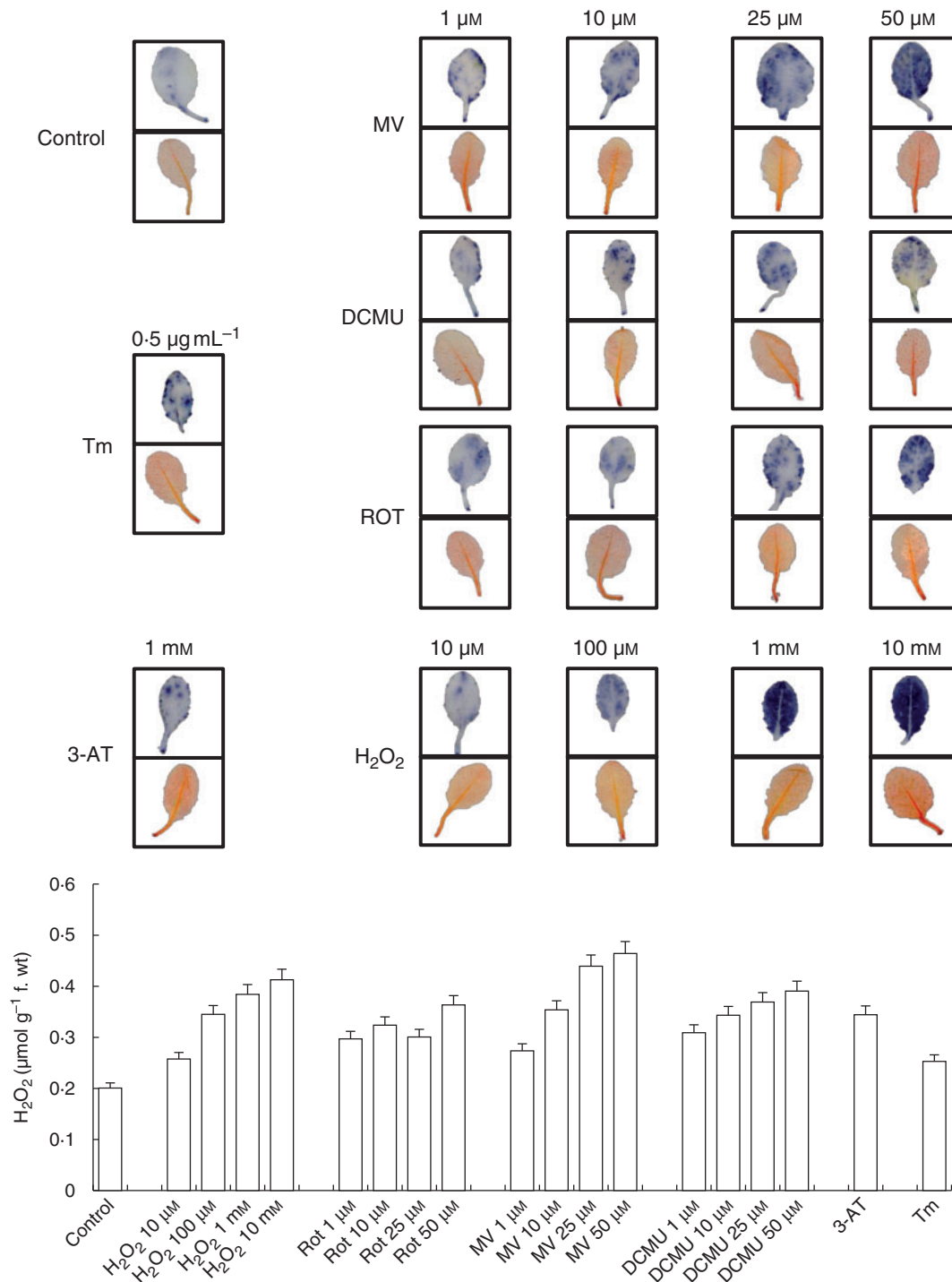


Fig. 1. Detection of O₂⁻ and H₂O₂ in leaves treated with H₂O₂, Rot, MV, DCMU, 3-AT and Tm by NBT (upper rows) and DAB (lower rows) staining. H₂O₂ content was also measured using a ferrous oxidation-xylenol orange (FOX) assay (below).

Transcription levels of ER stress responsive and protein folding helper BiP1 and BiP3 genes

Expression of the *BiP1* gene was increased 3- to 3.5-fold by H₂O₂ treatments, while the highest concentration of H₂O₂ (10 mM) decreased it. Rotenone at 1 and 10 μM also increased *BiP1* levels, while 25 and 50 μM did not change it, and nor did 3-AT treatment. By contrast, MV and DCMU treatments

decreased expression of *BiP1*, but treatment with the ER stress inducer Tm increased it (Fig. 4).

The level of *BiP3* transcript was increased by H₂O₂ treatment. This increase was 8-fold under 10 μM H₂O₂, and 6-, 5- and 3-folds under 100 μM, 1 mM and 10 mM H₂O₂ treatments, respectively. Rotenone and MV enhanced the levels of *BiP3* except with 50 μM MV. Similarly, 3-AT increased *BiP3*

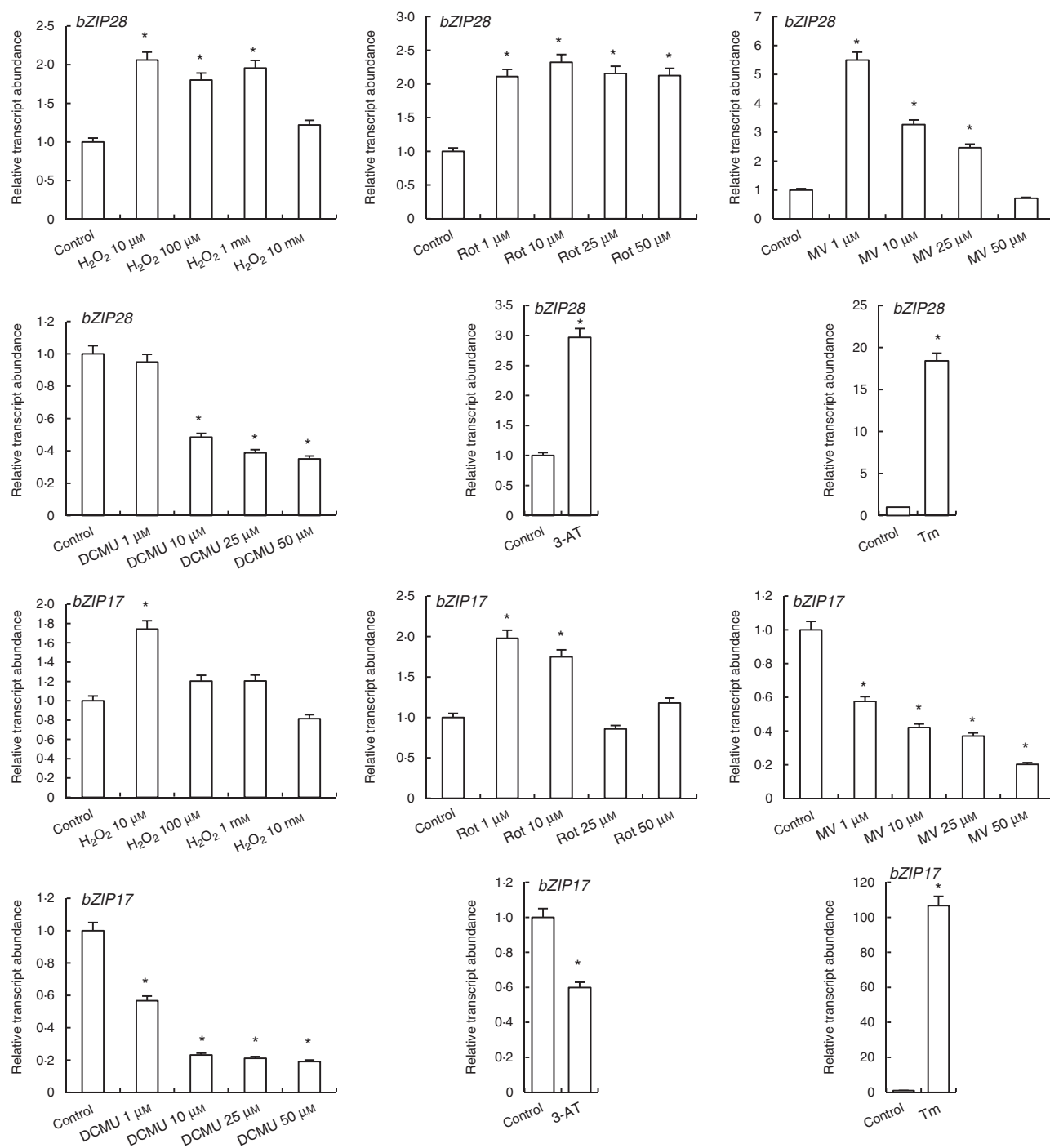


Fig. 2. qRT-PCR analysis of expression of ER stress sensor/transducer genes *bZIP28* and *bZIP17* in leaves treated with H_2O_2 , Rot, MV, DCMU, 3-AT and Tm. *Significant difference compared with the control group at $P < 0.05$.

expression 9-fold, while Tm increased it 660-folds as compared with controls (Fig. 4).

Transcription levels of protein folding machinery elements in the ER: CNX and ERO1

Transcription of *CNX* was increased by H_2O_2 and rotenone except with 10 mM H_2O_2 . The highest increases were observed in

10 μM H_2O_2 and 1 μM rotenone treatments. MV and DCMU treatments decreased *CNX* expression, while Tm induced it (Fig. 5).

H_2O_2 at 10 μM enhanced the levels of *ERO1* transcripts by 7-fold, while 100 μM and 1 mM H_2O_2 increased it by 4- and 5-fold, respectively. By contrast, rotenone treatments increased transcription abundance of *ERO1* 2- to 4-fold. MV treatments increased *ERO1* expression except in the 50 μM treatment group, while DCMU treatments decreased it. 3-AT and Tm

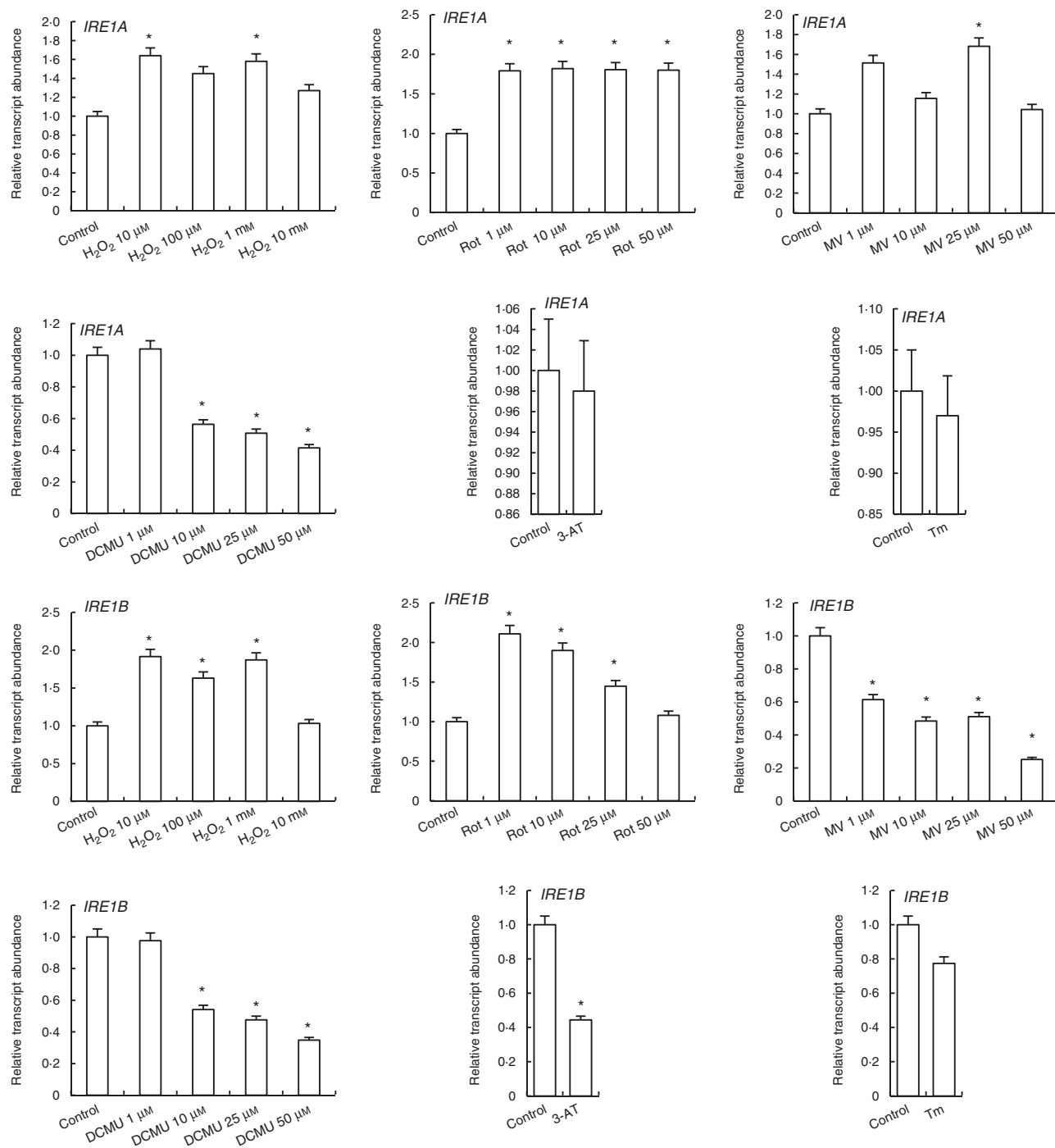


Fig. 3. qRT-PCR analysis of expression of ER stress sensor/transducer genes *IRE1A* and *IRE1B* in leaves treated with H₂O₂, Rot, MV, DCMU, 3-AT and Tm. *Significant difference when compared with the control group at $P < 0.05$.

treatments enhanced *ERO1* transcript levels 3- and 10-fold, respectively (Fig. 6).

Expression of ERAD-related *HRD1*, *SEL1*, *DER1* and *UBC2* genes

H₂O₂ at 10 μM slightly increased *HRD1* transcript levels, while other concentrations did not affect its expression. *HRD1* expression was decreased by rotenone treatments except at

1 μM concentration, which did not change it. By contrast, MV, DCMU and 3-AT treatments decreased *HRD1* transcripts, while TM increased it 2-fold (Fig. 7).

Transcription of *SEL1*, another protein degradation related gene, increased 3.7-fold under 10 μM H₂O₂. *SEL1* expression was also increased by 1 μM (2.5-fold) and 10 μM (2-fold) rotenone treatments. By contrast, 10 μM and higher concentrations of MV decreased *SEL1* transcription. Moreover, 1 μM DCMU enhanced *SEL1* transcription, while other concentrations of

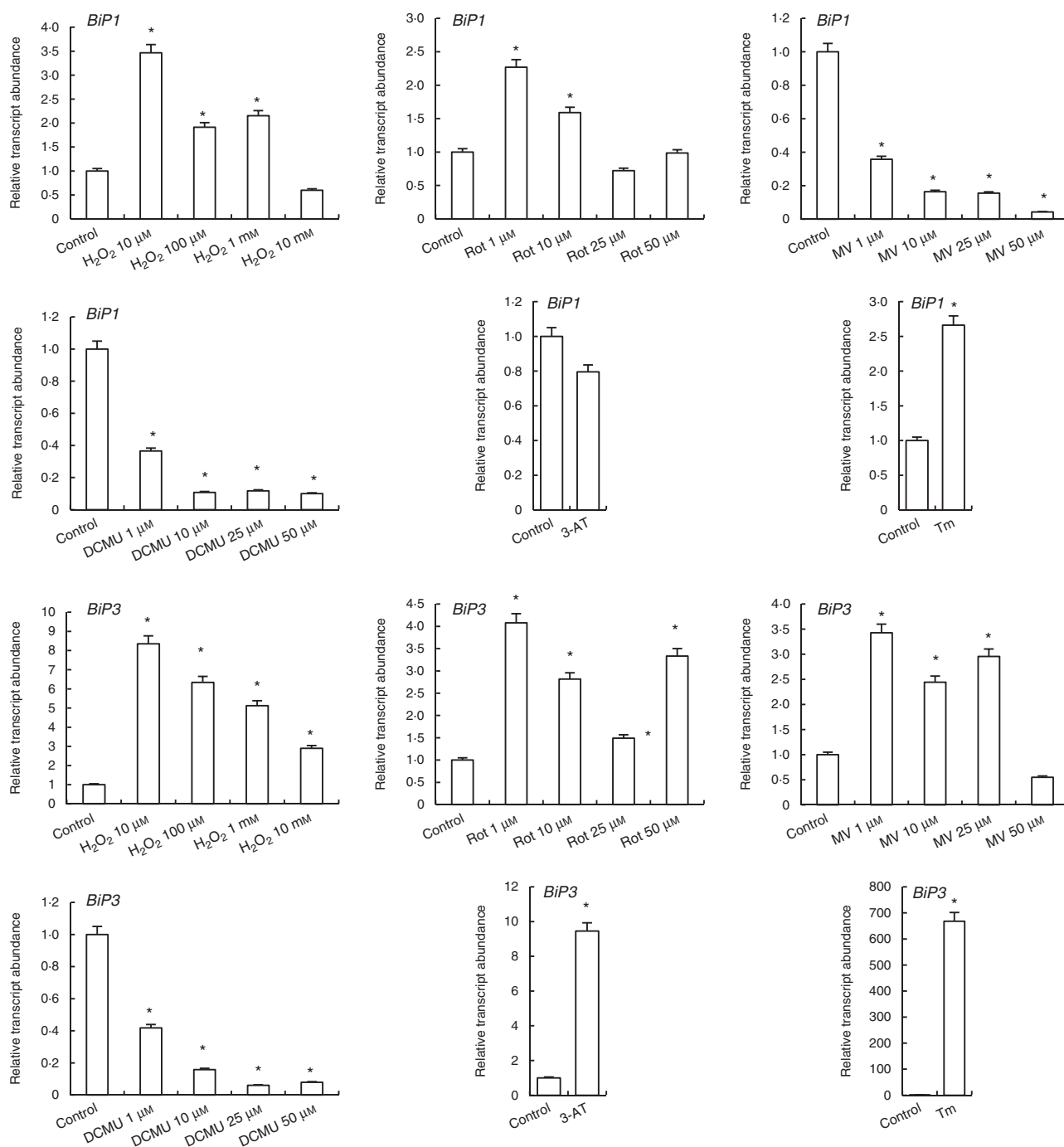


Fig. 4. qRT-PCR analysis of expression of ER folding helper/quality control genes *BiP1* and *BiP3* in leaves treated with H₂O₂, Rot, MV, DCMU, 3-AT and Tm. *Significant difference when compared with the control group at $P < 0.05$.

DCMU decreased it. Treatment with the ER stress inducer Tm increased *SEL1* transcripts 2-fold (Fig. 7).

H₂O₂ and rotenone treatments increased *DER1* and *UBC32* transcripts. MV at 1 μM increased expression of *DER1*, while all MV treatments decreased expression of *UBC32* genes. Expressions of both *DER1* and *UBC32* were enhanced by 1 μM DCMU treatments, while higher concentrations decreased them. 3-AT decreased both *DER1* and *UBC32* transcripts, while

Tm treatment had an inducing effect on expression of these genes (Fig. 8).

DISCUSSION

The main aim of this study was to elucidate the effects of induced organellar (mitochondria, chloroplast and peroxisomes) ROS on ER stress response in *Arabidopsis thaliana*. As

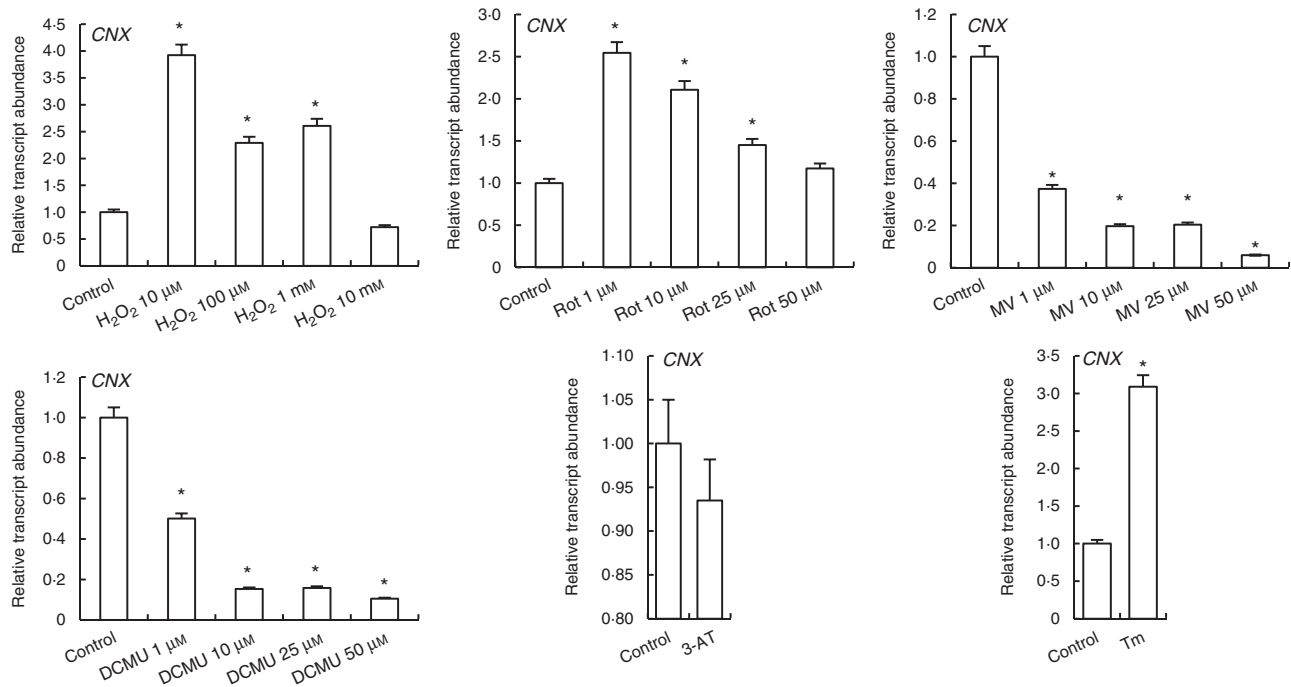


Fig. 5. qRT-PCR analysis of expression of ER folding helper/quality control gene *CNX* in leaves treated with H₂O₂, Rot, MV, DCMU, 3-AT and Tm. *Significant difference when compared with the control group at $P < 0.05$.

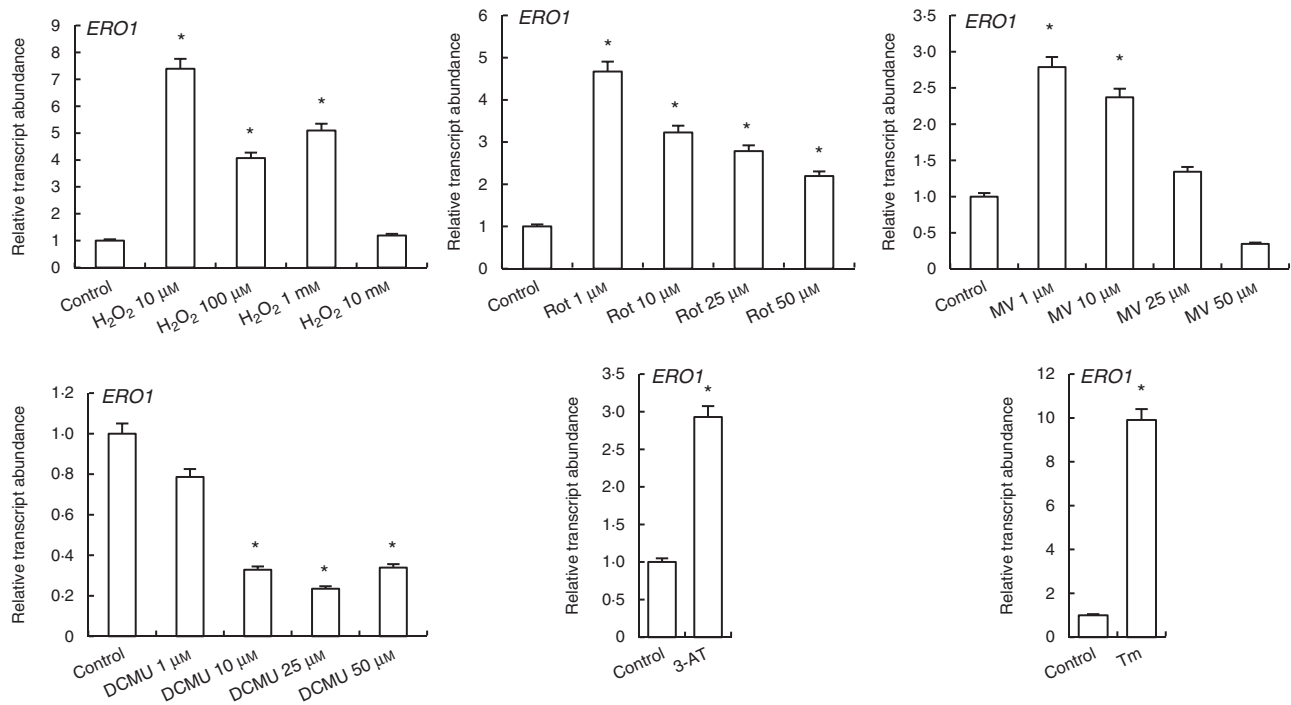


Fig. 6. qRT-PCR analysis of expression of *ERO1*, which is responsible for regulation of the oxidative state of ER, in leaves treated with H₂O₂, Rot, MV, DCMU, 3-AT and Tm. *Significant difference when compared with the control group at $P < 0.05$.

previously mentioned, ER is the centre for protein folding in the cell. Adverse environmental conditions can cause the accumulation of unfolded proteins in the ER, a phenomenon termed ER stress. ER stress can be identified by enhanced expression

of unique transcription factors and genes, which are members of the UPR. By inducing UPR, plants can prevent accumulation of the misfolded and unfolded proteins in the ER. By contrast, if the ongoing stress conditions cause accumulation of massive

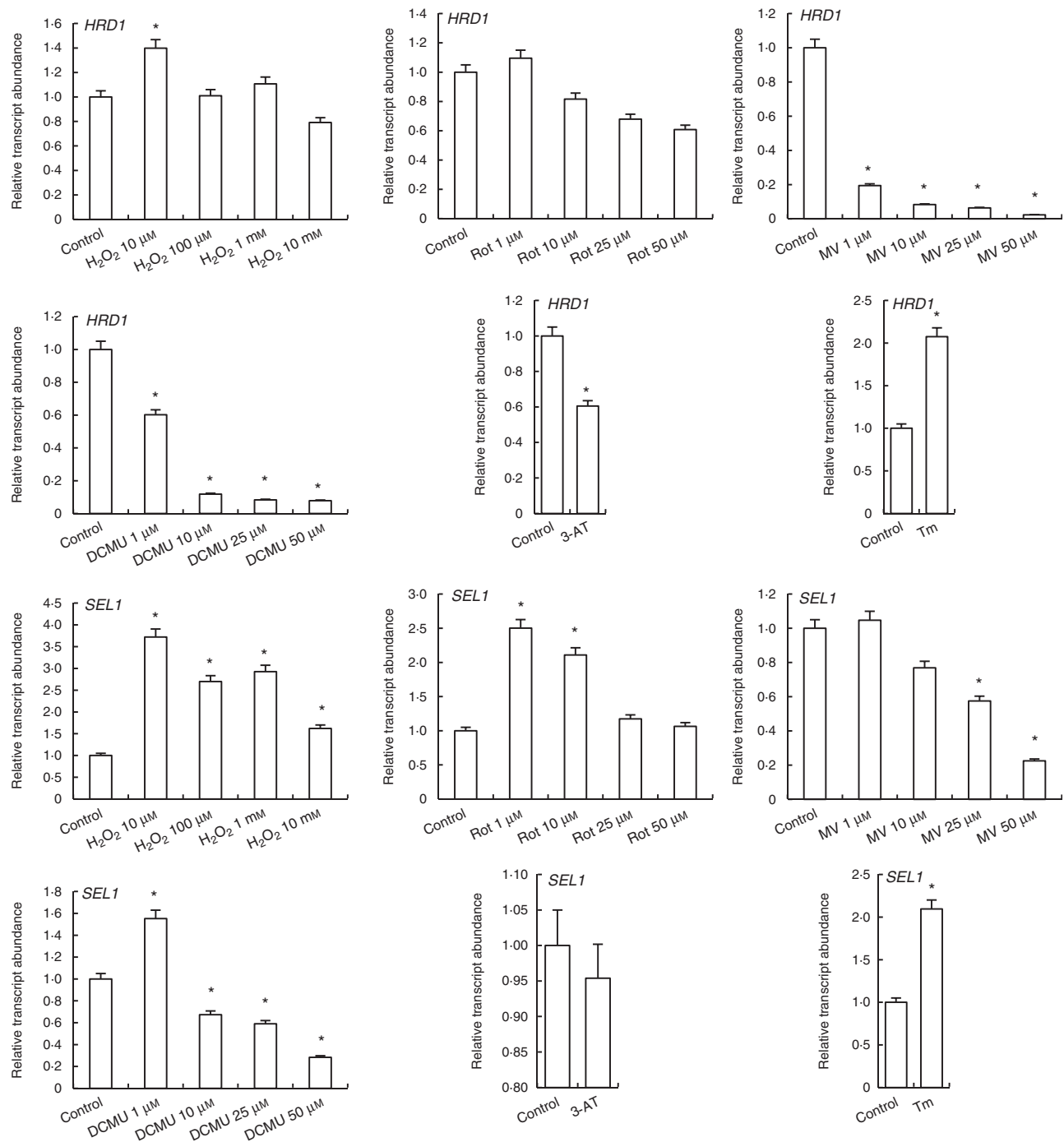


Fig. 7. qRT-PCR analysis of expression of ERAD genes *HRD1* and *SEL1* in leaves treated with H₂O₂, Rot, MV, DCMU, 3-AT and Tm. *Significant difference when compared with the control group at $P < 0.05$.

amounts of misfolded or unfolded proteins, expression of ERAD genes can be induced to overcome this issue.

ROS originating from different compartments of the cell have different effects on the cellular response to stress, which was also previously shown in different transcriptomic studies (Gadjev *et al.*, 2006; Laloi *et al.*, 2007; Sewelam *et al.*, 2014). Accordingly, in the present study, 11 of 12 genes were upregulated by rotenone treatment, while 5 of 12 and 3 of 12 genes were upregulated by MV and DCMU treatment, respectively.

As can be seen from Fig. 9, there is a clear distinction in the ER stress response against mitochondria (due to Rot treatment), chloroplast (due to MV and DCMU treatments) and peroxisome (due to 3-AT) originated ROS.

Many protein folding and other enzymatic and protein degradation reactions that occur in the ER require ATP (Marzec *et al.*, 2012). However, there is no local source of ATP in the ER and therefore ATP supply depends on import from the cytosol. As mitochondria are the major source of ATP for reactions

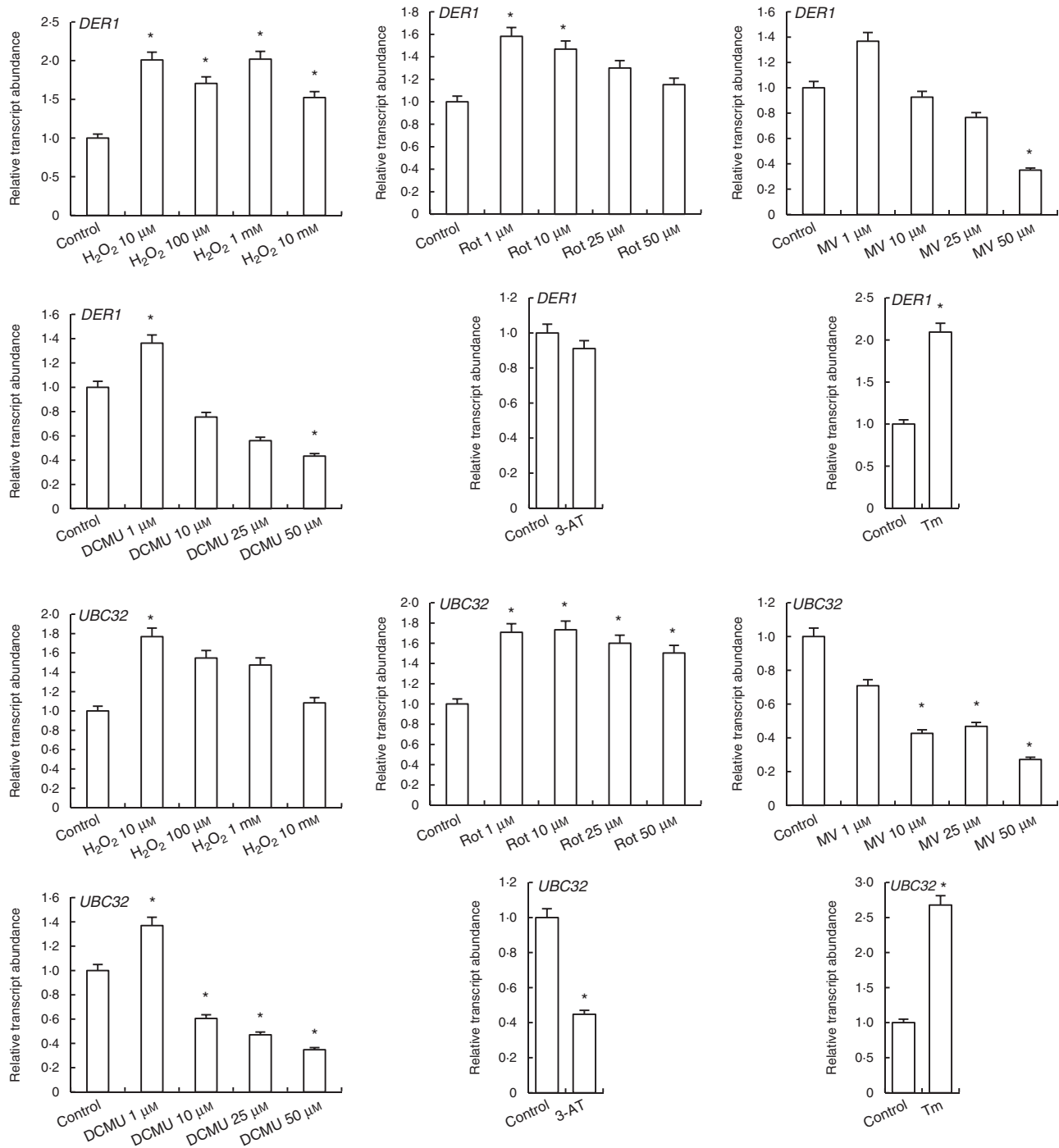


Fig. 8. qRT-PCR analysis of expression of ERAD genes *DER1* and *UBC32* in leaves treated with H₂O₂, Rot, MV, DCMU, 3-AT and Tm. *Significant difference when compared with the control group at $P < 0.05$.

that take place in the cell, it is inevitable that induction of ROS production in mitochondria causes an imbalance in mitochondrial metabolism and affects the availability of ATP in the cell. HSP70-like proteins BiP1 and BiP3 have ATPase activities and can release their protein ligands only in the presence of ATP (Vitale and Denecke, 1999). Similarly, CDC48, a motor protein that is responsible for removal of proteins from the ER to cytosol for degradation, also shows ATPase activity (Marshall

et al., 2008). Therefore, in the present study, the induction of ER stress-related genes by mitochondrial ROS production might be attributed to accumulation of unfolded proteins in the ER due to decreased folding and ERAD capacity caused by insufficient ATP.

Generation of chloroplastic ROS downregulated the expression of genes related to ER stress and protein folding, which might indicate a decrease in the rate of protein synthesis. It was

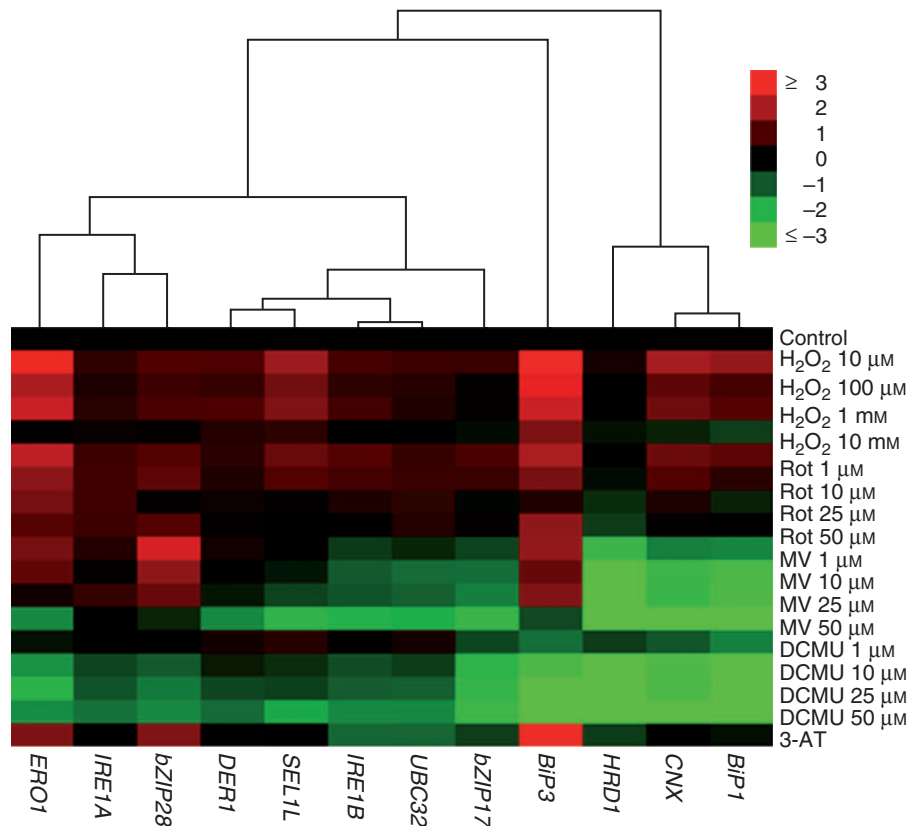


FIG. 9. Hierarchical clustering and heat map of ER stress-related genes in leaves treated with H₂O₂, Rot, MV, DCMU and 3-AT (average linkage and Euclidean distance as similarity measure). qRT-PCR data for each gene were log₂*n* transformed, so that controls have a value of 0, increased expressions have positive and decreased expressions have negative values. Genes were grouped according to their expression patterns.

previously shown that protein synthesis can be inhibited under extreme environmental conditions such as drought stress in plants (Dhindsa and Cleland, 1975; Cramer *et al.*, 2011). Like other stresses, drought also causes metabolic imbalance in chloroplasts due to decreased availability of CO₂ and increased ROS production. Although response to drought is a complex process, MV and DCMU treatments have some similar consequences to drought in chloroplasts. Therefore, a decrease in the availability of carbon backbone for synthesis of amino acids and proteins might reduce protein synthesis and decrease load on the protein folding machinery.

UPR is initiated by increased levels of ER stress sensor/transducer genes such as *bZIP28*, *bZIP17*, *IRE1A* and *IRE1B*. Liu *et al.* (2007a, b) found that salt stress response in arabidopsis requires *bZIP17*. Activation of *bZIP17* upregulates salt stress responsive genes and this suggests that the salt stress response is associated with some of the UPR elements. Moreover, a *bZIP17* single mutant was more sensitive to salinity (Liu *et al.*, 2007a, b). Also, expression of the heat stress-induced *bZIP17* and *bZIP28* (Che *et al.*, 2010) and their single mutants showed higher sensitivity to heat stress (Gao *et al.*, 2008). It is also known that, upregulation of some ER stress-related genes, including *BiP3*, *BiP1* and *CNX*, in part depends on *bZIP28* (Liu and Howell, 2010a). In this study, H₂O₂ treatment and mitochondrial ROS production induced both *bZIP28* and *bZIP17*, while peroxisomal ROS increased *bZIP28* but decreased *bZIP17*.

Three *BiP* genes have been identified in *A. thaliana* (Maruyama *et al.*, 2014). Among them, expression of *BiP3* was observed only under ER stress (Noh *et al.*, 2003). Earlier studies showed that overexpression of *BiP* genes caused osmotic tolerance in tobacco, confirming its link to stress conditions (Alvim *et al.*, 2001). Both *BiP1* and *BiP3* are key regulators for *bZIP28*-activated unfolded protein response signalling (Srivastava *et al.*, 2013). *BiP3* induction is an important switch for UPR during ER stress conditions and abiotic stress conditions such as heat. Moreover, *BiP3* induction is dependent on *IRE1B* activity (Deng *et al.*, 2011). In this study, exogenous H₂O₂ application and ROS originating from mitochondria enhanced expression levels of *BiP1* and *BiP3*. Peroxisomal ROS production via 3-AT treatment increased *BiP3* but not *BiP1*. A closer look at chloroplastic ROS production showed that both MV and DCMU application decreased *BiP1*, while MV enhanced *BiP3*. These results showed that *BiP3* was more responsive than *BiP1* to ROS production in the organelles investigated.

ER-resident calnexin (CNX) functions with calreticulin in protein folding, especially of glycoproteins. Several studies have shown that its expression was enhanced by ER stress inducer agents such as Tm and dithiothreitol (Kamauchi *et al.*, 2005). Similarly, various environmental stress conditions changed *CNX* expression in soybean (Nouri and Komatsu, 2010). Moreover, Sarwat and Naqvi (2013) found that expression of rice *CNX* was induced by drought in tobacco. Also,

Garcia de la Garma *et al.* (2015) showed that under salinity tobacco BY-2 cells produced ROS in the mitochondria and this probably induced ER stress. Moreover, *CNX* and calreticulin genes of BY-2 cells were significantly induced by salinity and the ultrastructure of ER was highly altered. However, it is not known how *CNX* expression is affected directly in response to ROS treatments. In our study, we have shown that *CNX* expression was increased by H₂O₂ treatment and mitochondrial ROS production, but not by chloroplastic ROS sources. Induction of *CNX* by mitochondrial ROS production (rotenone treatment) is consistent with the results obtained by de la Garma *et al.* (2015).

ERO acts as a regulator of ER redox state and responds to unfolded proteins (Dixon *et al.*, 2003; Onda *et al.*, 2009). Previously, it was shown that expression of *ERO1* was enhanced under salt stress (Ozgur *et al.*, 2014). Moreover, ROS-induced *ERO* expression depends on H₂O₂ but not on ¹O₂, as evident from the DCMU-treated plants (Fig. 6).

Remarkably, enhanced expressions of *BiP3* and *ERO1* were tightly associated with the availability of H₂O₂ or an ROS that can be converted to H₂O₂ such as O₂^{•-}. Independent of the compartment of H₂O₂ production, expression of *BiP3* and *ERO1* increased in this study, except in DCMU-treated groups. ROS produced by DCMU is ¹O₂, which cannot be enzymatically converted to any other ROS, and damage done by ¹O₂ is strictly localized to chloroplasts. Due to its inability to diffuse long distances it does not have a signalling role on its own but with its oxidation products. These findings indicate that H₂O₂ has a signal role to induce *ERO1*, which is the regulator of redox status of the ER.

Liu *et al.* (2011) found that salt stress increased ERAD capacity in arabidopsis. Salinity and other environmental conditions increased the accumulation of unfolded and misfolded proteins, which are eventually removed by ERAD (Howell, 2013). *A. thaliana* plants with a defect in HRD3, an ERAD element, were sensitive to salt stress but not to osmotic stress. These plants also accumulated more ROS upon paraquat application (Liu *et al.*, 2011). SEL1 forms a complex with HRD3 and this complex interacts with DER1 to initiate ERAD (Su *et al.*, 2011). This ERAD mechanism observed in plants is also conserved in organisms through yeast to human (Kaneko and Nomura, 2003). Peroxisomal ROS accumulation did not have significant effect on the ERAD system. However, ROS produced by DCMU in chloroplasts induced SEL1, DER1 and UBC32. To the best of our knowledge, this is the first report on induction of ERAD via ROS in a plant system.

As evidenced by transcriptomic studies, transcription of *UBC32*, an important ERAD component, was enhanced under salt, mannitol and drought stresses (Cui *et al.*, 2012). In this study, H₂O₂ and chloroplastic and mitochondrial ROS increased the transcription level of *UBC32*.

In conclusion, we have shown how organellar (chloroplast, mitochondria and peroxisome) ROS production affects the ER stress response in a plant system for the first time. Exogenous H₂O₂ treatments induced transcription of *UPR* and *ERAD* genes, but excess amounts of H₂O₂ (10 mM) did not show a similar effect in the cell. In general, low concentrations of ROS induced the transcription of ER stress-related genes, which might indicate involvement of ROS as secondary messengers during ER stress. ROS originating from mitochondria caused

induction of all the investigated genes to some level. Moreover, *ERO1* and *BiP3* were clearly induced by H₂O₂ accumulation. In addition, there was a clear distinction between the roles of chloroplastic and mitochondrial ROS in response to the ER stress response.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of **Figure S1**: relative transcript abundance for genes in attached and detached leaves.

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