RESEARCH PAPER

Impaired PSII proteostasis triggers a UPR-like response in the var2 mutant of Arabidopsis

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Abstract

Cellular protein homeostasis (proteostasis) is maintained through the balance between de novo synthesis and proteolysis. The unfolded/misfolded protein response (UPR) that is triggered by stressed endoplasmic reticulum (ER) also plays an important role in proteostasis in both plants and animals. Although ER-triggered UPR has been extensively studied in plants, the molecular mechanisms underlying mitochondrial and chloroplastic UPRs are largely uncharacterized despite the fact that these organelles are sites of production of harmful reactive oxygen species (ROS), which damage proteins. In this study, we demonstrate that chloroplasts of the Arabidopsis yellow leaf variegation 2 (var2) mutant, which lacks the metalloprotease FtsH2, accumulate damaged chloroplast proteins and trigger a UPR-like response, namely the accumulation of a suite of chloroplast proteins involved in protein quality control (PQC). These PQC proteins include heat-shock proteins, chaperones, proteases, and ROS detoxifiers. Given that FtsH2 functions primarily in photosystem II proteostasis, the accumulation of PQC-related proteins may balance the FtsH2 deficiency. Moreover, the apparent up-regulation of the cognate transcripts indicates that the accumulation of PQC-related proteins in var2 is probably mediated by retrograde signaling, indicating the occurrence of a UPR-like response in var2.

Keywords: Photosystem II, PSII repair, proteostasis, retrograde signaling, singlet oxygen, unfolded protein response, damaged protein response

Introduction

The unfolded/misfolded protein response (UPR) was first characterized in the endoplasmic reticulum (ER) (Kozutsumi et al., 1988). This ER-mediated UPR (erUPR) is activated when protein folding is impared at the luminal side under ER stress conditions. This response is ubiquitously conserved in eukaryotic cells and is crucial to eliminate misfolded/unfolded proteins, thereby maintaining protein homeostasis (proteostasis) (Walter and Ron, 2011). Similarly, mitochondria also activate a mitochondrial UPR (mtUPR) under oxidative stress conditions (Aldridge et al., 2007; Pellegrino et al., 2013). Both erUPR and mtUPR lead to the accumulation of proteins involved in proteostasis (Aldridge et al., 2007; Iwata et al., 2008; Pellegrino et al., 2013). These proteins include various chaperones and proteases, which are induced via a process called organelle-to-nucleus retrograde signaling (RS). A chloroplast-mediated UPR (cpUPR) has been found in the green unicellular alga Chlamydomonas reinhardtii through use of a repressible chloroplast gene expression system (Pérez-Martín...
ecotype and were harvested on the same day from plants grown together. Arabidopsis thaliana Plant material and growth conditions

Materials and methods

Plant material and growth conditions

All the Arabidopsis thaliana seeds were derived from the Columbia (Col-0) ecotype and were harvested on the same day from plants grown together under of conditions continuous light (CL; 80 µmol m−2 s−1 at 20±2 °C). Seeds for the var2 knock-out allele (SAIL_253_A03) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). The WT and var2 seeds were surface-sterilized and plated on Murashige and Skoog medium (Duchefa Biochemie) with 0.8% (w/v) agar and supplemented with 0.5% (v/v) sucrose. Seeds were stratified for 3 d at 4 °C in darkness and then placed under CL. At 5 d old, seedlings were transferred to soil and grown under CL until sampling.

Chloroplast isolation and tandem mass spectrometry

Chloroplasts were isolated from 3-week-old plants of the WT and var2 grown under CL as described previously (Kauss et al., 2012). Briefly, rosette leaves of mature plants (90 plants for WT and 180 plants for var2) were homogenized in a Waring blender in chloroplast isolation buffer [50 mM Hepes-KOH, pH 8, 5 mM MgCl2, 5 mM EDTA pH 8, 5 mM EGTA pH 8, 10 mM NaHCO3, and 0.33 M D-sorbitol, supplemented with SIGMAFAST™ Protease Inhibitor (1 tablet per 100 ml)]. The homogenate was filtered through four layers of Miracloth and centrifuged at 400 g for 8 min at 4 °C. The pellets were suspended in isolation buffer and loaded onto a two-step Percoll gradient (40:80%) to separate intact and broken chloroplasts. Intact chloroplasts enriched between the two Percoll steps were carefully collected and washed twice with HS buffer (50 mM Hepes-KOH, pH 8, and 0.33 M D-sorbitol). The integrity of the chloroplasts was checked under a microscope (Supplementary Fig. S1 at JXB online). Intact chloroplasts corresponding to equal amounts of chlorophyll were lyzed, and the proteins extracted using 6 M guanidine hydrochloride buffer (guanidine hydrochloride dissolved in 100 mM Tris, pH 8.5). The lysed samples were sonicated in an ice bath for 1 min with a pulse of 3 s on and 5 s off, heated at 95 °C for 5 min, and then centrifuged at 21 000 g for 30 min at 4 °C. Total protein content was estimated using a Pierce™ BCA protein assay kit (ThermoFisher Scientific). For MS analysis, equal amounts of total protein (2 µg µl−1) from three independent biological samples were denatured using 10 mM DTT at 56 °C for 30 min followed by alkylation in 50 mM iodoacetamide at room temperature for 40 min in the dark. The proteins were then desalted using a Nanosep membrane (Pall Corporation, MWCO 10K) in 200 µl digestion buffer (40 ng µl−1 trypsin in 100 mM NH4HCO3, pH 8.5). The digested peptides were dried in a refrigerated CentriVap concentrator (Labconco, Kansas, MO). The dried peptides were resuspended in 0.1% (v/v) formic acid (FA) solution, and separated using a nanoAcquity Ultra Performance LC (Waters, Milford, MA) equipped with a 20-mm trap column (C18 5 µm resin, 180 µm I.D., Waters) and a 250-mm analytical column (C18 1.7 µm resin, 75 µm I.D., Waters). The peptide mixture reconstituted in 0.1% FA was loaded onto the trap column with a flow rate of 3 µl min−1 for 10 min, followed by elution to the analytical column for further separation under the following conditions with a flow rate of 250 nl min−1: (i) 0 min gradient from 8–25% of solvent B (Acetonitrile, ACN), (ii) 15 min gradient from 25–40% of solvent B, (iii) 5 min gradient from 40–90% of solvent B, (iv) 5 min washing at 90% of solvent B, and finally (v) equilibrating with 97% of solvent A for 15 min (solvent A: 0.1% FA; solvent B: 99.9% ACN/0.1% FA). The separated peptides were analysed using a Q Exactive Mass Spectrometer (ThermoFisher Scientific). A full MS survey scan was carried out at a resolution of 70 000 at 400 m/z over an m/z range of 300–1800, with an automatic gain controls (AGC) target of 3×106 and a maximum ion injection time (IT) of 30 ms. The top 20 multiply charged parent ions were selected using data-dependent MS/MS mode, and fragmented by higher-energy collision dissociation (HCD) with a normalized collision energy of 27% in the m/z scan range of 200–2000. MS/MS detection was carried out at a resolution of 17 500 with an AGC target value of 5×106 and a maximum IT of 120 ms. Dynamic exclusion was enabled for 30 s.

Label-free quantification

Raw MS data files were processed and analysed using the MaxQuant software (v. 1.5.8.3) with label-free quantitation (LFQ) and the
intensity-based absolute quantification (ibaQ) algorithm enabled as described previously (Luber et al., 2010; Schwanhäusser et al., 2011). Parent ion and MS/MS spectra were searched against the FASTA format database at TAIR (http://www.arabidopsis.org/). The precursor ion tolerance was set at 7 ppm with an allowed fragment mass deviation of 20 ppm. Carbamidomethylation of cysteine was set as a fixed modification while N-terminal acetylation and oxidation of methionine and tryptophan were defined as variable modifications. Peptides of a minimum of six amino acids and a maximum of two missed cleavages were allowed. False discovery rate (FDR) was set to 0.01 for both peptide and protein identification. The LFQ and ibAQ intensity values were used to calculate the protein expression and abundance. Proteins were considered as being expressed if the intensity values were detected in at least two of the three replicates in at least one of the independent biological samples. Expression matrices of the proteins were represented as heat maps prepared using Multi-Experiment viewer (MeV4.9.0). After log2-transformation of the intensity values and data imputation (replacing missing values by normal distribution), proteins exhibiting at least a 2-fold accumulation with P < 0.05 (Student’s t-test) were considered as differentially accumulated in var2 in comparison with the WT. The oxidation (Oxi-PTM) in the proteins was calculated using the intensities of the individual oxidized peptides of the respective proteins. Gene Ontology (GO) enrichment analysis of differentially expressed proteins was carried out using the Generic GO Term Finder tool (http://go.princeton.edu/cgi-bin/GOTermFinder) to determine the significantly enriched GO terms in the category of biological processes (Katari et al., 2010) with a significance of P < 0.05.

Western blot analyses
The total chloroplast proteins were separated by 10% SDS-PAGE gels and blotted onto Immuno-Blot PVDF membrane (Bio-Rad). HSP70, CPN60A, CPN60B, and RbcL proteins were immunochemically detected using rabbit anti-HSP70 (1:10 000 dilution), rabbit anti-CPN60A (1:10 000), rabbit anti-CPN60B (1:10 000), and rabbit anti-RbcL (1:10 000) antibodies, respectively (all obtained from Agrisera).

RNA extraction and quantitative RT-PCR (qRT-PCR)
Total RNA was extracted from three independent biological replicates of 3-week-old plants of the WT and var2 grown under CL using a Spectrum Total RNA Kit (Sigma-Aldrich). Samples of 1 μg RNA were treated with RQ1 RNase-free DNase I (Promega). First-strand cDNA was synthesized with oligo(dT)$_{18}$ primers (Promega) and Improm II reverse transcriptase (Promega) according to the manufacturer’s protocol. qRT-PCR was carried out using a QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems) and iTaq Universal SYBR Green PCR master mix (Bio-Rad). The relative transcript level of each gene was determined with the comparative delta-ΔCt method and normalized to the transcript level of PP2A (At1g13320). The primer sequences used in this study are listed in Supplementary Table S1.

Results
Changes in the chloroplast proteome in var2
Inactivation of the FtsH2 protease disturbs PSII proteostasis (Patel and Latterich, 1998; Ogura and Wilkinson, 2001; Yu et al., 2004; Nishimura et al., 2016). In spite of this, var2 mutant plants lacking FtsH2 are viable, indicating that chloroplasts may activate certain signaling pathway(s) to compensate for the defective proteostasis. We were therefore interested in learning more about this adaptive mechanism. To this end, we compared the total chloroplast proteome of var2 with that of the WT by using a label-free quantitation assay (Fig. 1A). We normalized each protein sample based on total protein content, since there was an apparent correlation between the WT and var2 samples with regards to chloroplast number (or chlorophyll content) and protein amount (Supplementary Fig. S1). Equal amounts of total protein extracted from intact chloroplasts were subjected to MS followed by protein identification using the MaxQuant quantitative proteomics software package (Fig. 1A) (Luber et al., 2010; Schwanhäusser et al., 2011). A total of 1474 proteins were detected in at least two replicates in either var2 or the WT. Among these, 263 proteins were specifically detected in var2, 401 proteins were WT-specific, and 810 proteins were present in both the WT and var2 (Fig. 1B, Supplementary Table S2). This quantitative analysis identified 603 differentially accumulated proteins (log$_2$ FC > 1, P < 0.05), of which 317 proteins had higher and 286 proteins had lower accumulation in var2 as compared to WT (Fig. 1C; Supplementary Tables S3, S5). To gain insights into these differentially accumulated proteins in var2, the 603 proteins were subjected to Gene Ontology (GO) term enrichment analyses (P < 0.05) for Biological Processes. The results obtained indicated that proteins involved in stress responses and chloroplast PQC were significantly overrepresented among the up-regulated proteins in var2 (Fig. 2A; Supplementary Fig. S2A). Conversely, the representation of proteins involved in photosynthesis, chlorophyll metabolism, and protein import was reduced (Supplementary Tables S4, S6). Many other biological processes, including carbohydrate and amino acid metabolism, ATP synthesis, and transcription/translation-related components, were also affected. Among the up-regulated proteins, increased accumulation of chloroplast transcription/translation-related proteins such as TypA translation elongation GTPase (SVR3), pentatricotide repeat-containing protein (SVR7), and elongation factor-G (EF–G) was consistent with previous reports where knockdown or complete loss of either of these proteins has been found to suppress the leaf variegation in var2 (Miura et al., 2007; Liu et al., 2010; Zoschke et al., 2013). Proteins involved in photosynthetic electron transport and in the degradation of PSII core proteins (such as FtsH proteases) were considerably down-regulated (Supplementary Table S5). The observation that all subunits of the FtsH protease were reduced in var2 confirms that FtsH2 is dispensable for the assembly of the FtsH protease. Strikingly, a suite of light-harvesting antenna proteins in PSII were markedly under-represented in var2, implying that proteins involved in genome-coupled expression may function in var2 to repress the expression of photosynthesis-associated nuclear genes (PhANGs). Although a substantial number of proteins involved in various biological processes were differentially accumulated, we focused our analyses on those that were directly involved in maintaining or reinstating proteostasis.

Proteins involved in chloroplast PQC accumulate in the chloroplasts of var2
The GO enrichment analysis of proteins highly accumulated in var2 revealed a suite of proteins associated with PQC (Fig. 2A, Supplementary Fig. S2A, Supplementary Tables S3, S4), the functions of which were similar to those of proteins implicated in the eUPR (Iwata et al., 2008; Walter and Ron, 2011; Duwi Fanata et al., 2013). For example, the heat-shock protein Hsp60
family is linked to folding and assembly of their target proteins (Koumoto et al., 2001; Bonshtien et al., 2007; Salvucci, 2008). Indeed, HSPs such as Cpn10, Cpn20, Cpn60A, Cpn60B1, Cpn60B2, and Cpn60B4 were highly accumulated in var2, and there was also notable accumulation of the heat-shock proteins ClpB3 (an Hsp100 family protein), Hsc70-1 (Hsp70-6), Hsc70-2 (Hsp70-7), and Hsp90C, which prevent the misfolding of functional proteins or refolding of damaged proteins (Pulido et al., 2016) (Fig. 2A; Supplementary Fig. S2A, B; Fig. 3; Supplementary Tables S3, S4). In addition, two peptidyl-prolyl cis-trans isomerases, cyclophilin 20–3 (CYP20-3) and FKBP16-1, which facilitate protein folding by catalysing the cis-trans isomerization of proline imidic peptide bonds in oligopeptides, were accumulated (Fig. 2A). CYP20-3 also regulates cellular redox homeostasis under certain stress conditions by activating sulfur assimilation, leading to increased cellular thiol content and reduction potential (Park et al., 2013).

In addition to these chaperones and HSPs, several proteases involved in protein degradation and processing also exhibited higher levels in var2 (Fig. 2A, Supplementary Fig. S2A, Supplementary Tables S3, S4). These included Clp, Deg-protease, pr sequence protease 1 (PreP1), stromal processing peptidase (SPP), and organellar oligopeptidase (OOP). Interestingly, the protein levels of all subunits comprising the Clp protease were elevated (Fig. 2A, Supplementary Fig. S2A, Supplementary Tables S2–S4), including the five catalytic subunits (ClpP1, ClpP3, ClpP4, ClpP5, ClpP6), four non-catalytic subunits (ClpR1, ClpR2, ClpR3, ClpR4), two accessory subunits (ClpT1, ClpT2), and one adaptor subunit (ClpF) (Adam et al., 2006). By contrast, the auxiliary components ClpC1, ClpC2, and ClpD, which are involved in unfolding the substrates for subsequent degradation by the Clp protease (Rosano et al., 2011), remained unchanged in var2 compared to the WT (Supplementary Table S2). Since chloroplasts also require ClpC1/2 and ClpD for protein import.
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(Proano et al., 2011), unchanged levels of these chaperones probably ensures the regulation of protein import into the chloroplasts. The accumulated Clp protease may degrade the damaged/misfolded proteins in var2, counterbalancing the deficiency of FtsH2. The proteome analysis consistently revealed a reduced accumulation of some of the known targets of the Clp protease involved in chlorophyll biosynthesis, including chlorophyllide a oxygenase (CAO) (Sakuraba et al., 2009), HEMA1/GluTR (Apitz et al., 2016), and phytoene synthase (PSY) (Welsch et al., 2018) (Fig. 2B; Supplementary Table S5). This augmented Clp

| Proteins related to protein quality control (PQC) are highly accumulated whereas photosynthesis-related proteins are significantly reduced in the Arabidopsis var2 mutant. (A) Heat maps showing the expression of PQC-related proteins in var2 as compared with the wild-type (WT). GO analysis of the proteins highly accumulated in var2 compared with the WT revealed a significant enrichment in protein folding, proteolysis, detoxification, and chloroplast organization processes (Supplementary Fig. S2A). A complete GO analysis of proteins accumulated in var2 is shown in Supplementary Table S4. (B) Heat maps showing the expression of photosynthesis-related proteins in var2 as compared with the WT. GO analysis of the proteins reduced in var2 revealed a significant enrichment of proteins involved in photosynthetic protein import, PSI and PSII assembly, PSII repair, photosynthetic electron transport chain (PETC), and chlorophyll biosynthesis (Supplementary Fig. S2B). A complete GO analysis of down-regulated proteins in var2 is shown in Supplementary Table S6. |
activity might produce a large quantity of short peptides, which then undergo further degradation in the var2 mutant as a result of the concomitant accumulation of OOP (Kmiec et al., 2013). The increased levels of SPP and PreP1, involved in the processing of signal peptides (Stahl et al., 2002; Zhong et al., 2003), may promote maturation of newly imported proteins involved in PQC in var2.

The Deg proteases, which are ATP-independent serine endopeptidases, function in the degradation of photodamaged PSI proteins under excess light conditions (Hausühl et al., 2001; Chi et al., 2012). Among the five chloroplast Deg proteases, DegP1, DegP5, and DegP8 are present at the luminal side, whereas DegP2 and DegP7 are located in the stroma (Chi et al., 2012). We anticipated an accumulation of DegP1, DegP2, DegP5, and DegP8 because of their involvement in D1 degradation (Hausühl et al., 2001), which would balance the FtsH2 deficiency in var2. However, we found that the luminal Deg proteases were either down-regulated (DegP1 and DegP5) or remained unchanged (DegP8). Only the stromal DegP2 showed a higher accumulation in var2 (Fig. 2A, Supplementary Tables S2, S3, S5). Despite controversy as to whether DegP2 cleaves damaged D1 or not (Hausühl et al., 2001), it might be interesting to examine the role of DegP2 in var2 in this regard in future studies. Collectively, our results indicated that the lack of FtsH2 lead to remarkable changes in the accumulation of proteins associated with chloroplast PQC.

Proteins related to detoxification and redox homeostasis are accumulated in var2

Impaired proteostasis results in the alteration of the redox state in chloroplasts, which may thus impair ROS homeostasis (Kato et al., 2009; Rochaix and Ramundo, 2018). Indeed, chloroplasts of var2 exhibit increased levels of both superoxide radicals (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) relative to the WT (Kato et al., 2009). These increased levels of ROS may cause the accumulation of damaged proteins in the chloroplasts. Thus, not surprisingly, a group of proteins primarily involved in ROS detoxification were accumulated in var2, including CuZn-superoxide dismutase 2 (CSD2), Fe–superoxide dismutase 1 (FSD1), stromal ascorbate peroxidase (sAPX), monodehydroascorbate reductase 6 (MDAR6), dehydroascorbate reductase 3 (DHAR3), and glutathione reductase 2 (GR2) (Fig. 2A, Supplementary Fig. S2A, Supplementary Tables S3, S4). Similarly, var2 exhibited an elevated level of the glutathione S–transferase F8 (GSTF8), which scavenges secondary and tertiary oxidized derivatives of lipids and flavonoids, thereby mitigating the deleterious effects of reactive carbonyl species derived from lipid peroxide (Dixon and Edwards, 2010; Yamauchi et al., 2011). Proteins acting in redox maintenance, such as 2-Cys peroxiredoxin A and B (2CPA and 2CPB), peroxiredoxin-II–E (PRXIIE), NADPH-dependent thioredoxin reductase C (NTRC), and thioredoxin-like protein CDSP32 (CDSP32), were also up-regulated in var2.

Under oxidative stress conditions, peroxiredoxins containing a redox–active cysteine directly reduce H$_2$O$_2$ and organic hydroperoxides into water and alcohol. During this scavenging process, the cysteine is oxidized into sulfenic acid, which is then reduced back into its active form by thioredoxins, including CDSP32 (Broin et al., 2002) and NTRC (Moon et al., 2006). CDSP32 induced under oxidative stress conditions protects the photosynthetic apparatus against oxidative damage (Broin et al., 2002). NTRC is also involved in ROS-scavenging during chlorophyll biosynthesis in chloroplasts, thus possibly protecting the function of Mg–protoporphyrin monomethyl ester cyclase (CHL27) (Stenbaek et al., 2008). Increasing the amount of proteins involved in detoxification and redox maintenance might be crucial for mitigating photodamage in var2 chloroplasts.

Proteins associated with photosynthesis are reduced in var2

Whilst many proteins involved in protection, maintenance, and detoxification were up-regulated in var2, it was apparent that proteins involved in PSI and PSII assembly, the photosynthetic electron transport chain, chlorophyll synthesis, and chloroplast protein import were down-regulated in var2 compared to the WT (Fig. 2B, Supplementary Fig. S2B, Supplementary Tables S5, S6). Even though many of the proteins associated
with photosynthesis were reduced in var2 compared to the WT, it was noteworthy that the PSII RC proteins PsbA/D1, PsbB/CP47, PsbC/CP43, and PsbD/D2, as well as some of the PSI RC proteins, including PsaA, PsaB, and PsaL, were accumu-
lated in var2. Like PSII RC proteins, PSI RC proteins are prone to oxidative damage (Zivcak et al., 2015; Takagi et al., 2016). Therefore, the accumulation of PSI RC proteins together with PSII RC proteins in var2 suggests that the FtsH protease might also be involved in PSI repair or assembly. Interestingly, proteins involved in the dark reaction or the Calvin cycle, including Rubisco subunits, phosphoglycerate kinase 1 (PGK1), and glyceraldehyde 3-phosphate dehydro-
genase subunits, also accumulated in var2 (Supplementary Fig. S2A, Supplementary Table S3, S5). This could be an adaptive strategy of energy-deprived var2 plants to improve carbon fixation, thus producing more sugars to fulfill the energy demand, at least to some extent.

The outer envelope translocon protein TOC33, which is involved in the import of photosynthesis-associated proteins (PhAPs) (Jarvis and López-Juez, 2013), was down-regulated in var2 (Fig. 2B, Supplementary Fig. S2B, Supplementary Table S5). TOC33, TOC34, and TOC159 act as receptors for preproteins, whereas TOC75–III forms a β-barrel cation-selective channel through which the preproteins cross the outer membrane (Jarvis and López-Juez, 2013). The TOC33/159/75 complex is responsible for the import of PhAPs, while the TOC34/120/75 complex imports housekeeping proteins (Jarvis and López-Juez, 2013). The down-regulation of the TOC complex, especially of those subunits involved in the import of photosynthetic preproteins, may be one of the photoprotection mechanisms in var2 in addition to the increased levels of proteins related to PQC. Indeed, it has previously been demonstrated that under oxidative stress conditions the chloroplast import machinery undergoes a rapid E3 ligase-dependent turnover, which reduces the supply of photosynthetic proteins and subsequently minimizes ROS production by the photosystems (Ling et al., 2012; Ling and Jarvis, 2015).

Accumulation of PQC-related proteins in var2 is transcriptionally regulated

Since the majority of chloroplast proteins are encoded in the nucleus, any perturbations in the chloroplast may activate the process of retrograde signaling (RS) to regulate gene expression and to sustain chloroplastic homeostasis (Apel and Hirt, 2004; Fischer et al., 2007; Tripathy and Oelmüller, 2012; Ashraf and Harris, 2013; Chan et al., 2016). It is possible that the up-regulation of nuclear-encoded plastid proteins involved in PQC and detoxification and the down-regulation of photosynthesis-related proteins in the dysfunctional chloroplasts of var2 results from RS. To test whether this was the case, the transcript levels of ClpR2, ClpR4, CPN10, CPN60A, FSD1, and CSD2 were assessed in the WT and var2 seedlings using qRT-PCR. All the examined transcripts were significantly up-regulated in var2 compared to the WT (Fig. 4A, B), indicating that the accumulation of these PQC- and detoxification-related proteins seemed to be transcriptionally controlled.

The heat-stress transcription factor A-2 (HSFA2) drives the expression of HSPs by binding to the palindromic HSF-binding motif present in their promoter regions (Nishizawa et al., 2006; Schramm et al., 2006). Interestingly, HSFA2 was up-regulated in var2 (Fig. 4C), coinciding with a substantial accumulation of HSPs. Previous reports of the existence of a chloroplast UPR were based on the analysis of both transcript and protein abundance in cells in which protein-folding stress was elicited by knockdown of the stromal Clp protease and/or treatment with LIN (Rabindro et al. 2014; Llamas et al., 2017). In both studies, the genes encoding ClpB3 and Hsp21 (direct targets of HSFA2) were the two most highly up-regulated. While ClpB3 was up-regulated in var2 (Fig. 2A), the Hsp21 protein could not be detected in our chloroplast proteome data, probably due to the limitations of MS in detecting either small-sized or low-abundant (or rapidly turned-over) proteins. Nevertheless, the confirmation of higher transcript levels of both ClpB3 and Hsp21 in var2 compared to the WT was indicative of a possible function of RS in priming a cpUPR-like response.

Chloroplast proteome changes induced by clp and var2 are largely comparable

The accumulation Clp in the var2 mutant may compensate for the deficiency in var2, which includes impaired PSII repair. This would suggest that a deficiency of either of these proteases may induce a cpUPR-like response. To test this hypothesis, we compared the chloroplast proteome of var2 with the available chloroplast proteomes of different Arabidopsis clp mutants, such as clpR2, clpR4, and clpP3 (Kim et al., 2009, 2013a; Zybałów et al., 2009). Loss of any of these subunits results in the loss of Clp activity. A total of 82 proteins were significantly changed in these mutants, among which the abundances of 57 proteins were increased whilst those of 25 proteins were reduced by at least 2-fold (Supplementary Table S7). Among the 57 up-regulated proteins in the clp mutants, 54 were also up-regulated in var2 and of the 25 significantly down-regulated proteins, 16 were also down-regulated in var2. The majority of the 54 proteins up-regulated in both clp and var2 function in protein folding and ROS detoxification, whereas the down-regulated proteins are mostly implicated in photosynthesis.

Loss of FtsH2 also affected the abundance of plastid-encoded proteins. For instance, ClpP, and PSI and PSII core proteins were up-regulated, whereas a significant portion of plastid-encoded proteins involved in photosynthesis as well as in housekeeping were down-regulated (Supplementary Table S2). This down-regulation of plastid-encoded proteins could be due to the decreased accumulation of the plastid-encoded DNA polymerases (PEP, RPOA, RPOC1, and RPOC2) (Supplementary Table S5). However, in the clp mutants, except for the photosynthetic apparatus, other plastid-encoded proteins remained almost unchanged (Kim et al., 2013a). Interestingly, two plastid-encoded proteins, YCF3 and YCF4, which are involved in the PSI assembly (Krech et al., 2012), showed opposing expression: while YCF3 was reduced, YCF4 was strongly accumulated in var2 (Fig. 2A, B). This difference could be attributable to the relative relevance of these proteins.
For instance, YCF4 acts as an auxiliary and a non-essential assembly factor, whereas YCF3 is indispensable for PSI assembly in higher plants (Krech et al., 2012). This also suggests that, besides PSI assembly, YCF4 may have additional functions in chloroplasts deficient in PSII repair.

The var2 cpUPR-like response results in the accumulation of enzymes involved in the MEP pathway

$^{1}$O$_2$ mainly generated by chlorophyll molecules in PSII is the prime cause of PSII damage (Aro et al., 1993; Santabarbara et al., 2007; Triantaphylidès et al., 2008; Telfer, 2014; Dogra et al., 2018). Therefore, chlorophyll biosynthesis needs to be tightly regulated (Meskauskiene et al., 2001; Krieger-Liszay, 2005; Triantaphylidès and Havaux, 2009). Chlorophyll is composed of a chlorin ring and an isoprenoid phytol tail that are synthesized via the tetrapyrrole and methylerythritol 4-phosphate (MEP) pathways, respectively (Kim et al., 2013b). At the final step of chlorophyll biosynthesis, the chlorophyll synthase catalyses the esterification of chlorophyllide with the geranylgeranyl diphosphate (GGPP) synthesized via the MEP pathway (Oster et al., 1997; Wu et al., 2007). Inhibition of the MEP pathway results in the loss of the stoichiometric ratio between GGPP and chlorophyllide, causing the accumulation of free chlorophyllide (Arakane et al., 1996; Meskauskiene et al., 2001). Because free tetrapyrrole molecules generate $^{1}$O$_2$ in the presence of light, the coordinated regulation of the MEP and tetrapyrrole pathways is essential to avoid its generation (Kim et al., 2013b). In addition, several enzymes in the MEP pathway are tightly regulated (Sauret-Güeto et al., 2006; Kim

Fig. 4. The accumulation of proteins related to proteostasis in the Arabidopsis var2 mutant is transcriptionally regulated. (A) Relative abundance of proteins exhibiting a higher accumulation in var2 compared with the wild-type (WT). The ClpD protein is shown as the control in which the steady-state levels remained unchanged. (B) Relative transcript levels of the proteins accumulated in var2 were examined using qRT-PCR. (C) The expression of stress transcription factor A2 (HSFA2) and its downstream target genes Hsp21 and ClpB3 are up-regulated in var2. The relative abundance of the proteins was calculated using mean protein intensities. The relative transcript levels were calculated by qRT-PCR using PPA2 as a control. The data are means (±SD) of n=3 replicates. Significant differences between mean values were determined using Student’s t-test (*P<0.05).
et al., 2013b; Pulido et al., 2016). For example, deoxyxylulose 5-phosphate synthase (DXS), the first enzyme in the MEP pathway, readily aggregates under oxidative stress conditions, resulting in its inactivation. The J-protein J20 interacts with the inactive DXS and enables association with Hsp70 for either refolding (reactivation) or degradation (Pulido et al., 2013). This regulatory process largely relies on additional Hsp100 chaperones such as ClpB3 and ClpC1. When Hsp70-DXS interacts with ClpC1, DXS is unfolded and subsequently degraded via the Clp protease (Fig. 5A). In contrast, when Hsp70-DXS interacts with CLPB3 it leads to the refolding of DXS (Fig. 5B) (Pulido et al., 2016). DXS and other MEP pathway enzymes, including DXR, ISPD, ISPE, ISPG, and ISPH, are potential substrates of the Clp protease, as manifested by their accumulation in the clp mutants (Kim et al., 2013a). Given the increased accumulation of the catalytic core subunits of the Clp protease in var2, we anticipated that there would be concurrently reduced levels of the MEP enzymes. However, they either remained stable or instead accumulated (Supplementary Tables S2, S3). The DXS level was almost comparable between var2 and the WT, which was consistent with a previous report (Pulido et al., 2016). This unforeseen phenotype may be partly explained by the higher accumulation of ClpB3 and HSP70 (HSC70-1 and HSC70-2), which may protect these enzymes against proteolysis (Fig. 5C).

**Accumulation of damaged chloroplast proteins in var2**

Transcriptional induction and subsequent accumulation of proteins related to proteostasis suggested a probable action of chloroplast-to-nucleus RS in var2. We further assumed that this RS pathway, if triggered, may have been partly caused by an accumulation of the substrates of the FtsH protease, including the photodamaged PSII RC proteins. In response to excess light, PSII core proteins as well as light-harvesting proteins of the PSII RC are prone to oxidation at certain tryptophan (Trp) residues (Dreaden Kasson et al., 2012; Kale et al., 2017). Various oxidized forms of Trp, namely oxindolylalanine, N-formylkynurenine, and kynurenine with their corresponding

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**Fig. 5.** The cpUPR-like response contributes to the refolding of enzymes involved in the MEP pathway in the Arabidopsis var2 mutant. (A, B) DXS is the first enzyme in the MEP pathway and is a prone-to-aggregate protein under oxidative stress conditions. Interaction of Hsp70 with ClpC1/ClpC2 results in the unfolding and degradation of DXS (A). In contrast, Hsp70–ClpB3 interaction assists the refolding and reactivation of DXS (B). (C) Label-free quantitation indicating the steady-state levels of ClpB3, Hsp70 (Hsc70-1 and Hsc70-2), ClpC1, ClpC2, and DXS in var2 as compared to the wild-type (WT). The data are means (±SD) of n=3 replicates.
mass shifts of +16, +32, and +4 Da, respectively, were detected for those proteins (Supplementary Table S8). Given that PSII repair is a default process and that ROS are by-products of photosynthesis, photodamaged PSII proteins may accumulate in the chloroplasts of var2 mutant plants grown under normal light conditions. In agreement with this, we found at least 1.8-fold higher accumulation of PSII proteins in var2 compared to the WT (Fig. 6A; Supplementary Table S2). The relative proportion of oxidized to non-oxidized PSII proteins was higher in var2 compared to the WT (Fig. 6B; Supplementary Table S8). In addition to PSII proteins, the HCF136 protein involved in the assembly of PSII (Meurer et al., 1998) exhibited a similar Trp oxidation, which was more intensified in var2. Two PSI proteins, PsbH2 and PsbB, as well as four proteins involved in the Calvin-Benson cycle, namely RCA, RbcL, RbcS1A, and FBP1, also exhibited higher levels of Trp oxidation.

Discussion

Under unfavorable environmental conditions, plant cells often produce ROS because of disturbed oxygenic metabolism. This leads to changes in the cellular redox status and the accumulation of unfolded/misfolded/damaged proteins in various subcellular compartments (Martínez and Chrispeels, 2003; Duwi Fanata et al., 2013). These stressed compartments, such as the ER and mitochondria, then activate the UPR to eradicate the inactivated proteins (Martínez and Chrispeels, 2003; Aldridge et al., 2007; Iwata et al., 2008; Walter and Ron, 2011; Duwi Fanata et al., 2013; Pellegrino et al., 2013). Recent studies have shown that chloroplasts lacking Clp protease activity also exhibit a similar response (Schmollinger et al., 2013; Ramundo et al., 2014; Llamas et al., 2017). Since the Clp protease is one of the major components of PQC, its failure presumably results in the accumulation of its misfolded/unfolded or aggregated substrates (Kim et al., 2009, 2013a; Zybailov et al., 2009). In return, chloroplasts trigger a UPR-like response to reinstate proteostasis. Like the Clp protease, the membrane-bound FtsH metalloprotease also contributes to chloroplast proteostasis, especially for PSII RC proteins (Zaltsman et al., 2005; Kato et al., 2009; Nishimura et al., 2016). Hence, inactivation of the FtsH protease impairs PSII proteostasis, potentiating ROS accumulation in the chloroplasts (Kato et al., 2007; Miura et al., 2010), which then results in an altered chloroplast

![Figure 6](image/png)

**Fig. 6.** Trp-oxidized photosynthetic proteins are significantly accumulated in the Arabidopsis var2 mutant. (A) Steady-state levels photosynthetic proteins and GAPA-2 in var2 and the wild-type (WT). All proteins except HCF136 and PsAH exhibited at least 1.8-fold higher levels in var2. The data represent mean intensity values of the related peptides (n=3 replicates) (Supplementary Table S2). (B) Oxidation levels of the proteins. These proteins exhibited at least 1.6-fold higher oxidation levels in var2 as compared with the WT. The levels of oxidation were calculated using total intensities of peptides carrying oxidized Trp residues (Supplementary Table S8). The data represent the means of n=3 replicates. GAPA-2, a non-photosynthetic protein, was also found to undergo Trp-oxidation but showed no change in expression or in oxidation levels in var2 as compared with the WT.
proteostasis in terms of both quality and quantity (Fig. 1B, C). One of the changes (which takes place in this organelle) is the accumulation of damaged proteins. In turn, enhanced expression of proteins related to PQC and detoxification in the chloroplasts of var2 might be required in order to eliminate both ROS and damaged proteins (Figs 2A, 3) and to maintain redox homeostasis.

The substantial accumulation of catalytic core subunits of the Clp protease (Fig. 2A, Supplementary Tables S2, S3) suggests its complementary role in var2. Contrary to this notion, however, some MEP pathway enzymes, including DXS, that have previously been demonstrated to be substrates of Clp remained unchanged or even accumulated in var2 (Supplementary Table S2, S3). One explanation might be that the up-regulation of ClpB3 and other chaperones may protect DXS and other MEP enzymes from the Clp-mediated degradation (Figs 4, 5). However, some other known targets of Clp, including CAO, GluTR, and PSY, did not seem to be protected. The ORANGE (OR) protein, a holdase chaperone, protects PSY from Clp-mediated degradation (Welsch et al. 2018). Even though the OR protein was highly accumulated in var2 (Supplementary Table S3), it did not seem to protect PSY from degradation. One possible explanation for the down-regulation of PSY is that the increased Clp activity prevails over the OR activity. Zybailov et al. (2009) suggested that reduced or impaired photosynthetic electron transport (PET) may facilitate the accumulation of enzymes involved in the MEP pathway. Since PET is reduced in var2 due to impaired PSII repair, this may consequently cause the accumulation of MEP enzymes.

The chloroplast proteome in var2 was largely similar to that observed in different clp mutants (Supplementary Table S7). This suggests that any deficiency of PQC may generally affect chloroplast proteostasis, which consequently triggers cpUPR to reinstate proteostasis. The onset of cpUPR in the clp and var2 mutants, caused by the reduced PQC, might explain the previously demonstrated epistatic complementation of the leaf variegation in var2 by the loss of ClpC2 activity (Park and Roderme 2004). The deficiency of ClpC2 may potentiate cpUPR in var2, leading to the suppression of the leaf variegation. Despite the great similarity between the var2- and the clp-induced chloroplast proteomes, we noticed that whilst FtsH proteases remained unchanged in the clp mutants (Kim et al., 2013a), almost all subunits of the Clp protease were accumulated in var2 compared to the WT (Fig. 2; Supplementary Table S3). This indicates that the var2 cpUPR-like response is slightly different from the cpUPR established in the clp mutants, which coincides with their phenotypic differences (e.g. var2 leaf variegation). Given that the inactivation of FtsH2 resulted in the accumulation of damaged proteins (Fig. 6B) and ROS (Kato et al., 2007) in chloroplasts, the var2-conferring cpUPR-like response may be more precisely referred to as a damaged protein response (DPR).

Despite the non-stressful light conditions, the accumulation of Trp-oxidized PSII proteins was apparent in both the WT and var2 (Fig. 6B), supporting the notion that generation of $^{1}\text{O}_2$ and photodamage of PSII are inevitable during photosynthesis (Krieger-Liszkay, 2005). The $^{1}\text{O}_2$-induced oxidation of PSII R.C proteins has been previously reported as a signature of photodamage under conditions of photoinhibition (Dreaden Kasson et al., 2012; Kale et al., 2017). In our study, we observed this Trp-oxidation for the first time also in other proteins, such as those constituting PSI and enzymes involved in the Calvin–Benson cycle. Given that $^{1}\text{O}_2$ is mainly generated at the PSI R.C in the appressed region of the grana (the grana core) and that it is unable to travel long distances owing to its extremely short life span (Gorman and Rodgers, 1992), this result was quite puzzling. Furthermore, the vast majority of the PSI complex is located at the non-appressed regions of the grana (the grana margin) and the stroma lamellae (Andersson and Anderson, 1980; Wang et al., 2016). Although it is possible that the Trp-oxidation of these proteins resulted from in vitro oxidation, the non-appressed region of grana may serve as an alternative source of $^{1}\text{O}_2$ generation, as has been proposed recently (Wang et al., 2016; Dogra et al., 2018). In this regard, perhaps enzymes involved in the Calvin–Benson cycle may reside close to the non-appressed region of the grana in order to use the chemical energy generated via the light-dependent photosynthetic reaction. This proximity may cause Trp-oxidation. Highly enriched chlorophyll-synthesis enzymes along with PSII proteins and the de novo protein synthesis machinery in the grana margin also suggest that chlorophyll or its precursors synthesized during PSII reassembly may act as photosensitzers (Wang et al., 2016; Dogra et al., 2018).

Since photooxidative stress conditions directly affect the chloroplast PQC, cpUPR/DPR-like responses must play important roles in WT plants (Fig. 7). Hence, determining the underlying mechanisms of cpUPR/DPR-like responses, especially in the context of RS, would shed light on adaptive responses of plants to photooxidative stress. Even though we are unable to provide any direct evidence as to whether chloroplast-to-nucleus RS triggers a cpUPR-like response in var2 (Fig. 7), given the repression of light-harvesting antenna proteins of PSII (Supplementary Table S2) it is rational to assume that Genomes Uncoupled 1 (GUN1), a central regulator of the expression of PhANGs, may be involved in the cpUPR-like response in var2. However, given that GUN1 has no obvious function in either the clp- or LIN-confounded cpUPR (Llamas et al., 2017), it is possible that, if GUN1 acts in var2, its function may be restricted to the repression of photosynthesis-associated nuclear genes. In addition, the enhanced levels of ROS in var2 (Kato et al., 2007) may lead to an accumulation of oxidized products of lipids and carotenoids, some of which are known to trigger RS. Reactive electrophile species such as OPDA and phytoprostanes can also induce detoxification-related genes (Mueller et al., 2008). In addition, H$_2$O$_2$ may diffuse out of the chloroplast to activate HSFAs (Yu et al., 2012). Given that Trp-oxidized proteins accumulated in var2 and that Trp-oxidation is mainly dependent on $^{1}\text{O}_2$, RS mediated by EXECUTER1 (EX1, a putative $^{1}\text{O}_2$ sensor)– (Wang et al., 2016) may contribute to the var2-conferring cpUPR-like response. However, since EX1-mediated $^{1}\text{O}_2$ signaling requires an active FtsH2 protease, this signaling is likely to be dormant in var2. Forward genetic studies such as second-site mutagenesis of the var2 mutant.
may provide a pathway towards revealing the molecular components involved in RS priming of a cpUPR/DPR-like response. In addition, inactivation and/or overexpression of the PQC-related proteins that differentially accumulate in var2 as compared to the WT would provide further insights into how chloroplasts adapt to photooxidative stress conditions.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Amounts of chlorophyll and proteins are shown to be comparable with respect to chloroplast number in var2 and the wild-type.

Fig. S2. GO analysis of up- and down-regulated proteins in var2 relative to the wild-type.

Table S1. List of the primers used in the qRT-PCR analysis.

Table S2. List of the chloroplast proteins detected in var2 and/or the wild-type.

Table S3. List of the proteins with at least 2-fold higher accumulation in var2 relative to the wild-type.

Table S4. GO analysis of proteins with at least 2-fold higher accumulation in var2 relative to the wild-type.

Table S5. List of proteins showing increased levels of Trp oxidation in var2 as compared with the wild-type.

Table S6. GO analysis of proteins with at least twofold down-regulation in var2 relative to the wild-type.

Table S7. Comparison of dp– and var2–conferred proteomes.

Table S8. List of proteins showing increased levels of Trp oxidation in var2 as compared with the wild-type.

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