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Authors

Walley, Justin
Xiao, Yanmei
Wang, Jin-Zheng
et al.

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Plastid-produced interorganellar stress signal MEcPP potentiates induction of the unfolded protein response in endoplasmic reticulum

Justin Walley[a,1,2], Yanmei Xiao[b,1], Jin-Zheng Wang[b,1], Edward E. Baidoo[c,d], Jay D. Keasling[c,d,e], Zhouxin Shen[a], Steven P. Briggs[a], and Katayoon Dehesh[b,3]

[a]Section of Cell and Developmental Biology, University of California at San Diego, La Jolla, CA 92093; [b]Department of Plant Biology, University of California, Davis, CA 95616; [c]Lawrence Berkeley National Laboratory, Joint BioEnergy Institute, Emeryville, CA 94608; [d]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94608; and [e]Department of Chemical and Biomolecular Engineering, Department of Bioengineering, University of California, Berkeley, CA 94720

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Cellular homeostasis in response to internal and external stimuli requires a tightly coordinated interorgannellar communication network. We recently identified methylerythritol cyclodiphosphate (MEcPP) as a novel stress-specific retrograde signaling metabolite that accumulates in response to environmental perturbations to relay information from plastids to the nucleus. We now demonstrate, using a combination of transcriptome and proteome profiling approaches, that mutant plants (*ceh1*) with high endogenous levels of MEcPP display increased transcript and protein levels for a subset of the core unfolded protein response (UPR) genes. The UPR is an adaptive cellular response conserved throughout eukaryotes to stress conditions that perturb the endoplasmic reticulum (ER) homeostasis. Our results suggest that MEcPP directly triggers the UPR. Exogenous treatment with MEcPP induces the rapid and transient induction of both the unspliced and spliced forms of the UPR gene *bZIP60*. Moreover, compared with the parent background (P), *ceh1* mutants are less sensitive to the ER-stress-inducing agent tunicamycin (Tm). P and *ceh1* plants treated with Tm display similar UPR transcript profiles, suggesting that although MEcPP accumulation causes partial induction of selected UPR genes, full induction is triggered by accumulation of misfolded proteins. This finding refines our perspective of interorgannellar communication by providing a link between a plastidial retrograde signaling molecule and its targeted ensemble of UPR components in ER.

methylerythritol cyclodiphosphate | retrograde signaling | interorgannellar communication | unfolded protein response | endoplasmic reticulum

The endoplasmic reticulum (ER) function is crucial to adjustment and maintenance of a balance between protein loads and folding capacity in response to frequently changing intracellular and environmental conditions. To maintain balance (homeostasis) under stressful conditions, the ER activates conserved intracellular signal transduction pathways collectively termed the unfolded protein response (UPR) (1). The UPR monitors ER protein-folding capacity and communicates the ER status to gene expression programs that up-regulate genes encoding components of the protein folding machinery or the ER-associated degradation system (1, 2). In plants, two distinct and parallel branches of the UPR signaling pathway have been identified. One pathway involves two integral membrane-bound transcription factors (bZIP17 and bZIP28). The other pathway involves an ER membrane-localized dual-functioning (kinase/ribonuclease) protein, inositol-requiring protein-1 (IRE1). IRE1 catalyzes unconventional cytoplasmic splicing of mRNA encoding basic leucine zipper 60 (bZIP60), the transcription factor responsible for the induction of ER quality control genes (3, 4). Stress causes activation and nuclear relocation of bZIP17 and bZIP28, and activation of IRE1 responsible for splicing of *bZIP60* mRNA that encodes transcriptionally active nuclear localized bZIP60. These activated transcription factors induce transcription of target genes, including genes that mediate the UPR.

Protein folding is coupled to many biological processes, including the trafficking of molecules to specific cellular locations and the regulation of cellular growth and differentiation. As such, coordination between the UPR and other stress response pathways is essential for maintaining cellular homeostasis and integrity. Recent studies have highlighted roles for membrane contact sites (MCSs) between the ER and other organelles as important sites for intracellular signaling, and by extension potential role of MCSs in modulating ER functions in response to changes in cellular environment or metabolic requirements (5, 6). The discovery of these physical connections, specifically between the ER and the outer envelope membrane of plastids (7–9), invites speculation on their function as import sites of as-yet-unknown signaling molecules potentialy involved in fine tuning of ER function in response to stress.

Our recent studies, focused on understanding how plants sense and respond to environmental stresses, led to the discovery of the

Significance

A defining characteristic of living organisms is dynamic alignment of cellular responses to stress through activation of signal transduction pathways essential for fine-tuning of interorgannellar communication. Uncovering these communication signals is one of the prime challenges of biology. We have identified a chloroplast-produced retrograde signal, methylerythritol cyclodiphosphate (MEcPP), as a trigger of unfolded protein response (UPR) required for restoration of protein-folding homeostasis in the endoplasmic reticulum (ER). Increased levels of MEcPP via genetic manipulation or exogenous application potentiate expression of a sub-set of UPR genes, and alter plant's resistance to the ER stress inducing agent. These findings provide a link between a plastidial retrograde signal and transcriptional reprogramming of ER genes critical for readjustment of protein-folding capacity in stressed cells.

Author contributions: Z.S. and K.D. designed research; Y.X., J.-Z.W., E.E.B., and Z.S. performed research; J.D.K. and S.P.B. contributed new reagents/analytic tools; J.W., Y.X., J.-Z.W., and K.D. analyzed data; and J.W., S.P.B., and K.D. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE61675). The raw spectra for the proteome data have been deposited in the Mass Spectrometry Interactive Virtual Environment (MassIVE) repository (massive.ucsd.edu/ProteoSAFe/static/massive.jsp) (accession ID MSV000078868).

[1]J.W., Y.X., and J.-Z.W. contributed equally to this work.

[2]Present address: Department of Plant Pathology and Microbiology, Iowa State University, Ames, IA 50011.

[3]To whom correspondence should be addressed. Email: kdehesh@ucdavis.edu.

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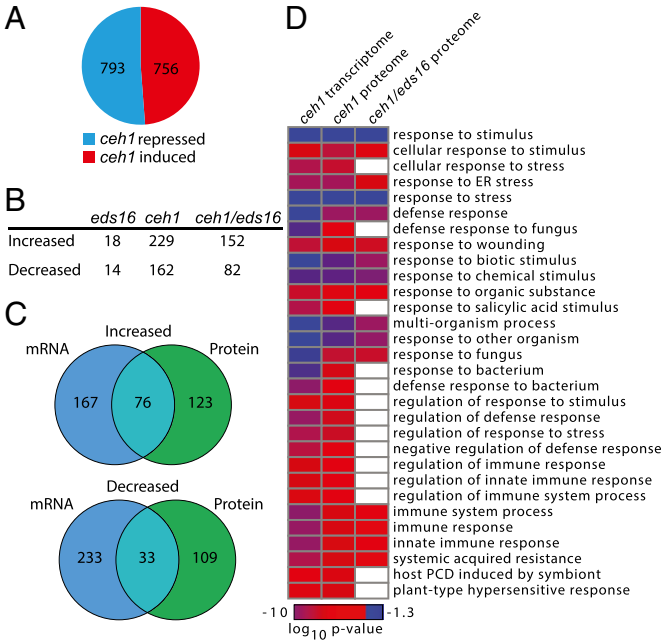

Fig. 1. Overview of the MEcPP dependent transcriptome and proteome. (*A*) Number of transcripts with altered levels in *ceh1*. (*B*) Number of proteins with altered abundance in *eds16*, *ceh1*, or *ceh1/eds16* double mutants. (*C*) Overlap between the transcriptome and proteome of *ceh1* for the gene-products that were reliably detected at the transcript and protein level. (*D*) GO Biological Process terms that are overrepresented ($P < 0.05$) in both the induced *ceh1* transcriptome and proteome profiling datasets. White boxes represent terms that are not overrepresented in the *ceh1/eds16* double mutant.

small metabolite 2-C-methyl-D-erythritol 2, 4-cyclodiphosphate (MEcPP), an isoprenoid intermediate and a dynamic plastidial stress-specific signal produced by the methylerythritol phosphate (MEP) pathway (10). Specifically we showed that MEcPP functions as a rapid plastid-to-nucleus communication signal. To gain a better understanding of perception and signal transduction of the MEcPP signaling cascade here we performed transcriptomic and proteomic comparisons of wild type to the high MEcPP accumulating mutant plants designated as *ceh1* (*constitutively expressing HPL*). These analyses revealed that MEcPP induces expression of selected UPR genes and accumulation of their respective proteins, thereby expanding the retrograde signaling function of MEcPP from plastid-to-nucleus to interorgannellar communication. This finding sets the stage for dissection of previously unidentified molecular links facilitating plastid to ER communication, critical for maintenance of cellular homeostasis under stressful conditions.

Results

Identification of MEcPP-Dependent Transcriptome and Proteome Alterations. Previously, we showed that MEcPP, an intermediate of isoprenoids (11–13), acts as a retrograde signaling metabolite to coordinate the transcription of selected stress-responsive nuclear encoded genes (10, 14). To extend our knowledge of gene expression changes mediated by MEcPP we performed transcriptome and proteome profiling of *ceh1* plants, which accumulate high MEcPP levels due to a mutation in HMBPP synthase (HDS) (10). The "wild-type" control background for all experiments is the Parent line (P) used to identify *ceh1*, which contains a Pro_{HPL}:*LUC* transgene (10). This approach resulted in the identification of 1,549 transcripts and 391 proteins with significantly altered abundance in the *ceh1* mutants (Fig. 1 *A* and *B* and Datasets S1 and S2). Furthermore, we looked at the subset of genes for which both mRNA and protein levels were quantified, and observed that 76 of the 243 *ceh1* induced transcripts also exhibited an increase in their corresponding protein (Fig. 1*C* and Dataset S1). We have previously shown that the level of the defense hormone salicylic acid (SA) is highly increased in *ceh1* (10). Therefore, we also profiled the proteome of SA deficient *eds16* (*eds16-1* allele) mutants (15) as well as *ceh1/eds16* double mutants to uncover SA-independent MEcPP effects. This approach enabled the identification of 234 proteins whose levels were altered by MEcPP in an SA-independent manner (Fig. 1*B* and Dataset S1).

We determined enrichment of Gene Ontology (GO) terms to better understand biological processes impacted by MEcPP induced alterations in gene expression. In particular, we were interested in biological processes impacted in both the *ceh1* transcriptome and proteome datasets as they represent MEcPP regulated transcriptional responses that penetrate through to the proteome. This analysis revealed that biological processes enriched in both the transcriptome and proteome of *ceh1* mutants are predominantly stimuli/stress related (Fig. 1*D* and Dataset S2). Furthermore, many of the enriched GO terms were also enriched in the *ceh1/eds16* induced proteins. These findings collectively demonstrate MEcPP-dependent but SA-independent activation of selected stress response pathways.

The Unfolded Protein Response Is Activated by MEcPP. We were intrigued by enrichment of the GO term "response to ER stress" in nonstressed *ceh1* and *ceh1/eds16* mutants. Typically, the ER stress response is activated under specific circumstances such as environmental stresses or other conditions resulting in accumulation of unfolded proteins, a key step in readjustment of the ER protein folding capacity to meet cellular needs (3, 4). Thus, we hypothesized that "response to ER stress" enrichment may represent a direct or indirect effect of altered MEcPP levels, independent of external cues.

To better define which ER stress response genes are induced by increased MEcPP levels, we examined the transcript levels of key UPR signaling and response components and determined that the expression levels of a subset of UPR genes are elevated in *ceh1* mutant plants (Dataset S1). Additionally, we verified the microarray data by quantitative RT-PCR (RT-qPCR) (Fig. 2). Next, we examined whether the activation of the UPR in *ceh1* is due to: (*i*) the specific signaling activity of MEcPP; (*ii*) the high levels of SA in the *ceh1* mutant; (*iii*) or a general response to perturbations of the MEP pathway. Recent reports of SA involvement in the UPR (16, 17), led us to examine not only *ceh1*

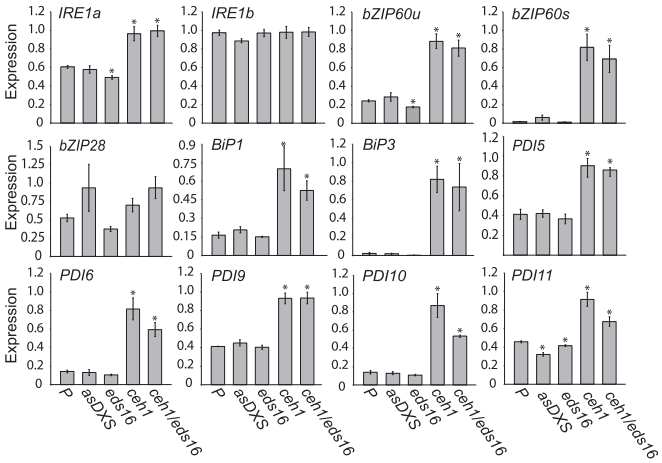

Fig. 2. UPR transcripts are induced in a MEcPP-specific manner. Normalized transcript levels of UPR genes in nonstressed Parent (P), *asDXS*, *eds16*, *ceh1*, and *ceh1/eds16* genotypes. RT-qPCR data are means of $n = 3 \pm$ SEM. Asterisks denote significant differences ($P < 0.05$) from the P line (i.e., wild-type) as determined by *t* tests.

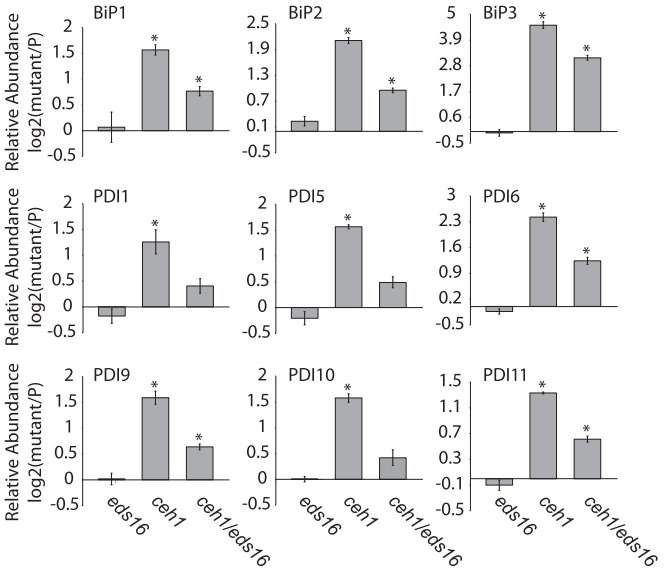

Fig. 3. UPR proteins are induced in a MEcPP-specific manner. Normalized iTRAQ protein abundance ratios of detected UPR proteins in mutants (*eds16*, *ceh1*, and *ceh1/eds16*) relative to the P line plants. Data are means of $n = 3 \pm$ SEM. Asterisks denote a significant difference ($P < 0.05$) from the P line as determined by *t* tests.

plants (high in MEcPP and SA), but also *ceh1/eds16* lines that contain high MEcPP but negligible SA levels (10). Additionally, to determine whether UPR induction is specific to the *ceh1* mutant or could also be triggered by general perturbation of the MEP pathway, our analyses included plants whose initial MEP pathway enzyme, DXP synthase (DXS) (18), was silenced by RNAi (*asDXS*) (10). Analyses of these genotypes by RT-qPCR established that the expression levels of the UPR signaling gene *IRE1a*, but not its paralog *IRE1b*, is elevated in all *ceh1* mutant backgrounds irrespective of SA levels and independently from general MEP pathway perturbation (Fig. 2). It is well established that ER stress activation of IRE1a and IRE1b results in production of the spliced (active) form of bZIP60 (19–22). We therefore assayed transcript levels of unspliced (*bZIP60u*) and spliced (*bZIP60s*) forms of *bZIP60* and found that both forms were increased in all of the *ceh1* backgrounds, but not in the *asDXS* lines (Fig. 2). Taken together, these results show that MEcPP causes selective induction of the UPR genes.

We extended these analyses to downstream UPR genes including binding proteins (*BiP1* and *3*) (23) and protein disulfide isomerases (*PDI 5*, *6*, *9*, *10*, and *11*) (24). We observed elevated transcript levels of these genes in all of the *ceh1* mutant backgrounds regardless of SA levels or perturbation of *DXS* expression levels (Fig. 2).

Thus, MEcPP-mediated induction of selected UPR signaling and downstream genes is independent of both SA and of a general stress response triggered by MEP pathway perturbation.

We used proteomics to measure the levels of UPR proteins corresponding to the above genes. We found that, although UPR signaling proteins were below the detection levels, the downstream UPR protein levels, including binding proteins (BiP1, 2, and 3) (23), and protein disulfide isomerases (PDI1, 5, 6, 9, 10, and 11) (24), were higher in *ceh1* and *ceh1/eds16* mutants compared with the P plants (Fig. 3). Notably, these same proteins are reduced in abundance in *ceh1/eds16* mutants compared with *ceh1*, albeit to different degrees. These data indicate that, although MEcPP-regulated transcriptional responses are SA-independent, the MEcPP-regulated protein accumulation is partially SA-dependent, thereby revealing the multilayered nature of UPR regulation (Figs. 2 and 3 and Dataset S1).

Exogenous Application of MEcPP Induces *bZIP60*. To directly test if MEcPP can induce UPR signaling genes and to further delineate the timing of potential induction we treated P plants with either mock or 100 μM MEcPP by two different methods of application, spraying or placing a drop on leaves. Subsequently we monitored transcript levels of *IRE1a*, *bZIP60u* and *bZIP60s* throughout a 180 min time course. Both approaches displayed similar outcomes, and hence here we only present the data from sprayed leaves showing that whereas *IRE1a* levels were not significantly altered, transcript levels of *bZIP60u* and *bZIP60s* were notably and significantly ($P < 0.05$) induced at 15 and 30 min, respectively, in MEcPP treated compared with mock treated plants (Fig. 4). These data substantiate a signaling function of MEcPP in induction of selected UPR genes. The disparity between this transient versus steadily induced expression profiles of the corresponding genes in *ceh1* plants may be the result of prolonged exposure to the constitutively high MEcPP levels in the mutant background.

Taken together these results demonstrate that MEcPP acts as a signaling molecule that rapidly and transiently induces *bZIP60* levels and splicing. The lack of an increase in *IRE1a* suggests that induction of this gene requires either greater levels or prolonged exposure to MEcPP, as occurs in *ceh1* mutant plants.

***ceh1* Plants Are Resistant to the ER Stress Inducer Tm.** The increased levels of UPR components in *ceh1* led us to test whether this conferred ER stress tolerance. Thus, we exposed P, *eds16*, *ceh1*, and *ceh1/eds16* genotypes to a commonly used ER stress inducing agent tunicamycin (Tm) (23). The similarity of root lengths of these genotypes under our experimental conditions prompted us to exploit this phenotype as a reliably uniform parameter for examining and comparing their tolerance to Tm (Fig. S1). Thus, we measured the relative root growth 24 h post Tm treatment compared with the corresponding root length of untreated seedlings (Fig. 5*A*). These data clearly show that the root growth in P and *eds16* compared with the *ceh1* and *ceh1/eds16*

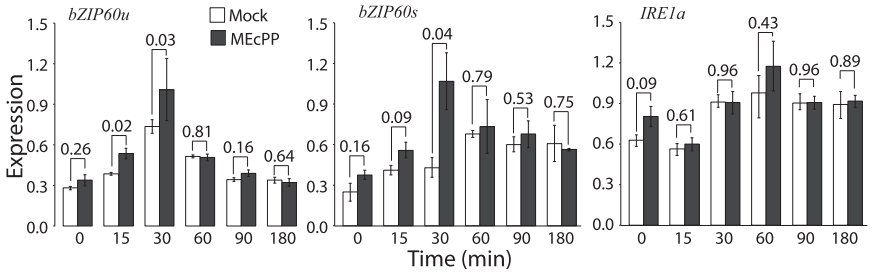

Fig. 4. Exogenous application of MEcPP induces *bZIP60*. Normalized transcript levels of genes in the P line treated with mock (0.01% Silwet 77) or 100 μM MEcPP. RT-qPCR data are means of $n = 3 \pm$ SEM. The values above the bars represent the *P* value for each comparison as determined by *t* tests.

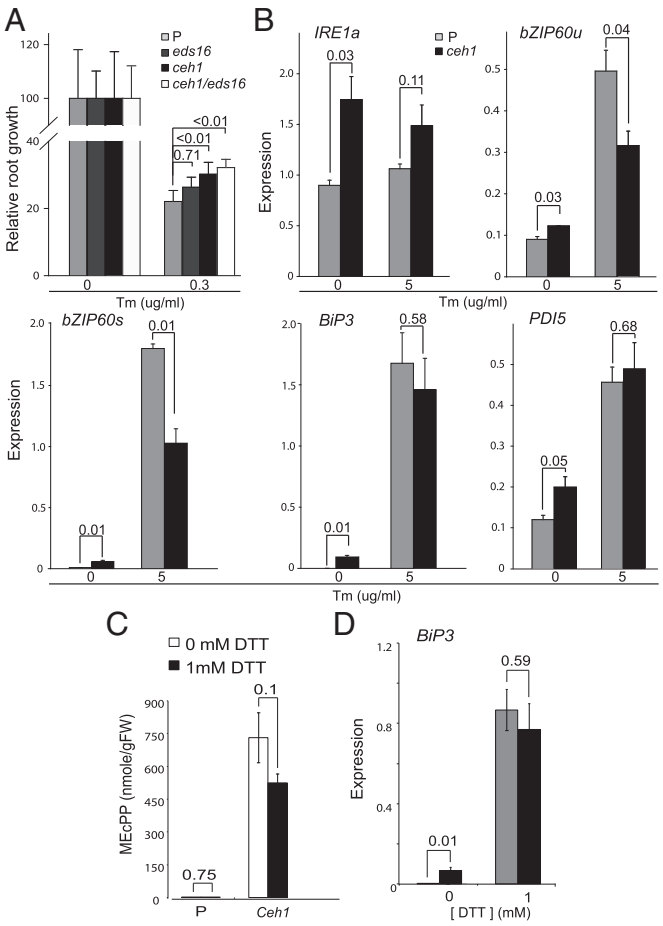

Fig. 5. *Ceh1* plants are more tolerant to tunicamycin. (*A*) P, *eds16*, *ceh1*, and *ceh1/eds16* plants were grown vertically on 1/2MS plates containing 1% sucrose for 6 d and subsequently transferred onto plates with or without 0.3 μg/mL Tm. Root growth was measured 24 h post Tm treatment, and presented as the percentage length of Tm treated roots to the corresponding untreated roots. Data are means of $n = 30 \pm$ SEM. (*B*) RT-qPCR of P and *ceh1* plants treated with 0 or 5 μg/mL Tm for 2 h. Data are means of $n = 3 \pm$ SEM. (*C*) MEcPP levels of P and *ceh1* plants grown on 0 or 1 mM DTT for 16 d. Data are means of $n = 6 \pm$ SEM. (*D*) RT-qPCR of *Bip3* in P and *ceh1* plants treated with 0 or 1 mM DTT for 2 h. Data are means of $n = 3 \pm$ SEM. The values above the bars represent the *P* value for each comparison as determined by *t* tests.

genotypes is more vulnerable to Tm treatment. Moreover, the similar growth rate of roots in *ceh1* and *ceh1*/*eds16* genotypes is a clear indication of an SA-independent MEcPP-mediated higher tolerance to Tm.

To explore how MEcPP affects UPR gene induction during ER stress caused by Tm, we monitored UPR transcript levels in P and *ceh1* plants treated 2 h with 0 or 5 μg/mL of Tm. Similar to our previous results, in nonstressed plants the levels of UPR transcripts are increased in *ceh1* relative to P plants (Fig. 5*B*). However, the transcript level of *bZIP60u*, *bZIP60s*, *PDI5*, and *BiP3* is higher in both P and *ceh1* plants treated with 5 μg/mL Tm. These results show that high MEcPP levels trigger partial induction of UPR genes and that full induction results from severe ER stress caused by accumulation of misfolded proteins.

Because of the experimental nature of short-term exposure of plants to TM treatment, we set to examine the effect long-term treatment of yet another less specific but commonly used UPR eliciting agent DTT (DTT) (20, 25, 26) on alteration of MEcPP levels. Therefore, we measured MEcPP levels in P and *ceh1* plants grown on plates with either 0 or 1 mM DTT for 14 d. No significant differences in MEcPP levels due to DTT treatment were detected (Fig. 5*C*). Furthermore, the heightened MEcPP levels in *ceh1* relative to P were maintained in plant exposed to long-term treatment DTT. Next, we examined whether the transcript levels of the downstream UPR gene *BiP3* in P and *ceh1* plants would alter in response to short-term (2 h) DTT treatment. Monitoring *BiP3* transcript levels in P and *ceh1* plants treated with 0 or 1 mM DTT shows the same trend as those of the Tm treated plants whereby in nonstressed plants the levels of UPR transcripts are higher in *ceh1* relative to P plants (Fig. 5*D*). In addition, these transcript levels are higher in both P and *ceh1* plants treated with 1 mM DTT. These results further substantiate role of high MEcPP levels as partial inducer of UPR genes.

Collectively, these findings indicate that the UPR to ER stress caused by accumulation of misfolded proteins in the ER is not through MEcPP. Rather, MEcPP may act as an interorgannellar communication signal during environmental stress to induce UPR components in advance of the accumulation of misfolded proteins, thereby potentiating this pathway.

Discussion

In response to cellular stress, tightly controlled interorgannellar communication enables organisms to activate signal transduction pathways that relay, integrate, and ultimately reestablish cellular homeostasis. Identification of these communication signals has thus far remained as one of the prime challenges of modern biology.

Using global transcriptome and proteome profiling we have shown that MEcPP produced in the chloroplast acts as an interorgannellar signaling metabolite that induces expression of select stress responsive genes. This result extends our previous finding defining MEcPP as a critical and stress-specific retrograde signaling metabolite inducing transcriptional activation of targeted nuclear stress-responsive genes encoding plastidial proteins (10). In particular, combinatorial molecular genetics and pharmacological approaches have established that MEcPP or a MEcPP-mediated signal(s) reprograms expression of a subset of UPR genes to increase levels of the core UPR proteins in the absence of ER stress. The observation that some but not all UPR genes are induced in *ceh1* implies that high MEcPP levels in *ceh1* do

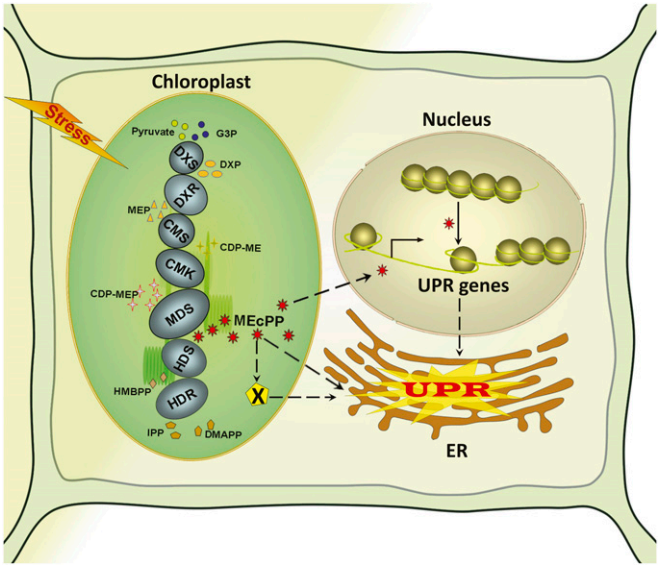

Fig. 6. Schematic model depicting alternative routes by which MEcPP potentiates induction of selected UPR genes. The MEcPP signal may function in the nucleus by first altering chromatin architecture and functional dynamics or by directly modulating a regulator of UPR. Alternatively, the MEcPP signal may, directly or indirectly, potentiate activation of selected UPR genes directly in ER.

not result in accumulation of unfolded proteins thereby triggering actual ER stress, but rather that MEcPP potentiates induction of selected UPR genes. Our findings provide strong support for a dynamic function of MEcPP as an interorgannellar communication signal that transmits information to prime the UPR machinery, thereby poising the cell to better handle ER stress resulting from adverse conditions. Consistent with this notion, abiotic stresses that induce UPR genes, such as light and wounding (27–29), also increase MEcPP levels (10).

Although elevated levels of MEcPP induce UPR genes, it is unclear how this occurs. We favor two potential mechanisms: transcriptional activation in the nucleus or direct UPR signaling activation in the ER; both could be involved. In the schematic model presented (Fig. 6), we propose that MEcPP may modulate transcription of the targeted UPR genes either by altering the functional organization of nuclear domains in response to stress leading to changes in chromatin architecture and functional dynamics (10), or by activating and/or repressing factors that directly regulate transcription of these genes. Alternatively, MEcPP or its signals may regulate selected proteins directly in the ER resulting in transcript splicing and translation (Fig. 6). Indeed the established presence of membrane contact sites between chloroplast and ER (7–9, 30, 31) offers a possible route for MEcPP export from the chloroplast to the ER followed by activation of UPR signaling. Deciphering the mode and site of MEcPP action will usher us into uncharted areas of interorgannellar signaling, and will provide a deeper understanding of the communication mechanisms between plastid and ER pathways linked to cellular adaptation. Additionally these studies provide new insight into the key role of the UPR as a cellular mechanism underlying systems robustness under stress conditions.

Materials and Methods

Plant Growth Conditions and Treatment. *Arabidopsis thaliana* (*Arabidopsis*) Col-0 ecotype were grown in a 16-h-light/8-h-dark cycle at 22 °C. Protein measurements were performed with 3-wk-old soil grown plants, and all of the other analyses were carried out with 2-wk-old plants grown on 1/2× Murashige and Skoog (MS) plates.

Exogenous MEcPP applications were performed as described (10).

Tm and DTT Treatment Assays. Tunicamycin tolerance experiments were performed by growing seedlings on 1/2 MS followed by selection and transfer of seedlings with same root length to plates containing DMSO as the control or 5 μg/mL Tm plates. For each treatment, root length of 30 seedlings was examined using Image J software. For short-term treatment, Tm was applied on 14-d-old seedlings for 2 h followed by tissue collection for RT-qPCR analyses.

For long-term DTT treatment, surface sterilized seeds were grown in 1/2× MS medium with DTT or water as mock for 16 d to collect tissue for MEcPP measurement. For short-term treatment, DTT or water as the control were applied on 14-d-old seedlings for 2 h followed by tissue collection for RT-qPCR analyses.

Microarray Profiling. The analyses were carried out on total RNA was isolated by TRIzol extraction (Life Technologies) and further purified using the Qiagen RNeasy kit followed by RNA quality and concentration assay using a Nanodrop spectrophotometer (NanoDrop, Thermo Scientific) and a 2100 Bioanalyzer (Agilent Technologies). The microarray analyses were carried out according to the manufacturer's instructions using Affymetrix *Arabidopsis* Genome Array ATH121501 Gene Chips, and subsequent analyses were performed with Affymetrix Microarray Suite version 5.0 software. An unpaired *t* test was used to test the difference in genotypes P line versus *ceh1*. Transcripts with more than a 1.5-fold change and *P* value less than 0.05 were considered significantly changed.

RT-qPCR Expression Analyses. Total RNA was extracted and used for RT-qPCR as described (32, 33). Dual normalization was carried out using the internal controls At4g34270 and AT4G26410 (34). Primers used to detect *bZIP60u* (AT1G42990), *bZIP60s*, *IRE1a* (AT2G17520), *IRE1b* (AT5G24360), *BiP1* (AT5G28540), and *BiP3* (AT1G09080) were described (21). Additional primers are listed in Dataset S3.

Proteomic Analyses. Protein isolation and mass spectrometry methods were carried out as described (35). Protein ratio between the P and each mutant line were calculated by taking the ratios of the total iTRAQ intensities from the corresponding iTRAQ reporter. Protein ratios were then $\log_2$ converted. Proteins that significantly changed in each mutant, relative to the P line, were determined using *t* tests (two tailed, paired). Proteins with more than 1.5-fold change and *P* value less than 0.05 were considered significantly changed.

Functional Category Enrichment. GO term enrichment was carried out using Virtual Plant 1.3 (virtualplant.bio.nyu.edu/cgi-bin/vpweb/virtualplant.cgi) (36). The background set for the microarray data were all genes with unambiguous probes on the ATH1 array, whereas the background set for the proteome data were all proteins detected by mass spectrometry. Enrichment was calculated using the Fisher Exact Test with FDR correction. GO terms with a *P* value less than 0.05 were considered enriched.

Metabolite Measurement. Measurements of MEcPP levels were carried out as described (10, 37).

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