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Plastidial metabolite MEcPP induces a transcriptionally centered stress-response hub via the transcription factor CAMTA3

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The general stress response (GSR) is an evolutionarily conserved rapid and transient transcriptional reprograming of genes central for transducing environmental signals into cellular responses, leading to metabolic and physiological readjustments to cope with prevailing conditions. Defining the regulatory components of the GSR will provide crucial insight into the design principles of early stress-response modules and their role in orchestrating master regulators of adaptive responses. Overaccumulation of methylerythritol cyclodiphosphate (MEcPP), a bifunctional chemical entity serving as both a precursor of isoprenoids produced by the plastidial methylerythritol phosphate (MEP) pathway and a stress-specific retrograde signal, in *ceh1* (*constitutively expressing hydroperoxide lyase1*)-mutant plants leads to large-scale transcriptional alterations. Bioinformatic analyses of microarray data in *ceh1* plants established the overrepresentation of a stress-responsive *cis* element and key GSR marker, the *rapid stress response element* (*RSRE*), in the promoters of robustly induced genes. *ceh1* plants carrying an established *4×RSRE:Luciferase* reporter for monitoring the GSR support constitutive activation of the response in this mutant background. Genetics and pharmacological approaches confirmed the specificity of MEcPP in *RSRE* induction via the transcription factor CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3 (CAMTA3), in a calcium-dependent manner. Moreover, CAMTA3-dependent activation of *IRE1a* (*inositol-requiring protein-1*) and *bZIP60* (*basic leucine zipper 60*), two *RSRE* containing unfolded protein-response genes, bridges MEcPP-mediated GSR induction to the potentiation of protein-folding homeostasis in the endoplasmic reticulum. These findings introduce the notion of transcriptional regulation by a key plastidial retrograde signaling metabolite that induces nuclear GSR, thereby offering a window into the role of interorgannellar communication in shaping cellular adaptive responses.

MEcPP | retrograde signals | GSR | RSRE | CAMTA3

Stress-triggered transcriptional reprogramming plays fundamental roles in transducing stress signals and ultimately enabling adaptive responses through readjustments of the appropriate physiological and metabolic processes. The initial transcriptional reprograming known as the "general stress response" (GSR), at times referred to as the "cellular stress response" or "core stress response," is a recognized evolutionarily conserved stress response present across kingdoms (1–5).

The GSR, a rapid and transient transcriptional reprogramming, is induced by a wide variety of stresses imposed upon organisms by environmental forces on macromolecules such as membrane lipids, proteins, and/or DNA (6). Bioinformatic analysis of the promoters of the rapid wound-response genes (5 min after mechanical damage) in plants led to the identification of an overrepresented functional *cis*-element, the *rapid stress response element* (*RSRE*), which is analogous to the yeast *stress response element* (*STRE*) (4, 7). A reporter line containing luciferase (LUC) driven by a synthetic promoter with four copies of the *RSRE* (*4×RSRE:LUC*) has confirmed the multistress responsive nature of *RSRE* induction and established the line as suitable for readout of stress-induced rapid transcriptional responses (4). Indeed, exploiting the *4×RSRE:LUC* line unraveled roles for $[Ca^{2+}]_{cyt}$, reactive oxygen species (ROS), MAPK signaling, and hormone signaling in modulating this stress-responsive transcriptional hub (4, 8–10) and further identified a member of the calmodulin-binding transcriptional activator (CAMTA) family, CAMTA3, as the predominant transcription factor that activates *RSRE* and, by extension, induces the GSR (10, 11).

Forward genetic studies directed at unraveling the components of stress-signaling networks in plants led to the discovery of the small stress-specific plastidial retrograde signaling metabolite methylerythritol cyclodiphosphate (MEcPP), which functions both as an intermediate of the methylerythritol phosphate (MEP) pathway for isoprenoids biosynthesis and as a communicator of environmental perturbations from the plastid to the nucleus (12, 13). The *ceh1* (*constitutively expressing hydroperoxide lyase1*) mutant accumulates large quantities of MEcPP and displays various stress-associated phenotypes such as compromised growth, high salicylic acid (SA) content, and constitutive activation of the unfolded protein response (UPR) required for restoration of protein-folding homeostasis in the endoplasmic reticulum (ER) (13–15). We performed bioinformatic analyses of global microarray expression profiles and identified overrepresentation of the *RSRE* motif in the promoters of robustly induced genes in the *ceh1* mutant, including key UPR-regulatory genes such as the ER membrane-localized *inositol-requiring protein-1* (*IRE1a*) and the transcription factor basic leucine zipper 60 (*bZIP60*) responsible

Significance

A dual-function plastidial metabolite, methylerythritol cyclodiphosphate (MEcPP), an intermediate of the plastidial pathway for isoprenoids production and a retrograde signaling metabolite, transduces signals to activate general stress-response (GSR) genes by inducing a transcriptionally centered stress hub. Specifically, MEcPP mediates the induction of the rapidly and transiently stress-responsive functional *cis*-element *rapid stress response element* via the calmodulin-binding transcriptional activator CALMODULIN-BINDING TRANSCRIPTION ACTIVATOR 3 in a calcium-dependent manner. MEcPP-mediated induction of this hard-wired GSR circuitry is a prime example of the integration of this metabolite into transcriptional networks and, by extension, modulation of downstream responses such as protein-folding capacity. This finding provides a foundation for investigating mechanisms of biological responses to stress and the roles of metabolites beyond the expected classical biochemical pathways.

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for the induction of ER quality control (15). Additional molecular genetics, pharmacological, and metabolic studies provided unequivocal evidence supporting the MEcPP-mediated induction of the *RSRE* and *RSRE* containing genes such as *IRE1a* and *bZIP60* via the transcription factor CAMTA3, in a calcium dependent manner. These results highlight the regulation of nuclear transcription by a small plastidial effector molecule and demonstrate the integration of a plastidial retrograde signaling metabolite in a stress-induced transcriptional network including the UPR responsible for maintenance of protein-folding homeostasis in the ER.

Results

Constitutive Activation of the GSR Is Triggered Specifically by Perturbation in the *Hydroxymethylbutenyl Diphosphate Synthase*. We previously established that accumulation of MEcPP in the *ceh1* mutant results in constitutive expression of a number of otherwise stress-responsive genes (13). To define MEcPP-mediated responses at the transcriptional level, we examined global transcriptional profiles in *ceh1* mutants versus the parent plants (15), followed by bioinformatic analyses. The analyses revealed overrepresentation of the *RSRE* in the promoters of robustly induced genes, but not suppressed genes, in the *ceh1* mutant (Fig. 1*A*). Gene Ontology (GO) term-enrichment analyses implicated the induced *RSRE*-containing genes as stress responsive (Fig. 1*B* and Table S1). To examine the validity of the bioinformatic analyses *in planta*, we used homozygous plants generated from crossing *ceh1* mutants to the line established for functional readout of stress-induced rapid transcriptional response, *4×RSRE:LUC*, hereafter referred to as the "parent line" (P) (4, 8, 10). Subsequent luciferase-activity assays determined that the otherwise stress-inducible *RSRE* is significantly and constitutively active in the *ceh1*/*RSRE:LUC* line (hereafter for simplicity designated "*ceh1*") (Fig. 1 *C* and *D*).

We previously have reported that the *ceh1* mutant, in addition to expressing high MEcPP levels, contains notably elevated levels of the stress-inducible hormone SA (13). To test whether high SA levels could contribute to the constitutive induction of *RSRE*, we generated homozygous lines containing *4×RSRE:LUC* in SA-deficient backgrounds. Specially, we crossed P into the *eds16-1* line, an SA-deficient mutant encoding a dysfunctional *isochorismate synthase1* (*ics1*) gene (16), and into *ceh1*/*eds16-1* double-mutant lines representing high MEcPP-expressing but SA-deficient plants (13). Luciferase-activity assays confirmed the presence of constitutively induced *RSRE* in both *ceh1* and *ceh1*/*eds16-1* backgrounds but not in the P and *eds16-1* lines (Fig. 1 *E* and *F*). The slight reduction in *RSRE* activation in *ceh1*/*eds16-1* compared with that in *ceh1* suggests that although constitutive SA contributes modestly to GSR activation in *ceh1*, it is not the predominant factor. Moreover, this result positions MEcPP as a potential candidate metabolite contributing to induction of the *RSRE*.

Next, we questioned whether the constitutive induction of the *RSRE* is the result of general stress caused by alteration of the MEP pathway or is caused specifically by the modulation in the activity of hydroxymethylbutenyl diphosphate synthase (HDS) enzyme that catalyzes conversion of MEcPP to HMBPP (13). To address this question, we sought to identify representative rapidly and generally stress-inducible genes whose promoters contain the GSR *cis*-element *RSRE*. Specifically, we found two genes, *CRK14* (*cysteine-rich receptor-like protein kinase 14*) and *WRKY48* (*WRKY DNA-binding protein 48*), with up-regulated expression levels in *ceh1* and rapid and transient induction by a number of stresses, including UV-B exposure and wounding, an archetypal *RSRE* activity profile (Fig. S1) (17). We then quantified the relative expression levels of these two genes in various genotypes, including reported lines of parent background for *ceh1* (P), *ceh1*, complemented *ceh1* (CP), and *HDS* cosuppression (*csHDS*), and the RNAi line of deoxy-xylulose-5-phosphate synthase (*asDXS*), encoding the enzyme catalyzing the first step of the MEP pathway (13, 18, 19). These data confirmed the exclusive presence of high basal levels of these two otherwise stress-inducible genes in the *ceh1* and *csHDS* lines and not in the other genotypes (Fig. S2). Most notably, despite the display of the stress phenotype of variegated leaves in both the *csHDS* and *asDXS* lines, the basal expression levels of the two

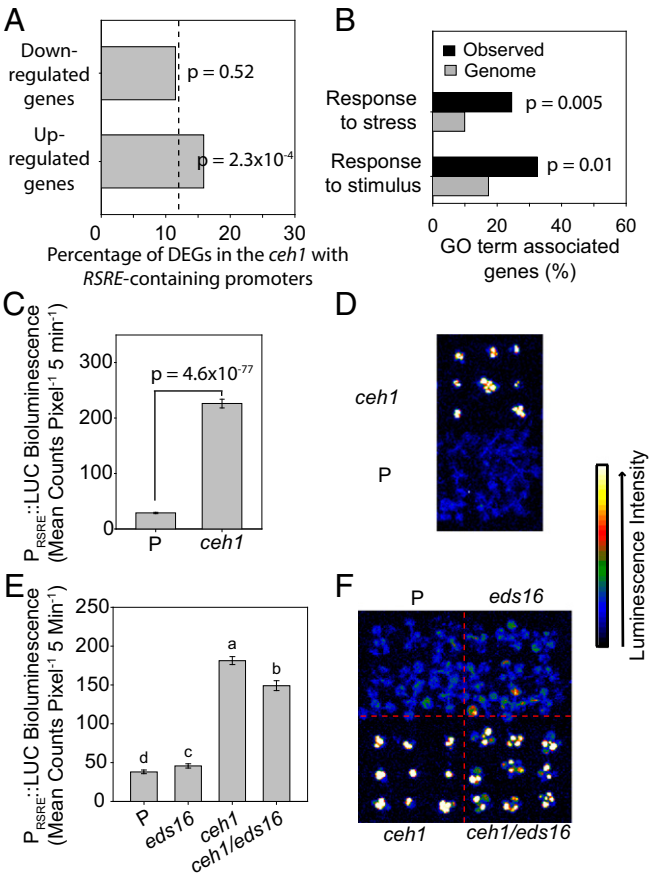

Fig. 1. The constitutive activation of GSR is specific to the *ceh1* and does not depend on SA. (*A*) Proportion of DEGs in the *ceh1* mutant containing an *RSRE* in the proximal 500 bp of the promoter region. The dashed line represents the proportion of genes on the ATH1 genome array with *RSRE*-containing promoters. *P* values were determined via the hypergeometric distribution. (*B*) Enrichment of selected GO terms in *RSRE*-containing genes induced in *ceh1* (observed); *P* values were determined using false-discovery rate (FDR)-corrected Fisher's exact test. (*C*) Basal *RSRE:LUC* activity in parent (P) and *ceh1* seedlings. Data are presented as means ± SEM ($n \geq 123$) with the *P* value determined using a two-tailed Student's *t* test. (*D*) Representative darkfield images of P and *ceh1* plants expressing *RSRE:LUC*. (*E*) Basal *RSRE:LUC* activity in the listed genotypes. Data are presented as means ± SEM ($n \geq 123$). Bars that do not share a letter represent statistically significant differences as determined by Tukey's honestly significant difference (HSD) test ($P < 0.05$). (*F*) Representative darkfield images of plants of each genotype expressing *RSRE:LUC*. The color-coded bar displays the intensity of LUC activity.

stress-responsive genes in *asDXS* are similar to the levels present in the P and CP plants (Fig. S2). These results therefore provide evidence for the specificity of altered HDS enzyme activity, rather than the general perturbation of the MEP pathway, in inducing GSR.

In Plants Containing High Levels of MEcPP the GSR Is Induced via the Transcription Factor CAMTA3. We previously identified a member of the CAMTA family, CAMTA3, as the key transcriptional activator of the *RSRE* (10). In light of this result, we questioned whether this function of CAMTA3 is preserved in the *ceh1*-mutant background or is replaced, in part or in totality, by other transcriptional modules. To address this question, we used the previously generated *camta3*/*RSRE* line (10) and crossed it to *ceh1*/*RSRE* to obtain *ceh1*/*camta3*/*RSRE*. As above, for simplicity the mutant lines containing the *4×RSRE:LUC* reporter are referred to simply by mutant name (Fig. 2*A*). One notable phenotypic disparity between these lines is their difference in growth: *ceh1*/*camta3* seedlings are larger than *ceh1* seedlings but still are smaller than seedlings in the P or *camta3* backgrounds (Fig. 2 *A* and *B*).

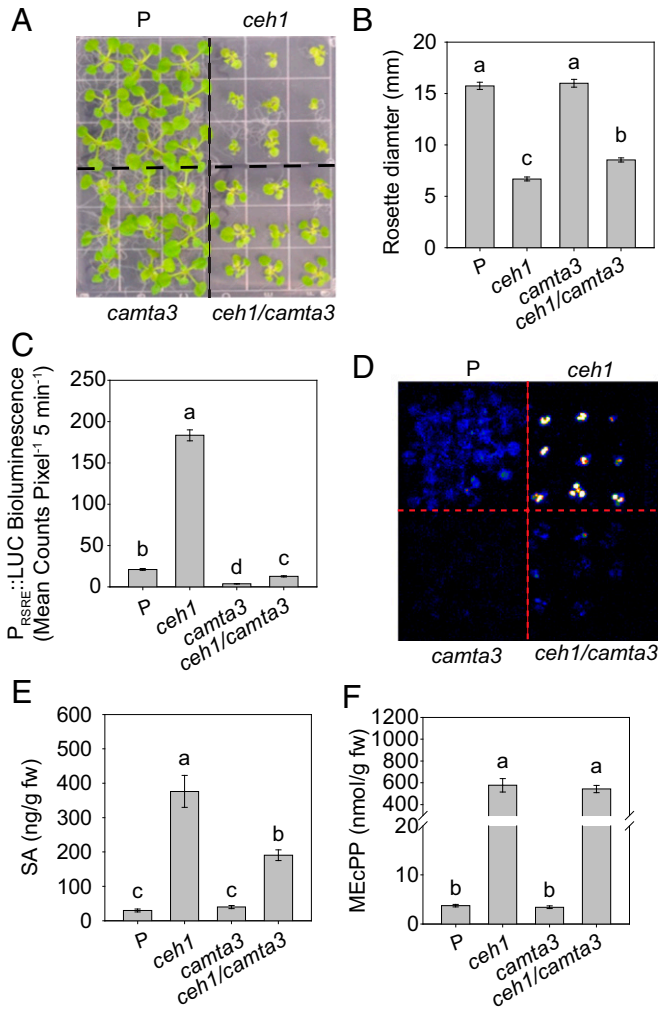

Fig. 2. In plants containing high levels of MEcPP, the GSR is induced via the transcription factor CAMTA3. (*A* and *B*) Representative images of the examined genotypes (P, *ceh1/RSRE*, *camta3/RSRE*, and *ceh1/camta3/RSRE*) (*A*), and average rosette diameter (*B*). Data are presented as means ± SEM ($n \geq 65$). (*C*) Basal *RSRE:LUC* activity in aforementioned seedlings. Data are presented as means ± SEM ($n \geq 123$) with the *P* value determined using the two-tailed *t* test. (*D*) Darkfield image analysis of representative plants expressing transcriptional *RSRE:LUC*. (*E* and *F*) Levels of SA (*E*) and MEcPP (*F*) in the genotypes examined. Data are presented as the means ± SEM of four independent biological replicates. Bars that do not share a letter represent statistically significant differences as determined by Tukey's HSD test ($P < 0.05$).

Next, we tested the luciferase activity in the aforementioned backgrounds and established that, compared with the respective parent backgrounds, both basal and constitutive levels of *RSRE* activity are highly reduced in the *camta3* and *ceh1/camta3* lines (Fig. 2 *C* and *D*). This finding confirms CAMTA3 as the prime *RSRE* transcriptional activator, independently of the genotype examined.

Given previous results reporting CAMTA3 as a suppressor of SA production in *Arabidopsis* (20–23), we examined the SA levels along with a panel of other hormones as controls, namely jasmonic acid (JA), abscisic acid (ABA), and auxin (IAA) in the P, *ceh1*, *camta3*, and *ceh1/camta3* genotypes (Fig. 2*E* and Fig. S3*A*). These measurements provided evidence for the absence of any significant variation in the levels of two of the control hormones, namely IAA and JA. Interestingly, however, in contrast to ABA, whose lower level in *ceh1* is recovered in *ceh1/camta3*, there is a substantial decrease in SA levels in the *ceh1/camta3* genotype relative to *ceh1*. It is of note that SA levels in *ceh1/camta3* are still higher than the levels in P plants, potentially supported by other transcriptional activator(s) in the *ceh1*-mutant background (Fig. 2*E*). Moreover, the reduced SA levels align with the decreased expression levels of the SA-marker gene, *PR1* (Fig. S3*B*). These data therefore substantiate the specificity of CAMTA3 as a positive regulator of SA levels in the *ceh1* mutant under the growth temperature used (24–25 °C). Indeed, the suppressing effect of CAMTA3 on SA at 19–22 °C has been reported previously to disappear at 24–25 °C and to be overcome at 4 °C (20–23). These results thus add another instance of a conditional and complex interaction between CAMTA3 and SA regulation.

Next, we measured the levels of MEcPP in the aforementioned backgrounds to test the potential influence of CAMTA3 in determining the metabolite's levels and, by extension, to delineate the contributions of MEcPP levels to deactivation of *RSRE* in the *ceh1/camta3* background (Fig. 2*F*). The similar MEcPP levels in *ceh1* and *ceh1/camta3* clearly demonstrate the irrelevance of CAMTA3 functionality to MEcPP levels.

Next, we questioned whether the expression levels of *RSRE*-containing genes display similar dependency on CAMTA3. For these experiments, we targeted the aforementioned GSR model genes *CRK14* and *WRKY48* and the *RSRE*-containing UPR genes, *IRE1a* and *bZIP60*, previously shown to be up-regulated in the *ceh1* mutant (15). Analyses of the transcript levels of these genes in the four genotypes of P, *ceh1*, *camta3*, and *ceh1/camta3* clearly display the key role of CAMTA3 in determining the expression levels of all four genes. Specifically, the data show induced expression levels of these genes in the *ceh1* mutant and their reduced expression (albeit to different degrees) in *ceh1/camta3* relative to the *ceh1* background (Fig. 3). Thus the data verify CAMTA3 as the key transcriptional activator of the *RSRE* and further establish the role of this transcriptional activator in the induction of stress-responsive genes, including those of the UPR regulating the ER protein-folding capacity.

Exogenous Application of MEcPP Rapidly Induces *RSRE* via CAMTA3. We next examined the specificity of MEcPP in potentiating *RSRE* by exogenous treatment of the *4×RSRE:LUC* parent line with MEcPP or water as the solvent control (Fig. 4, upper curves). For these

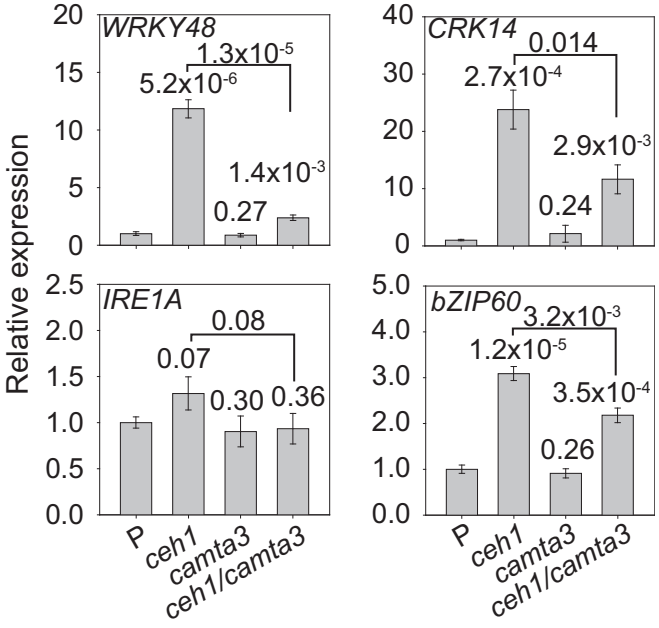

Fig. 3. The expression of GSR and UPR genes in the *ceh1* line is induced predominantly via CAMTA3. Relative expression levels of two GSR marker genes (*WRKY48* and *CRK14*) and two of the UPR genes (*IRE1a* and *bZIP60*) analyzed by quantitative RT-PCR (qRT-PCR) in P, *ceh1*, *camta3*, and *ceh1/camta3* lines. Data are presented as means ± SEM of four independent biological replicates. The numbers above each pair of bars are *P* values from one-tailed *t* tests relative to the P line, unless otherwise indicated.

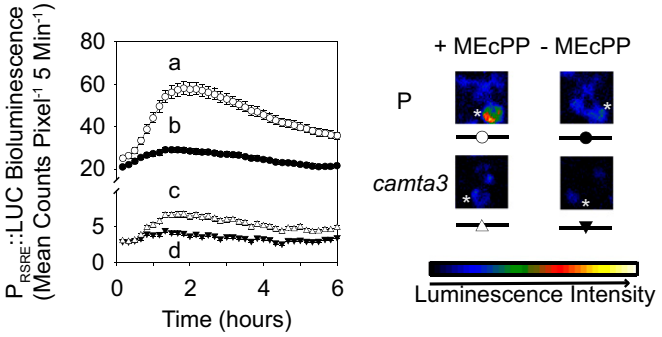

Fig. 4. Exogenous application of MEcPP rapidly induces *RSRE* via CAMTA3. (*Left*) Activity of *RSRE:LUC* in P (upper curves) and *camta3* (lower curves) leaves following application of MEcPP (open circles and triangles) or water as the control (filled circles and triangles). Data are shown as means ± SEM ($n \geq 187$). Lines that do not share a letter represent statistically significant differences as determined by Tukey's HSD test at the 90-min time point ($P < 0.05$). (*Right*) Representative plants are shown at right, with the treated leaves labeled by a white asterisk. The color-coded bar displays the intensity of LUC activity.

experiments, we used two independent sources, commercially prepared and in-house chemically synthesized MEcPP. The results clearly show that direct application of either MEcPP preparation results in similar rapid and transient luciferase activity in P plants (Fig. 4 and Fig. S4*A*) that is reminiscent of the previously reported wound-induced *RSRE* activity profile (4, 8, 10). The observed activity in control plants treated with water likely results from touch-mediated activation of *RSRE* upon application of the droplet (Fig. 4 and Fig. S4*A*). As an additional control, we examined the impact of MEcPP treatment on *4×mRSRE:LUC*, a line previously developed to contain *RSRE* mutated in three of the six core nucleotides (4). The absence of luciferase activity in the *4×mRSRE:LUC* line treated with MEcPP, as opposed to the *4×RSRE:LUC*, displays the specificity of the *RSRE* in conferring the MEcPP-mediated response (Fig. S4*B*).

Next, we questioned whether this direct impact of MEcPP in potentiating *RSRE* occurs via CAMTA3. Therefore we conducted MEcPP application experiments on the *camta3*/*RSRE* background (Fig. 4 and Fig. S4*A*, lower curves). The result mimics an *RSRE* induction profile similar to that of the P plants but much smaller in magnitude, providing additional support for the key role of CAMTA3 in MEcPP-mediated *RSRE* induction. However, the subtle but detectable luciferase activity in the *camta3* mutant supports the presence of an additional MEcPP-responsive *RSRE* transcriptional activator, here referred to as "factor X."

We next tested a potential molecular connection between MEcPP and CAMTA3 by examining the *CAMTA3* transcript level in the *ceh1* mutant and further examined whether such an alteration is specific to *CAMTA3* or extends to any of the other five members of the *CAMTA* family. These analyses identified *CAMTA3* as the only gene family member whose transcript level is statistically significantly increased in *ceh1* relative to P plants (Fig. 5*A* and Fig. S5). We further examined whether MEcPP could directly induce the expression of *CAMTA3* and, if so, whether such an induction occurs before or in concordance with the temporal dynamics of *4×RSRE:LUC* activity in response to the exogenous application of MEcPP (Fig. 5*B*). The temporal asynchrony between the activation of *RSRE*, peaking at 90 min, and MEcPP-induced expression of *CAMTA3* at 180 min in response to MEcPP application (Fig. 4*A* and Figs. S4 *A* and *B* and 5*B*) strongly suggests that, at least initially, function of MEcPP in potentiating *RSRE* activity is not via the induction of *CAMTA3* expression levels.

Next, we asked whether the accumulation of MEcPP in *ceh1* results in enhanced stability of CAMTA3 protein levels and, by extension, activation of *RSRE*, specifically in light of a report identifying SR1IP1 (CAMTA3/AtSR1 interaction protein 1) as an adaptor for ubiquitination of CAMTA3 in plants challenged with pathogens (24). Thus, we questioned whether SR1IP1 might be involved in GSR regulation and if the constitutive activation of *RSRE* in the *ceh1* mutant is associated with reduced expression levels of *SR1IP1* and thereby altered CAMTA3 stability. To address these possibilities, we first generated homozygous *sr1ip1*/*4×RSRE:LUC* lines and treated them with MEcPP, followed by luciferase-activity measurements (Fig. S6*A*). As evidenced by the data, SR1IP1 is not involved in MEcPP-mediated induction of the GSR. In addition, similar *SR1IP1* expression levels in the genotypes with varying MEcPP and SA levels, namely P, *ceh1*, *eds16-1*, and *ceh1*/*eds16-1* (Fig. S6*B*), exclude an association between SA or MEcPP levels and modification of the *SR1IP1* expression levels. Last, application of the bacterial elicitor flg22 (a 22-aa flagellin peptide) on *sr1ip1* resulted in induction of *RSRE* similar to that in the P plants (Fig. S6*C*). These findings collectively exclude the involvement of SR1IP1 in the regulation of the *RSRE*-mediated GSR.

SR1IP1 is not necessarily the only factor regulating the stability of CAMTA3 protein. Accordingly, we compared the CAMTA protein levels in P versus *ceh1* plants using our previously generated proteomic dataset (15) (massive.ucsd.edu/ProteoSAFe/static/massive.jsp) (Fig. 5*C*). These data clearly display similar CAMTA3 abundance in P and the *ceh1*-mutant background, thus excluding a significant contribution of differential CAMTA3 abundance to constitutive activation of *RSRE* in the *ceh1*-mutant background.

MEcPP Induction of *RSRE* Is Ca^{2+} Dependent. The role of Ca^{2+} as a secondary messenger in stress signaling in general and its established role in *RSRE* induction in particular (9, 10) led us to examine the dependency of MEcPP-mediated RSRE induction of this element using the Ca^{2+} chelator EGTA (Fig. 5*D*). The results show that *4×RSRE:LUC* plants treated with EGTA no longer respond to the inductive effect of MEcPP, thus providing clear evidence that Ca^{2+} is required in potentiating MEcPP-mediated transcriptional activation of RSRE.

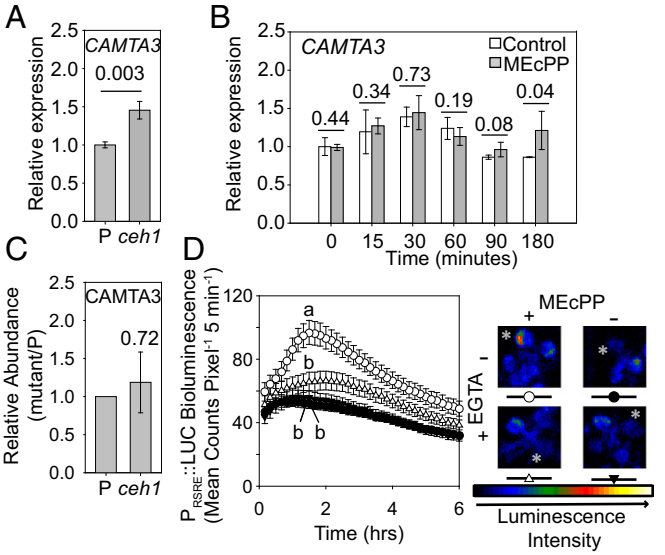

Fig. 5. Activation of *RSRE* by MEcPP is Ca^{2+} dependent. (*A*) Relative expression levels of *CAMTA3* analyzed by qRT-PCR in the P and *ceh1* lines. Data are presented as means ± SEM ($n = 3$), with the *P* value determined by the two-tailed *t* test. (*B*) Relative expression levels of *CAMTA3* analyzed by RT-qPCR in P plants at different times after the application of water (white bars) or MEcPP (gray bars) to the leaves. Data are presented as means ± SEM ($n = 3$). *P* values from one-tailed *t* tests are shown above each pair of bars. (*C*) Normalized iTRAQ CAMTA3 protein abundance in *ceh1* plants relative to P plants. Data are presented as means with the *P* value determined by the two-tailed *t* test. (*D*, *Left*) Activity of 4×*RSRE:LUC* in the P background following pretreatment with EGTA (triangles) or water (circles) and subsequent MEcPP application (open circles and triangles) or water as the control (filled circles and triangles). Data are shown as means ± SEM ($n \geq 111$). Lines that do not share a letter represent statistically significant differences as determined by Tukey's HSD test at the 90-min time point ($P < 0.05$). (*Right*) Representative plants are shown with the treated leaves labeled by a white asterisk. The color-coded bar displays the intensity of LUC activity.

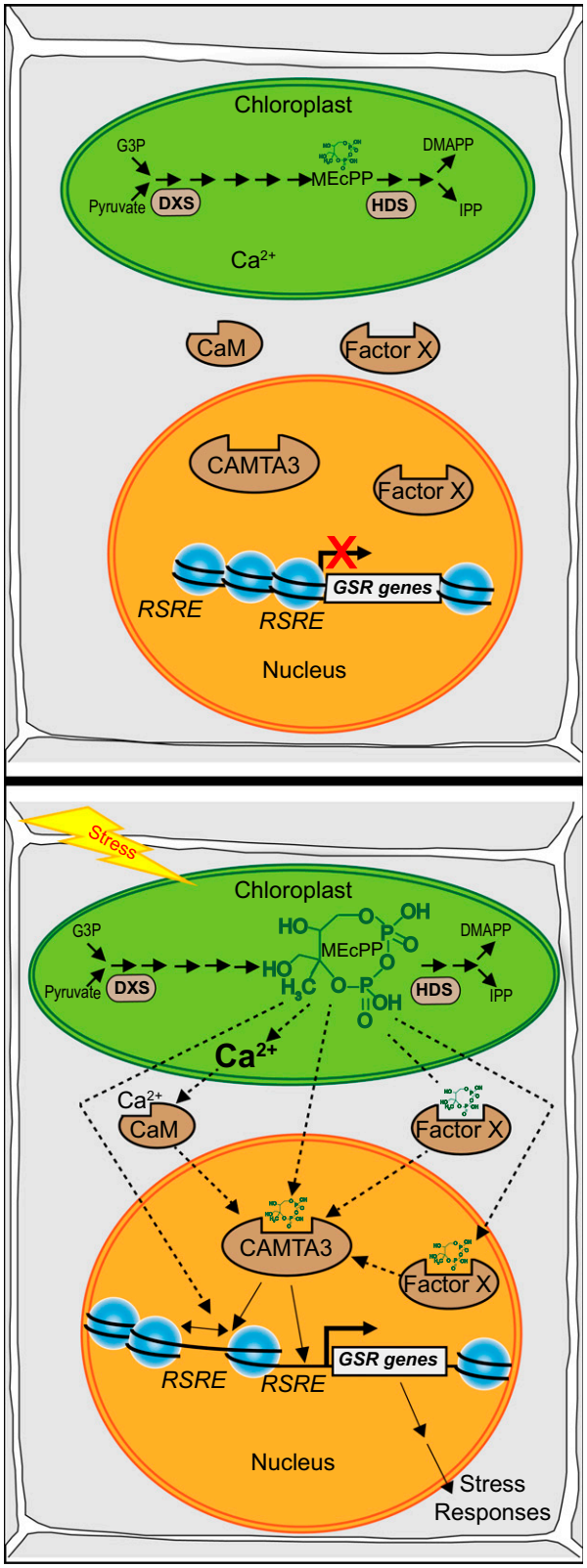

Fig. 6. Simplified schematic models of MEcPP mode(s) of action in potentiating the GSR. Schematic models of the MEcPP-mediated cellular responses before (*Upper*) and after (*Lower*) stress. Possible MEcPP signaling routes are depicted, including stress-mediated alteration in MEcPP levels functioning as a rheostat for the release of Ca^{2+} for the activation of CAMTA3; MEcPP-mediated alteration of chromatin architecture enabling the accessibility of *RSRE* for transcriptional regulators; MEcPP potentially functioning as an allosteric modulator of CAMTA3 or its potential coinducer; or a yet unknown transcriptional activator (factor X) binding to and activating RSRE, ultimately triggering the GSR.

Discussion

Organisms respond to environmental perturbations by altering gene expression and, by extension, activating integrated stress-response networks that ultimately reconfigure the cellular homeostasis vital to a timely and optimal adaptation to unfavorable conditions. One such integrated stress-response network is the GSR, which rapidly and transiently responds to a variety of stresses.

The dynamic role of biosynthetic metabolites in orchestrating master regulators of adaptive responses, although irrefutable, is not yet fully understood. Here we provide bioinformatic, molecular genetic, metabolomic, and pharmacological evidence for the integration of the stress-specific plastidial retrograde signaling metabolite MEcPP into the GSR transcriptional circuitry via activation of the *cis*-element *RSRE*, thereby inducing of a core set of stress-response genes.

The striking similarity between the rapid and transient activation of *RSRE* by external application of MEcPP and by wounding strongly supports the transcriptional regulation of this GSR element by the stress-specific plastidial retrograde signaling metabolite. Indeed the increase in the MEcPP levels in response to wounding or high light (13) correlates well with the activation of GSR genes with *RSRE* in their promoters, thus further substantiating the integration of a plastidial retrograde signaling metabolite in a stress-induced transcriptional network. The stress-mediated induction of MEcPP levels in bacterial cultures exposed to oxidative stress is supportive of the functional conservation of this metabolite in stress responses and even in the regulation of stress-responsive transcriptional networks (25).

Here, we deepen our understanding of the mechanism by which this well-conserved stress-signaling metabolite functions in plants by showing that MEcPP-mediated *RSRE* induction is via the transcriptional activator CAMTA3 in a Ca^{2}-dependent manner.

Our previous report on role of the stress-responsive retrograde signal MEcPP in activating the UPR in the ER did not address the molecular mechanisms of this action (15). Here we illustrate the critical role of CAMTA3 in the activation of *RSRE*-containing genes such as *IRE1a* and *bZIP60*, the two UPR genes responsible for maintaining the protein-folding capability of the ER, and thereby shed light on the molecular mechanisms of MEcPP action. However, understanding how this retrograde signaling metabolite integrates into the transcriptional network remains a challenge. Among several potential scenarios, one is that MEcPP functions as a rheostat for the release of Ca^{2+} for CAMTA3 activation, as presented in the simplified schematic model (Fig. 6). Indeed the dependency of MEcPP action on the presence of Ca^{2+} supports this scenario. Additionally or alternatively, MEcPP may function as an allosteric modulator by binding to and altering the conformation, and thus the affinity, of CAMTA3. However, this possibility requires an as yet unidentified MEcPP plastid–nuclear mode of transport, because the predominant presence of CAMTA3 in the nucleus is well established (11). Alternatively and/or additionally, MEcPP may function as an allosteric modulator of still another transcriptional activator, factor X, that supports subtle but significant residual *RSRE* activity in the absence of CAMTA3 while also potentially enabling stronger *RSRE* activation via interaction with CAMTA3 (Fig. 6). Finally, MEcPP could mediate dynamic architectural changes in chromatin structure through epigenetic regulation, similar to its direct function in nucleoid decondensation in chlamydial cultures by disrupting the interaction of histone-like proteins with DNA (26). In this case, chromatin becomes the receptor of the metabolic state of the cell, in part through fluctuation in MEcPP levels.

In summary, our finding unfolds the role of MEcPP as a key dynamic metabolic effector that orchestrates a complex stress-responsive transcriptional network, in part by activing *RSRE*. Moreover, our evidence bridging and extending the GSR to the UPR via CAMTA3-driven Ca^{2+}-dependent *RSRE* activation provides a new window for understanding how the induction of stress-response networks leads to the integration of interorgannellar communication, ultimately refining and shaping cellular adaptive responses.

Materials and Methods

***Cis* Element and GO Analysis.** *ceh1* differentially expressed genes (DEGs) with the *RSRE* motif within the first 500 bp of their promoters were identified by using BioVenn (27) for comparison with the list of previously described *ceh1* DEGs (15) and the list of all previously identified *RSRE*-containing promoters (10), followed by calculation of significance by the cumulative hypergeometric distribution. GO analysis was conducted with the BioMaps tool from VirtualPlant 1.3 (28).

Plant Growth and Treatments. All experiments were conducted on 2-wk-old seedlings grown in 16-h light/8-h dark cycles at 24–25 °C on 1/2× Murashige and Skoog medium. Luciferin treatment, flg22 application, and mechanical wounding were performed as described previously (4, 8, 10). EGTA was applied by placing a 2-μL drop of 5 mM EGTA or water (as a control), both with 0.01% SilWet L-77, on a leaf 30 min before the addition of 2 μL of either water or MEcPP (100 μM final concentration) to the pretreatment drop.

MEcPP Synthesis and Treatment. MEcPP, either a commercial (Echelon, catalog no. I-M054) or an in-house synthesized compound, was applied to a single leaf per plant as described previously (13). The in-house synthesized MEcPP was prepared as previously described (29) with a slight modification, namely the conversion of bisphosphate to pyrophosphate was with two instead of one equivalents of 1,1′-carbonyldiimidazole, and stoichiometric $Pd(OH)_2/C$ was used for the hydrogenation step.

Generation of *RSRE:LUC* Lines. The *ceh1/RSRE* line was generated by crossing *4×RSRE:LUC* (4) to *ceh1* segregated from the previously described original *HPL:LUC* marker (13). The *ceh1/camta3/RSRE* and *ceh1/eds16-1/RSRE* lines were generated by crossing *camta3/RSRE* (10) or *ceh1/eds16-1* (16) to *ceh1/RSRE*. The *sr1ip1/RSRE* line was generated by crossing *4×RSRE:LUC* to the Arabidopsis Biological Research Center line SALK_064178 (24). Genotyping primers used are listed in the Table S2.

Quantification of Gene Expression. Real-time PCR and data normalization were performed as described (4) using QuantPrime. All primer sequences are listed in the Table S2.

Luciferase-Activity Quantification. Quantification and statistical analysis of *RSRE:LUC* activity were performed as previously described (10).

Hormone and MEcPP Measurement. The analyses of hormones and MEcPP were conducted as previously described (14, 30).

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