Distinct Roles for Mitogen-Activated Protein Kinase Signaling and CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR3 in Regulating the Peak Time and Amplitude of the Plant General Stress Response

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To survive environmental challenges, plants have evolved tightly regulated response networks, including a rapid and transient general stress response (GSR), followed by well-studied stress-specific responses. The mechanisms underpinning the GSR have remained elusive, but a functional cis-element, the rapid stress response element (RSRE), is known to confer transcription of GSR genes rapidly (5 min) and transiently (peaking 90–120 min after stress) in vivo. To investigate signal transduction events in the GSR, we used a 4xRSRE::LUCIFERASE reporter in Arabidopsis (Arabidopsis thaliana), employing complementary approaches of forward and chemical genetic screens, and identified components regulating peak time versus amplitude of RSRE activity. Specifically, we identified a mutant in CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR3 (CAMTA3) with reduced RSRE activation, verifying this transcription factor’s role in activation of the RSRE-mediated GSR. Furthermore, we isolated a mutant in MITOGEN-ACTIVATED PROTEIN KINASE KINASE1 (mekk1-5), which displays increased basal and an approximately 60-min earlier peak of wound-induced RSRE activation. The double mekk1/camta3 mutant positioned CAMTA3 downstream of MEKK1 and verified their distinct roles in GSR regulation. mekk1-5 displays programmed cell death and overaccumulates reactive oxygen species and salicylic acid, hallmarks of the hypersensitive response, suggesting that the hypersensitive response may play a role in the RSRE phenotype in this mutant. In addition, chemical inhibition studies suggest that the MAPK network is required for the rapid peak of the RSRE response, distinguishing the impact of chronic (mekk1-5) from transient (chemical inhibition) loss of MAPK signaling. Collectively, these results reveal underlying regulatory components of the plant GSR and further define their distinct roles in the regulation of this key biological process.

To cope with persistent environmental stresses, plants have evolved intricate biochemical and physiological responses, adapting to a great variety of stresses over a wide range of time scales. Over time, the effects of these stresses are quite varied. However, the immediate cellular perturbations caused by diverse stresses, such as disruption of plant cell walls and membranes, are often shared (Kültz, 2005). Accordingly, plants like bacteria (Bacillus subtilis; Hecker et al., 2007) and yeast (Saccharomyces cerevisiae; Gasch et al., 2000) have both a variety of well-studied stress-specific responses and a rapid and transient general stress response (GSR) that is less well understood. The GSR modulates the transcription of a suite of stress-responsive genes, mediated in part by the rapid stress response element (RSRE). The RSRE responds to a range of biotic and abiotic stresses in a rapid and transient fashion, similar to the stress response element (AGGGG) mediating the GSR in yeast (Walley et al., 2007).

The in vivo functionality of the RSRE was investigated using 4xRSRE::LUCIFERASE (4xRSRE::LUC) transgenic Arabidopsis (Arabidopsis thaliana) reporter plants (Walley et al., 2007). Wound-induced 4xRSRE::LUC activity is rapid (5 min) and transient (reaching a peak at 90–120 min and returning to basal levels within 6–8 h), coinciding with the wound-induced transcriptional pattern of GSR genes with RSRE in their promoter (Walley et al., 2007; Walley and Dehesh, 2010). Furthermore, analysis of genes that are induced following a wide array of stresses, including microbe-associated molecular pattern (MAMP) recognition, osmotic stress, and UV-B light,
revealed strong RSRE overrepresentation in early stress-responsive genes, lending strong support to the general, rapid, and transient nature of RSRE-associated gene transcription (Walley et al., 2007; Zou et al., 2011; Benn et al., 2014). 4xRSRE:Luc plants, as a proven genetic tool, provide a platform to dissect the upstream transcription events regulating the GSR.

Among known regulators of the RSRE are the CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR (CAMTA) family of transcription factors (Bouché et al., 2002; Yang and Poovaiah, 2002; Benn et al., 2014). This family has been shown to bind to an RSRE-like DNA sequence in vitro (Yang and Poovaiah, 2002). Moreover, recent genetic approaches and transient expression-based assays have shown that CAMTA transcription factors are required for robust 4xRSRE:Luc activity, with CAMTA3 playing the predominant role of the examined family members (CAMTA1–CAMTA4; Benn et al., 2014). However, the specific nature of RSRE transcriptional regulation by CAMTAs has remained an enigma; these transcription factors are constitutionally transcribed and present in the nucleus, but strong RSRE-driven gene transcription is observed only in response to stress. One explanation for this inconsistency could be that rapid calcium ion fluxes, typical of many stresses, lead to CAMTA activation through calmodulin, despite that fact that CAMTA transcription factors do not appear to require calmodulin for either entry into the nucleus or DNA binding (Yang and Poovaiah, 2002). Calmodulin binding could lead to CAMTAs interacting with other transcription factors or may alter the inherent transcriptional activity of CAMTAs, alone or in conjunction with other posttranslational modifications. An E3 ubiquitin ligase involved in CAMTA3 degradation has recently been identified, but no activators of CAMTA have yet been reported (Zhang et al., 2014).

Reported rapid stress response signals that may directly or indirectly activate CAMTA include secondary messengers, such as the aforementioned Ca²⁺, changes in redox or pH homeostasis, or posttranslational modifications of signaling proteins (Padmanabhan and Dinesh-Kumar, 2010; Walley and Dehesh, 2010; Segonzac et al., 2011; Xiao et al., 2013). Alternatively, stress-induced GSR transduction pathways may result in remodeling of the chromatin structure and, hence, accessibility of RSRE. Regardless, to accommodate the rapidity of the response, there must be preexisting GSR regulators in the cell. A likely GSR regulatory candidate, known to be involved in multiple rapid stress signaling networks across kingdoms, is MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) signaling cascades, which consist of at least three levels of activating phosphorylation (MAPKKK, MAPKK, and MAPK; Ichimura et al., 1998; Zhang and Klessig, 2001; Nakagami et al., 2005; Andreason and Ellis, 2010; Rodriguez et al., 2010; Šamajová et al., 2013). Within MAPK signaling cascades, of particular note is MEKK1, a MAPKKKK implicated in myriad stress responses through multiple downstream signaling partners (Asai et al., 2002; Nakagami et al., 2005; Hadiarto et al., 2006). For example, in response to wounding, MEKK1 is necessary for MAPK4 activation through MEK1 and MEK2 (Ichimura et al., 2006). MEKK1 is also involved in, but not necessary for, MAPK3 and MAPK6 activation in response to elicitors/pathogens, through MEK4 and MEK5 (Asai et al., 2002; Suarez-Rodriguez et al., 2007).

Here, we undertook two complementary approaches of forward and chemical genetic screening using 4xRSRE:LUC plants to gain insight into regulation of the RSRE-mediated GSR. The results of this combinatorial strategy highlight the importance of MAPK cascades in controlling both the peak time and the amplitude of RSRE activity and further confirm the role of CAMTA3 as a regulator of amplitude but not the timing of RSRE peak activity in the GSR. These findings expand our repertoire of GSR regulators and demonstrate the utility of the 4xRSRE:LUC system for identifying functional modules that control plant adaptive response pathways.

**RESULTS**

A Forward Genetic Screen Identifies CAMTA3 and MEKK1 as Regulators of RSRE

To identify components of stress signaling leading to RSRE-driven gene transcription, we conducted a forward genetic screen using a population of ethyl methanesulfonate (EMS)-mutagenized 4xRSRE:LUC Arabidopsis. Seedlings were screened for misregulation of stress-induced LUC activity, using wounding as a model stress that is easily applied and immediately perceived (Walley et al., 2007). We identified two distinct groups of mutants, one with CONSTITUTIVE ACTIVITY OF RSRE (CAR; seven independent lines identified) and the other with REDUCED ACTIVITY OF RSRE (RAR; four independent lines identified), in response to wounding.

Among mutants with reduced RSRE activity, RAR71 shows approximately 70% diminished basal and wound-induced LUC activity relative to that of 4xRSRE:LUC parent plants, while the time of peak activity and the duration of RSRE induction remain unchanged (Fig. 1, A and B). Map-based cloning placed the RAR71 causative mutation near the top of chromosome 2, between markers C2-2 (3.52 Mb) and C2-3 (6.4 Mb; Jander et al., 2002). The presence of CAMTA3, a known activator of RSRE activity (Benn et al., 2014), within this region prompted us to sequence this gene in the RAR71 background, revealing a single C-to-T transition resulting in a premature stop codon in CAMTA3 (Supplemental Fig. S1), confirming that the RAR71 phenotype results from the defect in CAMTA3, herein designated camta3-4. The success of the forward genetic screen in isolating this known activator of RSRE verified the suitability of our screening approach.

Of the constitutive activation mutants, CAR98 showed reduced size and strong (approximately 4-fold above parent) basal LUC activity (Fig. 2A). It should be noted that constitutive activation of RSRE in CAR98 is not the
sheer result of reduced size in this line, as several mutant plants with reduced size did not constitutively express 4xRSRE: LUC and other CAR mutant lines were not reduced in size. This higher LUC basal activity was not extended to a stronger wound-induced response; both the parent and the mutant genotypes showed similar peak values in response to wounding, potentially caused by signal saturation. Interestingly, however, the peak time of wound-induced 4xRSRE: LUC activity in CAR98 is shifted to an earlier time, reaching a maximum at 40 to 60 min rather than 90 to 120 in the parent (Fig. 2B; Supplemental Movie S1).

To clone CAR98, we used a mapping-by-sequencing approach in a population of backcross F2 seedlings (James et al., 2013; Henry et al., 2014). Sequence analysis revealed a C-to-T transition in the first exon of MEKK1, leading to a Pro (P)-to-Leu (L) amino acid change (Fig. 2C). This amino acid is not in the catalytic domain of MEKK1 but rather is predicted to lie in an α-helix structural domain by the Protein Homology/Analogy Recognition Engine (Kelley and Sternberg, 2009) and is predicted to disrupt protein function by the Protein Variation Effect Analyzer (score of −5.682; Choi, 2012; Choi et al., 2012). For further confirmation, CAR98 was crossed to the null mekk1-1 transfer DNA insertion line (SALK_052557; maintained as a heterozygote), which failed to complement this mutation (Supplemental Fig. S1). Therefore, CAR98 is renamed mekk1-5.

The pattern of 4xRSRE: LUC activity in mekk1-5 implicates MEKK1, and by association downstream MAPKK and MAPK, in the regulation of both peak time and basal level of RSRE activity.

**mekk1-5 Is a Partial Loss-of-Function Mutant in MEKK1**

The distinct phenotypes of mekk1-5 relative to mekk1-1 prompted us to examine these mutants more closely. Our studies corroborate the previous findings showing that mekk1-1 has a growth defect, overaccumulates hydrogen peroxide (H2O2) and other reactive oxygen species (ROS), and displays spontaneous programmed cell death (PCD; Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007). For each of these features, mekk1-5 displays intermediary phenotypes.
between those of the parent line and the mekk1-1 mutant (Fig. 3, A–C). For example, at 14 to 18 d, mekk1-5 is smaller than parent plants, a difference that becomes more severe with age, while remaining much larger than mekk1-1 seedlings throughout development (Fig. 3A; Supplemental Fig. S2). Moreover, mekk1-5 accumulates more \( \text{H}_2\text{O}_2 \) than parent plants, although less than mekk1-1, as visualized using 3,3′-diaminobenzidine (DAB; Fig. 3B). This accumulation of ROS may account for the RSRE phenotype of mekk1-5, as evidenced by the ability of ROS, in the form of singlet oxygen induced by methyl viologen, to activate RSRE (Supplemental Fig. S3). Finally, mekk1-5 seedlings retain slightly more of the vital stain Trypan Blue than parent seedlings, but again less than mekk1-1 (Fig. 3C).

The reduced size and enhanced ROS and PCD in mekk1-1 are hypothesized to be caused by a constitutive hypersensitive response (HR) associated with the accumulation of high levels of the stress hormone salicylic acid (SA; Ichimura et al., 2006; Suarez-Rodriguez et al., 2007). Thus, we quantified SA levels in the three genotypes to test the possible association of mekk1-5 phenotypes with a constitutive HR. mekk1-5 accumulated significantly more SA than parent plants but significantly less than mekk1-1 (Fig. 4). The intermediately elevated levels of SA, in a pattern similar to those of ROS, PCD, and plant size, strongly suggest a constitutive HR in this mutant and suggest that mekk1-5 is a partial loss-of-function mutant in MEKK1.

The mekk1-5 mutation is within the first 166 amino acids of MEKK1, a region that when deleted (MEKK1Δ166) results in constitutive kinase activity in a protoplast system (Hadiarto et al., 2006) and has been implicated in the control of substrate specificity of this kinase (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007). The proposed function of this region raised the possibility of altered regulation and substrate specificity/preference of MEKK1 in mekk1-5. To investigate this possibility, we examined the phosphorylation profiles of known downstream targets of MEKK1, namely MAPK3, MAPK4, and MAPK6, as well as MAPK11, using an antibody that recognizes phosphorylated MAPKs in a western-blot assay. The similar mobility between MAPK4 and MAPK11 did not allow the separation of these two polypeptides (Fig. 3D; Bethke et al., 2012). In this assay, all genotypes displayed faint uninduced reactive bands for phosphorylated MAPK3 and MAPK4/MAPK11 and a much stronger reactive band for MAPK6. To induce MAPK phosphorylation, we used the bacterial flagellin-derived MAMP peptide flagellin22 (flg22), which activates MAPK within 15 min (Schwessinger et al., 2011; Bethke et al., 2012; Feng et al., 2012; Kong et al., 2012). Fifteen minutes after treatment, all three phosphorylated MAPK reactive signals on the western blot became stronger, comparably across all three genotypes. Therefore, unlike MEKK1Δ166 (Hadiarto et al., 2006), enzyme regulation and substrate preference do not appear to be altered in mekk1-5.

The camta3/mekk1 Double Mutant Establishes Distinct Roles of CAMTA and MAPK in RSRE Activation

To begin to assemble the functional module(s) of the signaling events upstream of RSRE activation, we generated the camta3-4/mekk1-5 double mutant line. In the camta3-4/mekk1-5 double mutant, 4xRSRE:LUC activity is reduced similarly to camta3-4 single mutants, confirming that CAMTA3 lies downstream of MEKK1 in activating RSRE. However, like mekk1-5, the peak time of wound-induced 4xRSRE:LUC activity in the double mutant is shifted earlier by approximately 60 min. In addition, the double mutant mekk1-5/camta3-4 displays a comparable plant size to mekk1-5, and the SA levels are further increased (Fig. 5; Supplemental Fig. S2). Therefore, the elevated SA, reduced plant size, and accelerated RSRE peak are independent of CAMTA3, whereas the amplitude of the RSRE response depends on this transcription factor. This suggests two functions of MEKK1, the first suppressing SA accumulation and delaying RSRE activation in a CAMTA3-independent manner and the second suppressing RSRE activation through CAMTA3.

Chemical Genetic Screening Supports MAPK’s Role in the Regulation of RSRE

To complement the forward genetic screen, we performed a chemical genetic screen using 4xRSRE:LUC plants. Specifically, we searched for chemical inducers of 4xRSRE:LUC activity, particularly those with altered peak time. In this screen, we examined 360 chemicals that have been shown to affect pollen tube growth, 123 of which affect endomembrane trafficking (Drakakaki
et al., 2011). One chemical, previously shown to affect PIN localization (Drakakaki et al., 2011), caused a small but significant induction of RSRE, with an approximately 0.5-h delayed peak, and is herein designated RSRE-MODULATING1 (RM1; Fig. 6A). To determine the specificity of RM1 in the induction of RSRE activation, and to test whether the delayed peak of RSRE activity in response to RM1 treatment is an inherent property of this compound or due to the time of absorption, we examined the effects of other chemical inhibitors. Specifically, we assayed a collection of 35 similar chemicals known to inhibit a variety of classes of plant kinases and phosphatases (Supplemental Table S1; Franz et al., 2013). The compound with the most pronounced effect, PD98059 (here designated RM2), caused an approximately 1.5-h delay in the peak of flg22-induced 4xRSRE:LUC activity (Fig. 6B). A similar, although less pronounced, delay (0.5 h) was observed in wound-induced 4xRSRE:LUC peak activity (Supplemental Fig. S4). RM2 is known to be a potent inhibitor of mammalian MKK1 (Cohen, 1997) and is an established inhibitor of MAPK signaling in plants (Romeis et al., 1999; Zhang et al., 2006; Chang and Nick, 2012). In contrast to mekk1-5, the delayed RSRE peak in RM2-treated seedlings suggests that MAPK signaling is necessary for the rapidity of transcription in the GSR.

Surprisingly, U0126, another MAPK inhibitor commonly used in plants, with a slightly different substrate preference than RM2 (Favata et al., 1998; Asai et al., 2002; Zhang et al., 2006; Rodriguez et al., 2010), did not alter the peak time of 4xRSRE:LUC activity (Supplemental Fig. S5). These two inhibitors may affect distinct plant response pathways because of their differing substrate preferences.

DISCUSSION

Plants have evolved an exquisite array of mechanisms to adapt to environmental stresses. This adaptation is coupled to a combination of general followed by specific stress responses. The GSR is the initial rapid signaling cascade preceding the induction of consequent stress-specific signaling activities. Here, we have used a combination of complementary screening approaches to identify signal transduction components of the GSR using a functional cis-element associated with the plant GSR, the RSRE. We have both confirmed the importance of CAMTA3 and identified a function for MEKK1 as well as established these proteins’ distinct roles in the control of amplitude and timing of peak activity in this GSR-associated transcriptional response.

Here, we affirmed CAMTA3 as a key inducer of RSRE, in agreement with previous work (Benn et al., 2014), and further established the specific role of CAMTA3 in regulation of the amplitude but not the peak time of the RSRE response.

In addition, we identified mekk1-5, a partial loss-of-function allele in MEKK1 that affects plant size, SA levels, PCD, and ROS less severely under our growth conditions than reported for other mekk1 mutants (Ichimura et al., 2006; Nakagami et al., 2006; Suarez-Rodriguez et al., 2007; Gao et al., 2008). Unlike previously reported mutants, the P92L substitution in mekk1-5 does not disrupt the catalytic domain of the protein but rather a region within the first 166 amino acids...
suggested to play a role in the enzyme’s regulation and substrate recognition (Hadiarto et al., 2006; Suarez-Rodriguez et al., 2007). Under our experimental conditions, mekk1-5 did not display constitutively phosphorylated MAPK3, MAPK4, and MAPK6 as reported for MEKK1D166 (Fig. 3; Hadiarto et al., 2006). On the contrary, the MAPK phosphorylation pattern of both mekk1-1 and mekk1-5 resembles that of the parent control plants. Because of the similar mobility of MAPK4 and MAPK11, we could not specifically determine MAPK4 phosphorylation (Bethke et al., 2012). However, the essential role of MEKK1 in MAPK4 phosphorylation (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007) led us to surmise that the reactive band in mekk1 mutant lines belongs to phospho-MAPK11, activated in part through some kinase(s) other than MEKK1 (Ichimura et al., 2006).

Beyond MAPK phosphorylation, MEKK1 has other known roles that could be disrupted in mekk1-5. For example, MEKK1 has been shown to both bind to the promoter of WRKY53 and phosphorylate this protein (Miao et al., 2007). Moreover, the kinase activity of MEKK1 seems to be dispensable for its function (Suarez-Rodriguez et al., 2007; Pitzschke et al., 2009), indicating that MEKK1 function is not solely dependent on the phosphorylation of downstream MAPK. Structure-function analyses of MEKK1, for which mekk1-5 provides a valuable experimental tool, may offer insight into these varied roles.

The early-shifted wound-induced 4xRSRE:LUC peak in mekk1-5 suggests that MEKK1, directly or indirectly, acts to delay signaling upstream of RSRE responses. An approximately 20-min peak shift in peak was also observed in the flg22 induction of mekk1-5 (Supplemental Fig. S4), but this shift was not statistically significant, suggesting that the presence and degree of the shift may depend on the nature of the stress. In contrast to the early peak time of RSRE in mekk1-5, chemical inhibition of MAPK signaling by RM2 (PD98059), an established inhibitor of MAPKK activity in plants, causes a delayed peak (Fig. 6; Supplemental Fig. S4; Romeis et al., 1999; Zhang et al., 2006; Rodriguez et al., 2010; Chang and Nick, 2012). This difference between the impacts of chemical inhibition of MAPK signaling and mutation in a MAPKK may be caused by two alternative scenarios. The first is that RM2 acts on substrate MAPKK other than those downstream of MEKK1. For example, U0126, a MAPKK inhibitor with slightly different substrate preference than RM2 (Favata et al., 1998), did not alter the time of RSRE peak activation (Supplemental Fig. S4), supporting the idea of MAPK pathway specificity in RSRE activation. On the other hand, MAPK signaling is an established positive regulator of rapid stress responses across multiple kingdoms (Posas et al., 2000; Kyriakis and Avruch, 2001; Proft and Struhl, 2004; Roux and Blenis, 2004; Kyriakis and Avruch, 2012), supporting the hypothesis that the greater basal and more rapid RSRE peak in mekk1-5 is not caused by downstream MAPK signaling but rather by some other role(s) of this kinase.

An interaction with plant immune responses, particularly the HR, may explain the mekk1-5 phenotype.

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MAPK proteins are important in MAMP recognition signaling and have been shown to be targets of bacterial effectors, making them promising candidates for immune monitoring (Zhang et al., 2007, 2012). For instance, the activity of MAPK4 (downstream of MEKK1) has been shown to be necessary for suppressing a constitutive immune response mediated by SUMM1 and SUMM2 (Kong et al., 2012; Zhang et al., 2012). The reduced size and constitutive immune responses in the mekk1 mutants, therefore, may be due to the disruption of MAPK4 phosphorylation (Gao et al., 2008) or immune monitoring at any other step of the signaling cascade via as-yet-unknown resistant proteins (Fig. 7). Like mekk1-1, mekk1-5 displays PCD and enhanced SA and ROS, hallmarks of the HR, supporting a link between MEKK1 function and the immune response.

Although a mutation in MEKK1 may lead to a constitutive HR, how that interacts with 4xRSRE:LUC regulation is unclear. The mekk1-5/camta3-4 double mutant has greatly reduced 4xRSRE:LUC activity but still displays reduced plant size and enhanced SA, indicating that the mekk1-5-mediated HR is not dependent on RSRE activation. Conversely, it may be that HR-associated elevated H2O2 in mekk1-5 leads to the elevated basal RSRE levels, in line with the ability of singlelet oxygen induced by methyl viologen to induce RSRE activity (Supplemental Fig. S3). A constitutive HR may even lead to the shift in RSRE activity peak time, analogous to systemic acquired resistance, the more rapid pathogen response following distal pathogen attack (Conrath, 2006). Similarly, an accelerated physiological response to salt treatment is observed in yeast cells previously salt stressed (Mettetal et al., 2008). In the model presented (Fig. 7), we propose that constitutive HR in mekk1-5 leads to both the observed elevated basal RSRE activity and the more rapid peak in response to wounding, albeit through independent mediators.

CONCLUSION

Here, we have confirmed CAMTA3 as an activator in the RSRE-driven GSR as well as demonstrated a role for MAPK signaling in controlling both the level and peak time of RSRE-driven transcription, either directly or via an association with immune signaling. Additionally, our mekk1 allele provides a novel tool for investigating the role and regulation of this protein and its downstream partners in the GSR. Finally, through a combination of forward genetics and chemical genetics, we were able to decipher differences between the effect of chronic and transient loss of MAPK signaling. Together, these results fine-tune our understanding of the regulation of timing and amplitude in the plant GSR.

MATERIALS AND METHODS

Plant Growth and LUC Imaging

Arabidopsis (Arabidopsis thaliana) growth and LUC imaging were performed as described previously (Walley et al., 2007; Benn et al., 2014). Briefly, surface-sterilized Arabidopsis seeds were plated on one quarter-strength Murashige and Skoog basal salt medium (Sigma) and grown under a 16-h-light/8-h-dark cycle at 22°C. For LUC imaging, seedlings were sprayed with 1 mM luciferin (Promega) and 0.1% (v/v) Tween 20 at 16 to 24 h prior to imaging and moved from growth chamber to bench at 5 AM on the day of imaging, 4 h prior to treatment and imaging starting at 12 PM. One 5-min exposure was taken every 10 min using an Andor DCU345-BV CCD camera (Andor Technology) and analyzed using Andor Solis image-analysis software (version 15; Andor Technology; Walley et al., 2007; Savchenko et al., 2010). Quantification of LUC activity was performed as described previously (Benn et al., 2014). Briefly, 4 × 4-pixel regions of interest were placed on either a single wounded leaf or on both cotyledons of chemically treated seedlings. Means and SE values for each genotype/treatment combination were extracted from the raw Andor Solis data files using custom Perl scripts (http://www-phb.ucdavis.edu/labs/dehesh/dehesh-lab-code.html). Peak time was extracted using the Excel Max function, and ANOVA tests were performed on time of peak or log-transformed LUC data in R using the AOV function. Means separation was performed using Tukey’s honest significant difference test, via the HSD.test function from the agricolae package in R.

SALK lines were obtained from the Arabidopsis Biological Resource Center and genotyped using the primers listed in Supplemental Table S2 (Rein and Skaletsky, 1998; Neff et al., 2002; Alonso et al., 2003).

Forward Genetic Screening

Fourteen-day-old M2 EMS-mutagenized 4xRSRE:LUC seedlings, plated approximately 1.3 cm apart, were wounded by hemostat and screened for aberrant LUC activity. More than 20,000 M2 seedlings from more than 150 pools of M1 plants harvested together were assayed. Putative mutants with altered LUC expression, indicating disrupted RSRE activation, were removed to fresh plates, grown for an additional 5 d, and imaged again. Seedlings that consistently displayed reduced wound-induced activation of RSRE (designated RAR; four independent lines identified) or constitutively active RSRE (designated CAR; seven independent lines identified) were selected for further analyses. Each line’s phenotype was confirmed in the M3 before crossing to either Landsberg erecta or the parent for cloning.

Map-Based Identification of Causal Mutations

We used a combination of low-resolution recombinant mapping of F2 seedlings from a Landsberg erecta cross and cloning by sequencing using pooled F2 seedlings (100–150 seedlings) from a cross with the parent line (James et al., 2013). Sequencing data were analyzed using the Genotyping and Mutation Discovery pipeline by the Comai laboratory (Henry et al., 2014).

Chemical Genetic Screening

Seeds were plated on 100 μL of medium one per well on a 96-well plate. One-week-old seedlings were treated with one 10-μL drop of approximately 20 μM solution of individual chemicals dissolved in 0.5% (v/v) dimethyl sulfoxide (DMSO) applied to each well. At least four replicate seedlings were assayed for each chemical; confirmed chemical hits were retested with three experiments each consisting of at least 18 seedlings. In screening kinase and phosphatase inhibitors, seedlings were treated with one 10-μL drop of 40 μM solution in 0.5% (v/v) DMSO in combination with either wounding or 1 μM g22.

Tissue Staining

DAB staining was performed according to a slightly modified procedure reported previously (Nakagami et al., 2006). Briefly, 18-d-old seedlings were cut at the hypocotyl-root junction and vacuum infiltrated with DAB staining solution (10 mM Na2HPO4, 0.05% (v/v) Tween 20, and 1 mg mL−1 DAB; made fresh) for 2 min. Plates were covered and incubated 2 h with gentle shaking, followed by a 95% (v/v) ethanol wash overnight. The ethanol waschanged for a final 4-h wash before imaging. Representative seedlings are shown in the figures; at least four seedlings per genotype were assayed in each of two separate experiments. Trypan Blue staining was performed by a similar procedure to DAB staining, except that lactophenol Trypan Blue solution was used without vacuum infiltration.
**MAPK Phosphorylation Assay**

Activated MAPKs were detected as described previously with minor alterations (Feng et al., 2012). Briefly, 18-d-old seedlings were sprayed with either 1 μM flg22 (Genscript) or water containing 0.02% (v/v) Silwet L-77, incubated 15 min, and flash frozen in liquid nitrogen. Proteins were then extracted in phosphatase-inhibiting buffer and their concentrations determined using the Bradford reagent to normalize protein loading (Bradford, 1976). Approximately 50 μg of protein was loaded per well on a 10% (v/v) SDS gel. Following transfer, membranes were stained with Ponceau S for a loading control before western analysis supplemented with MPK11 by the anti-p44/42 MAPK; Cell Signaling catalogue no. 4307).

**SA Extraction and Analysis**

SA was extracted and analyzed as described previously (Engelberth and Engelberth, 2009; Savchenko et al., 2010).

**Supplemental Data**

The following materials are available in the online version of this article.

- **Supplemental Figure S1.** Complementation tests.
- **Supplemental Figure S2.** mekk1/camta3 mutants resemble mekk1-5 mutants in size and SA content.
- **Supplemental Figure S3.** Dose-dependent induction of the RSRE by methyl viologen.
- **Supplemental Figure S4.** mekk1-5 treated with flg22 and wounded parent plants treated with RM2 (PD98059).
- **Supplemental Figure S5.** The MAPK inhibitor U0126 does not affect the peak time of RSRE activation.
- **Supplemental Table S1.** Kinase and phosphatase modulators.
- **Supplemental Table S2.** Primer sequences used for genotyping.
- **Supplemental Movie S1.** Time-lapse movie of control, mekk1-5, cavata3-4, and mekk1-5/cavata3-4 wound-induced LUC activity.

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