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A key general stress response motif is regulated non-uniformly by CAMTA transcription factors

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SUMMARY

Plants cope with environmental challenges by rapidly triggering and synchronizing mechanisms governing stress-specific and general stress response (GSR) networks. The GSR acts rapidly and transiently in response to various stresses, but the underpinning mechanisms have remained elusive. To define GSR regulatory components we have exploited the *Rapid Stress Response Element* (*RSRE*), a previously established functional GSR motif, using Arabidopsis plants expressing a *4xRSRE::Luciferase* (*RSRE::LUC*) reporter. Initially, we searched public microarray datasets and found an enrichment of *RSRE* in promoter sequences of stress genes. Next, we treated *RSRE::LUC* plants with wounding and a range of rapidly stress-inducible hormones and detected a robust LUC activity solely in response to wounding. Application of two Ca^{2+} burst inducers, flagellin22 (flg22) and oligogalacturonic acid, activated *RSRE* strongly and systemically, while the Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) significantly reduced wound induction of *RSRE::LUC.* In line with the signaling function of Ca^{2+} in transduction events leading to activation of *RSRE*, we examined the role of CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATORs (CAMTAs) in *RSRE* induction. Transient expression assays displayed CAMTA3 induction of *RSRE* and not that of the mutated element *mRSRE.* Treatment of selected *camta* mutant lines integrated into *RSRE::LUC* parent plant, with wounding, flg22, and freezing, established a differential function of these CAMTAs in potentiating the activity of *RSRE.* Wound response studies using *camta* double mutants revealed a cooperative function of CAMTAs2 and 4 with CAMTA 3 in the *RSRE* regulation. These studies provide insights into governing components of transduction events and reveal transcriptional modules that tune the expression of a key GSR motif.

Keywords: *Rapid Stress Response Element*, general stress response, CALCIUM/CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR, secondary messenger Ca^{2+}, stress hormones.

INTRODUCTION

To counteract environmental perturbations, plants launch controlled and synchronized suites of signaling pathways that perceive and transduce extracellular signals into intracellular responses to help cells to adjust their physiological and metabolic status accordingly. Central to these adaptive responses is transcriptional reprogramming, initially comprised of a core set of general stress-responsive genes followed by stress-specific genes whose expression is in tune with the physiological needs dictated by the nature of stress. The general stress response (GSR), also referred to as the core stress response, is an evolutionarily conserved process through which diverse organisms rapidly respond to the initial effects of external perturbations (Kultz, 2005; Lopez-Maury *et al.*, 2008). However, while this fundamental response has been well studied in bacteria and yeast, little information is known about the GSR in plants.

To identify components of the GSR in plants, we have previously used a microarray-based approach to assess rapid transcriptional alterations (5 min) in response to mechanical wounding in *Arabidopsis thaliana* (*A. thaliana*). Promoter analysis of genes induced in this rapid wound response (RWR) led to the identification of an over-represented *cis*-regulatory element, which was designated the Rapid Stress Response Element (*RSRE;* CGCGTT) (Walley *et al.*, 2007). Additional studies using the *4xRSRE::LUC* line, as compared with the *mRSRE::LUC* plants expressing the mutated *RSRE* containing three nucleotide substitutions in the core sequence, confirmed the *in vivo* functionality of *RSRE*, and further established *RSRE* as a GSR element that is rapidly and transiently activated in response to diverse stresses, including abiotic (mechanical wounding and cold) and biotic (herbivory by *Pieris rapae*,

and infection by *Botrytis cinerea*) challenges (Walley *et al.*, 2007). The rapidity of RSRE activation suggests the presence of pre-existing regulatory components involved in transcriptional activation of this motif, but the nature and the extent to which this governing machinery interfaces with known stress signaling cascades is yet unknown.

Previous efforts have identified several candidate components of rapid stress-induced signaling cascades, including hormones, calcium bursts, reactive oxygen species (ROS) bursts, and kinase cascades (Fujita *et al.*, 2006). Elicitors that trigger rapid stress signaling cascades are critical tools for dissecting the core general mechanism(s) involved in perception of and early signal transduction of plant responses to environmental challenges. Indeed, previous reports have alluded to a potential transduction pathway initiated by oligogalacturonic acid (OGA), a damage-associated molecular pattern that induces *RSRE::LUC* activity (Walley *et al.*, 2007), as well as calcium, reactive oxygen species, and nitric oxide (Hahn *et al.*, 1981; Orozco-Cardenas and Ryan, 1999; Rasul *et al.*, 2012). This finding therefore leads to the question of whether OGA is unique in this function or if a similar transduction pathway as measured by *RSRE::LUC* activity can be initiated by other biotic elicitors such as flagellin22 (flg22), which is an established inducer of calcium burst, reactive oxygen species burst, kinase cascades, and ethylene production (Boller and Felix, 2009). While these elicitors induce a variety of downstream signaling processes, previous work demonstrating calcium regulation of a synthetic promoter containing the core *RSRE* motif *vCGCGb* (Whalley *et al.*, 2011), elevates the importance of exploring the role of this rapid and transient signaling messenger in the transduction of event(s) leading to *in vivo* activation of the GSR.

The *ACGCGT*-based synthetic promoter overlaps fully with the consensus sequence of the core (*vCGCGb*) binding motif of the CALCIUM/CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATOR (CAMTA) family of transcription factors, also referred to as signal responsive (SR) factors (Bouche *et al.*, 2002; Yang and Poovaiah, 2002). In *A. thaliana* there are six *CAMTA*s displaying differential and rapid (<15 min) expression responses to various stresses (e.g. cold, salinity) and hormones (e.g. ABA, jasmonic acid) (Yang and Poovaiah, 2002). Most studies on this family of TFs have been conducted on CAMTA3, an established repressor of salicylic acid (SA)-mediated plant defenses, shown to bind to the core *vCGCGb* motif of a number of genes including *non-race-specific disease resistance1 (NDR1)*, *enhanced disease susceptibility1 (EDS1)*, *C-repeat/dehydration responsive element binding factor 2* (*CBF2*, also known as *DREB1c*), as well as to the *ethylene insensitive3* (*EIN3*) promoter region *in vivo* (Doherty *et al.*, 2009; Du *et al.*, 2009; Nie *et al.*, 2012). The CAMTA3 activation of *CBF2* in response to cold together with impaired freezing tolerance of double *camta1*/3 establishes a role for CAMTAs in regulation of plant responses to cold (Doherty *et al.*, 2009). This role is expanded to CAMTA2 since it functions with CAMTA1 in concert with CAMTA3 at 4°C to induce peak transcript levels of *CBF1*, *2* and *3* (Kim *et al.*, 2013). The increased sensitivity of *camta1* to auxin is suggestive of an additional role for this TF in suppressing stress-induced plant responses to auxin (Galon *et al.*, 2010a). Moreover, several studies have established the overrepresentation of the *vCGCGb* element in the promoters of misregulated genes in *camta* mutants exposed to either drought or cold (Kim *et al.*, 2013; Pandey *et al.*, 2013). The relative importance of the element in regulating stress responses is reinforced by the detection of the element, or closely related variants thereof, in a number of global analyses of stress-responsive *cis*-regulatory elements (Zou *et al.*, 2011; Maruyama *et al.*, 2012; Korkuc *et al.*, 2014). Collectively, these findings support the notion of CAMTAs being integrators of the GSR through transcriptional modulation of early stress-induced genes.

Toward identification and subsequent integration of regulatory components of the GSR, we used publically available microarray datasets to investigate the presence of the *RSRE* motif in rapidly and transiently stress-responsive genes. The results demonstrate notable enrichment of this functional GSR motif in promoters of early stress-responsive genes. Next, we examined the ability of stress-induced phytohormones, elicitors that induce calcium burst, and CAMTA1 through four transcription factors in inducing *RSRE::LUC* activity in response to various stresses *in planta*. Our findings establish that while elicitors and calcium strongly induce *RSRE*, the phytohormones are ineffective in robust activation of this functional motif. Using transient expression assays, we show the sequence specificity of the *RSRE* core sequence in CAMTA3-mediated transcriptional activation of this element. We further demonstrate the differential, yet cooperative role of the CAMTA1–4 transcription factors in activation of this GSR transcriptional hub.

RESULTS

The *RSRE* is enriched in promoters of early stress-responsive genes

Comparative analyses of publically available microarray datasets that examine gene expression in plants at different time points after perturbations with various stresses is a useful strategy for differentiating between specific and GSR genes. To test the nature of stress and temporal dependency of induction of native promoters containing the *RSRE*, we first identified genes that were differentially expressed at six time points following wounding, UVB, or osmotic stress (Kilian *et al.*, 2007). These analyses revealed differential and temporal variations in genes induced by these stresses. Specifically, the highest percentage of

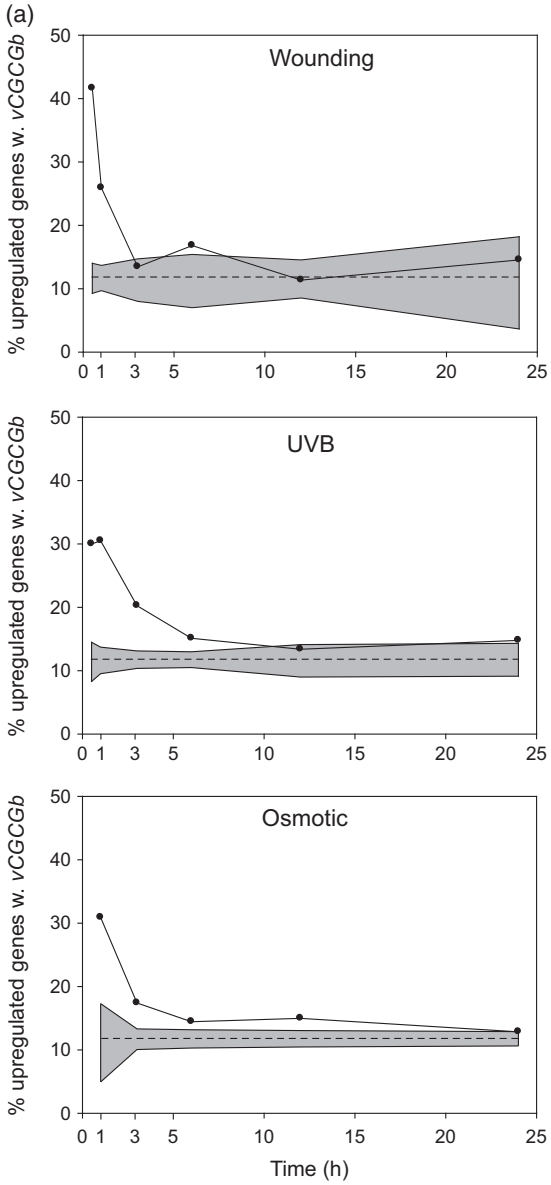
RSRE containing genes was detected at 30, 30–60, and 60 min post wounding, UVB and osmotic stresses, respectively. These temporal differences, however, may be due to the rapidity of perception rather than differential time of gene induction in response to these stresses (Figure 1a,b).

Further analysis of promoter sequences of the induced genes revealed significant enrichment of the *vCGCGb* motif in genes up-regulated at early time points (approximately 30–60 min), which was reduced and subsequently absent at later time points (Figure 1a). The apparent rapid and transient expression pattern of genes containing *vCGCGb* elements mimic the *in vivo* profile of the *RSRE::LUC* activity previously reported (Walley *et al.*, 2007). Subsequently, we expanded the analyses to microarray profiles of genes responsive to flg22, OGA, cold, heat, and gamma radiation (Denoux *et al.*, 2008; Yoshiyama *et al.*, 2009) (Figure 1b). These analyses reveal that, with the exception of heat treatment, *vCGCGb* containing genes constitute a statistically significant percentage (18–30%) of rapidly stress-induced genes (Figure 1b). Interestingly, this enrichment is limited to induced genes as the *vCGCGb* motif is not over-represented in genes down-regulated in the tissues exposed to wounding, flg22, OGA, cold, osmotic stress, UVB, or gamma radiation (Table S1). Collectively, these data lend further support to our earlier finding defining *RSRE* as a functional GSR element, present primarily in rapidly stress-induced genes (Walley *et al.*, 2007; Walley and Dehesh, 2010).

The *RSRE* is not robustly activated by stress-induced hormones

To counteract environmental stresses, plants utilize a number of different mechanisms including phytohormone-based pathways to signal for a stress response, which may ultimately lead to tolerance (Bostock, 2005; Chehab *et al.*, 2007). To test the role of these phytohormones in the GSR, we employed *RSRE::LUC* expressing plants, herein designated as parent (P), to examine the induction of this GSR motif by selected hormones known to be stress-inducible such as SA, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), methyl jasmonate (MeJA), and hexenyl

(a)



(b)

Over-representation of *vCGCGb* in shoot rapid stress-induced genes

Stress	% upreg w. *vCGCGb*	p-value
Wounding	25.89	0.00
Flg22	23.35	0.00
OGA	23.45	0.00
Cold	23.15	8.6E-04
Heat	13.95	0.12
Osmotic	30.86	5.4E-06
UVB	30.48	0.00
Gamma radiation	18.24	6.7E-05

Figure 1. The *RSRE* is enriched in promoters of early stress-responsive genes.
(a) Graphs display the percentage of up-regulated genes containing a *vCGCGb* element within 500 bp of the translational start site, at various time points post stress. The percentage of promoters containing the motif in the whole genome is indicated by the dashed line and the gray shading indicates the 95% confidence interval.
(b) Percentage of stress-induced genes containing the *vCGCGb* motif in their promoters. Gene expression was measured 1 h post all stresses, with the exception of gamma radiation, where measurements were at 90 min post treatment. *P*-values were calculated using the cumulative hypergeometric distribution with correction for multiple testing.

acetate (HA) (Saltveit and Dilley, 1978; Harris and Outlaw, 1991; Creelman *et al.*, 1992; Sharma *et al.*, 1996; Turlings *et al.*, 1998). Specifically, we quantified LUC activity 90 min post treatment of P plants with various concentrations of these phytohormones. These data indicate that while SA is ineffective, the other hormones induce *RSRE* activity up to 1.8-fold above the control levels (Figure 2a, b). This modest, but statistically significant induction of *RSRE*, however, is far below the levels found in response to wounding, suggesting that, within the hierarchy of the stress signaling network, stress-induced hormones are downstream of the early response genes.

RSRE::LUC is systematically induced by 13mer OGAs and flg22

The previous work demonstrated that application of OGAs to wound sites results in induction of *RSRE::LUC* activity in tissues that were not directly wounded (Walley *et al.*, 2007). The established importance of degree of polymerization (DP) of OGAs in inducing canonical OGA responses (Hahn *et al.*, 1981) led us to question whether the systemic activation of *RSRE::LUC* was caused by OGAs of certain DP (DP10–15), or by any OGAs present in the rough pectin preparation. Therefore, we examined systemic *RSRE* activity in P plants 90 min post application of purified OGAs, either as pure DP13 or as a mixture of DP7–22, to the wound site. We also performed these measurements after application of non-OGA polysaccharides of varying DP, namely chito- and manno-hexaose (DP6), and malto- and laminari-heptaose (DP7). The strongest systemic induction was in response to treatment of the wound site with DP13 OGA, followed by OGAs mixture of DP7–22 (Figure 3a,b). The reduced systemic activity of the *RSRE* in response to OGA 7–22 as compared with the pure DP13 sample is most likely due to reduced relative levels of DP13 species in this preparation. Application of other polysaccharides of shorter DP(6–7) was ineffective in the systemic induction of the *RSRE* (Figure 3a,b), thus confirming the established specificity of 13mer OGAs in inducing the activation of this GSR transcriptionally functional motif.

Furthermore, we tested the effect of another known biotic elicitor flg22, in systemic induction of *RSRE* and show that, indeed this elicitor induces systemic *RSRE::LUC* expression as effectively as DP13-OGA (Figure 3c).

Collectively, these data strongly support the role of the *RSRE* as a transcriptional hub in the GSR network, as well as it is suitability for dissecting the early stress response network(s).

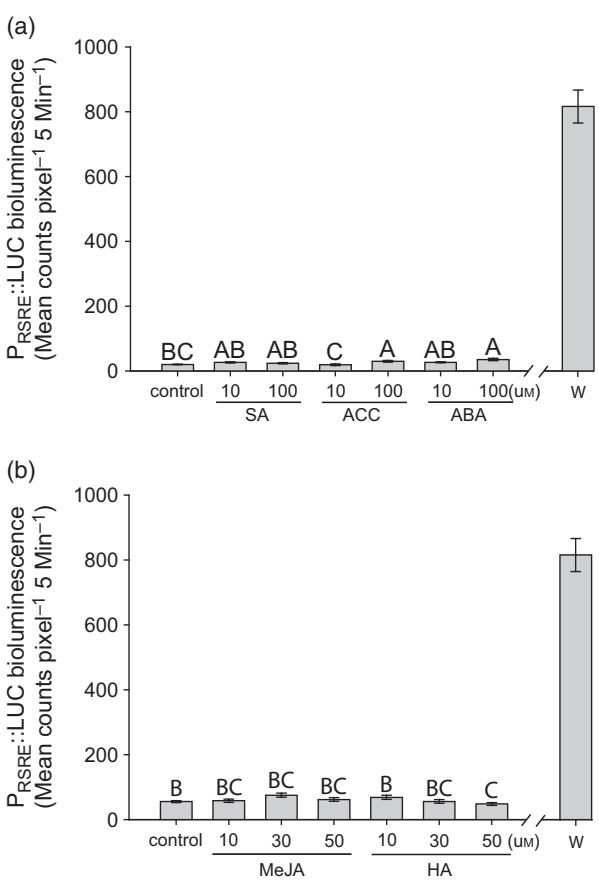


Figure 2. The *RSRE* is not robustly activated by stress-induced hormones. (a,b) *RSRE::LUC* activities were measured in the parent line before (control) or 90 min after treatment with the indicated concentrations of various hormones, or wounding. Data are means ± standard error of the mean (SEM) ($n \geq 63$) for the panel (a) and ±SEM ($n \geq 120$) for panel (b). Bars that do not share a letter represent statistically significant differences ($P \leq 0.05$).

Calcium-dependent signaling contributes to *RSRE* activity

Although *RSRE* is a functional GSR element, the role of stress transduction signals in activation of this element is unknown. Calcium is a secondary messenger implicated in initial biotic and abiotic signaling cascades, as well as those induced specifically by OGAs and flg22 treatment (Moscatiello *et al.*, 2006; Boller and Felix, 2009). To examine the role of calcium in induction of the *RSRE*, unwounded and wounded P plants were treated with ethylene glycol tetraacetic acid (EGTA), an effective chelator that limits calcium signaling in plants (Jones and Mitchell, 1989). These data show reduced (approximately 50%) LUC activity in wounded and EGTA-treated plants as compared with wounded and water-treated controls (Figure 4), thus confirming the signaling role of calcium in wound-induced expression of the *RSRE.*

CAMTA3 induces *RSRE::LUC* activity

The demonstrated functionality of calcium in the *RSRE* induction cascade led us to investigate the role of the calcium/calmodulin (CaM) binding transcription factors (CAMTAs; also referred to as *Arabidopsis thaliana signal responsive – AtSRs*) (Bouche *et al.*, 2002; Yang and Poovaiah, 2002), in transcriptional regulation of this motif. To this

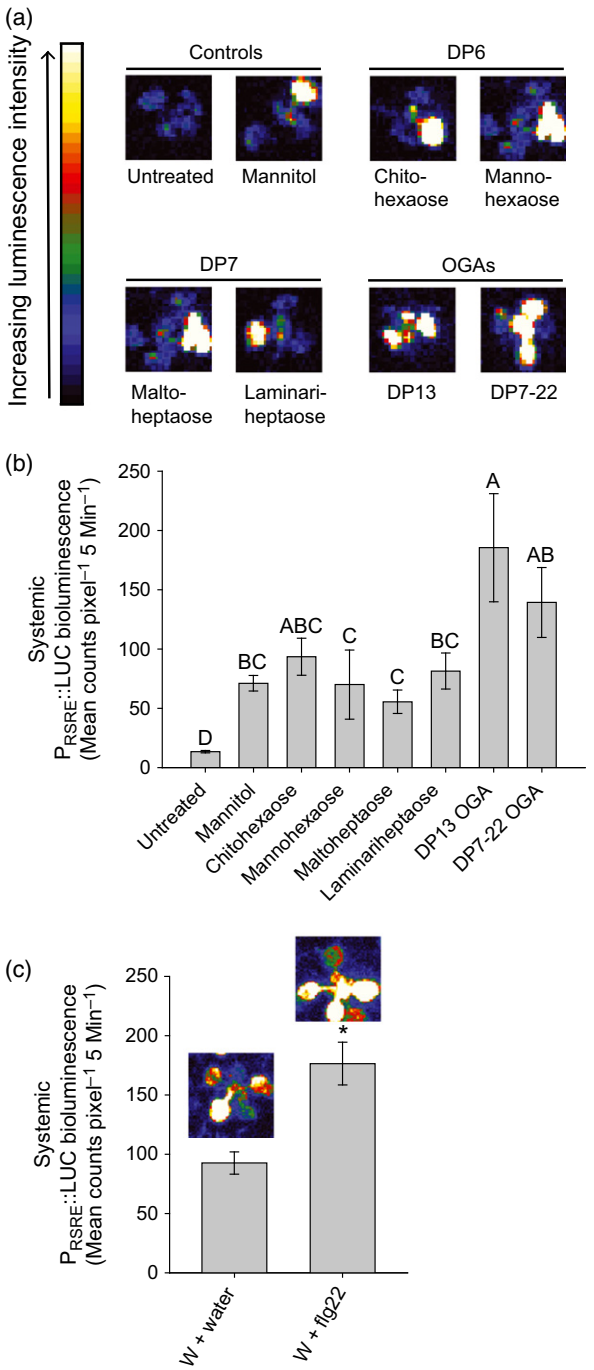


Figure 3. *RSRE::LUC* is systematically induced by 13mer OGAs and flg22.
(a) Dark field images of representative parent plants before (untreated) and 90 min after treatment of wound site with 10 μl of indicated chemical. The color-coded bar displays the intensity of LUC activity.
(b) Quantitative measurements of systemic LUC activity in untreated, and wounded plus elicitor-treated plants. Data are means ± standard error of the mean (SEM) ($n \geq 18$). Bars that do not share a letter represent statistically significant differences ($P \leq 0.05$).
(c) The histogram of systemic *RSRE::LUC* activity in parent plants 90 min after treatment of wound site with 10 μl of either water as control or 10 μM flg22. Asterisk indicates a significance determined by *T*-test ($P = 3.83E^{-07}$). Dark field image of representative plants are displayed above the corresponding histograms.

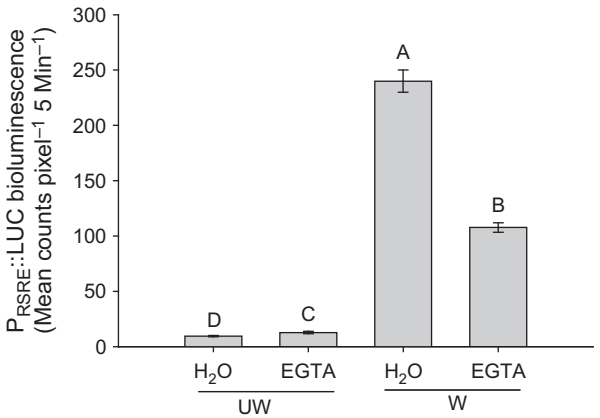


Figure 4. Calcium-dependent signaling contributes to *RSRE* activity.
Local *RSRE::LUC* activity was measured in leaves of unwounded (UW) or wounded (W) P plants 90 min post application of H_2O or the calcium chelator (EGTA). Bars that do not share a letter represent statistically significant differences ($P < 0.05$). Data are means ± standard error of the mean (SEM) ($n \geq 135$).

end, we selected CAMTA3, the most studied member of CAMTA family, and employed it in *Agrobacterium* (agro)-infiltration-based transient assays in *Nicotiana benthamiana*. Specifically, the *RSRE::LUC* construct and the known mutated dysfunctional *RSRE* (*mRSRE::LUC)* (Walley *et al.*, 2007) as the control, were infiltrated into *N. benthamiana* leaves independently, alone or together with *35S::CAMTA3.* This data clearly display strong LUC activity exclusively in leaves co-infiltrated with *RSRE::LUC* and 35S:: CAMTA3, and no activity in the assays with *mRSRE::LUC* construct (Figure 5). This finding, in agreement with our pervious report (Walley *et al.*, 2007), demonstrate that the integrity of RSRE core sequence is essential for CAMTA3-meidated transcriptional induction and by extension activation of the LUC reporter.

Local and systemic induction of *RSRE::LUC* is differentially regulated by CAMTAs

Next, we examined role of other CAMTAs in potentiating the transcriptional activation of *RSRE* in response to stress by generating *camtas 1* through *4* mutant lines containing stably integrated *RSRE::LUC* through crosses with the P line. Selection of the mutant lines was guided by the presence of T-DNA insertion in the exon sequences of the respective *CAMTA* (Figure S1).

Subsequently, we measured the local *LUC* activity in all the genotypes before and 90 min after wounding (Figure 6a,d). The low basal LUC activity in untreated tissue prohibits meaningful comparative analyses of these levels in the various genotypes. However, the data clearly display strong and differential wound-inducible levels of *RSRE* activity in these genotypes. Specifically, there is moderate reduction (approximately 30%) in wound-

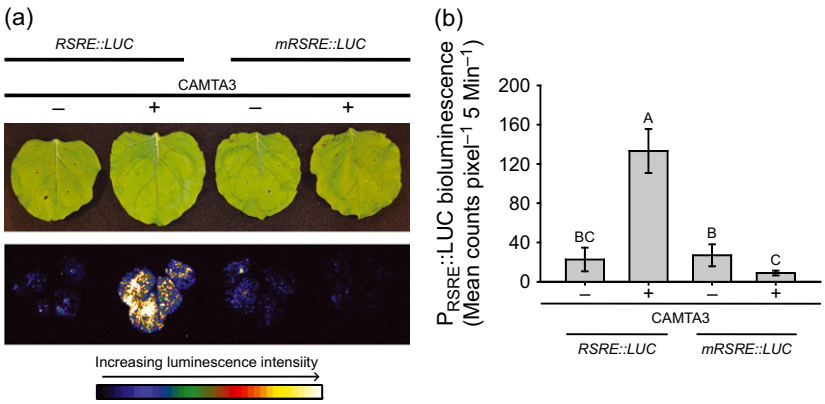


Figure 5. CAMTA3 induces *RSRE::LUC* activity. Transient expression assays in *Nicotiana benthamiana*.
(a) Representative assays displayed by bright field (upper panel), and dark field (lower panel) of leaves expressing *RSRE::LUC* or *mRSRE::LUC* alone, or together with *35S::CAMTA3*. The color-coded bar displays the intensity of LUC activity.
(b) Depicts means ± standard error of the mean (SEM) ($n = 8$) of *RSRE::LUC* and *mRSRE::LUC* activity alone, or with *35S::CAMTA3*, from two experimental replicates. Bars that do not share a letter represent statistically significant differences ($P < 0.05$).

induced LUC activity in the *camta1*, *2 and 4* and a strong reduction (approximately 65%) in *camta3* as compared with the corresponding levels in P lines, displaying non-uniform regulatory function of CAMTAs in regulating *RSRE*.

Next, we tested the systemic *RSRE::LUC* activity 90 min post wounding and application of either water or flg22 to the damaged site (Figure 6b,d). This result shows that application of either water, which can mimic touch response, or flg22 results in a moderate and strong systemic activation of *RSRE* in P line, respectively. This strong systemic LUC activity is reduced in the *camta* mutant lines, albeit disproportionally, with the lowest levels of induction in the *camta3* and the most in *camta1* as compared with the P line (Figure 6b,d).

Because of the established role of *CAMTAs1–3* in regulating plant responses to freezing (Doherty *et al.*, 2009; Kim *et al.*, 2013), we also tested *RSRE::LUC* activity in response to freezing in all the genotypes. These analyses established central roles of different CAMTAs in the induction of the *RSRE* in response to freezing, albeit at varying degrees. Specifically, LUC activity levels are reduced most strongly in *camta3*, followed by *camta4* and *camta2* and least impacted in the *camta1* as compared with the P line (Figure 6c,d).

Together, these data illustrate the suitability of *RSRE* for defining the specific role of CAMTAs in potentiating activation of early stress response regulatory networks.

Wounding-induced expression levels of CAMTA2–4 are independent of RSRE

Sequence analyses revealed the presence of the core *RSRE* motif *vCGCGb* in the promoters of *CAMTA2* and *CAMTA4* (Figure 7a). The established binding of CAMTA to this motif (Yang and Poovaiah, 2002; Galon *et al.*, 2010b), led us to explore the possibility of rapid alteration of *CAMTA* expression levels in response to stress.

The measurements of the *CAMTA* transcript levels before and 30 min after wounding revealed differential wound-inducible expression levels of these genes in the control P line (Figure 6b). In particular, wounding does not induce *CAMTA1* but increases the expression levels of the other three, with *CAMTA3* being the most strongly induced and *CAMTA2* as the least wound responsive (Figure 7b). Modest increases in the expression levels of *CAMTA2* and *4* as compared to the strong *RSRE::LUC* activity in response to wounding, in conjunction with the absence of the *RSRE* core motif in the *CAMTA1* and *3* promoters suggest an *RSRE*-independent transcriptional regulation of these TFs in response to wounding (Figures 2a,b and 7b). Furthermore, these data are in partial disagreement with previously published report illustrating that mechanical wounding-induced expression of all *CAMTAs* (Yang and Poovaiah, 2002). This discrepancy could be due to the lack of strict specificity of primer sequences previously used for amplification of these transcripts.

Expansion of these analyses to the mutant backgrounds also revealed limited transcriptional coregulation amongst the CAMTAs. Specifically, the data show partial dependence of wound-inducible expression levels of both *CAMTA1* and *CAMTA2* on *CAMTA3* (Figure 7b).

Wound induction of *RSRE* is via concerted function of CAMTAs

The lack of complete elimination of local as well as systemic *RSRE::LUC* activity in the *camta* single mutants (Figure 6a–d), led us to test functional redundancy of these TFs using double mutant lines. Wounding is a strong and synchronous stimulus which induces *RSRE* robustly. As such, we selected wounding as a stress method, and we generated homozygous double mutant lines for the three wound-inducible CAMTAs, namely *camta2/3*, *camta2/4*, and *camta3/4*. Comparison of LUC activity in response to wounding in the double, the single mutants, and the P line exhibits equally reduced *RSRE* expression levels in *camta2/3 and camta3/4* as compared with the P line, and the corresponding single mutants (Figure 8). In contrast, the LUC activity in *camta2/4*, although lower than in *camta2*, is

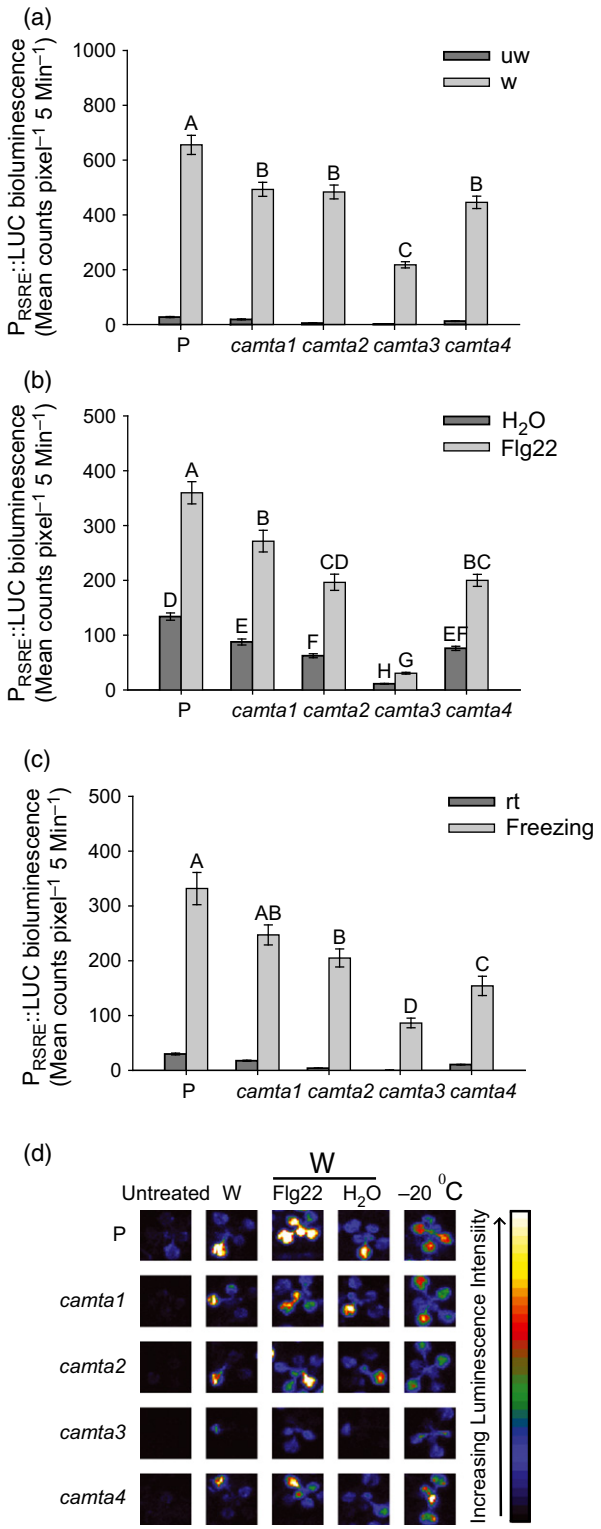


Figure 6. Local and systemic induction of *RSRE::LUC* is differentially regulated by CAMTAs.
Quantification of *RSRE::LUC* activity in unwounded (UW) or wounded (W) genotypes. Analyses of LUC activity measurements at 90 min post wounding and application of indicated stimuli.
(a) Local LUC activity in W and UW genotypes.
(b) Systemic LUC activity in response to application of water (control) and the biotic elicitor flg22 to the wound site.
(c) LUC activity in different genotypes 90 min post freezing (−20°C). (a–c) Depict means ± standard error of the mean (SEM) ($n \geq 72$). Bars that do not share a letter represent statistically significant differences ($P < 0.05$).
(d) Dark field images of representative plants of various genotypes before (untreated) or 90 min after treatment with indicated stresses. The color-coded bar display the intensity of LUC activity.

similar to the levels in the *camta4* single mutant (Figure 8). These data illustrate that CAMTA2 and 4 function in concert with CAMTA3 to activate the *RSRE* in response to wounding.

DISCUSSION

One of the defining characteristics of plants is their ability to maintain cellular homeostasis by constantly sensing and rapidly responding to environmental changes. Uncovering the mechanisms and signaling networks through which such processes occur is one of the challenges of modern plant biology. Here, we utilized the activity of a rapidly stress-inducible *cis*-regulatory motif to investigate the role of selected stress signaling and transcriptional regulatory components in modulating the plant GSR.

Comparative analyses of publically available microarray datasets demonstrated a stress-dependent bias for degree of enrichment of the *RSRE* motif in the promoters of rapidly inducible genes, alluding to the presence of other, yet to be identified classes of GSR motifs.

We also show that while stress-inducible hormones do not robustly induce the *RSRE*, calcium and calcium-inducing elicitors relay the stress signal in part to calcium/calmodulin regulated TFs that activate the GSR. Induction of *RSRE* by Ca^{2+} is in agreement with established role of this secondary messenger as an integrator of biotic and abiotic stress signaling cascades (Dodd *et al.*, 2010). This finding, although not surprising, has established a platform for gaining further insight into the poorly understood molecular mechanisms mediating Ca^{2+}-responsive gene expression.

In line with the signaling function of Ca^{2+} in transduction events leading to activation of the *RSRE*, and in agreement with previous reports establishing functional role of Ca^{2+}-dependent calmodulin-binding transcription factors, CAMTAs (Doherty *et al.*, 2009; Kim *et al.*, 2013), we used a transient assay to show the induction of *RSRE::LUC* but not *mRSRE::LUC* by CAMTA3. These data established the role of CAMTA3 in potentiating the transcriptional activity of *RSRE*. These studies do not show direct binding of CAMTA3 to the *RSRE*, yet such binding is well supported by the earlier reports showing that CAMTA3 binds to the core *vCGCGb* (Yang and Poovaiah, 2002; Du *et al.*, 2009; Nie *et al.*, 2012).

Additional genetic studies using mutant lines determined the functionality of additional CAMTAs in regula-

(a)

vCGCGb motifs in *CAMTA* promoters

Promoter	Motif	Location
CAMTA1	-	-
CAMTA2	*ACGCGG*	–616
	CCGCGG	–1951
CAMTA3	-	-
CAMTA4	*CCGCGG*	–1872

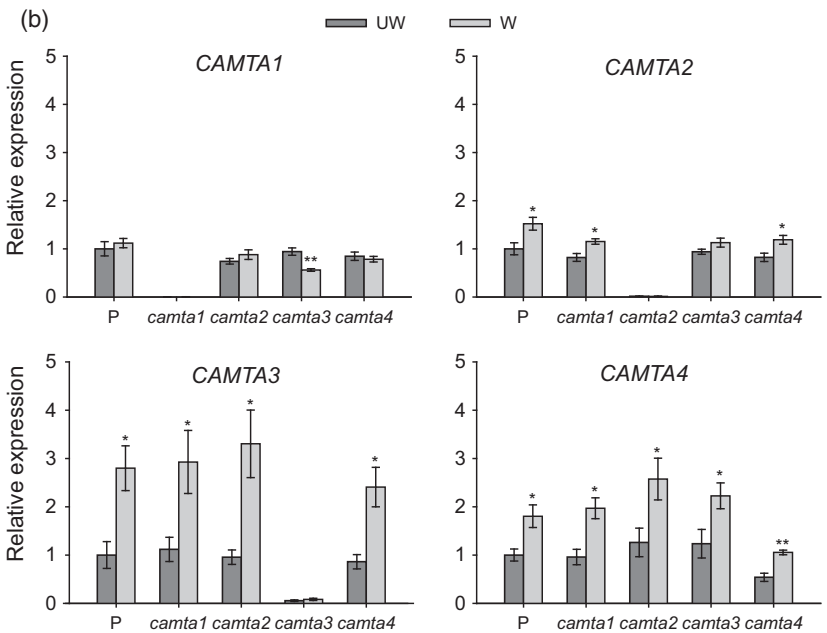


Figure 7. Wounding-induced expression levels of CAMTA2-4 are independent of RSRE.
(a) Presence and location of the *RSRE* core motif *vCGCGb* motif in the promoter sequences of *CAMTAs.*
(b) Relative expression levels of *CAMTA* genes in unwounded (UW) and 90 min post wounding (W) in various genotypes. Data are presented as expression levels relative to the reference gene. Data are means of four biological replicates ± standard error of the mean (SEM). Statistical significance was assessed using a two-tailed Student's *T*-test between each mutant and the P line with the corresponding treatment, $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

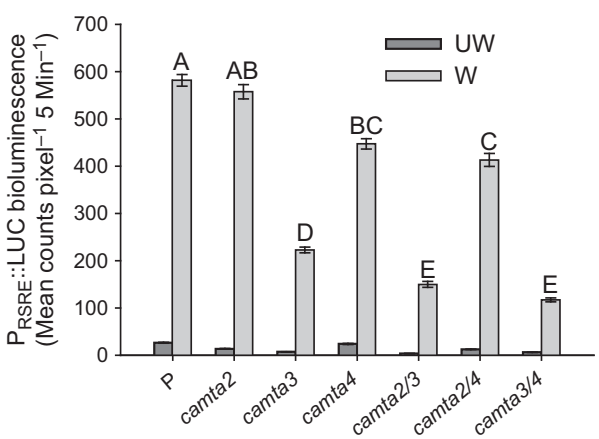


Figure 8. Wound induction of *RSRE* is via concerted function of CAMTAs.
Quantification of local *RSRE::LUC* activity in unwounded (UW) and wounded (W) P, single and double *camta* mutant lines 90 min after wounding. Bars that do not share a letter represent statistically significant differences ($P < 0.05$). Data are means ± standard error of the mean (SEM) ($n \geq 167$).

tion of the *RSRE.* Moreover, these studies display the functional redundancy of CAMTAs 1–4 in potentiating activation of the *RSRE* in response to freezing, wounding, and elicitor treatment. These TFs, however, elicit *RSRE* disproportionally, as CAMTA3 is the most potent and CAMTA1 is the least effective activator of this functional motif in response to all stresses examined. In addition to this hierarchal action, CAMTAs function cooperatively. That is, CAMTA3 works in concert with either CAMTA2 or 4 to potentiate induction of *RSRE* most robustly in response to wounding.

The rapidity of *RSRE* induction by the examined stimuli strongly supports existence of the CAMTAs prior the initiation of the stress. The presence of basal transcript levels of *CAMTA1–4* (Figure 7b) indeed support this notion, even though the concordance between the transcript and the corresponding protein levels is yet to be determined. Interestingly, despite the presence of basal *CAMTA* transcripts, *RSRE::LUC* activity is hardly detectable prior to perception of a stress signal. Several different stress-mediated scenarios may explain these results, such as post-translational modification of CAMTAs, removal of a repressor before CAMTA binding can occur, stress-induced nuclear translocation of a coactivator to function in concert with constitutively nuclear localized CAMTAs (Bouche *et al.*, 2002; Yang and Poovaiah, 2002), stress-mediated chromatin remodeling exposing the *RSRE* for binding, or Ca^{2+}-mediated binding of CAMTA by calmodulins, leading to altered CAMTA protein DNA-binding activity or interaction with transcriptional coactivators. Each of these potential scenarios

requires the action of one or more rapid stress-induced signaling processes; including production of Ca^{2+} signatures or reactive oxygen species bursts.

Ca^{2+} signatures are sensed and transmitted by a group of Ca^{2+}-dependent kinases and phosphatases. Amongst the plant Ca^{2+}-dependent protein kinases are the families of calcium-calmodulin-dependent kinases (CCaMKs), calcium-dependent protein kinases (CDPKs) that form an intricate cellular network for decoding Ca^{2+} signals and regulating cellular processes, including plant responses to stresses (Dodd *et al.*, 2010). For example, CDPKs are shown to regulate a burst of stress-induced ROS produced by NADPH oxidase (RBOH; for Respiratory Burst) (Kobayashi *et al.*, 2007). Our findings together with these reports suggest CCaMKS and CDPKs as relaying components of the Ca^{2+} signals necessary for potentiating transcriptional activation of the *RSRE.*

The other rapidly stress-inducible signaling process that could potentially regulate *RSRE* activity, either independently or in conjunction with Ca^{2+} is mitogen-activated protein kinase (MAPK) signaling. The MAPK cascade is a particularly attractive possibility, due to the strong induction of systemic *RSRE* activity by flg22 and OGA (Figure 3), both of which are known to induce MAPK cascades (Asai *et al.*, 2002; Chinchilla *et al.*, 2006, 2007; Suarez-Rodriguez *et al.*, 2007; Brutus *et al.*, 2010). A member of the Wall-Associated Kinase family (WAK1) is known to bind to OGAs (Brutus *et al.*, 2010), and we propose that WAK1 may bind specifically to 13mer OGAs to mediate this perception through MAPKs into downstream transduction cascade and ultimately activation of *RSRE.*

Based on our data and the previously published reports, we propose a simplified schematic model of stress sensing and transduction of convergent signaling pathways involved in activation of the GSR motif and transcriptional reprogramming of the GSR genes (Figure 9). Given this complexity, future work using this *RSRE::LUC* experimental platform integrated into various single and combined knockout lines will untangle the intertwined signaling web that enables convergence of multiple signals and TFs to activate a single functional motif in response to a wide range of stresses.

EXPERIMENTAL PROCEDURES

Plant growth conditions

All experiments were performed with 2-week-old plants grown on ¼ strength Murishige and Skoog (MS) basal medium (Sigma M0404, www.sigmaaldrich.com) with 1% phytoagar (plantMedia 40100072-2, www.plantmedia.com). Plants were grown at 22°C and 60% relative humidity in a growth chamber with a 16 h light/8 h dark photoperiod. Plants were moved from the growth chamber to the laboratory between 8 am and 10 am and all experiments were initiated between 12 pm and 2 pm.

Chemical treatments

Luciferin solutions were prepared as previously described (Walley *et al.*, 2008). Plants were sprayed with lucfierin approximately 20 h prior to initiation of imaging, to avoid activation of the reporter by mechanical stimulation from the spray. SA (Sigma S5922), ABA (A1049), MeJA (Sigma 392707), and HA (SAFC W317101, http://www.sigmaaldrich.com/safc.html) were dissolved in 80% ethanol and then diluted in de-ionized water. ACC (Sigma A3903) was dissolved in de-ionized water and diluted to the concentrations indicated. Plants were treated with SA, ABA, and ACC by the placement of a 10 μl drop on a single leaf per plant. MeJA and HA treatment was performed by placing 50 μl of the appropriate hor-

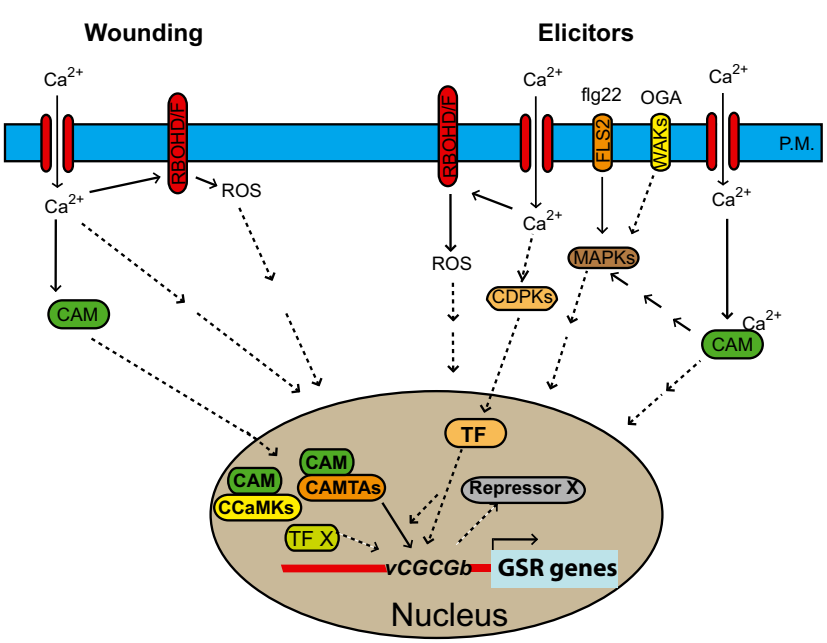


Figure 9. Simplified schematic model of stress sensing and signaling components involved in activation of the GSR motif and transcriptional reprogramming of the respective genes.
Biotic elicitors such as 22-amino-acid peptide flg22 and OGAs, and abiotic stresses such as wounding rapidly initiate convergent signaling pathways involved in induction of Ca^{2+} signatures, respiratory burst oxidase homolog D/F (RbohD) propagation of reactive oxygen species (ROS), and mitogen-activated protein kinase (MAPK) cascades, and ultimately transcriptional reprogramming of stress-responsive genes in part via activation of the general stress response (GSR) functional motif.
Abbreviations: CAM, calmodulin; CAMTA, calmodulin binding transcriptional activator; CCaMKs, calmodulin-dependent kinases; CDPKs, calcium-dependent protein kinases; FLS2, flagellin-sensitive 2; GSR,; P.M., plasma membrane; OGA, oligogalacturonic acid; TF, transcription factor; TFX, transcription factor X; WAKs, wall-associated kinases.

mone onto a cotton swab positioned in the center of the MS agar plate containing the plants. For elicitor treatments, 10 μl of the indicated elicitor or control was applied to a wound site on one leaf per plant. Flg22 (Genscript, www.genscript.com) was diluted in water to 10 μM. Oligo- and polysaccharides were the generous gift of Michael Hahn (Complex Carbohydrate Research Center, University of Georgia, Athens, GA, USA) and were dissolved in de-ionized water and diluted to 125 μg ml^{-1} EGTA (Sigma E4378) was dissolved in de-ionized water to pH 7.5 and diluted to 5 mM.

Luciferase quantification and analysis

Luciferase (LUC) activity of transgenic *4xRSRE::LUC* lines were measured using a charge-coupled device (CCD) camera (Andor DU-434BV, www.andor.com). Initial data analysis was conducted using Andor Solis software (version 15 www.andor.com). For measurements of local LUC activity, a 5 pixel by 5 pixel region of interest (ROI) was drawn over the center of the treated leaf, and the average counts for these 25 pixels was recorded as the LUC activity for that leaf. For measurements of systemic LUC activity, 5 by 5 pixel ROIs were placed on two systemic leaves, and the average counts for these 50 pixels was reported as the systemic LUC activity for that plant. Means and standard errors for each genotype/treatment combination were extracted from the raw Andor Solis data files using custom Perl scripts (http://www-plb.ucdavis.edu/labs/dehesh/dehesh-lab-code.html). Analysis of variance tests were performed on log-transformed data in R using the AOV function (R, www.r-project.org). Means separation was performed using Tukey's honest significant difference (HSD) test, via the HSD.test function from the agricolae package in R (http://cran.r-project.org/web/packages/agricolae/).

T-DNA insertion lines

camta1 (SALK_108806c), *camta2* (SALK_007027), *camta3* (SALK_001152), and *camta4* (SALK_013723) T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (Alonso *et al.*, 2003). The lines were genotyped using primer sequences (Table S2) designed using the Salk Institute Genomic Analysis Laboratory's T-DNA Primer Design tool (http://signal.salk.edu/tdnaprimers.2.html).

qRT-PCR

Total RNA was isolated via TRIzol (Life Technologies, www.lifetechnologies.com) extraction, and treated using a Plant RNeasy kit (Qiagen, www.qiagen.com) with DNase. SuperscriptIII (Life Technologies) was used to reverse transcribe 0.75 μg of RNA. qRT-PCR was performed with 6 μl of SYBR Green mix (Qiagen). Primers sequences are shown in Table S3. Reaction conditions and equipment are as described in Walley *et al.* (2007). Data analysis was performed using the $\Delta\Delta C_t$ method, as described in Walley *et al.* (2008), with the control AT4G26410 as the reference gene (Czechowski *et al.*, 2005).

Cloning and transient expression assays in *Nicotiana benthamiana*

CAMTA3 was initially cloned from Arabidopsis Col-0 cDNA into the pENTR D-TOPO vector (Life Technologies) and then recombined into pYL436 via the LR reaction (Liu *et al.*, 2004). For transient assays the *RSRE* and m*RSRE* with identical flanking sequences as previously described (Walley *et al.*, 2007) were cloned into pENTR D-TOPO and then recombined into pBGWL7 (Karimi *et al.*, 2005). Sequences of primers used for cloning are provided in Table S4.

Transient expression assays were performed using overnight cultures of *Agrobacterium* (GV3101) carrying *RSRE::LUC* or *35S::CAMTA3* were prepared in infiltration medium (2 mM Na_3PO_4, 50 mM 2-(N-Morpholino)ethanesulfonic acid hydrate (MES), 0.5% glucose, 100 μM acetosyringone) at OD_{600} = 0.2. The cultures were spot-infiltrated alone or together into 6–7-week-old *N. benthamiana* leaves.

Bioinformatic analyses

Lists of genes up-regulated by OGAs and flg22 were obtained from published microarray datasets (Denoux *et al.*, 2008). List of genes up-regulated by wounding, UVB, osmotic stress, cold, heat, and gamma radiation were extracted from public datasets (Kilian *et al.*, 2007; Yoshiyama *et al.*, 2009) using the R packages RMA and Limma with cutoffs of false discovery rate (FDR) <0.05 and fold change >2. Overlap between these lists was assessed using the Unix grep function. Searches for the *vCGCGb* motif were performed using a custom Perl script (http://www-plb.ucdavis.edu/labs/dehesh/dehesh-lab-code.html). Statistical significance was determined using the cumulative hypergeometric distribution, via the phyper function in R. These *P*-values were then corrected for multiple testing using the Benjamini–Hochberg procedure via the p.adjust function in R (Benjamini and Hochberg, 1995).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Schematics of insertion sties for each T-DNA in CAMTA genes.

Table S1. Percentages of the *vCGCGb* motif in genes down-regulated by various stresses in shoot.

Table S2. T-DNA Genotyping Primers.

Table S3. qRT-PCR and RT-PCR Primers.

Table S4. Primers used for cloning.

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