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Transcriptomic Analysis Reveals Calcium Regulation of Specific Promoter Motifs in *Arabidopsis* [W]

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Increases in intracellular calcium concentration ($[Ca^{2+}]_c$) mediate plant responses to stress by regulating the expression of genes encoding proteins that confer tolerance. Several plant stress genes have previously been shown to be calcium-regulated, and in one case, a specific promoter motif Abscisic Acid Responsive–Element (ABRE) has been found to be regulated by calcium. A comprehensive survey of the *Arabidopsis thaliana* transcriptome for calcium-regulated promoter motifs was performed by measuring the expression of genes in *Arabidopsis* seedlings responding to three calcium elevations of different characteristics, using full genome microarray analysis. This work revealed a total of 269 genes upregulated by $[Ca^{2+}]_c$ in *Arabidopsis*. Bioinformatic analysis strongly indicated that at least four promoter motifs were $[Ca^{2+}]_c$-regulated in planta. We confirmed this finding by expressing in plants chimeric gene constructs controlled exclusively by these *cis*-elements and by testing the necessity and sufficiency of calcium for their expression. Our data reveal that the C-Repeat/Drought-Responsive Element, Site II, and CAM box (along with the previously identified ABRE) promoter motifs are calcium-regulated. The identification of these promoter elements targeted by the second messenger intracellular calcium has implications for plant signaling in response to a variety of stimuli, including cold, drought, and biotic stress.

INTRODUCTION

Research over the past 35 years has shown calcium to be as important in plant cell signaling as it is in animal systems (DeFalco et al., 2010; Dodd et al., 2010; Kudla et al., 2010). Its role as a second messenger in plants has been well established in responses to many abiotic and biotic stresses (Galon et al., 2010a),; in response to hormones, for example, ABA (Kim et al., 2010); and during development, for example, in the growth of pollen tubes (Myers et al., 2009; Yan et al., 2009; Zhou et al., 2009) and root hair cells (Bibikova et al., 1997; Véry and Davies, 2000). Whereas animals respond to external stresses largely with behavioral changes, the major plant response to stress is affected through changes in gene expression, leading to reconfiguration of the proteome and resulting in improved stress tolerance. For this reason, most research into the role of calcium in plant stress responses has focused on the role calcium plays in regulating gene expression. Several stress signaling pathways use calcium as a second messenger. The expression of specific stress-responsive genes has been demonstrated to be calcium-regulated, for example, in response to cold (Knight et al., 1996; Knight et al., 1997; Tähtiharju et al., 1997; Galon et al., 2010a). The protein intermediates brokering calcium signals to regulate gene expression have also, in some cases, been identified, for example calmodulins (CaMs) (Takahashi et al., 2011), CaM-like proteins (Chiasson et al., 2005; Magnan et al., 2008; Xu et al., 2011), calcium-dependent protein kinases (CPKs) (Boudsocq et al., 2010; Coca and Segundo, 2010), CIPK/CBLs (Albrecht et al., 2003; Weinl and Kudla, 2009), and even transcription factors, such as CaM-binding transcription activators (CAMTAs) (Galon et al., 2008; Doherty et al., 2009; Du et al., 2009; Galon et al., 2010b).

As a result of the interest in studying responses to particular physiological stimuli, previous work on the calcium regulation of gene expression has focused largely on selected single or small numbers of genes per study. Such experiments do not allow prediction of the global effects of calcium on plant gene expression or reveal the extent to which it may play a role. In one exception, we have shown that a specific promoter motif, the abscisic acid responsive-element (ABRE) is calcium-regulated in *Arabidopsis thaliana* (Kaplan et al., 2006). This *cis*-element was so named because of its prevalence in genes responding to ABA-induced signaling pathways (Giuliano et al., 1988; Marcotte et al., 1989), and it contains the core ACGTG, which is known as the "G-box." This G-box has been described at length as a *cis*-acting promoter element involved in many diverse responses,

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including light, anaerobiosis, *p*-coumaric acid, ethylene, and methyl jasmonate (Menkens et al., 1995). The fact that the ABRE is calcium-regulated has implications for hundreds (if not thousands) of genes in all plant genomes. Calcium regulation of the ABRE motif was determined through transcriptomic and bioinformatic approaches followed by empirical confirmation (Kaplan et al., 2006). The power of this type of approach is that the data obtained allows a broader perspective and greater predictive power regarding calcium regulation of gene expression. However, it is clear from the diversity of genes that are known to be calcium-regulated that the ABRE cannot be the only calcium-regulated promoter motif. Our previous study (Kaplan et al., 2006) may have failed to identify other calcium-regulated motifs for many reasons. One major reason is that the study used microarray chips of only 9216 expressed sequences tag representing only ~6120 separate genes (Zik and Irish, 2003), ~23% of the genes in *Arabidopsis*. Another reason is that, in these experiments, the cellular concentration of calcium was artificially elevated (in the absence of external stress) by one specific treatment only. Different stresses and agonists are known to cause transient elevations in intracellular calcium with defined magnitude and duration; these different calcium profiles have been termed "calcium signatures." Some evidence suggests that specific signatures activate particular transcriptional responses (Dolmetsch et al., 1997; McAinsh et al., 1997; Dolmetsch et al., 1998; McAinsh and Pittman, 2009). It is therefore possible that the particular calcium signature we used may have favored activation of the ABRE over other promoter motifs. Thus, we hypothesize that the relatively shallow transcriptomic data pool and/or the limited characteristics of the calcium elevation applied limited the potential to identify more than one calcium-regulated promoter element. Therefore, to identify further promoter motifs and perform a more comprehensive study, we combined transcriptomic experiments using full genome chips (29,110 oligonucleotides representing 26,173 protein-coding genes), along with dendrimer technology to improve data depth, and combined this with the application of three calcium elevations of different characteristics. This approach revealed three more calcium-responsive promoter motifs, which we have confirmed as being calcium-regulated by empirical testing in planta. The implications of our findings, which broaden the picture on regulation of gene expression by this global ion in plants, are reviewed in the discussion.

RESULTS

Controlled Electrical Stimulations Elicit Specific Types of $[Ca^{2+}]_c$ Elevation

To interrogate the *Arabidopsis* genome to elucidate the full potential of intracellular calcium elevations to regulate gene expression, our first goal was to devise a system capable of producing calcium elevations of different characteristics (magnitude, duration, and multiple elevations) in *Arabidopsis*. Use of chemical agonists, such as ionophores, mastoparan, CaM antagonists, and DMSO, can lead to increases in $[Ca^{2+}]_c$ (Takahashi et al., 1998; Sangwan et al., 2002; Kaplan et al., 2006). However, the characteristics of these elevations cannot easily be controlled beyond altering the concentration of the agonist. We were aware of reports that the action potential produced by application of voltage is accompanied by an increase in intracellular calcium $[Ca^{2+}]_c$ in *Chara* spp (Williamson and Ashley, 1982) and that similar action potentials produced by voltage in higher plants can stimulate gene expression (Wildon et al., 1992; Herde et al., 1995). We therefore tested the hypothesis that application of voltage to *Arabidopsis* would cause an increase in $[Ca^{2+}]_c$, which we could subsequently use to study calcium-regulated gene expression. We were successful in stimulating $[Ca^{2+}]_c$ increases in seedlings expressing the calcium sensor aequorin (Knight et al., 1991; Knight and Knight, 1995) using voltage application (Figure 1). Moreover, we were able to control the characteristics of the $[Ca^{2+}]_c$ elevation using software to control the intensity and temporal dynamics of the voltage applied (see Methods). Using this system, it was possible to produce a single, transient elevation in $[Ca^{2+}]_c$ (Figure 1A), a series of transient elevations in $[Ca^{2+}]_c$ (Figure 1B), or to continually elevate $[Ca^{2+}]_c$ for a prolonged period (Figure 1C).

Imposed $[Ca^{2+}]_c$ Elevations Are Capable of Inducing Significant Changes in Gene Expression in *Arabidopsis*

We set out to measure global gene expression changes in response to the three different types of $[Ca^{2+}]_c$ elevation shown in Figure 1 (black lines). Each treatment was performed on two independent occasions, and seedlings were harvested for gene expression analysis 1 h after the end of each treatment, along with a matched unstimulated control (gray lines in Figure 1). For each treatment, microarray analysis was performed by comparing the treated sample with the untreated control in two dye-balanced biological replicates. For these experiments, we used microarray slides printed with the Operon *Arabidopsis* Genome Oligo Set Version 3 (Dr. David Galbraith, University of Arizona). As in the study by Okamoto et al. (2009), we chose to perform the microarray experiments using the Genisphere 3DNA Array 900 indirect cDNA labeling kit, because the indirect labeling method has many advantages over conventional microarrays, which use direct incorporation (Stears et al., 2000). For analysis, the raw data for each array was input into the online microarray database software BioArray Software Environment (BASE) (Saal et al., 2002). We used bespoke plug-ins developed by the Computational Biology Research Group (CBRG) in Oxford to perform many preanalytical steps on the raw data, including a cross-channel correction on each individual microarray spot and a global normalization procedure (see Methods). Because each treatment type was performed only in duplicate, we decided to use a statistical test based on Bayesian statistics, which has been shown to identify expression changes for experiments with low numbers of replicates more reliably than approaches based on a Student's *t* test or fold change (Long et al., 2001). This statistical approach is incorporated into the Cyber-T test (Baldi and Long, 2001; Long et al., 2001), which was integrated into the BASE program by the CBRG. Because validation experiments on the indirect labeling method for *Arabidopsis* have revealed a fold ratio resolution of 1.3- to 1.4-fold (based on a range of two times the SD), we chose to use a 1.5-fold ratio to the control as the

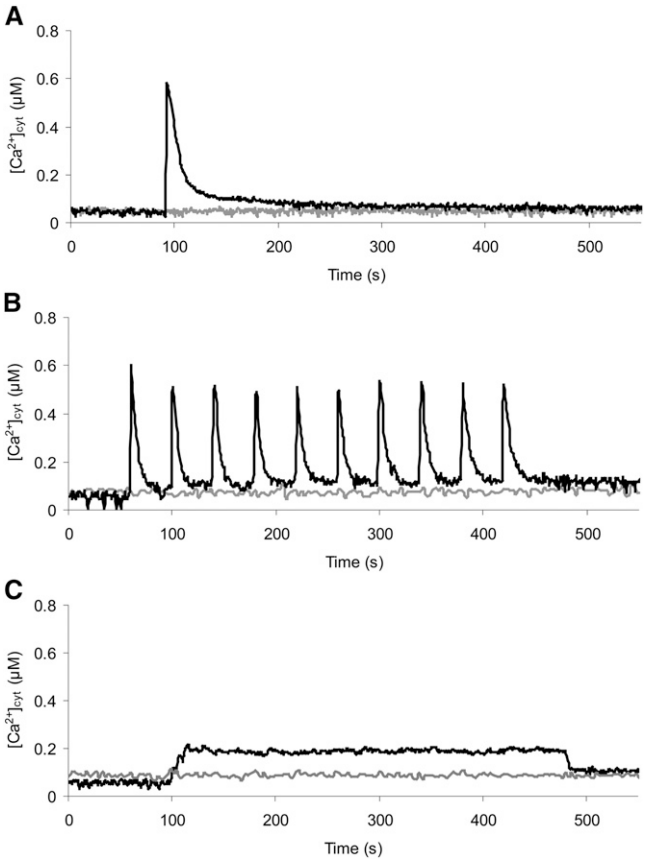


Figure 1. Calcium Elevations with Different Characteristics Used for Gene Expression Analysis.

Arabidopsis seedlings expressing cytosolic aequorin were reconstituted overnight in coelenterazine. Between 8 to 10 aequorin-expressing seedlings and four wild-type seedlings were floated in standard media in an electrostimulation cuvette for 2 h before treatment. Graphs show the average $[Ca^{2+}]_c$ response of the seedlings for unstimulated control (gray lines) and for seedlings stimulated by voltage (black lines) to produce a single transient $[Ca^{2+}]_c$ elevation **(A)**, a series of repeated transients in $[Ca^{2+}]_c$ **(B)**, or a prolonged $[Ca^{2+}]_c$ elevation **(C)**.

(A) to **(C)** A representative $[Ca^{2+}]_c$ trace (one of two replicates) is shown (five aequorin-expressing seedlings were frozen to calibrate the results). The average total $[Ca^{2+}]_{cyt}$ ($\pm$SD) mobilized for each treatment (minus the area of the control treatment) was **(A)** 18.05 $\pm$ 1.42 μM, **(B)** 40.46 $\pm$ 3.04 μM, and **(C)** 39.66 $\pm$ 0.22 μM. See Methods for details of applied electrical stimulus.

threshold to define a gene expression change. Table 1 shows the number of genes that were changed by more than 1.5-fold from the control with a Cyber-T P-value of <0.05 for each of the treatments (see Supplemental Data Set 1 online for the full gene lists).

The treatment with the greatest effect on gene expression was the oscillating $[Ca^{2+}]_c$ elevation, by which 256 genes were upregulated, and 97 genes were downregulated. By contrast, the prolonged $[Ca^{2+}]_c$ elevation led to the upregulation of only 10 genes and the downregulation of only 17 genes, despite the total area of $[Ca^{2+}]_c$ for this treatment being very similar to that of the oscillating treatment (Figure 1). Comparison of the upregulated gene lists from the single transient and oscillating $[Ca^{2+}]_c$ treatments revealed a large degree of overlap (47 of the 104 genes upregulated by the single transient elevation were also upregulated by the oscillating $[Ca^{2+}]_c$ treatment). Because we were interested in identifying all regulatory elements affected by $[Ca^{2+}]_c$ changes, we combined the transient and oscillation experiments in BASE to produce higher-confidence $[Ca^{2+}]_c$-regulated gene lists. We set the parameters to identify genes that were changed in at least two of the four experiments, so that genes that were absent in one replicate would now also be identified. This gave a list of 363 genes changed—of which 269 were upregulated, and 94 were downregulated (Table 1). This gene list can also be found in Supplemental Data Set 1 online.

To assess whether the genes upregulated by our $[Ca^{2+}]_c$ treatments overlap with responses to real biological stimuli, we performed functional classification analysis on our combined up- and downregulated gene lists, using the Bio-Array Resource Classification Superviewer (see Methods). The frequency (normalized to the frequency expected when considering all *Arabidopsis* genes) and P-value for the "Biological Process" (as well as the "Molecular Function and Cellular Component") classifications can be found in Supplemental Data Set 2 online. For the "Biological Process" classification, no significant ($P < 0.05$) frequencies were observed in the group of downregulated genes. However, the 269 upregulated genes gave a highly significant enrichment for genes involved in "response to stress" (3.71-fold, $P = 3.63 \times 10^{-18}$) and "response to abiotic or biotic stimulus" (3.46-fold, $P = 6.06 \times 10^{-14}$). To investigate this in more detail, we compared our upregulated gene list with publicly available AtGenExpress data for abiotic and biotic stress experiments. For this analysis, we used the Bio-Array Resource Expression browser, which returned all AtGenExpress experiments for which the genes in our list displayed an average fold change of twofold or greater. For the abiotic stresses, the twofold threshold was exceeded for microarray experiments performed at various time points after cold, drought, heat, osmotic, UV-B light, and wounding stresses (see Supplemental Table 1 online). Our upregulated genes also returned greater than twofold changes for some biotic experiments, including response to *Botrytis cinerea* infection (see Supplemental Table 2 online).

Known *cis*-Element Sequences Are Overrepresented in the Promoters of $[Ca^{2+}]_c$-Stimulated Genes

To identify potential calcium-activated DNA regulatory sites, we entered the 500-bp upstream regions of the genes from our amalgamated up- and downregulated gene lists into the Regulatory Sequence Analysis Tool (RSAT; http://rsat.ulb.ac.be/rsat). RSAT searches for overrepresented motifs using predefined oligonucleotide frequencies for *Arabidopsis* and a user-defined oligonucleotide length (van Helden et al., 1998; van Helden, 2003). No significantly overrepresented short sequences were found in the group of downregulated genes. However, several motifs were significantly overrepresented in promoter regions of the upregulated gene list (Table 2). It can be seen upon close inspection of these sequences that many of the overrepresented motifs are represented several times, because the motifs overlap

Table 1. Numbers of Genes Significantly Changed by the Three Characteristically Distinct $[Ca^{2+}]_c$ Elevations

Experiment (Total No. of Slides in BASE Experiment)	Upregulated	Downregulated	Total
Transient (2)	104	30	134
Oscillations (2)	256	97	353
Prolonged (2)	10	17	27
Oscillations and transient combined (4)	269	94	363

The software package BASE was used to analyze gene expression changes in the indicated experiments. The number of genes for each experiment that were >1.5-fold altered from the control and had a Cyber-T P-value of <0.05 are shown. For the single experiments (Transient, Oscillations, Prolonged), genes were required to be present in both of the two replicates to be considered significant. For the "Oscillations and transient combined" category, both replicates of these two experiments were combined into one experiment (to give four slides in total), and genes were required to be present in any two of the four experiments.

partially with other hexamers (see codes in "Seq" column of Table 2). Indeed, the RSAT program automatically aligns hexamers that contain overlapping sequences to produce a consensus sequence for each potential regulatory site. Two such alignments (see Supplemental Figure 1 online) seemed to contain two separate core sequences that, because of their similarity, had been aligned together. We therefore separated these into two sets of sequences and aligned these manually (Figures 2A and 2B). The first of these sequences, ACACGTG, contains the core sequence of the G-box or ABA-responsive element (ACGTG), which has been described on many occasions as a *cis*-acting promoter element involved in responses to many stimuli, for example light, anaerobiosis, *p*-coumaric acid, abscisic acid, ethylene, and methyl jasmonate (Menkens et al., 1995). This *cis*-element is activated by the ABRE-binding Factor (ABF) family of bZIP transcription factors (Choi et al., 2000). The second sequence, ACGCGT, is similar to the ABRE but instead has the core sequence CGCG, which is the binding site for CAMTAs (Bouché et al., 2002); we will refer to this site as the "CAM box." The two other alignments from RSAT (Figures 2C and 2D) also revealed sequences that we identified as previously described *cis*-regulatory elements. The sequence ACCGACAT (Figure 2C) matches the core sequence (CCGAC) of the C repeat (CRT) or drought response element (DRE), a *cis*-acting element that regulates the expression of genes responding to drought, low temperature, or high salt (Yamaguchi-Shinozaki and Shinozaki,

Table 2. Promoter Motif Analyses of Upstream Sequences of Genes Upregulated by Oscillations and Transient Calcium Elevations

Sequence	Identifier	Occurrence	Expected Occurrence	P-Value	E-Value	Z Score	Ratio
acacgt[a]	acacgt|acgtgt	105	48.09	8.9e-13	1.9e-09	8.21	2.18
ggccca[b]	ggccca|tgggcc	97	47.84	2.9e-10	6.0e-07	7.11	2.03
aacgcg[c]	aacgcg|cgcgtt	38	13.01	1.4e-08	2.9e-05	6.93	2.92
acgcgg[c]	acgcgg|ccgcgt	29	8.50	2.9e-08	6.0e-05	7.03	3.41
gccgac[d]	gccgac|gtcggc	35	13.04	3.6e-07	7.4e-04	6.08	2.68
accgac[d]	accgac|gtcggt	50	22.78	5.7e-07	1.2e-03	5.70	2.19
aacgtg[a]	aacgtg|cacgtt	70	37.89	1.9e-06	4.0e-03	5.22	1.85
acgtgg[a]	acgtgg|ccacgt	64	33.73	2.2e-06	4.7e-03	5.21	1.90
cacgtg[a]	cacgtg|cacgtg	52	25.48	2.7e-06	5.5e-03	5.25	2.04
cgcgta[c]	cgcgta|tacgcg	25	9.01	8.7e-06	1.8e-02	5.33	2.78
aacacg[a]	aacacg|cgtgtt	70	40.74	1.9e-05	4.0e-02	4.58	1.72
cacgtc[a]	cacgtc|gacgtg	48	24.75	2.2e-05	4.6e-02	4.67	1.94
acgcgt[c]	acgcgt|acgcgt	23	8.37	2.3e-05	4.7e-02	5.06	2.75
cccacc[b]	cccacc|ggtggg	42	20.87	3.1e-05	6.4e-02	4.63	2.01
acgcgc[c]	acgcgc|gcgcgt	24	9.14	3.1e-05	6.4e-02	4.92	2.63
ccgaca[d]	ccgaca|tgtcgg	44	22.35	3.3e-05	7.0e-02	4.58	1.97
atgggc[b]	atgggc|gcccat	77	47.12	4e-05	8.3e-02	4.35	1.63
atgtcg	atgtcg|cgacat	54	30.56	8e-05	1.7e-01	4.24	1.77
aaacgt[a]	aaacgt|acgttt	108	73.51	9.8e-05	2.0e-01	4.02	1.47

The 500 bp upstream regions of 269 genes upregulated in the combined oscillations and transient experiment were entered into the RSAT. Table shows all sequences identified by the RSAT program with an unadjusted P-value of $<10^{-05}$. Column heads designate the following: Sequence, overrepresented motif DNA sequence; Identifier, overrepresented motif DNA sequence and reverse complement; Occurrence, number of observed motifs; Expected Occurrence, expected number of observed motifs; Z score, log likelihood; Ratio, ratio of number of observed motifs versus expected number of motifs.

[a]Hexamer that forms part of the sequence for ABRE.

[b]Hexamer that forms part of the sequence for Site II.

[c]Hexamer that forms part of the sequence for CAM box.

[d]Hexamer that forms part of the sequence for CRT.

A

Alignment		Rev. cpl
.acacgt..	|	..acgtgt.
..aacgtg.	|	.cacgtt..
...acgtgg	|	ccacgt...
..cacgtg.	|	.cacgtg..
aacacg...	|	...cgtgtt
..cacgtc.	|	.gacgtg..
.aaacgt..	|	..acgttt.
ACACGTG	|	CACGTGT

B

Alignment		Rev. cpl
aacgcg..	|	..cgcgtt
.acgcgg.	|	.ccgcgt.
..cgcgta	|	tacgcg..
.acgcgt.	|	.acgcgt.
.acgcgc.	|	.gcgcgt.
ACGCGT	|	ACGCGT

C

Alignment		Rev. cpl	Score
accgac..	|	..gtcggt	2.12
gccgac..	|	..gtcggc	1.88
.ccgaca.	|	.tgtcgg.	0.72
.ccgacc.	|	.ggtcgg.	0.42
..cgacat	|	atgtcg..	0.02
accgacat	|	atgtcggt	2.12

D

Alignment		Rev. cpl	Score
aggccc..	|	..gggcct	0.41
.ggccca.	|	.tgggcc.	6.22
..gcccat	|	atgggc..	1.08
..gcccaa	|	ttgggc..	0.25
aggcccat	|	atgggcct	6.22

Figure 2. Alignments of Hexamer Sequences Overrepresented in the Promoters of Genes Upregulated by $[Ca^{2+}]_c$ Elevations Reveal Potential Calcium-Regulated *cis*-Elements.

The 500 bp upstream regions of 269 genes upregulated by $[Ca^{2+}]_c$ elevations were subject to promoter motif analysis using the RSAT as described in Methods.

(A) and **(B)** Manual alignments of hexamer sequences (Table 2) comprising portions of the ABRE **(A)** or CAM box **(B)** *cis*-element sequences. The consensus is taken as the most common base pair in that position (with a minimum of two of the same base pair required).

(C) and **(D)** Output alignments from the RSAT program of sequences comprising portions of the CRT **(C)** and Site II **(D)** *cis*-element sequences. The score is calculated by the oligo analysis tool by default and is equivalent to $-\log_{10}$ of the E-value (a score of 1.3 is approximately equal to an E-value of 0.05).

1994). Two families of proteins that bind to the CRT in promoters of genes responding to these stresses are C repeat binding factors (CBFs; also known as DRE binding factor 1s [DREB1s]) and DREB2s (Jaglo-Ottosen et al., 1998; Liu et al., 1998). The sequence AGGCCCAT (Figure 2D) contains the core of a sequence originally described as one of three *cis*-acting elements in the promoter of the proliferating cell nuclear antigen (PNCA) gene, originally in rice (*Oryza sativa*), which was thus designated Site IIa (Kosugi et al., 1995). Rice proteins identified as binding to this motif include bHLH transcription factors PCF1 and PCF2 (Kosugi and Ohashi, 1997). In *Arabidopsis*, the same Site II sequence GGCCCA and slight variations are bound by a family of transcription factors known as the TCP family, which share sequence similarity with rice PCF1/PCF2 (reviewed in Martín-Trillo and Cubas, 2010). The *Arabidopsis* TCP proteins, and their binding motif, have been described on many occasions as regulatory elements involved in regulating genes required for cell cycle control, growth, and development (Trémousaygue et al., 2003; Li et al., 2005; Welchen and Gonzalez, 2005, 2006; Hervé et al., 2009; Pruneda-Paz et al., 2009). To our knowledge, there have been no previous reports of the involvement of calcium signaling upstream of this *cis*-element or its transcription factors.

To help confirm these short sequences as bona fide *cis*-elements in our gene list, we mapped the positions of the central hexamer of each of the four alignments in the 500-bp upstream regions of our 269 upregulated genes. All four hexamers become enriched toward the start of transcription (see Supplemental Figure 2 online), as would be expected of functional *cis*-elements. We also analyzed association between the four different motifs by identifying genes (within the 269 upregulated) that contained the core hexamer of one or more of the motifs in their 500-bp upstream regions and calculating the proportion of association of each motif with the other three (see Supplemental Figure 3 online). Most (79%) gene promoters contained only one of the motifs, but associations were also found between all of the motifs, with CRT being associated with other motifs (specifically the ABRE and Site II) to a higher proportional extent than the others. We used the same gene lists that we used for the association analysis, as well as tools from the Bio-Array Resource, to investigate the biological function of the genes containing each of the motifs in their promoters. The Classification Superviewer revealed that the gene groups for all four motifs were significantly enriched for genes involved in "response to abiotic or biotic stimulus" and/or "response to stress" (see Supplemental Data Set 3 online). We subsequently compared these gene lists with AtGenExpress data for abiotic and biotic stress experiments using the Expression browser. This revealed that gene lists for each of the individual motifs displayed a greater than twofold change in AtGenExpress experiments following a range of abiotic stresses (see Supplemental Data Set 4 online). The highest fold change for the ABRE-, CRT-, and CAM box–containing genes was cold stress (52.33-fold, 45.66-fold, and 11.68-fold, respectively). The Site II–containing genes also showed up to an 8.98-fold upregulation in the cold experiments, but the highest fold change for this gene set was heat stress (20.91-fold). All of the gene lists, except that of the CAM box (which was the smallest gene list, with only 20 genes) also returned greater than twofold changes in response to biotic stimuli (see Supplemental Data Set 4 online).

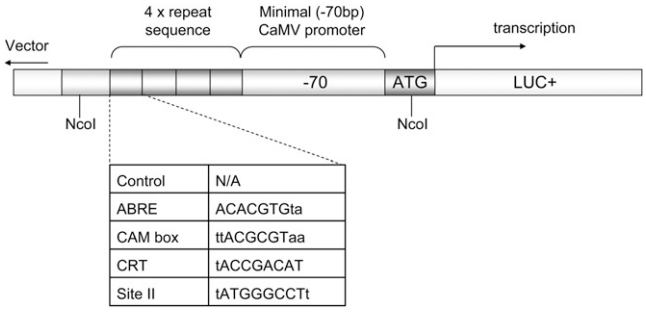
Because the motifs identified have all been previously described in the literature, and because their transcriptional binding proteins have been identified, we also searched our list of 269 upregulated genes for all known members of these families of transcription factors (CBFs, CAMTAs, TCPs, and ABFs). Only one of these genes, At3g02150, which encodes TCP13, was upregulated (Cyber-T p-value, 0.0057; median fold ratio, 1.78).

In Vivo Validation of Calcium-Responsive *cis*-Elements

Both ABRE and CAM box conform with the (A/C)ACG(T/C)G(T/G/C) consensus sequence identified previously as potentially being calcium-regulated (Kaplan et al. 2006). However, only the ABRE (not the CAM box) was subsequently shown using reporter

assays to be induced in response to calcium (Kaplan et al., 2006). To directly test calcium responsiveness of the CRT and Site II, along with the consensus ABRE and CAM box identified by microarray analysis, we first fused these regulatory elements to a luciferase (LUC) reporter gene. Our strategy was to produce chimeric genes with four copies of each element (Rushton et al., 2002) fused to the LUC+ reporter (Figure 3) and subsequently to use real-time PCR to detect *LUC*+ transcripts in response to $[Ca^{2+}]_c$. For these experiments, we decided to use DMSO and mastoparan, chemical agents previously shown to increase $[Ca^{2+}]_c$ in plants (Takahashi et al., 1998; Sangwan et al., 2002; Kaplan et al., 2006). DMSO is a commonly used polar aprotic solvent that has been shown to increase Ca^{2+} influx in plants because of its rigidifying effects on cell plasma membranes, which allows opening of calcium channels (Orvar et al., 2000). Mastoparan is a G protein–activating cationic-amphiphilic tetradecapeptide, which in mammalian cells leads to elevations in $[Ca^{2+}]_c$ through activation of PLC (Perianin and Snyderman, 1989). Although the mechanism of the $[Ca^{2+}]_c$ response to mastoparan in plants is still not fully understood, it is certainly different from the mechanism of DMSO (Takahashi et al., 1998); therefore, a response to both of these treatments would give a very strong indication of that our *cis*-elements are genuinely calcium-regulated.

To confirm that DMSO and mastoparan were capable of triggering $[Ca^{2+}]_c$ elevations in our experimental system, *Arabidopsis* seedlings, we tested their effects by measuring $[Ca^{2+}]_c$ using seedlings expressing aequorin (Knight and Knight, 1995). As can be seen in Figures 4A and 4B, DMSO and mastoparan both led to elevations in $[Ca^{2+}]_c$ in *Arabidopsis* seedlings. In the case of DMSO, this elevation was greater in magnitude and duration than with mastoparan, but mastoparan produced a more prolonged subsequent response. To confirm that these $[Ca^{2+}]_c$ elevations were capable of triggering the expression of $[Ca^{2+}]_c$-regulated genes, we used quantitative real-time PCR to test the effects of DMSO and mastoparan on the expression of three genes selected from our list of 269 upregulated genes. The transcripts of all three of these genes were increased in response to both DMSO and mastoparan to varying degrees (Figures 4C to 4E). Having demonstrated that these chemical agonists were capable of inducing expression of these candidate genes, we proceeded to test our chimeric CRT-, ABRE-, CAM box–, and Site II–*LUC*+ genes versus a similar construct lacking the *cis*-element repeats (control). We found that all four constructs showed increased *LUC*+ transcript levels in response to both DMSO and mastoparan, but the control displayed no such increase in expression (Figure 5). Two of the constructs, the ABRE and CAM box, were significantly more highly induced by mastoparan than DMSO (Figures 5A and 5B), whereas the CRT and Site II did not show a significant difference in their levels of *LUC*+ expression with the two $[Ca^{2+}]_c$ agonists (Figures 5C and 5D). These data strongly indicated that these *cis*-elements are calcium-regulated. We were also interested in whether the chimeric *cis*-element constructs would be responsive to real physiological stimuli. We therefore measured *LUC*+ expression from our chimeric genes after cold treatment. The CRT and CAM box were upregulated after 24 h of cold treatment, but the ABRE and Site II did not display increased expression in response to cold (see Supplemental Figure 4 online).



Control	N/A
ABRE	ACACGTGta
CAM box	ttACGCGTaa
CRT	tACCGACAT
Site II	tATGGGCCTt

Figure 3. Schematic of Constructs Used for Concatemer Studies.

The promoter region between the two *Nco*I sites was produced by PCR amplification. The *Nco*I-digested PCR fragment was then cloned into pDHLC+2, which contained the LUC+ coding region (the start codon of the LUC+ coding region is incorporated in the *Nco*I site). The promoter-LUC+ cassette was then cloned into the *Eco*RI site of pBIN19 for stable expression in plants. The exact sequence of the four repeats for each *cis*-element is shown in the table. A control construct, lacking the *cis*-element repeats, was produced in a similar way.

cis-Element Responses to Mastoparan Treatment Are $[Ca^{2+}]_c$-Dependent

The response of the *cis*-elements to treatments with two different calcium agonists strongly indicated that they were responsive to $[Ca^{2+}]_c$ elevations in the absence of physiological stimuli. However, to confirm that their induction was due to the $[Ca^{2+}]_c$ elevation specifically, it is necessary to control for other calcium-independent effects of the calcium agonists. By blocking the $[Ca^{2+}]_c$ elevation induced by an agonist, one can assess whether changes in $[Ca^{2+}]_c$ were truly responsible for the gene expression changes. Lanthanum chloride ($LaCl_3$) has been widely used to inhibit the action of calcium channels (Knight et al., 1992; Knight et al., 1996; Knight et al., 1997), usually at concentrations of 5 to 10 mM. However, at these concentrations, $LaCl_3$ itself induces a small elevation in $[Ca^{2+}]_c$ (Figure 6A), which may also affect gene expression. We therefore decided to test a range of $LaCl_3$ concentrations in combination with mastoparan (because in general this was more successful at inducing *LUC*+ expression than DMSO) (Figure 5) to identify the lowest concentration that would effectively block the $[Ca^{2+}]_c$ elevation while minimizing the effect of $LaCl_3$ alone on $[Ca^{2+}]_c$. Figure 6A shows that lanthanum treatment provoked a $[Ca^{2+}]_c$ increase in a dose-dependent manner. Lanthanum concentrations of 5 and 10 mM gave $[Ca^{2+}]_c$ elevations that were noticeably higher than at lower concentrations. There was no significant difference between the $[Ca^{2+}]_c$ elevations produced by $LaCl_3$ concentrations of 0.1 to 1 mM (Figure 6A). Inhibition of mastoparan-induced $[Ca^{2+}]_c$ was greater at 1 mM than at 0.5 mM and 0.1 mM (Figure 6B). Therefore, we chose to use 1 mM $LaCl_3$ to inhibit the $[Ca^{2+}]_c$ elevations induced by mastoparan for subsequent gene expression studies (incorporating controls that received only 1 mM $LaCl_3$ into all experiments). Figure 7 shows that mastoparan-induced *LUC*+ expression driven by CRT, ABRE, Site II, and CAM

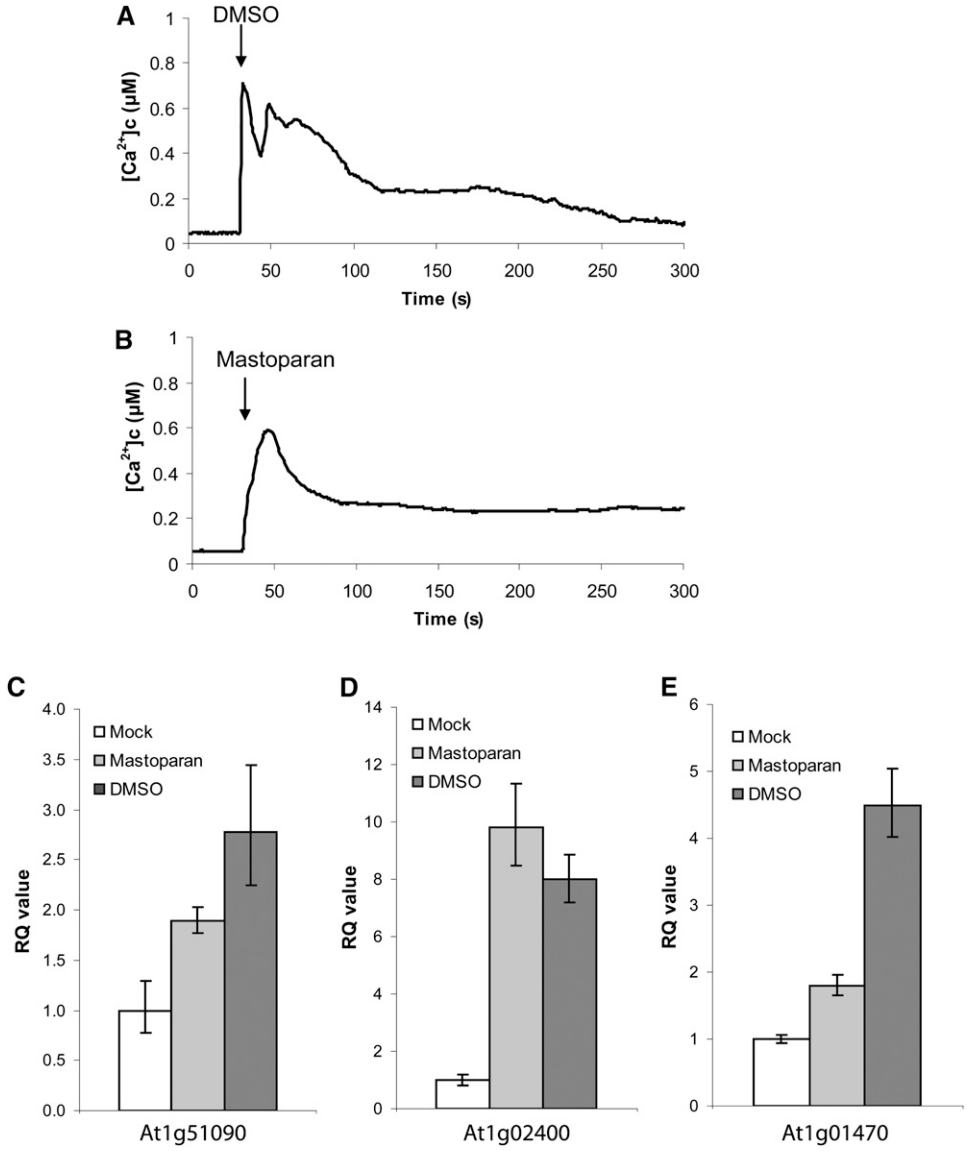


Figure 4. DMSO and Mastoparan Stimulate Increases in $[Ca^{2+}]_c$ and Induce Expression of Selected Genes That Were Upregulated by Electrically Stimulated $[Ca^{2+}]_c$ Elevations.

(A) and **(B)** *Arabidopsis* seedlings expressing cytosolic aequorin were reconstituted overnight in coelenterazine, and $[Ca^{2+}]_c$ measurements were performed by luminometry. DMSO (2% v/v) **(A)** and mastoparan (10 μM) **(B)** treatments were applied at the time shown by the arrow. The average calibrated $[Ca^{2+}]_c$ response of two seedlings is shown (representative of three experiments).

(C) to **(E)** Under the same conditions, seedlings were harvested after 3 h treatment compared with a water control (mock). RQ values for expression of At1g51090 **(C)**, At1g02400 **(D)**, and At1g01470 **(E)** measured using real-time PCR are shown. In each case, expression was normalized to expression values for *PEX4* (endogenous control). Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX}, which constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

box sites were all strongly inhibited by lanthanum. This demonstrates that mastoparan induction of expression through the CRT, ABRE, Site II, and CAM box promoter motifs is truly $[Ca^{2+}]_c$-dependent.

Finally, because the Site II and CAM box had not been previously demonstrated to be activated by $[Ca^{2+}]_c$ elevations induced by *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride (W7) (Kaplan et al., 2006), it was possible that these two motifs were differentially sensitive to the $[Ca^{2+}]_c$ signatures produced by DMSO and mastoparan compared with that produced by W7. To test this, we measured *LUC*+ expression driven by the Site II and CRT motifs in response to W7-induced $[Ca^{2+}]_c$ elevation in combination with $LaCl_3$ pretreatment, as in Kaplan et al. (2006). Figure 8 shows that Site II was clearly and

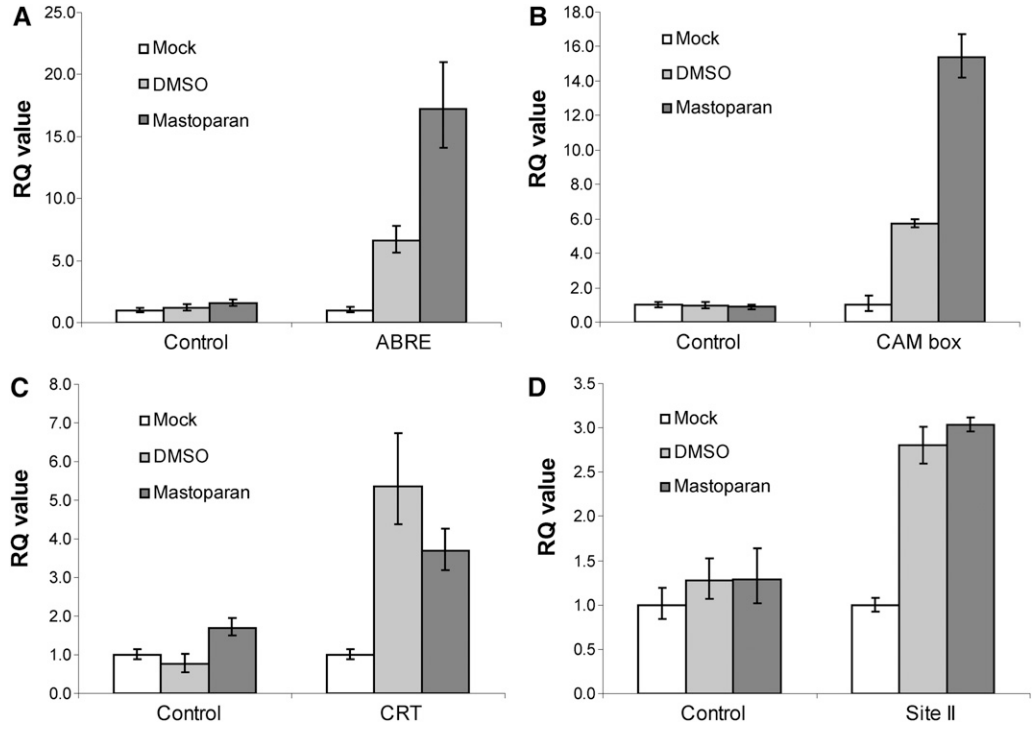


Figure 5. DMSO and Mastoparan Stimulate Increases in *ABRE*-, *CAM Box*–, *CRT*-, and *Site II*–*LUC*+ Expression.

Mastoparan (10 μM), DMSO (2% v/v), or water were added to *Arabidopsis* seedlings expressing LUC+ under the control of either a minimal promoter only (control) or four repeats of the *cis*-element sequences ABRE **(A)**, CAM box **(B)**, CRT **(C)**, or Site II **(D)**. Tissue was harvested 3 h after treatment. RQ values for *LUC*+ expression measured using real-time PCR are shown. *LUC*+ expression was normalized to expression values for *PEX4* (endogenous control). Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX}, which constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

strongly induced in response to W7 in a $[Ca^{2+}]_c$-dependant manner, but the CRT element was only very weakly induced.

DISCUSSION

Electrical Impulses Can Be Used to Generate "Designer" $[Ca^{2+}]_c$ Elevations

We describe a powerful approach to produce $[Ca^{2+}]_c$ elevations with user-defined parameters including magnitude, duration, and frequency of oscillation (Figure 1). A major advantage of this method over the application of different chemical calcium agonists to produce particular increases in $[Ca^{2+}]_c$ is that each $[Ca^{2+}]_c$ elevation is produced by the same primary treatment type (electrical impulse), thus reducing the likelihood of stimulus-specific secondary effects. Using electrical stimulation, we produced three distinct $[Ca^{2+}]_c$ elevations: single transients (Figure 1A), prolonged elevations (Figure 1C), and oscillations (Figure 1B).

We tested the ability of these distinct $[Ca^{2+}]_c$ elevations to induce gene expression in *Arabidopsis* seedlings. Calcium has been shown to be necessary as well as sufficient for expression of some plant genes, such as *GST1* in response to ozone (Clayton et al., 1999) and *KIN1* and *KIN2* in response to cold in *Arabidopsis* (Knight et al., 1996; Tähtiharju et al., 1997). In a previous study, we broadened the scope of this method of inquiry by testing the expression of 6120 genes in response to a chemical calcium agonist. This work resulted in the identification of the ABRE as a Ca^{2+}-regulated promoter motif (Kaplan et al., 2006). We wished to build upon this work, because there were several limitations to this original study. First, only one promoter motif was identified as $[Ca^{2+}]_c$-regulated (Kaplan et al., 2006). Given the number of genes reported in the literature to be calcium-regulated, and taking into consideration the number of regulons that likely include these genes (Galon et al., 2010a), it seemed probable that there remained other elements to be discovered. Second, the microarray analysis was performed with chips, allowing detection of transcript levels for only 6120 individual genes. Third, only one $[Ca^{2+}]_c$ signature was tested; it is possible that genes sensitive to particular parameters of an increase in $[Ca^{2+}]_c$ not represented by this specific elevation were not responsive to this treatment. Finally, the agonist used was the CaM inhibitor W7. In hindsight, this may have been a poor choice, because it inhibits both CaM and CPKs, and consequently is likely to interfere with the signal transduction process linking elevated $[Ca^{2+}]_c$ to gene expression. Therefore, we performed a new analysis on full genome chips (comprising

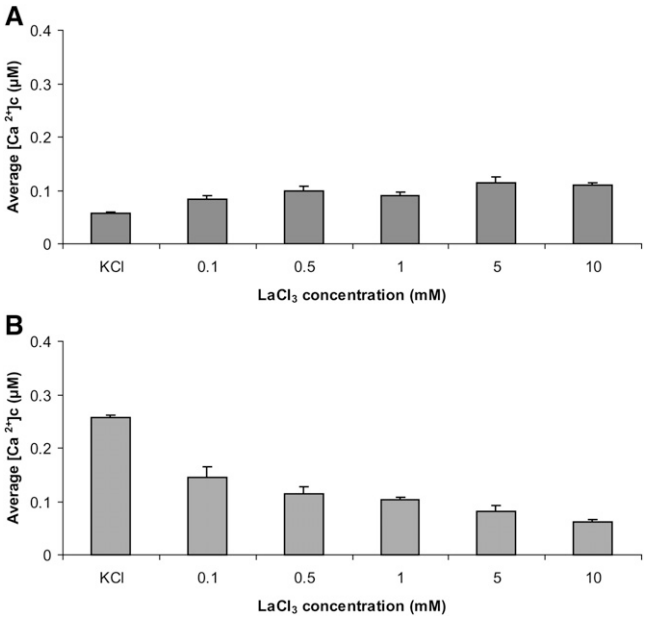


Figure 6. Optimization of $LaCl_3$ Concentration for Inhibition of Agonist-Stimulated $[Ca^{2+}]_c$ Elevations.

Two aequorin-expressing *Arabidopsis* seedlings were either treated with the indicated concentration of $LaCl_3$ or 20 mM KCl **(A)** or were incubated in a solution containing the indicated concentration of $LaCl_3$ or 20 mM KCl for 30 min before addition of 10 μM mastoparan **(B)** before calibration to calculate $[Ca^{2+}]_c$. The average concentration of $[Ca^{2+}]_c$ over 300 s is shown (n = 3; bars represent 1 SE).

29,110 70-mer oligonucleotides representing 26,173 protein-coding genes and 28,964 protein-encoding gene transcripts, as well as 87 microRNA gene precursors), and applied dendrimer technology to produce data with greater sensitivity and depth. Combining this with three different electrically induced $[Ca^{2+}]_c$ elevations to produce different $[Ca^{2+}]_c$ profiles dealt with all four of these limitations.

Comparing the genes that were induced in response to transient, prolonged, and oscillating $[Ca^{2+}]_c$ elevations revealed substantial differences in the number and identity of the genes expressed in response to each (Table 1; see Supplemental Data Set 1 online for full gene lists). It can be seen that the transient elevation of $[Ca^{2+}]_c$ induced an order of magnitude more genes (104) than the prolonged elevation (only 10 genes). This is despite the area under the curve of the prolonged $[Ca^{2+}]_c$ elevation being greater than that of the transient elevation (Figure 1). The most likely explanation for the greater potency of the transient in this case is that a certain threshold of $[Ca^{2+}]_c$ is required to induce expression of the additional genes. There are, however, other explanations that cannot be discounted; for instance, it may be that genes expressed in response to the transient $[Ca^{2+}]_c$ elevation show peak expression at a different time than those expressed in response to prolonged treatments. If this were the case, the time point chosen to measure expression in our experiments may have coincided better with the peak of expression of more genes in the case of the transient compared with prolonged $[Ca^{2+}]_c$ elevation. Investigations of differences in the temporal expression patterns of different genes in response to specific calcium elevations, as well as analysis of how genes respond to different thresholds of $[Ca^{2+}]_c$, would make interesting future studies. The most potent $[Ca^{2+}]_c$ elevation of all was the oscillations, which resulted in increased transcript levels for 256 genes (~2.5 times more than the transient $[Ca^{2+}]_c$ elevation and 25 times more than the prolonged $[Ca^{2+}]_c$ elevation). This is despite the total $[Ca^{2+}]_c$ mobilized in the oscillation treatment being very similar to that of the prolonged treatment (Figure 1). However, the difference in the number of genes deregulated by these two treatments could again be at least partially explained by the threshold and time point hypotheses above. Regarding the greater gene deregulation by the oscillations than the single transient, a simple argument of threshold is unlikely to explain this effect, because the peak heights of the $[Ca^{2+}]_c$ oscillations were not higher than those of the single transient (Figure 1). However, repeated transients that form part of the oscillations might be additive in effect and thus produce a more profound output. It has been shown in animal cells that very simple cellular mechanisms, even single proteins, such as mammalian CaM Kinase II (De Koninck and Schulman, 1998), can "decode" oscillations, and oscillations of certain frequencies can potentiate calcium-mediated effects to levels greater than single transients alone (Dolmetsch et al., 1997; Dolmetsch et al., 1998). Our data are consistent with the presence of such decoders in plants, although their identity remains unknown. It has been shown that certain single plant cell systems (where such work is possible), such as guard cells and root hairs, have the ability to decode $[Ca^{2+}]_c$ oscillations (Allen et al., 2001; Sun et al., 2007).

Although we were not attempting to mimic any natural $[Ca^{2+}]_c$ responses, the three profiles we generated have similarity to $[Ca^{2+}]_c$ reported in the literature. The single monophasic transient resembles the responses to osmotic stress, oxidative stress, and rapid cooling (Knight et al., 1996; Knight et al., 1997; Rentel and Knight, 2004), and prolonged responses have been reported in response to slow cooling (Plieth et al., 1999). Oscillations of calcium have been reported in guard cells responding to ABA and in legume root hairs responding to Nod factors (Wais et al., 2000; Allen et al., 2001), although it is important to note that the frequencies used here were not the same as those observed in guard and root hair cells. The $[Ca^{2+}]_c$-regulated genes we identified seem to show a bias toward genes induced by abiotic stresses (see Supplemental Data Set 2 online), including cold, osmotic, and oxidative stress (see Supplemental Table 1 online), which is consistent with the similarity of our $[Ca^{2+}]_c$ elevations to those produced by these types of stresses.

Identification of Four Promoter Motifs That Are Overrepresented in Genes Upregulated in Response to $[Ca^{2+}]_c$ Elevations

By combining the transient and oscillating $[Ca^{2+}]_c$ elevations into one experiment for analysis of gene expression changes, we were able to increase the potential for identification of *cis*-elements responding to a broader range of $[Ca^{2+}]_c$ elevations. By analyzing the promoter sequences of this combined list of genes induced by our electrical impulses, we found four distinct classes

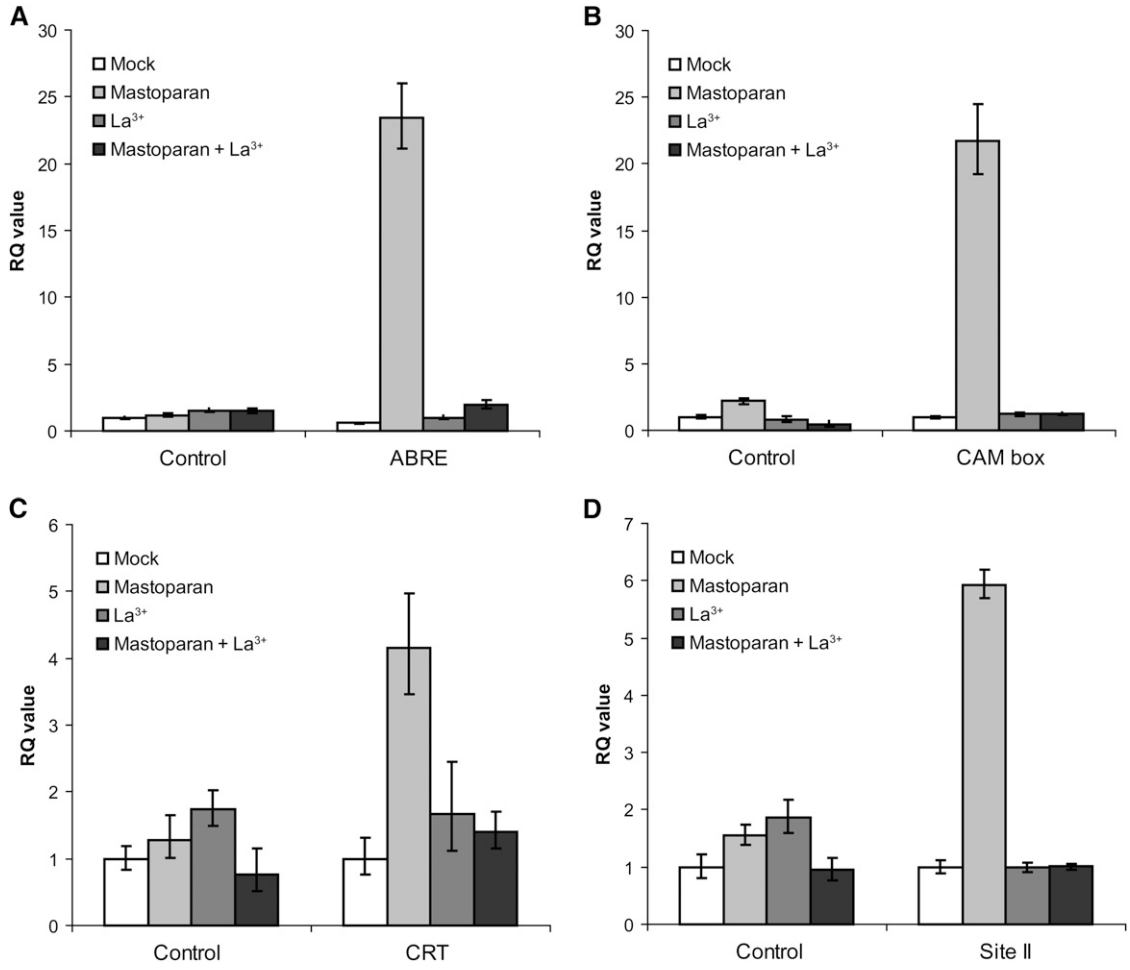


Figure 7. *ABRE*-, *CAM Box*–, *CRT*-, and *Site II–LUC*+ Expression Is $[Ca^{2+}]_c$-Regulated.

Arabidopsis seedlings expressing LUC+ under the control of either a minimal promoter only (control) or four repeats of the *cis*-element sequences ABRE **(A)**, CAM box **(B)**, CRT **(C)**, or Site II **(D)** were pretreated with either 1 mM $LaCl_3$ (La^{3+}) or water for 30 min before the addition of either water or mastoparan (to a final concentration of 10 μM). Tissue was harvested 3 h after treatment. RQ values for *LUC*+ expression measured using real-time PCR are shown. *LUC*+ expression was normalized to expression values for *PEX4* (endogenous control). Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX}, which constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

of short DNA motif that appeared at a frequency statistically higher than in the genome as a whole (Table 2, Figure 2). These four motifs most closely resembled motifs already described in the plant molecular biology literature, namely the CRT/DRE cold- and drought-responsive element (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997; Liu et al., 1998), the ABRE abscisic acid response element (Hobo et al., 1999), the binding site of the CAMTA transcription factors (which we have named CAM box) (Yang and Poovaiah, 2002), and finally the Site II motif (Trémousaygue et al., 2003). The ABRE (CACGTGT) and CAM box (ACGCGT) completely fit the consensus of the ABRE (CACGTG[T/C/G]) and ABRE coupling element ([C/A]ACGCG[T/C/A]) identified by our previous microarray experiment (Kaplan et al., 2006). The Site II and CRT motifs were not identified in the previous study (Kaplan et al., 2006), despite genes containing these elements being well-represented on the microarray used (89 of our 269 upregulated genes were present on the array and were significantly enriched for both CRT and Site II). One possible explanation is that these elements were differentially insensitive to $[Ca^{2+}]_c$ signatures generated by the W7 treatment used, whereas they are sensitive to DMSO- and mastoparan-induced $[Ca^{2+}]_c$ signatures. This seems to be true for the CRT, which showed only a very modest activation in response to W7 (Figure 8); it is therefore possible that the inhibitory effect of the W7 treatment upon CaM/CPK masks its activation. Indeed, evidence suggests that W7 reduces the expression of CRT/DRE-containing genes, such as *KIN1* and *KIN2* (Tähtiharju et al., 1997). The Site II element, however, showed substantial induction in response to W7 treatment (Figure 8); therefore, it is more surprising that this motif was not identified in the previous study. It is likely that a combination of the use of full genome chips (representing more than fourfold the genes on the array used in the

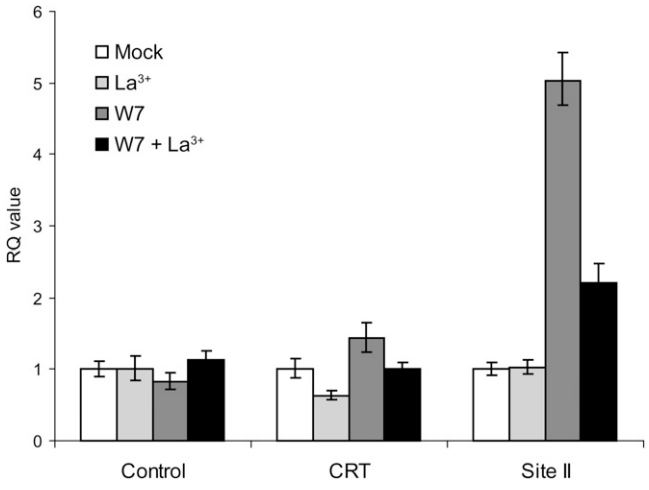


Figure 8. Response of CRT- and Site II–LUC+ Expression to W7 Treatment.

Arabidopsis seedlings expressing LUC+ under the control of either a minimal promoter only (control) or four repeats of the *cis*-element sequences CRT or Site II were pretreated with either 0.5 mM $LaCl_3$ (La^{3+}) or water for 15 min before the addition of either 0.3% DMSO control (mock) or W7 (to a final concentration of 600 μM). Tissue was harvested 3 h after treatment. RQ values for LUC+ expression measured using real-time PCR are shown. LUC+ expression was normalized to expression values for *PEX4* (endogenous control). Each value is the mean of three technical replicates. Error bars indicate RQ_{MIN} and RQ_{MAX}, which constitute the acceptable error for a 95% confidence limit according to Student's *t* test.

previous study) and the more sensitive labeling method, combined with optimized microarray analysis, allowed us to identify the Site II element in this study. Having identified four elements, it is still possible that there are more $[Ca^{2+}]_c$-regulated promoter motifs; identification of motifs statistically requires substantial numbers of genes containing that motif to be present in the list, and thus a small regulon of $[Ca^{2+}]_c$-regulated genes using another promoter motif might not be detected. In addition, because our analysis was performed at a single time point, it is possible that transcriptomic analysis at earlier or later time points would lead to the appearance of additional regulatory elements —a possibility which can be addressed in future studies.

The CRT/DRE, CAM Box, ABRE, and Site II Motifs Are Regulated by $[Ca^{2+}]_c$ in Planta

Having identified the CRT/DRE, Site II, CAM box, and ABRE motifs as being enriched in our list of genes induced by electrical impulse, we tested transactivation of these motifs in response to changes in $[Ca^{2+}]_c$ in planta. It is important to note that although activation of the ABRE was shown in our previous study (Kaplan et al., 2006), it was only tested in response to calcium agonists; without monitoring the effect of Ca^{2+} antagonists on this response, it remains possible that the effect observed was agonist-specific rather than calcium-specific. The ABRE coupling element (which resembles the CAM box) was not tested at all (Kaplan et al., 2006). To our knowledge, $[Ca^{2+}]_c$-regulation of CRT/DRE and Site II has not been previously reported. We used constructs with four copies of the motifs (Figure 3), each sequence having been empirically determined (Figure 2). We chose to use four copies of the motifs, because this number has previously been demonstrated to be the optimal compromise between motif construct accuracy and sensitivity in this type of construct (Rushton et al., 2002). We used two different calcium agonists, mastoparan and DMSO, both of which had previously been demonstrated to increase $[Ca^{2+}]_c$ in plant cells (Takahashi et al., 1998; Orvar et al., 2000). Both these agonists have different modes of action leading to $[Ca^{2+}]_c$ elevation and thus different secondary effects, so induction of expression in response to both provides strong evidence of calcium-regulation. We tested mastoparan and DMSO in our experimental system and detected significant elevations in $[Ca^{2+}]_c$ (Figure 4). We demonstrated that the expression of three candidate genes, chosen from our upregulated gene list, could be induced by mastoparan and DMSO, confirming that these agonists were appropriate for our study (Figure 4). All four motifs showed increases in expression in response to both mastoparan and DMSO (Figure 5). Interestingly, the ABRE and CAM box showed greater expression in response to mastoparan than DMSO, but Site II seemed to be equally responsive to both (Figure 5). CRT/DRE showed greater expression in response to DMSO, which is interesting because DMSO has been postulated to stimulate influx of cellular calcium by mimicking cold by rigidifying membranes and subsequently activating cold-regulated genes (Orvar et al., 2000). The relative differences in response to mastoparan and DMSO for each of the four motifs might be due to the cognate transcription factors responding to different specific characteristics of $[Ca^{2+}]_c$ elevations, which are more closely mimicked by either mastoparan or DMSO in particular cases (Figure 4).

The fact that all four motifs were induced in response to two chemical agonists with very different modes of action (and thus different secondary effects) was already strong evidence of $[Ca^{2+}]_c$ regulation of these motifs. However, to provide conclusive evidence, mastoparan-induction of all four motifs was tested in the presence of the calcium channel blocker lanthanum, which inhibits the mastoparan-induced $[Ca^{2+}]_c$ elevation (Figure 6). Figure 7 shows that, in all four cases, lanthanum inhibited the mastoparan-induced expression of the four promoter motifs. We conclude, therefore, that CRT/DRE, ABRE, CAM box, and Site II promoter motifs are $[Ca^{2+}]_c$-regulated in planta. We have demonstrated that calcium is both sufficient and necessary for expression via these motifs in response to DMSO and mastoparan. Our data suggest that these four promoter motifs are regulated by calcium in response to $[Ca^{2+}]_c$-inducing stimuli in nature, but they may well respond differentially to specific physiological stimuli. Indeed, the CRT and CAM box were upregulated by cold treatment, whereas the ABRE and Site II were not (see Supplemental Figure 4 online), suggesting the possibility of differential responses of the four motifs to the $[Ca^{2+}]_c$ elevation elicited by this treatment. The CRT is already well-known to respond to low temperature treatment (Stockinger et al., 1997), and it is interesting that the CAM box is also upregulated by cold, because CAMTAs have been shown to be

important in the cold-induced upregulation of the CBF transcription factor genes of *Arabidopsis* (Doherty et al., 2009). It is not clear what other signals induce the CAM box, although a role for the CAMTA transcription factors that bind this motif has also been shown in response to pathogens and auxin (Galon et al., 2008; Du et al., 2009; Galon et al., 2010b), which also provoke elevations in $[Ca^{2+}]_c$ (Felle, 1988; Knight et al., 1996; Grant et al., 2000). The CRT element is also induced in response to drought (Liu et al., 1998), and the ABRE is induced in response to ABA and drought (Hobo et al., 1999), which both induce $[Ca^{2+}]_c$ (McAinsh et al., 1990; Knight et al., 1996; Knight et al., 1997). It is not clear in which calcium-regulated process the Site II motif participates; therefore, correlating these conditions with $[Ca^{2+}]_c$ elevations is not currently possible. Future experiments with a variety of physiological treatments will help to further dissect how these promoter motifs differentially respond to various stimuli and their respective specific $[Ca^{2+}]_c$ signals. The regulation of CRT/DRE, ABRE, CAM box, and Site II is most likely through the CBF/DREB1/DREB2 (Jaglo-Ottosen et al., 1998; Liu et al., 1998), ABF (Choi et al., 2000), CAMTA (Finkler et al., 2007), and TCP transcription factor (Martín-Trillo and Cubas, 2010) families, respectively. We searched our upregulated gene list to test whether any of the members of these transcription factor families were themselves regulated transcriptionally by calcium. The gene encoding one TCP family member (TCP13) was slightly upregulated (1.78-fold), but none of the ABF, CBF/DREB1/DREB2, or CAMTA families were upregulated. These transcription factors may therefore be regulated by $[Ca^{2+}]_c$ on a posttranslational level. In some cases, potential pathways for posttranslational regulation are already evident; for example, the Ca^{2+}-binding protein CaM regulates CAMTAs, and ABF4 has been shown to interact with CPK32, one of the calcium-dependant kinases (Choi et al., 2005). Future research effort should therefore be directed toward revealing the precise molecular pathways by which calcium regulates these transcription factors and their interaction with these motifs in target gene promoters.

METHODS

Vector Construction

The LUC+ coding region was obtained from pGL3 (Groskreutz et al., 1995) and was cloned into pDH51 (Pietrzak et al., 1986) using *Sma*I and *Xba*I restriction sites to produce the construct pDHLC+1, which contained the LUC+ coding region between the 35S promoter and terminator. *Nco*I was then used to delete the region between the start of the 35S promoter and the start codon of LUC+ (which was within the *Nco*I restriction site). This gave a promoterless LUC+ construct, pDHLC+2, into which *Nco*I-*Nco*I promoter fragments could be cloned. Promoter fragments containing *cis*-element sequences were produced by PCR amplification from pDHLC+1. Forward primers containing an *Nco*I site and four repeats of the consensus *cis*-element sequence were designed to anneal to a region 70 bp upstream of the 3′ end of the 35S promoter. The reverse primer annealed to the *Nco*I site at the start codon of LUC+. The primer sequences can be found in Supplemental Table 3 online. The PCR products were cloned as *Nco*I-*Nco*I fragments into pDHLC+2. The whole chimeric cassettes were then cloned as *Eco*R1-*Eco*R1 fragments into the *Eco*R1 site of pBIN19 (Bevan, 1984) for stable expression in plants.

Plant Materials, Growth Conditions, and Transgenic Plant Construction

Arabidopsis thaliana ecotype Columbia (Col-0) and Col-0 ecotype seeds constitutively expressing apoaequorin under the 35S promoter were obtained from Lehle Seeds. Sterilized *Arabidopsis* seeds were sown on solid agar germination medium consisting of 0.8% (w/v) plant tissue culture grade agar supplemented with 1× Murashige and Skoog (MS) medium (Melford Laboratories) and an appropriate antibiotic if required. Sterilized seeds on MS plates were stratified for at least 48 h in darkness at 4°C. Plates were subsequently transferred to a growth chamber at a constant temperature of 21°C, with a 16-h photoperiod at a light intensity of 60 $\mu M\ m^{-2}\ s^{-1}$. Plants for seed production were transferred at 7 to 14 d old to peat and were moved to a greenhouse at 21°C with a 16-h photoperiod. Aracon tubes (BetaTech) were used to isolate individual mature plants. Plants were watered regularly until the siliques had fully developed, then were transferred to a drying room until dried to a sufficient level for seed collection. For plant transformation, binary vectors based on pBIN19-containing chimeric concatamer constructs were introduced into *Agrobacterium tumefaciens* C58C1 and transformed into Col-0 using the floral dip method (Clough and Bent, 1998). Primary T1 transformants were identified by kanamycin (50 mg/L) selection agar as described above, and subsequent analyses were performed on the T2 generation. LUC expression in the T2 generation was tested by imaging ~20 seedlings using a photon-counting camera. Expression levels varied by approximately one order of magnitude, because of positional effects. For each construct, lines were chosen that showed median levels of expression and had single insertions (as determined by segregation of LUC activity). Genomic DNA was extracted, and the transgenic was sequenced to confirm identity of construct.

Production of $[Ca^{2+}]_c$ Elevations by Application of Electrical Stimulation

Seedlings were floated in standard media (0.1 mM each of KCl, $CaCl_2$, $MgCl_2$) in a 1 cm × 10 cm trough with platinum electrodes. The cuvette was placed in a dark box and was allowed to rest for 2 to 4 h before treatment (to avoid any touch-induced calcium elevations close to the time of treatment). Voltage was applied via a PCL-728 Isolated 2-Channel Digital Analog Output Card (Advantech) inside a personal computer, controlled by the software Visidaq (Advantech). A program was written for the application of voltage in square wave format to allow manipulation of the variables ON time, OFF time, and amplitude. Because the maximum range of the PCL-728 was ± 10 V, the voltage was enhanced by connection through a fivefold voltage amplifier (generated by electronics workshop, Department of Plant Sciences, University of Oxford) when required. Specific regimens for each treatment were as follows: Transient calcium elevation; 40 V for 1.5 s. Oscillations; voltage was applied for 1 s every 40 s at increasing intensities, specifically 10 V, 12 V, 14 V, 15 V, 16.5 V, 17.5 V, 19.5 V, 21 V, 22.5 V, 24 V. Prolonged calcium elevation; initially, 2.5 V was applied for 100 ms every 1 s, then voltage was rapidly increased to account for attenuation. When the maximum voltage of 10 V was reached, the "ON" time of 100 ms was increased by 10-ms increments to further compensate for attenuation. Controls were not stimulated by voltage but were harvested alongside the treated samples.

Chemical and Cold Treatments

For all chemical treatments, plants were floated on water overnight, before chemical treatments the next morning. When $LaCl_3$ was used to inhibit $[Ca^{2+}]_c$ elevations by DMSO and mastoparan, it was added to

seedlings for a pretreatment period of 30 min. For measurement of $[Ca^{2+}]_c$ during chemical treatment for luminometry, double concentration of the chemical was added to the seedlings during measurement, giving a final concentration of 10 μM mastoparan and 2% w/v DMSO. For transcript analysis, seedlings were treated as above, and tissue was harvested by briefly drying on tissue paper and flash freezing in liquid nitrogen 3 h after treatment. For all experiments involving concatemer LUC+ fusions, experiments were performed in the T2 generation of at least two independent validated transgenic lines. For testing expression of LUC+ in response to W7, we followed the conditions described previously (Kaplan et al., 2006), namely 15 min preincubation with 0.5 mM $LaCl_3$, followed by 1 h treatment in 600 μM W7 or equivalent 0.3% DMSO control. For cold treatments, seedlings were transferred to a growth chamber at 5°C and were maintained there for 24 h, and (ambient) control samples were retained at 20°C under matched light conditions. Plants were harvested promptly after 24 h.

In Vivo Reconstitution of Aequorin and $[Ca^{2+}]_c$-Dependent Luminescence Measurements

To reconstitute aequorin before calcium imaging, seedlings expressing cytosolic apoaequorin were reconstituted by floating on water containing 10 μM coelenterazine (stock 1% [v/v] methanol; LUX Biotechnology) in the dark at 21°C for 12 to 24 h. For $[Ca^{2+}]_c$ measurements during chemical treatments, seedlings were transferred to a cuvette that was placed inside a digital chemiluminometer consisting of a 9829A photomultiplier tube powered by a PM28B high-voltage supply and cooled to −25°C with FACT50 air-cooled thermoelectric housing and an AD2 amplifier/discriminator (all from Thorn EMI). Output from the AD2 was channeled via a CTI computer counter board (Thorn EMI) to produce numerical output to a personal computer. Measurements of aequorin luminescence were taken every 1 s. To convert luminescence into $[Ca^{2+}]_c$, 2 M $CaCl_2$ and 30% ethanol were added to discharge the remaining aequorin. For $[Ca^{2+}]_c$ imaging during electrical stimulation, aequorin luminescence was recorded under a three microchannel plate-intensified charge-coupled device camera (Photek 216; Photek). The quantity of photons per unit area over time was measured by the camera, and the resulting cumulative image was established and stored by the acquisition and processing software (Image 32; Photek). Total aequorin for calibration was measured by removing the seedling(s) to a piece of wet filter paper (Grade 1; Whatman) and treating them with cold on the Peltier element as follows: the temperature was decreased to 0°C until aequorin luminescence returned to basal level, then was reduced to −20°C. When luminescence again decreased to a minimum, the temperature was increased up to room temperature. The aequorin luminescence measured during treatments was calibrated into $[Ca^{2+}]_c$ as described previously (Knight and Knight, 2000). For transcript analysis, tissue was harvested by briefly drying on tissue paper and flash freezing in liquid nitrogen 1 h after treatment.

Quantitative Real-Time PCR

A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to reverse transcribe cDNA from 1.5 μg total RNA extracted using Qiagen RNeasy plant mini kit (Qiagen) in conjunction with RNase-free DNase (Qiagen) to remove any genomic DNA contamination. Quantitative real-time PCR was performed on 6 μL of 1:50 diluted cDNA in a 15-μL reaction using an Applied Biosystems 7300 system. Relative levels of LUC+ transcript were measured using a custom-made gene-specific TaqMan Probe prepared by Applied Biosystems to the following specifications: forward primer, TGGGCTCACTGAGACTACATCA; reverse primer, CGCGCCCGGTTTATCATC; 6-carboxy-fluorescein–labeled probe, CCCCTCGGGTGTAATC; and expression levels were normalized to the expression of an endogenous control gene, *PEX4* (At4g25760; Applied Biosystems probe identifier At02304594_g1). Reactions were performed in an optical 96-well plate (Starlab) with three technical replicates for each sample. Gene-specific primer pairs were used with Fast Start SYBR Green Master with ROX (Roche Diagnostics) to measure relative expression of At1g02400 (forward, CCCATCTGACCCTACATGCT; reverse, TGGCTTCTTTGCTGTGTTTG), At1g01470 (forward, ATCAAGAGCCGTCATGTCCT; reverse, TCATTCGATTCCGATCTGTG) and At1g51090 (forward, CTATCCGCAAATTCCCTCAA; reverse, GCCTCTCAGGATCGTAACAAA), and expression levels were normalized to expression of *PEX4* (forward, TCATAGCATTGATGGCTCATCCT; reverse, ACCCTCTCACATCACCAGATCTTAG). In all cases, relative quantitation was performed by the $\Delta\Delta C_T$ (comparative cycle threshold) method (Livak and Schmittgen, 2001), and relative quantitation (RQ) values and estimates of statistical variation for each sample were calculated as described previously (Knight et al., 2009). The algorithm used is described in Relative Quantitation (RQ) Algorithms in Applied Biosystems Real-Time PCR Systems Software (Applied Biosystems Real-Time PCR Systems, 2007).

Preparation of Microarray Slides

Microarray slides were obtained from Dr. David Galbraith (University of Arizona, Tucson, AZ) and were printed with the Operon *Arabidopsis* Version 3 Array-Ready Oligo Set. The slides were baked for 40 min at 80°C and were immediately UV cross-linked twice in a Stratalinker 2400 (Stratagene) at 300 mJ. Prior to use, slides were prehybridized for 20 min at 65°C in a coplin jar containing 3.5× SSC, 0.1% SDS, and 10 mg/mL BSA. After prehybridization, slides were washed with shaking for 1 min in water and then for 1 min in isopropyl alcohol before drying with an airbrush (Paasche Airbrush Company).

Microarray Analysis

RNA was extracted using the RNeasy Plant Total RNA kit (Qiagen), and the quality of RNA samples was assessed using an Agilent 2100 Bioanalyser (Agilent Technologies). Between 1 and 2 μg total RNA was labeled using reverse transcriptase Superscript III (Invitrogen) and the Genisphere 3DNA 900 indirect labeling kit (Genisphere). cDNA was transferred evenly across the microarray using a "LifterSlip" (Erie Scientific Company), and hybridization was performed using an Advalytix SlideBooster SB400 (Advalytix AG) with a power of 27 and a pulse:pause ratio of 3:7 at 55°C for 16 h. After washing, the slides were hybridized in a similar way for a second time with the 3DNA dendrimer capture reagents supplied with the kit for 4 h, according to the manufacturer's instructions. After each hybridization, the microarrays were washed at 55°C in a solution of 2× SSC/0.2% SDS followed by room-temperature washes of 2× SSC and 0.2× SSC all for 10 min with shaking at 150 rpm. After the final wash, the slide was dried using an airbrush (Paasche Airbrush Company). Hybridized slides were scanned using a Perkin Elmer ScanArray Express HT (Perkin Elmer Wellesley) using 100% laser power and a variable photomultiplier tube setting determined by automatic sensitivity calibration with a signal target ratio of 98%.

Data Extraction

The resulting image files were transferred into the analysis program BlueFuse Version 3.0 or 3.2 (BlueGnome). Spots with insufficient signal above background and artifacts were removed from the data set both by manual flagging and by automatic exclusion of spots below a certain threshold (chosen manually) of an empirically determined BlueFuse "pON" score. The pON score is a measure of the probability of there being a hybridization signal for each spot independently of the other

channel. This score takes into consideration both the intensity of signal above background and the circularity and uniformity expected of a genuine microarray spot (Snyder and Saunders, 2006).

Data Normalization and Analysis Using BASE

Data output from Bluefuse were modified into a fused file format, which could be recognized by the BASE tool (Saal et al., 2002). The cross-channel correction tool (developed by the CBRG) was used to correct the intensities of spots with a one-channel bias by subtracting 2% intensity of channel one from channel two or vice versa (with a floor intensity of 200). Data were globally normalized to the median fold ratio of the central 60% of data, which had an intensity of >200 in both channels. The overall median fold ratio for the combined normalized data from biological replicates was determined using the fold change algorithm within BASE Statistical tests were performed using a local implementation of Cyber-T within BASE (using a sliding window size of 101 and a Bayes confidence estimate value of three times the number of slides in the experiment to a maximum of 10). The BASE plug-ins for cross channel correction, normalization, and Cyber-T test are available on request from the CBRG (http://www.molbiol.ox.ac.uk/CBRG_home.shtml).

Promoter Motif Analysis

Analysis of overrepresented hexamer sequences in 500 bp of upstream sequences (downloaded from The Arabidopsis Information Resource website; http://www.*Arabidopsis*.org/tools/bulk/index.jsp) was performed using the oligo analysis, pattern assembly, and DNA pattern matching tools available online at the RSAT site (http://rsat.ulb.ac.be/rsat) according to the developers' instructions (van Helden, 2003). Positional analysis of hexamer sequences was achieved in a similar way by downloading 500 bp of upstream sequences from The Arabidopsis Information Resource and transferring these to the pattern matching tool in RSAT.

Ontological Analysis

Gene lists were functionally classified using the Bio-Array Resource for Plant Biology Classification Superviewer (http://bar.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi), which uses Gene Ontology data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in *Arabidopsis* and assigns a P-value according to the strength of the enrichment. For details of normalized frequency and P-value calculation, please see the header on individual output Tables (see Supplemental Data Sets 2 and 3 online)

Comparison of Gene Lists with Existing Microarray Data

The list of 269 $[Ca^{2+}]_c$-regulated genes and four sublists representing genes containing either CRT/DRE (39 genes), ABRE (63 genes), CaM Box (20 genes), or Site II (97 genes) were compared with the AtGenExpress abiotic and biotic stress treatments (Kilian et al., 2007) using the Bio-Array Resource Expression browser (Toufighi et al., 2005) (http://bar.utoronto.ca/affydb/cgi-bin/affy_db_exprss_browser_in.cgi). Using this tool, we extracted the expression of genes from our lists that had corresponding data in the AtGenExpress series in response to specific abiotic and biotic stresses. Average fold values of expression for each treatment were calculated for each list.

Accession Numbers

The microarray experiments described herein are publicly available from www.ebi.ac.uk/arrayexpress with accession numbers E-MEXP-2659 (oscillations and prolonged calcium elevations) and E-MEXP-2661 (transient calcium elevation).

Supplemental Data

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

Supplemental Figure 1. RSAT Alignments of Hexamer Sequences Relating to the ABRE and CAM Box *cis*-Element Sequences.

Supplemental Figure 2. Positional Analysis of Promoter Motifs Upstream of Genes Responding to $[Ca^{2+}]_c$ Elevations.

Supplemental Figure 3. Association Analysis of Motifs Enriched in Upstream Regions of Genes Responding to $[Ca^{2+}]_c$ Elevations.

Supplemental Figure 4. Effect of Cold Treatment on ABRE-, CAM box–, CRT-, and Site II–*LUC*+ Expression.

Supplemental Table 1. Comparison of Genes Upregulated by $[Ca^{2+}]_c$ Elevations with Publicly Available AtGenExpress Data—Abiotic Stress.

Supplemental Table 2. Comparison of Genes Upregulated by $[Ca^{2+}]_c$ Elevations with Publicly Available AtGenExpress Data—Biotic Stress.

Supplemental Table 3. Primers for Production of Promoter Fragments Used in Our Study.

Supplemental Data Set 1. Genes Significantly Changed in Microarray Experiments.

Supplemental Data Set 2. Ontological Analysis of Up- and Down-regulated Combined Gene Lists.

Supplemental Data Set 3. Ontological Analysis of Gene Lists Containing Each of Four $[Ca^{2+}]_c$-Regulated Promoter Motifs.

Supplemental Data Set 4. Comparison of Gene Lists Containing Each of Four $[Ca^{2+}]_c$-Regulated Promoter Motifs with Publicly Available AtGenExpress Data for Abiotic and Biotic Stress Experiments.

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AUTHOR CONTRIBUTIONS

H.J.W. designed and performed microarray experiments and performed all microarray analyses, produced basic concatemer constructs, and wrote substantial parts of the article. A.W.S. produced transgenic plants expressing concatemer constructs and performed some of the real-time experiments. J.F.C.S. performed most of the real-time experiments. T.L. produced the binary constructs to express the concatemers in transgenic plants. R.L. performed some of the real-time experiments. N.J.S. developed the system for the dendrimer-based microarray experiments and optimised microarray analysis. H.K. performed some of the real-time experiments and wrote parts of the article. M.R.K. designed experiments and wrote substantial parts of the article.

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