Transcriptomic Analysis Reveals Calcium Regulation of Specific Promoter Motifs in *Arabidopsis* *a*

Helen J. Whalley, b,1 Alexander W. Sargeant, b John F.C. Steele, b Tim Lacoere, b Rebecca Lamb, b Nigel J. Saunders, c,2 Heather Knight, b and Marc R. Knight b,3

a Department of Plant Sciences, University of Oxford, Oxford OX1 3RB, United Kingdom  

b Plant Stress Lab, Durham Centre for Crop Improvement Technology, School of Biological and Biomedical Sciences, Durham University, Durham DH1 3LE, United Kingdom  

c Sir William Dunn School of Pathology, University of Oxford, Oxford OX1 3RE, United Kingdom

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., 2008; Yan et al., 2009; Zhou et al., 2009) and root hair cells (Bibikova et al., 1997; Very 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 (Chiaison 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,
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., 1998; McAinsh and Pittman, 2009) has confirmed as being calcium-regulated by empirical testing in planta. The implications of our findings, which broaden the picture on regulation of calcium-responsive promoter motifs, which we have confirmed as being calcium-regulated, are reviewed in the discussion.

RESULTS

Controlled Electrical Stimulations Elicit Specific Types of [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]c, which we could subsequently use to study calcium-regulated gene expression. We were successful in stimulating [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]c (Figure 1A), a series of transient elevations in [Ca²⁺]c (Figure 1B), or to continually elevate [Ca²⁺]c for a prolonged period (Figure 1C).

Imposed [Ca²⁺]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 [Ca²⁺]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
Calcium Regulation of Gene Expression

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\(^{2+}\)]\(_c\) (±SD) mobilized for each treatment (minus the area of the control treatment) was (A) 18.05 ± 1.42 μM, (B) 40.46 ± 3.04 μM, and (C) 39.66 ± 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 x 10\(^{-18}\)) and “response to abiotic or biotic stimulus” (3.46-fold, P = 6.06 x 10\(^{-4}\)). 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...
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, 1995).

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

<table>
<thead>
<tr>
<th>Experiment (Total No. of Slides in BASE Experiment)</th>
<th>Upregulated</th>
<th>Downregulated</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transient (2)</td>
<td>104</td>
<td>30</td>
<td>134</td>
</tr>
<tr>
<td>Oscillations (2)</td>
<td>256</td>
<td>97</td>
<td>353</td>
</tr>
<tr>
<td>Prolonged (2)</td>
<td>10</td>
<td>17</td>
<td>27</td>
</tr>
<tr>
<td>Oscillations and transient combined (4)</td>
<td>269</td>
<td>94</td>
<td>363</td>
</tr>
</tbody>
</table>

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.

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

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Identifier</th>
<th>Occurrence</th>
<th>Expected Occurrence</th>
<th>P-Value</th>
<th>E-Value</th>
<th>Z Score</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>acacgt(^a)</td>
<td>acacgt</td>
<td>105</td>
<td>48.09</td>
<td>8.9e-13</td>
<td>1.9e-09</td>
<td>8.21</td>
<td>2.18</td>
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<td>gcgcga(^b)</td>
<td>gcgcgcgtgcgc</td>
<td>97</td>
<td>47.84</td>
<td>2.9e-10</td>
<td>6.0e-07</td>
<td>7.11</td>
<td>2.03</td>
</tr>
<tr>
<td>aacgcg(^c)</td>
<td>aacgctaacgcg</td>
<td>38</td>
<td>13.01</td>
<td>1.4e-08</td>
<td>2.9e-05</td>
<td>6.93</td>
<td>2.92</td>
</tr>
<tr>
<td>acgcgg(^d)</td>
<td>acgcgcgtgcgc</td>
<td>29</td>
<td>8.50</td>
<td>2.9e-08</td>
<td>6.0e-05</td>
<td>7.03</td>
<td>3.41</td>
</tr>
<tr>
<td>gcgcgg(^e)</td>
<td>gcgcgcgtgcgc</td>
<td>35</td>
<td>13.04</td>
<td>3.6e-07</td>
<td>7.4e-04</td>
<td>6.08</td>
<td>2.68</td>
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<td>aaccg(^f)</td>
<td>aaccggtgcgc</td>
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<td>22.78</td>
<td>5.7e-07</td>
<td>1.2e-03</td>
<td>5.70</td>
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<td>70</td>
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<td>4.0e-03</td>
<td>5.22</td>
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<td>acgcgtgtgcgc</td>
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<td>3.1e-05</td>
<td>6.4e-02</td>
<td>4.63</td>
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<td>4.63</td>
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<td>2.0e-01</td>
<td>4.02</td>
<td>1.47</td>
</tr>
</tbody>
</table>

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^{-5}. 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.
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 [DREBs]) and DREB2s (Jaglo-Ottosen et al., 1998; Liu et al., 1998). The sequence AGGGCCCAT (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). As 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 shown experimentally to bind to the CRT cis-element or its transcription factor (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 shown experimentally to bind to the CRT cis-element or its transcription factor (see Supplemental Data Set 4 online).
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\(^{2+}\)]\(_i\). For these experiments, we decided to use DMSO and mastoparan, chemical agents previously shown to increase [Ca\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\) through activation of PLC (Perianin and Snyderman, 1989). Although the mechanism of the [Ca\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\) elevations in our experimental system, Arabidopsis seedlings, we tested their effects by measuring [Ca\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\) elevations were capable of triggering the expression of [Ca\(^{2+}\)]\(_i\)-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\(^{2+}\)]\(_i\) 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).

### cis-Element Responses to Mastoparan Treatment Are [Ca\(^{2+}\)]\(_i\)-Dependent

The response of the cis-elements to treatments with two different calcium agonists strongly indicated that they were responsive to [Ca\(^{2+}\)]\(_i\) elevations in the absence of physiological stimuli. However, to confirm that their induction was due to the [Ca\(^{2+}\)]\(_i\) elevation specifically, it is necessary to control for other calcium-independent effects of the calcium agonists. By blocking the [Ca\(^{2+}\)]\(_i\) elevation induced by an agonist, one can assess whether changes in [Ca\(^{2+}\)]\(_i\), 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\(^{2+}\)]\(_i\) (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\(^{2+}\)]\(_i\) elevation while minimizing the effect of LaCl\(_3\) alone on [Ca\(^{2+}\)]\(_i\). Figure 6A shows that lanthanum treatment provoked a [Ca\(^{2+}\)]\(_i\) increase in a dose-dependent manner. Lanthanum concentrations of 5 and 10 mM gave [Ca\(^{2+}\)]\(_i\) elevations that were noticeably higher than at lower concentrations. There was no significant difference between the [Ca\(^{2+}\)]\(_i\) elevations produced by LaCl\(_3\) concentrations of 0.1 to 1 mM (Figure 6A). Inhibition of mastoparan-induced [Ca\(^{2+}\)]\(_i\) 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\(^{2+}\)]\(_i\) 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

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**Figure 3. Schematic of Constructs Used for Concatemer Studies.**

The promoter region between the two Ncol sites was produced by PCR amplification. The Ncol-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 Ncol site). The promoter-LUC+ cassette was then cloned into the EcoRI 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.
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 \([\text{Ca}^{2+}]_c\)-dependent.

Finally, because the Site II and CAM box had not been previously demonstrated to be activated by \([\text{Ca}^{2+}]_c\) 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 \([\text{Ca}^{2+}]_c\) 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 \([\text{Ca}^{2+}]_c\) elevation in combination with LaCl3 pretreatment, as in Kaplan et al. (2006). Figure 8 shows that Site II was clearly and
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; Tålhind 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., 2010), 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} 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...
elevations to produce different $[\text{Ca}^{2+}]_c$ profiles dealt with all four combinations of
three different electrically induced $[\text{Ca}^{2+}]_c$ elevations, as well as 87 microRNA gene precursors), and applied dendrimer technology to produce data with greater sensitivity and depth. 29,110 70-mer oligonucleotides representing 26,173 protein-encoding genes and 28,964 protein-encoding gene transcripts, as well as 87 microRNA gene precursors, were 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$, would make interesting future studies. The most potent $[\text{Ca}^{2+}]_c$ elevation of all was the oscillations, which resulted in increased transcript levels for 256 genes ($\approx 2.5$ times more than the transient $[\text{Ca}^{2+}]_c$ elevation and 25 times more than the prolonged $[\text{Ca}^{2+}]_c$ elevation). This is despite the total $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ oscillations (Allen et al., 2001; Sun et al., 2007).

Although we were not attempting to mimic any natural $[\text{Ca}^{2+}]_c$ responses, the three profiles we generated have similarity to $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$-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 $[\text{Ca}^{2+}]_c$ elevations to those produced by these types of stresses.

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

By combining the transient and oscillating $[\text{Ca}^{2+}]_c$ 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 $[\text{Ca}^{2+}]_c$ elevations. By analyzing the promoter sequences of this combined list of genes induced by our electrical impulses, we found four distinct classes...
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 (Tremousaygue et al., 2003). The ABRE (CACGTGT) and CAM box (ACGCGT) completely fit the consensus of the ABRE ([C/A]ACGCG[T/C/A]) 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+}] signatures generated by the W7 treatment used, whereas they are sensitive to DMSO- and mastoparan-induced [Ca^{2+}] 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 (Tätährju et al., 1997). The Site II element, however, showed substantial induction in response to W7 treatment (Figure 8); it is therefore 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 7. ABRE-, CAM Box-, CRT-, and Site II-LUC+ Expression Is [Ca^{2+}]-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 LaCl3 (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.
expression values for PEX4 using real-time PCR are shown. LUC+ expression was normalized to harvested 3 h after treatment. RQ values for LUC+ expression measured control (mock) or W7 (to a final concentration of 600 mean of three technical replicates. Error bars indicate RQ MIN and RQMAX, without monitoring the effect of Ca²⁺ antagonists on this re-
et al., 2006), it was only tested in response to calcium agonists; activation of the ABRE was shown in our previous study (Kaplan 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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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 [Ca²⁺]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., 1986; Grant et al., 2000). The CRT element is also induced in response to abscisic acid (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, 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, 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-dependent 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 Smal and XbaI restriction sites to produce the construct pDHLC+1, which contained the LUC+ coding region between the 35S promoter and terminator. Ncol was then used to delete the region between the start of the 35S promoter and the start codon of LUC+ (which was within the Ncol restriction site). This gave a promoterless LUC+ construct, pDHLC+2, into which Ncol-Ncol promoter fragments could be cloned. Promoter fragments containing cis-element sequences were produced by PCR amplification from pDHLC+1. Forward primers containing an Ncol 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 Ncol site at the start codon of LUC+. The primer sequences can be found in Supplemental Table 3 online. The PCR products were cloned as Ncol-Ncol fragments into pDHLC+2. The whole chimeric cassettes were then cloned as EcoR1-EcoR1 fragments into the EcoR1 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 aequorin 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 µM 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. Aracan 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 \([\text{Ca}^{2+}]_c\) during chemical treatment for luminometry, double concentration of the chemical was added to the seedlings during measurement, giving a final concentration of 10 \(\mu\text{M}\) 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 concanamer 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 LaCl3, followed by 1 h treatment in 600 \(\mu\text{M}\) 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 \([\text{Ca}^{2+}]_c\)-Dependent Luminescence Measurements**

To reconstitute aequorin before calcium imaging, seedlings expressing cytosolic apoaequorin were reconstituted by floating on water containing 10 \(\mu\text{M}\) coelenterazine (stock 1% [v/v] methanol; LUX Biotechnology) in the dark at 21°C for 12 to 24 h. For \([\text{Ca}^{2+}]_c\), 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 \([\text{Ca}^{2+}]_c\), 2 M CaCl2 and 30% ethanol were added to discharge the remaining aequorin. For \([\text{Ca}^{2+}]_c\), 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 Pettr filter 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 \([\text{Ca}^{2+}]_c\), 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 \(\mu\text{g}\) 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 \(\mu\text{L}\) of 1:50 diluted cDNA in a 2-\(\mu\text{L}\) 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, TGGCGCTCACTGAGACTACATCA; reverse primer, CGGGCCGGTGTATCATC; 6-carboxy-fluorescein–labeled probe, CCCCTCGGGTGTAATC; and expression levels were normalized to the expression of an endogenous control gene, \(\text{PEX}_4\) (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 \(\text{At1g02400}\) (forward, CCCATGACCCCTATCATG; reverse, TGGCCTTTTGTGCTGTGGT), \(\text{At1g01470}\) (forward, ATCAAGAGCCGTCATGTCC; reverse, TCATTGATTCCGACTGTG) and \(\text{At1g51090}\) (forward, CTATCCGGAAATCCCTCA; reverse, GCTCTGAGGATGTAAACAA), and expression levels were normalized to expression of \(\text{PEX}_4\) (forward, CATAGATGTCGTCATCTC; reverse, ACCCTCTCATCATTACAGGATC). 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 Stratagene 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 isopropl 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 Bioanalyzer (Agilent Technologies). Between 1 and 2 \(\mu\text{g}\) total RNA was labeled using reverse transcriptase Superscript III (Invitrogen) and the Gemisphere 3DNA 900 indirect labeling kit (Gemisphere). 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 dendraimer 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 96%.

**Data Extraction**

The resulting image files were transferred into the analysis program BlueFuse Version 3.0 or 3.2 (BlueGene). 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 Supervisor (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 the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency in the Arabidopsis data to calculate the fold enrichment to particular gene classifications of the input gene list compared with the expected frequency

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 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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