

## Quantitative Analysis of *Cis*-Regulatory Element Activity Using Synthetic Promoters in Transgenic Plants

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### Abstract

Synthetic promoters, introduced stably or transiently into plants, are an invaluable tool for the identification of functional regulatory elements and the corresponding transcription factor(s) that regulate the amplitude, spatial distribution, and temporal patterns of gene expression. Here, we present a protocol describing the steps required to identify and characterize putative *cis*-regulatory elements. These steps include application of computational tools to identify putative elements, construction of a synthetic promoter upstream of *luciferase*, identification of transcription factors that regulate the element, testing the functionality of the element introduced transiently and/or stably into the species of interest followed by high-throughput *luciferase* screening assays, and subsequent data processing and statistical analysis.

**Key words** *Cis*-regulatory element, Synthetic promoter, *Luciferase* reporter, Stable transformation, Transient transformation

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### 1 Introduction

Identification and characterization of promoter regulatory elements is critical for understanding how cells control the timing, spatial patterning, and levels of gene expression, thereby facilitating the regulation of complex signaling and metabolic networks. As such, this endeavor continues to attract much attention, as it has since the discovery of functional promoter elements [1]. In plants, studies of promoter elements were initially carried out by construction of promoter fragments fused to reporter genes, such as chloramphenicol acetyltransferase (CAT) or  $\beta$ -glucuronidase (GUS), as a proxy for sequence activity [2–5]. The resolution of this approach was subsequently increased through random mutagenesis, or linker scan of the natural promoter, whereby a small (~5–12 bp) fragment of the promoter of interest is either randomly mutagenized or replaced with a linker sequence [6, 7]. While the identification of regulatory elements via analysis of promoter fragments remains viable, this approach is inherently lengthy and

cumbersome. This inefficiency, however, has been addressed by the recent development of a wide variety of computational approaches centered on comparison of promoter sequences, in combination with co-expression analyses, resulting in the rapid discovery of putative regulatory elements [8–11]. Functional verification of a putative *cis*-regulatory element can subsequently be carried out by construction of a synthetic promoter consisting of one to several copies of the element fused to a minimal promoter and reporter gene, followed by introduction of the construct in planta [11–14]. Many studies utilize constructs consisting of these plant synthetic promoters fused to the GUS reporter to examine the activity of putative regulatory elements [12, 14]. However, the inherent stability of GUS limits these analyses of element functionality to single time-point comparisons of levels and spatial patterns of expression, thus precluding detailed profiling of temporal changes in activity. Development of the luciferase reporter system [15] addressed this deficiency and enabled exquisitely detailed time course analysis of synthetic promoter activity in response to signals induced by a wide range of inputs including circadian rhythms, stress treatments, and chemical stimuli [11, 13, 16–18]. In addition, the employment of transient assays using synthetic promoters expanded the analytical capability for testing the functionality of specific transcriptional regulator(s) in controlling the activity of the *cis*-regulatory element [17].

Here we provide a summary of these combinatorial approaches (Fig. 1), which were instrumental in the recognition and characterization of the Rapid Stress Response Element (RSRE) as a general-stress-responsive *cis*-regulatory element [11], and the further elucidation of the non-uniform contribution of different members of the Calmodulin-Binding Transcriptional Activator (CAMTA) family in regulation of this functional response element [17].

## 2 Materials

All solutions should be prepared with ultrapure water (i.e. MilliQ).

### 2.1 Stable Transformation of *Arabidopsis thaliana*

1. Luria Broth (LB): 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, adjust pH to 7.0 with NaOH. Autoclave at 121 °C for 20–35 min and cool to at least 55 °C before adding antibiotics (*see* **Note 1**).

**Fig. 1** (continued) *4xRSRE:Luciferase* (*4xRSRE:LUC*) reporter construct. (**4a**) Image of luciferase activity in *Arabidopsis* plants stably expressing the *4xRSRE:LUC* construct. (**4b**) Image of luciferase activity in an *N. benthamiana* leaf that has been transiently transformed with the *4xRSRE:LUC* reporter construct and its transcriptional activator CAMTA3. (**5**) Graph showing time course of *LUC* activity in response to wounding in *Arabidopsis* plants stably transformed with the *4xRSRE:LUC* reporter construct

### 1. ID of regulatory elements by comparative promoter sequence analysis.

Motif 6	
CGCGTT	$p = 1.94\text{e-}07$
CGCGT	$p = 7.70\text{e-}05$
GCGCGT	$p = 3.48\text{e-}03$
CCGCGT	$p = 3.32\text{e-}04$

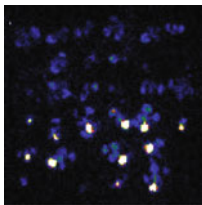
### 2. Selection of core element by statistical analysis and database searches.



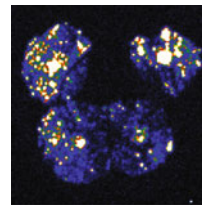
### 3. Creation of synthetic promoter:luciferase reporter construct.



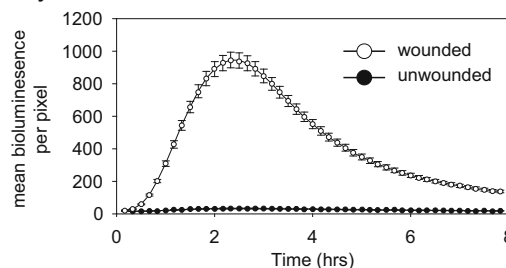
#### 4a. Stable transformation of synthetic promoter:luciferase construct into *A. thaliana*.



#### 4b. Transient transformation of the synthetic promoter:luciferase construct into *N. benthamiana*.



### 5. High-throughput screening in CCD camera, followed by data processing and statistical analysis.



**Fig. 1** Summary of protocol. Here, we summarize the steps required for identification of putative *cis*-regulatory elements and quantification of their activity in transient or stably transformed plant species. (1) Sample motif discovery readout. (2) Web Logo depiction of binding site of a TF, CAMTA3. (3) Simplified schematic of the

2. Sucrose solution: 5 % (w/v) solution in water. Add silwet L-77 to 0.05 % just prior to transformation of plants.
3. SOC media: 0.5 % yeast extract, 2 % tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose. Add all ingredients except the glucose and autoclave at 121 °C for 20–35 min and cool to at least 55 °C. Then, using sterile technique, add filter-sterilized glucose to 20 mM.

## 2.2 *Nicotiana benthamiana* Transient Assay

1. Luria Broth (LB): *see* [Subheading 2.1](#).
2. Infiltration buffer: 50 mM 2-(N-morpholino) ethanesulfonic acid hydrate (MES) pH 5.6, 100 μM acetosyringone, 2 mM Na<sub>3</sub>PO<sub>4</sub> (dodecahydrate), 0.5 % glucose.
3. SOC media: *see* [Subheading 2.1](#).

## 2.3 Imaging

1. MS media (½ strength MS with 0.8 % phytoagar): 2.22 g/L Murishige and Skoog (MS) basal medium, adjust pH to 5.7 using NaOH (1 M or lower). Add 0.8 % phytoagar and autoclave for 20–30 min at 121 °C. Allow solution to cool to ~60 °C and then pour into plates (100 mm × 100 mm × 15 mm) to a depth of 5–6 mm. 1 L MS media is sufficient for preparation of 30 plates.
2. Luciferin working solution: 1 mM luciferin, 0.01 % Tween 20. Solution may be prepared in advance and kept at 4 °C for up to 1 month.
3. Imaging system: Andor DU-484BV charge-coupled device (CCD) camera with Andor Solis software (v15). The camera is affixed to a light-tight box. Other imaging systems and software may be substituted.

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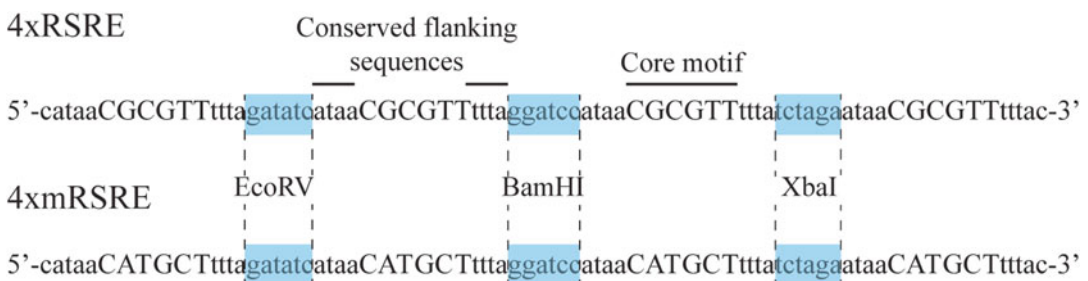
# 3 Methods

## 3.1 Design of the Synthetic Promoter

1. Identify a list of genes from which to identify over-represented promoter motifs—for example a set of genes induced in response to a specific treatment, a set of genes mis-regulated in a mutant, or a set of genes commonly induced by a set of related stimuli.
2. Obtain the promoter sequences of the genes (*see* [Note 2](#)).
3. Perform motif discovery analyses on the set of promoter sequences. Paste promoter sequences into motif discovery tool(s) and analyze for over-represented sequence motifs (*see* [Note 3](#)).
4. Select a motif for further analysis. Selection should be based on the *p*-values derived from different motif discovery tools for a given element. Elements with strongly significant *p*-values are more likely to contribute to the control of gene expression in the conditions under study. Motifs with high scores in the

discovery analysis should also be queried against both the scientific literature and specialized *cis*-element databases, in order to assess whether they are novel motifs or have been previously identified (*see* **Note 4**).

5. Identify which promoters in the input data set contain the sequence of the chosen motif, then acquire the 10 bp of flanking sequences on either side of each instance of the element. If the number of promoters is small, this may be done manually by searching for the motif and copying and pasting the sequences into a FASTA-formatted file (for larger promoter sets, *see* **Note 5**).
6. Paste the FASTA file containing the core and flanking sequences into an alignment and visualization tool, such as weblogo (<http://weblogo.berkeley.edu/logo.cgi>) [19]. To design the final sequence employed in further analyses, assess the consensus among the flanking sequences to specifically determine how many bases of the flanking sequence are potentially conserved and therefore should be added to core element (Fig. 2, *see* also **Note 6**). Finally, design two to four tandem repeats of the putative regulatory sequence for construction of the synthetic promoter.
7. Adequate spacing between the copies of putative functional motif sequences needs to be ensured by addition of short spacer sequences, such as DNA sequence targets of six-base cutter restriction enzymes, that separate the motifs in the synthetic promoter. These spacer sequences should be searched against the literature and regulatory element databases to confirm that they do not constitute functional elements, or that the sequence combination of the spacer and the flanking region do not result in inadvertent construction of previously known functional elements absent in the promoters of interest (*see* **Note 7**).
8. Design a control version of the synthetic promoter by substituting three to four nucleotides within the core sequence



**Fig. 2** Design of the tetrameric *RSRE* used in construction of synthetic promoter. Example of a multi-copy functional element (4xRSRE) and the mutated control (4xmRSRE) separated by recognition nucleotide sequences of different restriction enzymes, as previously described [11, 17]

(Fig. 2, *see* also **Note 8**). This approach will serve as a control for not only nucleotide specificity of the core sequence, but also as a vehicle to exclude inadvertent contribution of combined flanking and spacers sequences to the activity of the synthetic promoter.

### **3.2 Identification of Transcription Factors Interacting with Synthetic Promoters**

1. Perform a literature search to determine if the core motif used in the synthetic promoter matches previously described transcription factor (TF) binding sites (*see* **Note 9**).
2. In parallel with the literature search, look for the motif in databases of known TF-binding sites (*see* **Note 4**).
3. If the core motif is not found in the literature or TF-binding databases, select and employ an alternative approach for identification of TFs that interact with the motif. One approach is to use the synthetic promoter and mutated control as bait in a yeast 1-hybrid screen against a library of cloned TFs [20] (*see* **Note 10**). A second option, which may be run in parallel, is to use plants stably transformed with the synthetic promoter fused to the luciferase reporter (*see* **Subheading 3.3**) in a forward genetic screen (*see* **Note 11**). A third option is to use the synthetic promoter to screen a recombinant expression library (such as  $\lambda$ gt11 cloning system) as previously described [21, 22], followed by validation of specificity using the mutated version of the core sequence as control.

### **3.3 Stable Transformation of *A. thaliana***

The stable transformation protocol for *A. thaliana* (Arabidopsis) is a slightly modified version of the procedure previously developed by Andrew Bent's lab (<http://www.plantpath.wisc.edu/fac/afb/protocol.html>) [23].

1. Using standard molecular biology techniques, transform the synthetic promoter into an appropriate vector for stable transformation of Arabidopsis. The vector should include bacterial and plant selection genes, a cloning site upstream of a minimal promoter, and the luciferase reporter gene (*see* **Note 12**).
2. Transform the vector into an appropriate strain of *Agrobacterium tumefaciens* (*see* **Note 13**). Thaw 50  $\mu$ L competent *A. tumefaciens* on ice, then add 1–2  $\mu$ L of vector DNA. Freeze the cells in liquid nitrogen for 5' and then thaw in a 37 °C water bath for 5'. Add 1 mL 30 °C SOC media to tube and incubate in a 30 °C shaker for 2 h. Plate 50  $\mu$ L and 200  $\mu$ L of transformation reaction on LB plates with appropriate antibiotics and incubate at 28 °C for 2 days. Pick individual colonies and use PCR followed by sequencing to confirm successful transformation.
3. Grow Arabidopsis plants in pots of soil covered with standard charcoal fiberglass window screening or other similar mesh. To

stimulate flowering, plants should be grown under long day conditions (**Note 14**).

4. Cut the first set of inflorescences off and allow the plants to produce additional inflorescences. This increases the number of flowers and as such enhances transformation efficiency.
5. Add appropriate antibiotic to 250 mL LB in a 1 L flask and at room temperature. Inoculate culture with a toothpick that has been scraped across the surface of the previously prepared glycerol stock of the *A. tumefaciens* transformed with the synthetic promoter-luciferase construct (*see Note 15*). Cap culture flask with aluminum foil and incubate in a shaker at 28 °C for 16–18 h.
6. Take a sample from the culture and measure the optical density ( $OD_{600}$ ). The  $OD_{600}$  should be 0.8–1.2. Pour cultures into 500 mL centrifuge bottles and spin down at  $3000\times g$  for 15 min at 15 °C. Resuspend pellet in 5 % sucrose solution to  $OD_{600}=0.8$  (*see Note 16*).
7. Add Silwet L-77 to resuspended bacteria, to a concentration of 0.05 % and mix by inversion.
8. Wet two paper towels with deionized water and spread them out on the bottom of a standard-sized planting tray.
9. Pour *A. tumefaciens* solution into a shallow container (*see Note 17*). Depth of the solution should be about 4 cm.
10. Dip the bolts and rosettes into the *A. tumefaciens* solution and gently swirl for 3 s. Remove the plants from the culture and gently pat with a paper towel to remove excess *A. tumefaciens* solution (*see Note 18*).
11. Lay each treated pot on its side in the previously prepared planting tray. Cover with a second tray and move to growth chamber (*see Note 19*). After 24 h, upright the pots and move them to a fresh tray. From this point, grow the plants using standard light and watering conditions. Plants should be kept separate by staking up inflorescences. Once the seeds are set, reduce watering, and then withhold water once all seeds have matured.
12. Once plants have fully senesced, harvest seeds. Keep seeds from individual plants separate.
13. Harvest seeds and select transformants using an appropriate antibiotic or herbicide, depending on the vector used (*see Note 20*).

### 3.4 *Nicotiana benthamiana* Transient Assay

The transient transformation protocol is a modified version of the procedure previously described by Jurgen Denecke's lab (<http://www.plants.leeds.ac.uk/jd/pdf/Agrobacterium%20infiltration.pdf>).

1. Grow *N. benthamiana* (tobacco) plants on soil until 4–6 weeks old (*see* **Note 21**).
2. Using the appropriate standard molecular biology techniques, clone the transcription factor(s) of interest and synthetic promoter(s) into appropriate vectors for transient expression in tobacco (*see* **Note 22**).
3. Transform the vectors into a suitable strain of *A. tumefaciens* (*see* **Note 13**) following the procedure outlined in [Subheading 3.3, step 2](#).
4. Culture the transformed *A. tumefaciens* overnight in 3–5 mL of LB medium with appropriate antibiotics (*see* **Note 23**).
5. Centrifuge 1 mL of the cultures at  $3000\times g$  for 5 min at room temperature. Discard the supernatant.
6. Resuspend in 1 mL of infiltration buffer and repeat **step 5**. Repeat once.
7. Measure the OD<sub>600</sub> of the *A. tumefaciens* and then adjust to an approximate OD<sub>600</sub> of 0.1 using infiltration buffer (*see* **Note 24**).
8. Move the plants to the lab and select leaves for infiltration. Leaves that are large, but not the oldest on the plant, should be used (*see* **Note 25**).
9. Use a P200 pipette tip to make a small hole in the leaf where each infiltration site will be. We typically do four infiltrations of the same construct on a single leaf. Use a permanent marker to draw a circle, approximately 30 mm in diameter, around each hole. Label the leaf with the constructs with which it will be infiltrated.
10. Mix the cultures of the two corresponding transformed *A. tumefaciens* strains, one with the TF containing strain and the other containing the synthetic promoter, at a 1:1 ratio.
11. Draw up 1 mL of the combined bacterial culture into a 1 mL syringe (without tip). Place a finger over the hole on the upper side of the leaf, then press the syringe into the hole from the bottom side of the leaf and inject bacterial culture into the leaf. This should produce a region of discoloration radiating out from the hole—continue with injection until the discoloration fills the circle drawn on the leaf. This will require approximately 250  $\mu$ L of bacterial culture per site. Repeat for the other three sites on the leaf.
12. After all infiltrations have been completed, return the plants to normal growth conditions for 2 days.
13. Measure luciferase activity in the infiltration sites, following the procedure described in **steps 2–9** of [Subheading 3.5](#) (*see* **Note 26**).



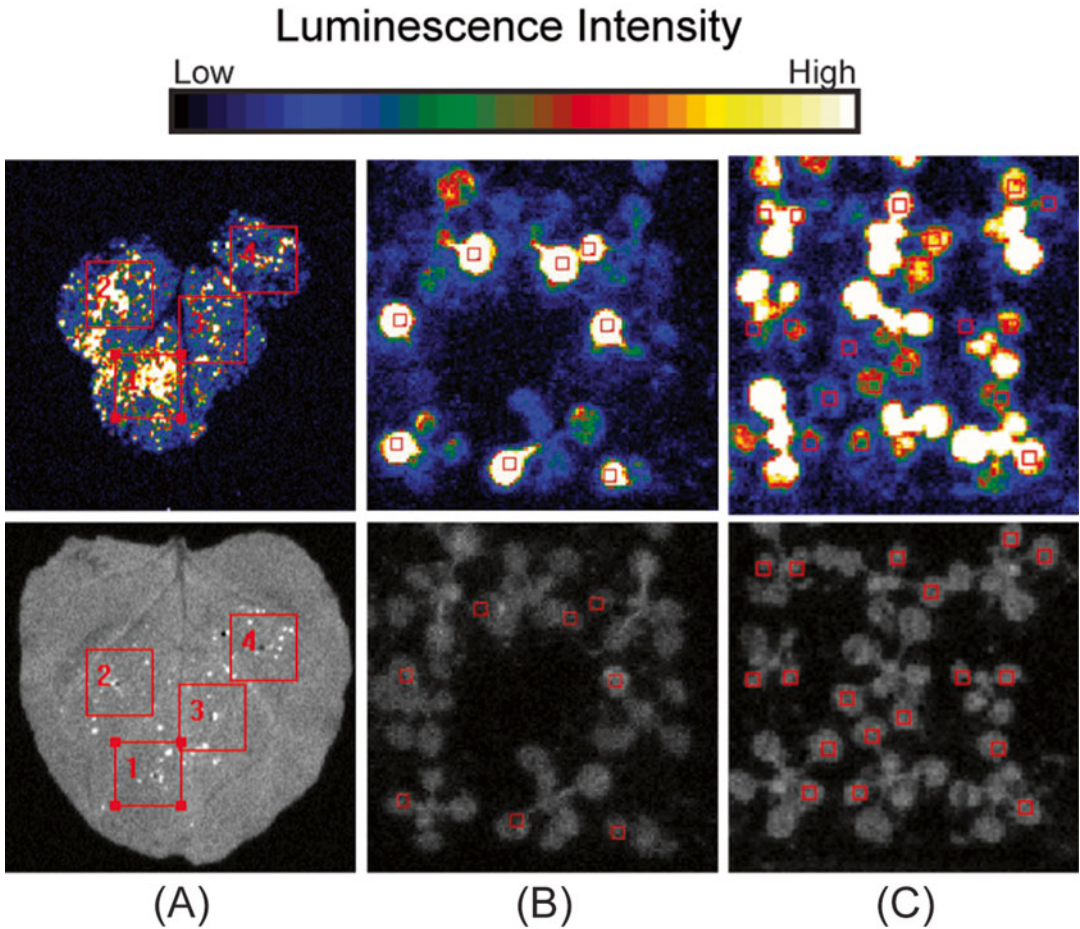
### 3.5 Imaging and Analysis

1. Plate sterilized *Arabidopsis* seeds (T<sub>3</sub> or subsequent generations) on MS media (*see* **Note 27**). After a 4 day stratification period at 4 °C, place plates in a growth chamber set to standard conditions suitable for your specific needs. For manageable number of genotypes and treatments, it is advisable to use each plate as an experimental block (i.e. replicate all genotypes and treatments on one plate).
2. Prior to imaging, spray plants with approximately 500 µL of 1 mM luciferin solution per plate. Carefully spray plants evenly and thoroughly (*see* **Note 28**).
3. Move plants from growth chamber to a location near the CCD camera imaging box (*see* **Note 29**). Turn on CCD camera and allow it to cool down to working temperature.
4. Use an old plate of plants to focus the camera. Do this by placing the plate into the camera system, leaving the door of the light-tight imaging box open, and running the camera on a real-time imaging setting. Adjust the focus by turning the focusing ring on the camera lens (*see* **Note 30**).
5. Remove the plate from the camera, shut the door, and take a background reading (*see* **Note 31**).
6. Place the experimental plates in the imaging box and image the plants for the desired length of time. Save the resulting file.
7. Use appropriate software to measure bioluminescence (*see* **Note 32**). In the image processing software, place regions of interest (ROI) over the tissue—these will calculate the average light intensity for the pixels within the region. If interested in local responses to stimuli, place one ROI on the treated tissue (Fig. 3b). If interested in systemic responses to stimuli, multiple ROI may be placed on tissues distal from the treated area, and then averaged together to give one systemic LUC expression value for that plant (Fig. 3c).
8. Reformat the data output from the image analysis software into a layout amenable for visualization and statistical analysis (*see* **Note 33**).
9. Perform appropriate statistical analyses (*see* **Note 34**).

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## 4 Notes

1. LB may be prepared in advance of transformation and stored at room temperature. However, to ensure sterility, we recommend making a dedicated batch of LB for the transformation.
2. For *A. thaliana*, we recommend using the promoter retrieval tool from the lab of Matthew Hudson (University of Illinois at Urbana-Champaign): <http://stan.cropsoci.uiuc.edu/prom.php>.



**Fig. 3** Selection of regions of interest (ROI) for quantification of luciferase activity is dependent on experimental design. *Top panel* shows images of leaves with luciferase activity, as indicated by the scale above the images. *Bottom panel* shows the same leaves autofluorescing. Each *red box* is a single ROI placed on the image using the Andor Solis software. The software reports an average light intensity for all pixels within each ROI. Images are from experiments described in Benn et al. [17]. (a) ROI for *N. benthamiana* transient assay demonstrating activation of *4xRSRE:LUC* by the transcriptional activator CAMTA3. (b) ROI for measurement of local *4xRSRE:LUC* activity in wounded leaves of *A. thaliana* displaying local response. (c) ROI for measurement of systemic *4xRSRE:LUC* activity in *A. thaliana* plants where treatment of wounded leaves with flg22 has generated a systemic response

This tool retrieves the 2000 base pairs (bp) sequence immediately 5' to the translational start site. Alternatively, sequences may be retrieved from TAIR at <https://www.arabidopsis.org/tools/bulk/sequences/index.jsp>, however these sequences exclude the 5' UTR [24].

3. We used the motif discovery tool from the lab of Matthew Hudson (University of Illinois at Urbana-Champaign): <http://stan.cropsci.uiuc.edu/cgi-bin/sift/sift.cgi> [8]. A variety of other tools exists for motif discovery, such as DREME

(<http://meme.nbcr.net/meme/tools/dreme>) and the TAIR motif discovery tool (<http://www.arabidopsis.org/tools/bulk/motiffinder/index.jsp>) [24, 25]. We recommend running the promoter set of interest through several motif discovery algorithms, to ensure that motifs selected for further analysis are not artifacts of a particular algorithm.

4. To assess whether identified motifs match characterized elements, we recommend using motif discovery with DREME (see **Note 3**) followed by application of the TOMTOM (<http://meme-suite.org/tools/tomtom>) tool to search for top elements in the JASPAR database (<http://jaspar.genereg.net/>) [26, 27]. Candidate elements may also be searched against the AGRIS database (<http://arabidopsis.med.ohio-state.edu/AtcisDB/bindingsites.html>) [28].
5. For large number of promoters (>50), we recommend using a script to extract the flanking sequences. An example of a script with this functionality (among others), *cisfinder.v2.pl*, is available at our lab's github page (<https://github.com/DeheshLab/Cis-Element-Tools>).
6. In the case of the *4xRSRE* containing synthetic promoter, 4 base-pairs of flanking sequence was used on either side of the core motif (Fig. 2).
7. In the *4xRSRE* containing synthetic promoter, we used restriction enzyme recognition sites as spacers, specifically EcoRV, BamHI, and XbaI (Fig. 2).
8. For the mutated *4xRSRE* promoter, we altered three base-pairs in the core motif (Fig. 2).
9. In the case of the *RSRE*, a literature search revealed that a similar element, *vCGCGb*, had been identified via an oligo selection experiment as the binding site for a TF known as *CAMTA3* [29]. Furthermore, a specific instance of the *vCGCGb* element, *CCGCGT*, had been shown to be required for *CAMTA3* activation of the *CBF2* gene [30]. Both of these findings strongly suggested *CAMTA3* as a likely binding partner for the *RSRE*.
10. The yeast 1-hybrid approach may be hindered if the synthetic promoter is similar to yeast *cis*-regulatory elements, leading to auto-activation of the synthetic promoter. This was the case with the *4xRSRE* promoter, which was strongly auto-activating in yeast (unpublished data).
11. The forward genetic screen approach was successfully used with the *4xRSRE* promoter, allowing the identification of *CAMTA3* and *MEKK1* as regulators of the element [18].
12. We used pATM-NOS as our cloning vector for stable transformation of *Arabidopsis* [11]. This vector contains the LUC+ reporter gene, an improved version of firefly luciferase.

13. We used the GV3101 strain of *A. tumefaciens* for both stable transformation of Arabidopsis and transient transformation of *N. benthamiana*.
14. Plants used for the transformation assay are grown in Sunshine mix soil in growth chambers set to 22 °C and 16 h light.
15. It is critical that all aspects of the **step 9** procedure are performed sterilely. The flask, toothpicks, and pipette tips should be autoclaved prior to use and all work should be performed in a sterile hood. However, once the culture has reached the desired OD, all subsequent steps need not be done sterilely.
16. If the OD<sub>600</sub> of the culture is such that the resuspension volume will exceed that of the centrifuge bottle, you may spin down only part of the culture. Resuspension may be accomplished via vortexing in a small volume, though we find that using a P1000 pipette is sometimes necessary. However, pipetting should be limited to reduce damage to the cells from shearing.
17. The container used for plant transformation should be wide enough to allow dipping of the plants into the solution. We use shallow Tupperware containers. For smaller pots, pipette tip boxes may be used.
18. 3 s of swirling the plants in the culture of transformed *A. tumefaciens* works well in our lab. This exposure time may be varied as needed, however less time may result in fewer transformants, whereas longer times may produce multiple insertions. Patting plants with paper towels will remove excess bacterial culture on the stems and leaves. Flowers should still be visibly wet after this step.
19. Use of an opaque planting tray as a lid is to maintain high humidity but reduce buildup of heat, which in turn can reduce transformation efficiency.
20. If *A. tumefaciens* containing the pATM-NOS vector is used for transformation, we recommend the selection method of Harrison et al. [31]. Sterilize and plate seeds on ½ strength MS media with 50 µg/mL kanamycin. High plating density may reduce the efficiency of selection. Seeds should be evenly distributed and planted at a density of ~100 seeds per plate (100 mm × 100 mm). Stratify at 4 °C for 2 days, expose to light for 6 h, then place the plates in dark for 2 days. Following the 2 day dark treatment, move plates to light for 48 h, after which transformants will display increased growth and almost normal greening, while non-transformants will be smaller and chlorotic.
21. We grow *N. benthamiana* on Sunshine mix soil under light and humidity conditions similar to those used for Arabidopsis in our lab. The optimal developmental stage for transient transformation is just post flowering time.

22. For the transcription factors, we use the pYL436 vector and for the synthetic promoters, we use the pBGWL7 vector [32, 33].
23. Depending on the construct and strain of *A. tumefaciens* used, time to reach the required OD may vary from 18 to 48 h.
24. The ideal OD<sub>600</sub> used in transformation depends upon the particular construct being used. In the case of *4xRSRE:LUC* construct, which expresses well and is highly responsive to environmental perturbations, an OD<sub>600</sub> of 0.1 works well. In the case of a less responsive element in a construct that is also expressing poorly, OD<sub>600</sub> values from 0.2 to 0.5 may be employed.
25. We use up to four leaves from a single plant for transient assays. We recommend using different stages of leaf maturity as a controlled experimental factor—i.e. if the older pair of leaves is used for a particular construct on the first repeat, use younger leaves for the same construct on the second repeat.
26. Leaves are detached prior to imaging. We typically set a single, large, region of interest (ROI) to cover each infiltration site for image quantification (Fig. 3a).
27. We find that conducting experiments on MS media (as opposed to soil) produces more consistent results, likely due to the stress-inducible nature of many of the promoter:*LUC* fusion constructs used in our lab. Seeds should be plated in a sterile hood on MS media made to the standard specifications of the lab (typically ¼ or ½ strength MS with 0.8–1.0% phytoagar).
28. Plants may be sprayed up to 18 h in advance of imaging. For constructs predicted to be wound or touch-inducible, plants should be sprayed the day prior to imaging, so that any spray-mediated induction of the reporter luciferin returns to basal levels prior to the initiation of the experiment.
29. If the synthetic promoters are expected to be highly responsive to environmental conditions, plants should be moved close to the CCD camera setup in advance of the experiment. We find that moving *4xRSRE:LUC* plants 4 h prior to the start of imaging is sufficient.
30. For our system, focusing is done with the following settings: acquisition mode = real time, readout mode = imaging, readout time per pixel (µs) = 1, shutter time (s) = 0.3, external shutter = fully auto, data type = counts.
31. The optimal exposure time must be empirically determined for each construct. The exposure should be at the shortest time that still allows for clear detection of the luciferase signal. In our lab, the *4xRSRE:LUC* activity is imaged using a 5' exposure, while the less active *pHYDROPEROXIDELYASE::LUC* construct requires a 15' exposure. For the *4xRSRE:LUC* in our system, the background reading is taken with the following settings (for

an 8 h run): acquisition mode=kinetic series, readout mode=imaging, readout time per pixel ( $\mu$ s)= $32 \times 2 \times \text{Gain}$ , shutter time (s)=300, number of accumulations=1, number in kinetic series=48, kinetic cycle time (s)=600, external shutter=fully auto, data type=counts (bg corrected).

32. To measure bioluminescence in the Andor Solis software, it is first necessary to change the default false coloring settings to allow viewing of the plants. This is done by opening the data histogram tool, followed by changing the mode to “range”, and finally altering the range until plants are visible (we use a range of 0–200 for *4xRSRE:LUC*). Toggling through the available color palettes using the change palette tool enables selection of most suitable color palette for the clearest visualization of the plants. Once the plants are clearly visible, use the ROI tool to place an ROI on the image by clicking the “Add ROI” button. ROI can then be dragged over the tissue to be measured and appropriately resized by clicking and dragging the corners of the ROI box. Repeat this process until ROI have been placed for all plant leaves in a particular experimental unit (i.e. wild-type, untreated) (Fig. 3). Then select all of the data in the ROI window and copy it into a spreadsheet utility, such as Microsoft Excel.
33. Reformatting of the data may be done by manually copying out and pasting the relevant data (i.e. mean luminescence at each time point) from the output of the image analysis software. If many experiments are to be performed, this process can become tedious, and prone to error. As such, we recommend using a processing script to convert the raw data into a form suitable for analysis. We have developed scripts for conversion of the raw Andor Solis ROI data into a format suitable for analysis in R. These scripts (*Andor\_parseR.pl* and *Andor\_parseR.systemic.pl*) are available at <https://github.com/DeheshLab/CCD-camera-data-analysis>.
34. Depending on the nature of the particular construct, the data may need to be log-transformed prior to analysis. For example, the *4xRSRE:LUC* reporter produces data that can range over several orders of magnitude, necessitating log-transformation to achieve normality and homogeneity of variance. Alternatively, nonparametric statistical tests may be used.

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