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SHORT COMMUNICATION

Identification and functional characterization of the *Marshallia* (Asteraceae) Clade III Cytokinin Response Factor (CRF)

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ABSTRACT

Cytokinin Response Factor (CRF) genes are a subgroup of AP2/ERF domain-containing transcription factors that are defined by the CRF domain, from which five clades of CRF genes have been identified. Clade III CRFs are strongly induced by cytokinin, as well as other abiotic stress factors, such as oxidative stress. While this appears well studied for the Clade III CRFs in Arabidopsis and tomato, there have been almost no studies done outside of these model systems. This study expands upon that and represents the first CRF research in the Sunflower family, Asteraceae. Fifty Asterid Clade III CRF protein sequences were examined, and novel Clade III CRF C-terminus motifs were identified. Clade III CRF genes of *Marshallia mohrii* and *M. caespitosa* were assembled from genome-skimming and transcriptomic data. Expression experiments were conducted on *M. caespitosa* to test responsiveness to both cytokinin and oxidative stress. Low levels of basal expression for the *McCRF1* were found to be strongly induced in both treatment groups. These are the first experiments to show regulation of a nuclear gene in a *Marshallia* species, and these results suggest there is broad conservation in the sequence, form, and regulation of Clade III CRF genes and proteins.

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Introduction

Cytokinin Response Factors (CRFs) are a subfamily of AP2/ERF domain-containing transcription factors with strong connections to cytokinin, as several members were originally identified as being highly induced in *Arabidopsis thaliana* (L.) Heynh by cytokinin.[1,2] CRFs proteins have a family specific AP2/ERF DNA-binding domain, a subfamily-specific CRF domain involved in protein–protein interactions as well as a predicted mitogen-activated protein kinase (MAPk) site.[2,3] Phylogenetic analyses using the CRF and AP2/ERF domains of CRF proteins across a wide range of flowering plant lineages have indicated that there are at least five major clades of CRFs, each containing a unique C-terminus motif that is well conserved.[2] While CRFs do have related expression patterns, such as preferential localization to vascular tissue, individual CRF clades appear to also have distinct regulation, such as induction by cytokinin.[2,3]

Perhaps the best-studied CRF clade is Clade III, yet, surprisingly, this really consists of an examination of only three genes: *AtCRF5* and *6* from Arabidopsis and *SlCRF5*, a tomato (*Solanum lycopersicum* L.) CRF.[2,4–7] While there has been a greater examination of *AtCRF6* than the others, genes showing direct connections to leaf senescence, it appears that features common to all Clade III CRF members are strong induction by cytokinin and oxidative stress, with possible connections to other abiotic stress responses.[2,4–6]

This study aimed to further our knowledge of Clade III CRFs beyond Arabidopsis and tomato. To do this, we looked for conserved motifs unique to Clade III CRF proteins and characterized the Clade III CRF gene for the genus *Marshallia* Shreb. (Asteraceae) for a response to cytokinin and oxidative stress to test if broad regulation of Clade III exists. This also represents the first research to identify and characterize regulatory mechanisms of any nuclear genes in *Marshallia*.

Materials and methods

Asterid clade III CRF data

Asterid Clade III CRF protein sequences were collected from public databases (e.g., NCBI and OneKP (One Thousand Plant Project)) and aligned manually in SeaView v. 4.4.2.[8,9] Sequences of low quality (e.g., missing large regions or short sequences) were excluded from further analysis. ClustalO v. 1.2.0 was used to align the remaining sequences to compare to manual alignment.[10] Fifty unaligned sequences were submitted to MEME motif analysis (See Supplemental Table 1 for species and accession information).[11] Parameters for MEME analysis were occurrence of motif = zero to one, number of motifs = 10, minimum number sites = 35, maximum number sites = 50, minimum width = five, and maximum width = 100.

Extraction and sequencing

DNA from all species of *Marshallia* were extracted from fresh leaf tissue using a modified 2X CTAB protocol as described by

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Supplemental data for this article can be accessed on the publisher's website.

Table 1. Sequences of the *McCRF1* and *MmCRF1* gene reading frames. SNPs between the *McCRF1* and *MmCRF1* coding regions are highlighted in yellow.

McCRF1	ATGAAACTAGACTTCATGGGATCTTCTCCTAAATTTAGAGTCAATCTC
MmCRF1	ATGAAACTAGACTTCATGGGATCTTCTCCTAAATTTAGAGTCAATCTC
	ACCGTCACCATCAAACAATCGGAACTCGATTCACCAAAAACGGTAACG
	ACCGTCACCACCAAACAATCGGAACTCGATTCACCGAAAACGGTAACG
	ATTTCCATGAACGATCGCGATGCTACGGACTCTTCAAGTGACGAAGAC
	ATTTCCATGACCGATCGCGATGCTACGGACTCTTCAAGTGACGACGAC
	CACAACGAATTGGGTCACCGAAAAATTAAAAGGTATGTAAATGTGATT
	CACAACGAATTGGGTCACCGGAAAATTAAAAGGTATGTAAATGTGATT
	CAGTTCGAAGACAACTGTTGTGGGAGAAATTTGAGTGGAAGTGATGGT
	CAGTTCGAAGACAACTGTTGTGGGAGAAATTTGAGTGGAAGTGATGGT
	AGTGATAAAGGGAAGAAAAAACAGAGTCGCCGGATGAAAGAACCAGTA
	AGTGATAAAGGGAAGAAAAAACAGAGTCGCCGGATGAAAGAACCAGTA
	AGTTCGGGAACCGAACGGAAGTTTAGGGGAGTAAGGCGACGGCCATGG
	AGTTCGGGAACCGAACGGAAGTTTAGGGGAGTAAGGCGACGGCCGTGG
	GGAAGGTGGGCGGCGGAGATTCGTGATATGGGGGTGAGGGTATGGTTG
	GGAAGGTGGGCGGCGGAGATTCGTGATATGGGGGTGAGGGTATGGTTG
	GGTACATACGATACGGCGGAGGAGGCGGCATTAGCTTATGATCGGAGA
	GGTACATACGATACCGCGGAGGAGGCGGCATTAGCTTATGATCGGAGA
	GCGATTGAACTACATGGATGGAAAGCTCAGACGAACTTTTTGCAACCA
	GCGATTGAACTACATGGATGGAAAGCTCAGACGAACTTTTTGCAACCA
	CCGCGTTCAGAAGTTGCAGTACCGGTGATAGCTTCTGGTTCTGTGTCC
	CCGCGTTCAGAAGTTGCAGTACCGGTGATAGCTTCTGGTTCTGTGTCC
	GATCAGTGTTCCGGGAAGGAGTTGCGGGGTGTTTCCTCGCCGACCTCC
	GATCAGTGTTCCGGGAAGGAGTTGCGGGGTGTTTCCTCGCCGACCTCC
	GTGTTACGGTTTGGTAAAACAGAGGCGGAGTCTGAGAAGCTGGATGAA
	GTGTTACGGTTTGGTAAAACAGAGGCGGAGTCTGAGAAGCTGGATGAA
	CAGAAGCAAAGTGAATCAAATGTCAGAGATGATGATTTCGGGTATGAT
	CAGAAGCAAAGTGAATCAAATGGCAGAGATGATGATTTCGGGTATGAT
	TGGGATTTGGAATACGATTTCTTAGACTTTCGGATAGCGTCTCCGATG
	TGGGATTTGGAATACGATTTCTTAGACTTTCGGATACCATCTCCGATA
	ATGGTGGAAGAGATTGATTTGGGAAGAAGAATGATGTGGGAGGTAGAG
	ATGGTTGAAGAGATTGATTTGGGAAGAGGAATGATGTGGGAGGTAGAG
	GACGATATGAAACCAAGAGTGTGGGATGTGGATGGTTGCTTCCAAGAT
	GACGATATGAAATCAAGAGTGTGGGATGTGGATGGTTGCTTCCAAGAT
	CCTGTGATTGGAGAGTGGTTAGATGATTAA
	TCTGTGGTTGGAGAGTGGTTAGATGATTAA

Doyle and Doyle (1987) or E.Z.N.A. kits (Omega Bio-tek, Inc., Norcross, GA) per manufacturer protocol.[12] RNA was extracted from fresh leaf material of five individuals of *Marshallia* Schreb. (two *M. mohrii* Beadle and F.E. Boynt., one *M. caespitosa* Nutt. Ex D.C., one *M. obovata* (Walt.) Beadle and F.E. Boynt., and one *M. trinervia* (Walt.) Trel.) and from fresh shoots and roots of *M. caespitosa* using Plant RNA extraction kit (Qiagen, Hilden, Germany) per manufacturer protocol. DNA samples were submitted to HudsonAlpha Institute for Biotechnology (Huntsville, AL) for paired-end library prep and 100 bp sequencing via an ILLUMINA (ILLUMINA Inc., San Diego, CA) HiSEQ 2000 platform. RNA samples were submitted to the Auburn University Genomics and Sequencing Laboratory (Auburn, AL) where cDNA libraries were prepared using an Illumina mRNA TruSeq kit and sequenced on an ILLUMINA HiSEQ 1500 platform.

Assembly and description

Reads from unassembled genome-skimming readsets were identified using BLAST and assembled using CAP3 for the coding region of Clade III CRF gene.[13,14] RNA reads from *Marshallia* accessions M2.9 (*M. trinervia*), M3.9 (*M. obovata*), M10.1.1 (*M. caespitosa*), M20r, and M21r (*M. mohrii*) were assembled using Trinity v20131110.[15] Both DNA and RNA reads were mapped to the resulting DNA contig and RNA transcript using Bowtie2 v. 2.1.0 with the flags – local, – qc-filter, and – no-unal.[16] Maps were visualized using Tablet 1.14.04.10 to assess the quality of the mapping and to assess the accuracy of the assemblies.[17]

5′ UTR sequence assembly was performed by identifying appropriate reads from all data sets via BLAST followed by hand assembly in SeaView. Bowtie2 was used to map read pairs from all data sets to further identify the reads of the 5′ UTR. Within the mapping, reads were selected that were properly mapped, along with their pair, and that exhibited soft clipping of the 5′ region. These reads were then extracted from the database and added to the assembly in an iterative process. The cis-element analysis was examined using the New PLACE cis-element web tool and manual examination of the sequence.[18]

Characterization of expression

Individuals of *Marshallia caespitosa* were grown in the Auburn University Plant Research Center greenhouses. Cypselae were planted in Sunshine (Sun Gro Horticulture, Agawam, MA) mix #8 potting soil just

below the surface of the soil and covered with a thin layer peat moss. A weekly watering/fertilizing regime and natural light and photoperiod were utilized. Plants were grown for approximately one year and then harvested while in the rosette growth stage. Whole harvested plants were used for oxidative stress and cytokinin induction tests. Three plants were sprayed with a control spray, three plants were sprayed with 0.5 µM solution of benzyladenine (BA), and three were sprayed with a 41.65mM or 0.5% solution of hydrogen peroxide. Plants were given a 6-h period to allow for uptake of the solutions and for changes in expression to occur. Each plant was separated into shoots and roots for RNA extractions using a Qiagen Plant RNA extraction kit (Qiagen, Hilden, Germany) per manufacturer protocol. cDNA was prepared using Quanta qScript cDNA supermix (Quanta BioSciences, Inc., Gaithersburg, MD, USA) per manufacturer protocol with an Eppendorf Thermal Cycler (Eppendorf, Hamburg, Germany). q-PCR was performed using two technical replicates of three biological replicates per experimental group in an Eppendorf Realplex with Quanta Sybr Taq mix per a modified protocol (10µl G-Bio taq, 0.4 µl primer mix, 9.6 µl template) using the following program: one cycle at 95.0°C, followed by 40 cycles of T_m = 95.0 for 30s, T_a = 55.0°C for 20s, and T_e = 68.0°C for 30s; melting curve program was set to 95.0°C for 15 s, 60.0°C for 15 s, a 20-m temperature increase period followed by 95.0°C for 15 s. Primers were designed using NCBI BLAST Primer and were prepared by Eurofins Scientific (*McCRF1* Forward = GCTTCTGGTTCTGTGTCCCGA, *McCRF1* Reverse = CCAAACCGTAACACGGAGGT, EF1A Forward = GATGATTCCCACCAAGCCCA, EF1A Reverse = CAAACAACCGACGAACCCAC). Mean Ct was calculated for each biological replicate. Mean Ct of EF1A was subtracted from mean Ct of the respective experimental replicate to calculate ΔCt. ΔΔCt was calculated by subtracting ΔCt of the experimental group from the ΔCt of the control group. Fold change (FC) was calculated by the equation 2^ΔΔCt.

Results and discussion

Clade III CRF genes have been found to be strongly induced by both the plant hormone cytokinin and oxidative stress, yet there has been little to no experimental study of these responses outside of Arabidopsis and tomato. Here, we aligned and analyzed 50 Asterid Clade III CRFs to provided valuable insight into the structure of these genes across a broader phylogenetic context and identified several conserved motifs. MEME analysis identified motifs common to all CRF proteins, including the CRF domain motif (VRI[SY]VTD[CG]DATD; e-value = 7.3e-066; Figure 1(a)) and the putative MAPk motif (SPTSVLRFD; e-value of 6.1e-054; Figure 1(b)). The most strongly supported motifs in the MEME analysis were those of the C-terminus. The first C-terminus motif was found to be [LY]D[QS]CFL[NK][DE][FY]FDFRSPSP[LI][IM]Y[ED]E (e-value of 2.2e-586; Figure 1(c)). The second was found at the end of the C-terminus and featured a WDV[DN]DF[FL] sequence (e-value = 9.3e-06; Figure 1(d)). These motifs are unique to Clade III CRFs and likely play an important role in the activation of the gene by acting as a trans-activation site.[2,19,20]

Assembly of Clade III CRF genes from non-model plants will help increase our understanding of conserved structure within their sequences and help determine if work done in model systems truly serves as a model for other plants. Here, we used novel sequence analysis from the non-model genus *Marshallia* to fully examine this premise. Assembly of a *Marshallia* Clade III CRF from genome-skimming data was difficult due to low coverage. However, using Trinity, a Clade III CRF transcript from start to stop codons successfully assembled, with some up- and downstream elements, from the M21r RNA read set (henceforth referred to as *MmCRF1*, GenBank accession MF687408; refer to Table 1 for coding sequence). The Trinity assembly of the M10.1.1

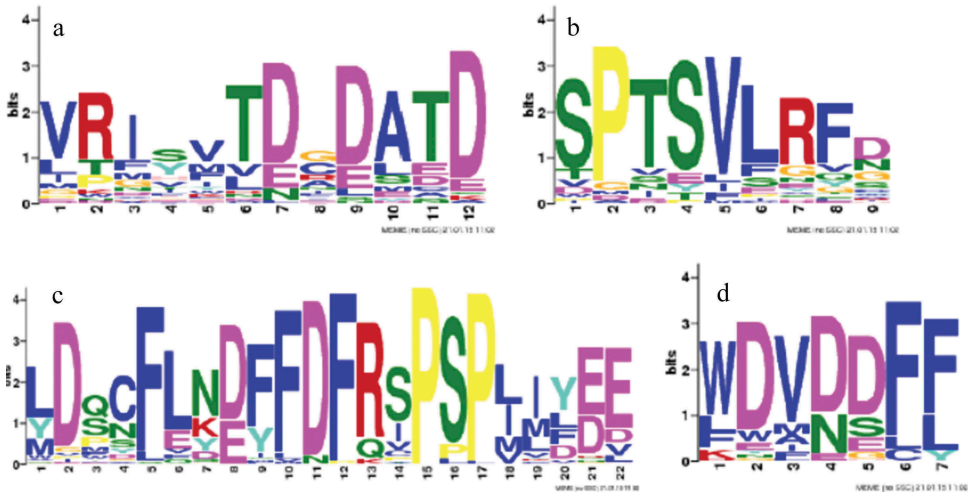

Figure 1. MEME analysis output for the CRF (a), putative MAPk (b), and putative 3′ trans-activation domain (c and d) motifs. The CRF domain motif (a) is the first of the motifs identified by the MEME analysis to occur in the amino acid sequence and is supported by an e-value of 7.3e-066. The putative MAPk site motif (b) occurs downstream of the AP2/ERF domain and was supported by an e-value of 6.1e-054. The first putative 3′ trans-activation domain (c) motif occurs downstream of the MAPk site and was supported by an e-value of 2.2e-586. The second putative 3′ trans-activation domain (d) typically occurs just upstream of the stop codon and was supported by an e-value of 9.3e-0.6.

(*M. caespitosa*) RNA read set provided a near-complete transcript, from just downstream of the CRF domain to just downstream of the stop codon. The top BLAST hit from the Trinity output provided a transcript that contained all elements of Clade III CRFs. To extend the *M. caespitosa* transcript, *M. caespitosa* DNA reads were mapped to the M21r transcript via previously described methods and a majority-rule consensus was called (henceforth referred to as *McCRF1*, GenBank accession MF687407; refer to Table 1 for coding sequence). The resulting consensus matched the Trinity transcript where overlap occurred and extended the sequence to the start codon. Other accessions provided only a truncated transcript (M20r) or no assembly at all (M2.9 and M3.9). Mappings of DNA readsets offered low and incomplete coverage. When compared to other genes, such as the tubulin genes, the read depth was quite low, reaching only ~50% for some readsets. Attempts to assemble 5′ UTR produced a contig approximately 500 bp in length (Table 3). A region either lacking read data or containing highly repetitive sequence was encountered in each data set that precluded assembly. Approximately 500 nucleotides were assembled upstream of the start codon, from which an analysis of cis-promoter elements (New PLACE) revealed a number of potentially conserved cis-element promoter element motifs.[18] Importantly, several of these can be generally linked to various stresses, as well as four ARR1AT-motifs connected to cytokinin regulation and two motifs (TCTCT/AGAGA) found in other CRFs linked to vascular localization (Table 3).[2,18]

Both *McCRF1* and *MmCRF1* protein sequences were highly similar to each other, yet exhibit some variation in the conserved domains of the protein (Figure 2). When compared to *SlCRF5*, the CRF domain of *McCRF1* varied in 18 positions while *MmCRF1* varied in 15 positions, with strong conservation of the core of the motif, D[CG]DATDDD. Within the most conserved region of the C-terminus motif, the *MmCRF1* protein was found to differ in three positions and the *McCRF1* protein varied in four. The core of this motif was a strongly conserved DF[FL]DFR[SI]PSP[LI]M pattern. The MAPk site showed no variation within its sequence, exhibiting the typical SP[TS]SVL motif, with a T in the third position. The small motif at the end of the C-terminus varied in three of five positions, from KWAND in *SlCRF5* to VWDVD in both *Marshallia* Clade III CRFs (Figure 3). *McCRF1* and *MmCRF1* featured 16 SNPs relative to each other, with 10 resulting in amino acid changes (Tables 1 and 2). NCBI BLASTn was used to calculate the percent similarity between *McCRF1, MmCRF1*, and *SlCRF5*. The *McCRF1* and *MmCRF1* proteins had a 96.5% similarity with each other and a 36.55% and 37.94% similarity with *SlCRF5*, respectively.

Table 2. Amino acid sequences for the *McCRF1* and *MmCRF1* proteins. Amino acid differences between *McCRF1* and *MmCRF1* proteins are highlighted in yellow.

McCRF1	MKLDFMGSSPKFRVNLTVTIKQSELDSPKTVTISMNDRDATDSSSDEDHNELGH
MmCRF1	MKLDFMGSSPKFRVNLTVTTKQSELDSPKTVTISMTDRDATDSSSDDDHNELGH
	RKIKRYVNVIQFEDNCCGRNLSGSDGSDKGKKKQSRRMKEPVSSGTERKFRGVR
	RKIKRYVNVIQFEDNCCGRNLSGSDGSDKGKKKQSRRMKEPVSSGTERKFRGVR
	RRPWGRWAAEIRDMGVRVWLGTYDTAEEAALAYDRRAIELHGWKAQTNFLQPPR
	RRPWGRWAAEIRDMGVRVWLGTYDTAEEAALAYDRRAIELHGWKAQTNFLQPPR
	SEVAVPVIASGSVSDQCSGKELRGVSSPTSVLRFGKTEAESEKLDEQKQSESNV
	SEVAVPVIASGSVSDQCSGKELRGVSSPTSVLRFGKTEAESEKLDEQKQSESNG
	DDDFGYDWDLEYDFLDFRIASPMMVEEIDLGRRMMWEVEDDMKPRVWDVDGCFQ
	DDDFGYDWDLEYDFLDFRIPSPIMVEEIDLGRGMMWEVEDDMKSRVWDVDGCFQ
	DPVIGEWLDD
	DSVVGEWLDD

Table 3. Sequence of 5′ UTR assembled from all *Marshallia* spp. readsets. This region extended approximately 500 bps upstream of the start codon and includes both a known vascular-localized motif (TCTCT/AGAGA) in yellow and a known cytokinin-regulated motif ARR1AT (NGATT/NCTAA) in green.

GGAGAGAGAGAGGTCTCAATGTGGGAGTAGCACAAGGTCTTATTTTTTCAATGTG
GGATGGGGCTATTTCAATATTAAAAAAAGTAAATGTGTCTAGATGTTGGAGAGAG
AGGGGATTAAGAAACACCTTATGTGCTACATTTGAAGCAGCATGTAAACGTACAT
TATGTAGTTCTTACCGTTCTTAACACAAAATGACTTCTTGCATAAGCGTTCCTCTAT
ACATATATATAGGGTGCNGTTCATGCGAGAACCAAGTTTATGCCGAGAATCACGA
GAACNATTGGTATTATTGTAATTAATTACAAATTACATAAAATATATTTTCGGTTC
AACCAAAAACTAATCGACATATATGCATCCAACGAACGTAACCAAATCAAANGAT
CTTTCCTACGATCTCTTCTTCATCTTTAGCTTCCACCGTCGTACACATCTTTTCCGG
CGATCAATTACTTCTATTCTATTT

Figure 2. Relative position map of conserved domains/motifs, highlighted in red, of the *Marshallia* Clade III CRF proteins.

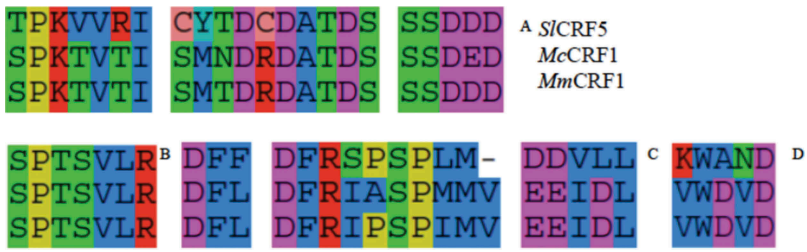

Figure 3. (a) Comparison of the CRF domains of *SlCRF5* (top), *McCRF1*, and *MmCRF1* (bottom) Clade III CRF proteins. This motif occurred in amino acid positions 26–47 in *SlCRF5* and 27–48 in *McCRF1* and *MmCRF1*. (b) Comparison of the putative MAPk site motif in *SlCRF5* (top), *McCRF1*, and *MmCRF1* (bottom) Clade III CRF proteins. This motif occurred in amino acid positions 180 – 186 in *SlCRF5* and 188–195 in *McCRF1* and *MmCRF1*. (c) Comparison of strongly conserved motif in putative trans-activation domain in *SlCRF5* (top), *McCRF1*, and *MmCRF1* Clade III CRF proteins. This motif occurred in amino acid positions 237–253 in *SlCRF5* and 230–247 in *McCRF1* and *MmCRF1*. (d) Comparison of 3′ motif of trans-activation domain in *SlCRF5* (top), *McCRF1*, and *MmCRF1* Clade III CRF proteins. This motif occurred in amino acid positions 283–287 in *SlCRF5* and 263–267 in *McCRF1* and *MmCRF1*.

Examination of expression levels by qPCR analysis found while *McCRF1* has a low basal level it is very strongly induced by both cytokinin (0.5 µM Benzyl Adenine) and oxidative stress (41.65 mM H_2O_2). Average Fold Change (FC) was calculated for samples achieving exponential increase during amplification and showed that *McCRF1* was strongly induced by both oxidative stress and cytokinin treatment in roots and shoot tissues. The largest increase of 259.31 FC expression was found in shoot tissue from cytokinin treatment. This strong increase is consistent with previous work on Clade III CRFs, which showed the strongest induction in aerial organs in cytokinin treatment groups, as well as the identification of the ARR1AT cytokinin responsive cis-elements in the promoter (Table 3).[2,3] There was also very strong 72.11 FC increase in shoots after oxidative stress treatment, which would be similar to previous findings for *AtCRF6*.[7] While the average FC increases in the roots were lower than for the shoots they were still strong; 11.13 and 65.61 for oxidative stress and cytokinin treatment, respectively. The results of this analysis show that there is conservation in *Marshallia* for, at least, cytokinin and oxidative stress regulation found in the other examined Clade III CRFs, *AtCRF5, AtCRF6*, and *SlCRF5*.

This work has increased our understanding of the structure and conserved C-terminus motifs that are unique to the Clade III CRFs. It has also demonstrated that these genes have conservation in regulatory mechanisms across a broader phylogenetic breadth than previously known. Furthermore, we have investigated genetic processes in *Marshallia* for the first time, increasing our understanding of this clade and expanding analyses into CRF genes across a greater phylogenetic context.

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Disclosure of Potential Conflicts of Interest

The authors have no interests to disclose.

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