RESEARCH PAPER

Solanum lycopersicum cytokinin response factor (SlCRF) genes: characterization of CRF domain-containing ERF genes in tomato

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Abstract

Cytokinin is an influential hormone in growth and developmental processes across many plant species. While several cytokinin-regulated genes have been well characterized in Arabidopsis, few have been identified in tomato, Solanum lycopersicum. Here a tomato family of 11 highly related cytokinin response factor genes designated as SlCRF1–SlCRF11 (Solanum lycopersicum cytokinin response factor) are identified and characterized. SlCRFs are AP2/ERF transcription factors and generally orthologous to Arabidopsis CRF clade members (AtCRFs). Some SlCRF genes lack a direct Arabidopsis orthologue and one SlCRF has a unique protein domain arrangement not seen in any other CRF protein. Expression analysis of SlCRF1–SlCRF11 revealed differential patterns and levels across plant tissues examined (leaf, stem, root and flower). Several SlCRFs show induction by cytokinin to various degrees, similar to AtCRFs. Additionally it is shown that some SlCRFs can be regulated by other factors, including NaCl, ethylene, methyl jasmonate, and salicylic acid. Examination of SlCRF proteins in transient Agrobacterium infiltration experiments indicates they can be nuclear localized in planta. Using a bimolecular fluorescence complementation (split-yellow fluorescent protein) system, it is also shown that SlCRF proteins can interact to form homo- and heterodimers. Overall this work indicates that some SlCRFs resemble previously identified CRFs in terms of structure, expression, and cytokinin regulation. However, SlCRFs have novel CRF protein forms and responses to abiotic factors, suggesting they may have a diverse set of roles in stress and hormone regulation in tomato.

Key words: CRF, cytokinin, cytokinin response factor, SlCRF, tomato.

Introduction

Cytokinin is an essential plant hormone known to be involved in numerous plant growth and developmental processes (Mok and Mok, 2001; Werner and Schmülling, 2009). Over the last decade, a model of cytokinin signalling in plants resembling bacterial two-component systems has become well established (To and Kieber, 2008; Werner and Schmülling, 2009). In this model, the binding of a sensor histidine kinase-like receptor to cytokinin initiates a multi-step phosphorelay. Upon autophosphorylation, the receptor transfers the phosphoryl group to a histidine-containing phosphotransfer protein (HPt), which then transfers the phosphate to one of two types of response regulators (RRs) localized in the nucleus. Type-B RRs, transcription factors, then activate the expression of their target genes mediating cytokinin-regulated growth and developmental processes or other aspects of plant life, whereas type-A RRs act as part of a feedback control loop to regulate this process (To and Kieber, 2008).

Recently the cytokinin response factors (CRFs) were identified as several highly related AP2/ERF transcription factors induced by cytokinin from global expression analyses in Arabidopsis (Hoth et al., 2003; Rashotte et al., 2003; 2006; Brenner et al., 2005; Kiba et al., 2005; Hirose et al., 2007). CRFs appear to form a branch pathway of the
cytokinin signalling pathway and may regulate downstream cytokinin targets independently or in conjunction with type-B response regulators (Rashotte et al., 2006; Werner and Schmülling, 2009). CRFs form a unique group of ERF proteins containing a clade-specific CRF domain that is always accompanied by an AP2/ERF DNA-binding domain. Furthermore, CRF domain-containing proteins are present in all land plants, but not in green algae, indicating that they may play important roles specific to land plants (Rashotte and Goertzen, 2010). Mutant analyses in Arabidopsis have implicated CRFs in the development of cotyledons, leaves, and embryos, as indicated by reduced size of cotyledons of the crf1,2,5 triple mutant and the embry-lyletal phenotype of the crf5,6 double mutant (Rashotte et al., 2006). In general, little is known of the function of CRFs outside of Arabidopsis, and very few CRF genes from other species have been examined in any detail. The genes that have been studied, PTI6/SlCRF1 and TSI, are linked to processes other than cytokinin regulation, including disease resistance and stress responses (Zhou et al., 1997; Park et al., 2001; Gu et al., 2002). This study was conducted to completely identify and characterize all CRF genes in tomato Solanum lycopersicum, which here are designated as SlCRF genes. Eleven SlCRF genes were identified through a combination of existing sequence comparison and rapid amplification of cDNA ends (RACE)-PCR. Once SlCRF genes were identified, their expression was examined in different plant tissues, as was regulation by cytokinin, salt, and other hormones. In addition, the cellular localization of SlCRF genes in planta and the ability of SlCRF proteins to form homo- and heterodimers with each other was determined. Together this study generates a first complete picture of all CRF genes in any species, suggesting a broader function for CRF beyond cytokinin regulation and allowing functional parallels to be made between related clades of CRFs across species.

Materials and methods

Plant materials and growth conditions

The tomato dwarf cultivar Micro-Tom was used for all experiments. Plants were grown in Sunshine Mix #8 soil under a 16:8 h light:dark photoperiod at 25 °C. Plants were grown in Sunshine Mix #8 soil under a 16:8 h light:dark photoperiod at 150 μE m⁻² s⁻¹ and a 26 °C night (dark) temperature.

RNA isolation, cDNA synthesis, and expression analysis

Leaves, stems, flowers, and roots were harvested from 52-day-old Micro-Tom plants, and immediately flash-frozen in liquid nitrogen. RNA was extracted using a Qiagen RNeasy Kit according to the manufacturer’s instructions. A 500 ng aliquot of the total RNA was used for each tissue type in the subsequent reverse transcription with Qiagen qScript cDNA supermix. The first strand of cDNA was diluted 10 or 20 times before it was used in the reverse transcription-PCR (RT-PCR). PCR conditions were initiated for 2 min at 95 °C, followed by cycles of 30 s at 94 °C, a 30 s annealing step, a 35 s extension at 72 °C, and a 5 min final extension at 72 °C. RT-PCR was conducted for SlCRF1–SlCRF5, SlCRF11, and TIP41 over 29 cycles with a 56 °C annealing temperature step, and for SlCRF6–SlCRF10 over 35 cycles with a 54 °C annealing temperature step. SlCRF-specific primers used in the RT-PCR are as follows: SlCRF1 forward, 5’-GGAAA ATTCAGTCGCCGTTGA-3’; SlCRF1 reverse, 5’-AAATTGTGAACGCCTGCA-3’; SlCRF2 forward, 5’-GGTTGATGCTGCTGTCGCT-3’; SlCRF2 reverse, 5’-CTTCCAGGCAAGACGGTCC-3’; SlCRF3 forward, 5’-AAATGACCCGAGACGCA-3’; SlCRF3 reverse, 5’-CTTCCAGGCAAGACGGTCC-3’; SlCRF4 forward, 5’-TCAATTCCCTCTGTTCACCA-3’; SlCRF4 reverse, 5’-GTTTGGCCTATTTCCACTGCT-3’; SlCRF5 forward, 5’-AACGATGACGCAGCAGAAG-3’; SlCRF5 reverse, 5’-CTGACACCGGCAAATTTTT-3’; SlCRF6 forward, 5’-GGTTGATGATGCCAGAAGA-3’; SlCRF6 reverse, 5’-GAAGAAACCTCCTGGTGGATAAG-3’; SlCRF7 forward, 5’-AUGTCTACAAAGAAATGAGTT-3’; SlCRF7 reverse, 5’-GGTGTGATGGGGTCACATTTC-3’; SlCRF8 forward, 5’-CCACCAAGATGTAGCTAA-3’; SlCRF8 reverse, 5’-GGTGCCAGCTGGTTATGG-3’; SlCRF9 forward, 5’-TGAAAGAAAATGGGAAATATG-3’; SlCRF9 reverse, 5’-TGTGTAC-3’; SlCRF10 forward, 5’-AAGTCCGTTGGTGGTAAAG-3’; SlCRF10 reverse, 5’-TAATGGTTGGTGGTGGTAAAG-3’; SlCRF11 forward, 5’-AGATGACGACGAGAGGAAT-3’; SlCRF11 reverse, 5’-CTGACACCGGCAAATTTTT-3’.

SlCRF gene expression in response to hormone or salt treatment, as described below, was examined using RT-PCR initiated with 2 min at 95 °C, followed by 29-40 cycles of 30 s at 94 °C, 45 s at 57 °C, and 40 s at 72 °C, and a 5 min final extension at 72 °C. RT-PCR at different cycle lengths was performed for genes of varying intensities: SlCRF3 (29 cycles), SlCRF1, SlCRF2, SlCRF4, SlCRF5, SlCRF6, SlCRF10, and SlCRF11 (30 cycles), SlCRF5 (30 cycles for salt, 35 for other treatments), SlCRF7 (35 cycles for methyljasmonate (MeJA), 40 for other treatments), and SlCRF8 and SlCRF9 (40 cycles). Primers used to examine SlCRF3–5 and TIP41 were as noted above. RT-PCR primers for SlCRF1, SlCRF2, and SlCRF6–11 are as follows: SlCRF1 forward, 5’-AAGATGACGACGAGAGGAAT-3’; SlCRF1 reverse, 5’-CTGACACCGGCAAATTTTT-3’; SlCRF2 forward, 5’-AAATGACCCGAGACGCA-3’; SlCRF2 reverse, 5’-CTGACACCGGCAAATTTTT-3’; SlCRF4 forward, 5’-AGATGACGACGAGAGGAAT-3’; SlCRF4 reverse, 5’-CTGACACCGGCAAATTTTT-3’; SlCRF5 forward, 5’-TGAAAGAAAATGGGAAATATG-3’; SlCRF5 reverse, 5’-TGTGTAC-3’; SlCRF6 forward, 5’-CCACCAAGATGTAGCTAA-3’; SlCRF6 reverse, 5’-GGTGCCAGCTGGTTATGG-3’; SlCRF7 forward, 5’-AUGTCTACAAAGAAATGAGTT-3’; SlCRF7 reverse, 5’-TTACCCCTGTCACAAAAAC-3’; SlCRF8 forward, 5’-TGAAAGAAAATGGGAAATATG-3’; SlCRF8 reverse, 5’-TGTGTAC-3’; SlCRF9 forward, 5’-AAGTCCGTTGGTGGTAAAG-3’; SlCRF9 reverse, 5’-TAATGGTTGGTGGTGGTAAAG-3’; SlCRF10 forward, 5’-AAGTCCGTTGGTGGTAAAG-3’; SlCRF10 reverse, 5’-AAATGACCCGAGACGCA-3’; SlCRF11 forward, 5’-AGATGACGACGAGAGGAAT-3’; SlCRF11 reverse, 5’-CTGACACCGGCAAATTTTT-3’.

For quantitative real-time PCR (qRT-PCR) analysis, total RNA was extracted from cytokinin- or dimethylsulphoxide (DMSO) control-treated leaves using the same reagents and protocol as described for RT-PCR. A 2 μl aliquot of a 20-fold cDNA dilution was used for each reaction in the following qPCR. qPCR was performed with the SYBR-Green chemistry in a Eppendorf Mastercycler ep realplex with the manufacturer’s instructions. A 500 ng aliquot of the total cDNA was converted into cDNA with Qiagen qScript cDNA supermix. In the following qPCR, qPCR was performed with the SYBR-Green chemistry in a Eppendorf Mastercycler ep realplex with the same set of primers used for examining salt or hormone responses except SlCRF1 and SlCRF2. Primers for SlCRF1 and SlCRF2 are the same as used in the first RT-PCR experiment. Each reaction contains 9 μl of SYBR-Green supermix, 2 μl of cDNA template, 3 μl of 4 μM primers, and 3 μl of sterile water. The qPCR program consists of one cycle at 95 °C, followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, and 35 s at 68 °C. The relative
expression data used in the figure represent means±SE of two biological replicates. All samples are compared with the control gene TIP41 (Exposito-Rodríguez et al., 2008).

Hormone and salt treatments
For all hormone and salt (NaCl) treatments, plants were grown as described above and then leaves or other tissues were excised from 15-day-old Micro-Tom plants, placed in water, and gently shaken for 2 h prior to treatment. Then treatments or appropriate controls were added to shaking tissue for various times as indicated: 5 μM cytokinin (N6-benzyladenine; BA), 100 μM MeJA, and 2 mM SA (salicylic acid), each with the carrier solvent DMSO, and 200 mM NaCl and 1 mM Ethephon (of which ethylene is a breakdown product) with the appropriate level water controls. After designated treatment times (1 h or 3 h) leaves were removed from solution, patted dry, and immediately flash-frozen in liquid nitrogen, and stored at –80 °C until RNA extraction.

Phylogenetic analysis
Full-length sequences of SICRF genes were originally identified by making use of existing sequence data from the four full-length SICRF genes (SICRF1, SICRF3, SICRF4, and SICRF5) that were previously known either through 3' RACE-PCR analysis of partial unigene constructs (SICRF3, SICRF4, and SICRF5) or from an existing gene sequence for SICRF1, also known as PTI6. BLAST analysis of the tomato unigene collection and now fully sequenced tomato genome was conducted using these four SICRF genes and additional CRF sequences from other species, primarily Arabidopsis, at http://solgenomics.net using publicly available genome sequence data from the International Tomato Genome Sequencing Project and from the Kazusa Full-length Tomato cDNA Database at http://www.pgb.kazusa.or.jp/kalfom. Searches were done primarily using conserved AP2/ERF- or CRF-specific domain regions of the known SICRF genes in a manner similar to that done in the identification of CRF genes in a wide range of plant species (Rashotte and Goertzen, 2010). Once all full-length SICRF gene sequences were found, they were translated and aligned as proteins in CLC Sequence Viewer v6.5.1 using default parameters. A phylogenetic cladogram was generated using the Neighbor-Joining method via bootstrap analysis of full-length aligned SICRF proteins again in CLC Sequence Viewer v6.5.1 using default parameters. Arabidopsis genes examined herein are designated as follows: CRF9 (At1g49120), CRF10 (At1g68550), CRF11 (At3g25890), and CRF12 (At1g25470); and were previously noted as B-clade members of the CRF genes in Rashotte and Goertzen (2010), CRF9=CRF-B1, CRF10=CRF-B3, CRF11=CRF-B4, and CRF12=CRF-B2.

Protein examination
Vector construction: All plasmids for BiFC (bimolecular fluorescence complementation) were generated using the Invitrogen GATEWAY™ cloning system according to the manufacturer’s instructions. Entry clones for SICRF1, SICRF2, SICRF3, and SICRF5 were prepared/generated via a BP reaction using the pDONR221 and the att-B PCR product containing att-B adaptor sites and full-length cDNA sequence except the stop codon. Through an LR reaction, coding sequence was transferred to destination vectors pSAT4-DEST-n (1–174) EYFP-C1 and pSAT5-DEST-c (175–end) EYFP-C1 which have N- and C-terminal parts of the yellow fluorescent protein (YFP) gene, respectively. These destination clones were later used to transform Micro-Tom protoplasts. To examine cellular localization in planta, SICRF1, SICRF2, and SICRF5 were transferred, through an LR reaction, to the 35S:SICRF-GFP (green fluorescent protein) constitutive expression destination vector pMDC84. These destination clones were later used to transform Agrobacterium tumefaciens that was injected into tobacco leaves. All destination vectors were obtained through the ABRC at Ohio State University.

Protoplast isolation and transformation for BiFC analysis
For isolating leaf protoplasts, leaves were taken from 15-day-old plants, cut into thin strips, and placed in enzyme solution [2% Cellulase R10, 1% Macerozyme R10, 0.6 M mannitol, 20 mM KCl, 25 mM MES solution, pH 5.7 which was heated at 55 °C for 10 min, then cooled down to room temperature before adding 10 mM CaCl2 and 1% bovine serum albumin (BSA)] under vacuum for 30 min. Next, leaf strips were gently shaken for 4 h or overnight at 40–60 rpm before increased shaking at 90–100 rpm for 10 min to release protoplasts. Enzyme solution containing the protoplasts was filtered with a 40 μm cell sifter into a 50 ml conical tube and spun at 100 g for 2 min to pellet the protoplasts. Pelleted protoplasts were resuspended in 2 ml of cold wash solution (0.6 M mannitol, 5 mM MES pH 5.7, 20 mM KCl, 10 mM CaCl2) and spun again. Then the pellet was resuspended in wash solution to obtain the final volume for electroporation and kept on ice until transformation. Electroporation of protoplasts was performed as in Rashotte et al. (2006) and then they left undisturbed in the dark at room temperature overnight prior to microscopic observation.

Agrobacterium infiltration and transformation for in planta examination of cellular location
Tobacco (Nicotiana tabacum) plants were grown under a long day 16 h light 26 °C, 8 h dark 22 °C cycle. Destination vectors used for transformation (SICRF genes in pMDC84, as described above) were transformed into A. tumefaciens (C58-C1) by a method similar to that used in Rashotte et al. (2006), leading to a floral dip. However, once properly antibiotic-selected individual colonies were identified, further grown up in liquid culture, and spun down, they were then resuspended in infiltration media (10 mM MgCl2, 10 mM MES, 100 μM acetosyringone) and left at room temperature for 3 h similar to the method of Liu et al. (2002). Agrobacterium was then infiltrated into the abaxial side of 14- to 21-day-old plant leaves using a needleless 2 ml syringe. Plants were then examined for transient transformation and GFP expression 48–72 h after injection using epifluorescence microscopy as in Cutcliffe et al. (2011).

Epifluorescence microscopy
BiFC and Agrobacterium-infiltrated tobacco leaves were examined using a Nikon Eclipse 80i epifluorescence microscope with a UV source in transformed protoplast. A standard UV filter was used in addition to 1 ng ml–1 of Hoechst 33342 dye initially to observe and identify nuclei in intact cells as a measure of the cell viability. A YFP filter that blocks both chlorophyll fluorescence and Hoechst 33342 fluorescence was used to examine the localization of any split-YFP fusions that occur due to BiFC between proteins. Cytokinin (2 μM BA) was routinely added to protoplasts prior to examination. A GFP filter that blocks both chlorophyll fluorescence and Hoechst 33342 fluorescence was used to examine cellular localization of any cells expressing GFP in Agrobacterium-infiltrated tobacco leaves. All photos were taken with a Qimaging Fast 1394 digital camera and are presented as composite images using Adobe Photoshop CS3 without altering the original integrity of the picture.

Results
Identification of novel tomato CRF genes (SICRF genes)
A family of 11 CRF genes from tomato, known as Solanum lycopersicum cytokinin response factor genes or SICRF1–SICRF11, have been identified and characterized (Fig. 1, Table 1; Supplementary Table S1 available at JXB online).
These genes are members of the AP2/ERF transcription factor family, specifically related to clade VI and VI-L of the ERF subfamily of genes, known in Arabidopsis as AtERF genes (Sakuma et al., 2002; Nakano et al., 2006; Rashotte and Goertzen, 2010). These genes were identified from a combination of BLAST searches of emerging tomato genome sequence resources using previously identified CRF genes in tomato, orthologous AtCRF sequences, and 3’ RACE of incomplete expressed sequence tag (EST) unigene builds of SlCRF genes. Previous work identified transcription of four SlCRF sequences (SlCRF1, SlCRF3, SlCRF4, and SlCRF5), including the existing PTI6 gene, that has also been designated as SlCRF1 (Rashotte and Goertzen, 2010). From this base, 10 novel full-length expressed CRFs (SlCRF2–SlCRF11) have been identified, comprising all proteins in tomato containing a CRF domain, a defining characteristic of CRF proteins (Fig. 1, Table 1; Supplementary Table S1). In several cases 3’ RACE was used to generate full-length gene transcripts from assembled unigenes lacking a 3’ end region. Subsequent genome assemblage and sequenced bacterial artificial chromosome (BAC) contigs have verified the determined sequence identified from 3’ RACE experiments. Full-length transcripts for SlCRF1–SlCRF11 are presented (Supplementary Table S1). SlCRFs at a protein level fall into three classifications (Fig. 1A). One is a standard CRF protein (SlCRF1, SlCRF2, SlCRF4–SlCRF6, and SlCRF9–SlCRF11), which contains both a CRF and AP2 DNA-binding domain in addition to a putative mitogen-activated protein kinase (MAPK) phosphorylation motif, as seen in a wide range of plant species (Rashotte and Goertzen, 2010). The second is a shortened CRF protein (SlCRF7 and SlCRF8), which contains the CRF and AP2 DNA-binding domain but lacks the 3’ third of the protein and the phosphorylation motif, as is also seen in other species such as Arabidopsis (CRF7 and CRF8). The final classification is a unique CRF protein (SlCRF3), containing two CRF and AP2 DNA-binding domains in an alternating pattern. This is the only known CRF protein that contains more than a single CRF domain and is expressed, from >250 identified CRF proteins examined across all land plants. Interestingly its chromosomal position is very close to the highly related SlCRF8, only 9125 bp away, suggesting a possible gene duplication event (Table 1).
Alignment of these proteins revealed high similarity in domain regions, such as the core conserved region DPDATDSSSD of the CRF domain (Fig. 1B), similar to that seen in previous alignments of CRF proteins from a wide range of land plants (Rashotte and Goertzen, 2010). For ease of alignment and phylogenetic analyses in this study, the full-length SlCRF3 was split into N- and C-terminal parts each containing a CRF and AP2 domain, although a full-length version yielded similar results (data not shown). Phylogenetic analysis based on similar domain sequences indicates that some SlCRFs have a paired relationship, suggesting an ancient duplication, as well as most SlCRFs having an Arabidopsis orthologue (Fig. 1C; D). Tomato and Arabidopsis do not have directly orthologous phylogenetic protein pairs since, in some cases, a single SlCRF protein is grouped with two Arabidopsis proteins (SlCRF2 with AtCRF1 and AtCRF2; SlCRF5 with AtCRF5 and AtCRF6). Additionally, SlCRF1 has no orthologous Arabidopsis gene partner (Fig. 1D), although it is part of a related subclade of CRF proteins found in a number of other species (Rashotte and Goertzen, 2010).

SlCRF genes are expressed in different plant tissues

Previous work identified four SlCRF genes (SlCRF1, SlCRF3, SlCRF4, and SlCRF5) as expressed in leaf tissues (Rashotte and Goertzen, 2010). Here it is shown that SlCRF3–SlCRF11 are expressed in multiple different plant tissues throughout the plant (leaf, stem, root, and flowers) to varying degrees (Fig. 2). Generally, SlCRF expression levels were consistent across plant tissues examined. However, some genes showed preferential tissue expression, as seen for roots in SlCRF4 and SlCRF5 and for stems in SlCRF8 and SlCRF11 (Fig. 2).

SlCRF transcript levels are regulated by cytokinin and salt

Knowing that several CRFs in Arabidopsis have previously been shown to be induced by cytokinin, the regulation of SlCRF genes by cytokinin was examined. Tomato leaves (15 d old) were treated with cytokinin (5 \( \mu \)M BA) or DMSO as a vehicle control for 1 h and 3 h and examined using real-time PCR. Three SlCRF genes (SlCRF2, SlCRF3, and SlCRF5) were found to be strongly (4- to 6-fold) induced by cytokinin (Fig. 3A). SlCRF2 showed rapid induction by cytokinin at 1 h after treatment to 6-fold over untreated levels and by 3 h was still induced, although at this point only ~3.5-fold over control levels. Both SlCRF3 and SlCRF5 showed no induction at 1 h, but were highly induced (4- to 5-fold) after 3 h of cytokinin treatment. A few other SlCRF genes showed weaker levels (1.5- to 2-fold) of induction at 3 h of cytokinin treatment (SlCRF1, SlCRF6, SlCRF7, SlCRF8, and SlCRF9), whereas SlCRF4, SlCRF10, and SlCRF11 showed no change in expression (Fig. 3A). The results follow a pattern similar to that seen for AtCRF genes whereby some, but not all, members of this group are transcriptionally regulated by cytokinin (Rashotte et al., 2006).

SlCRF genes were also examined for changes in response to salt and other hormones in leaves treated at 1 h and 3 h versus controls using RT-PCR. The results revealed expression changes in several genes, although many showed little to no alterations (Fig. 3). Expression analysis of salt treatment (200 mM NaCl) revealed induction of SlCRF1, SlCRF4, and SlCRF6 at both 1 h and 3 h as well as a minor induction of SlCRF2, SlCRF5, and SlCRF7 at 3 h (Fig. 3B). This suggests a new potential role for SlCRF genes in stress regulation. Expression analysis of ethylene treatment

| Table 1. SlCRF gene description |
|--------------------------------|
| **Gene name** | **Chromosome/position** | **Gene model** | **Size (amino acids/bp)** |
|----------------|-------------------------|----------------|--------------------------|
| SlCRF1/PT16    | Ch 6 (44654446–44653700)| Solyc06g082590| 248/747                  |
| SlCRF2         | Ch 8 (62045738–62046757)| Solyc06g081960| 340/1023                 |
| SlCRF3         | Ch 1 (2911579–2910313)  | Solyc01g008890| 344/1035                 |
| SlCRF4         | Ch 3 (2016125–2014935)  | Solyc03g007460| 396/1191                 |
| SlCRF5         | Ch 1 (78502891–78503773)| Solyc01g005500| 293/882                  |
| SlCRF6         | Ch 6 (32043471–32044523)| Solyc06g051840| 387/795                  |
| SlCRF7         | Ch 1 (14596809–14596333)| Solyc01g014720| 174/525                  |
| SlCRF8         | Ch 1 (2901188–2900649)  | Solyc01g008890| 175/540                  |
| SlCRF9         | Ch 3 (62191449–62190256)| Solyc03g119580| 397/1194                 |
| SlCRF10        | Ch 5 (3622457–3621438)  | Solyc05g009450| 339/1020                 |
| SlCRF11        | Ch 4 (874453–875505)    | Solyc04g007180| 350/1053                 |

**Fig. 2.** SlCRF expression patterns in various tomato tissues. RT-PCR analysis of SlCRF1–SlCRF11 in leaf, stem, root, and flower tissues of 52-day-old plants is shown. The TIP41 gene serves as an internal control.
(1 mM Ethephon) showed some induction of SlCRF1 and SlCRF4 at both 1 h and 3 h, while SlCRF2 was repressed at both 1 h and 3 h and SlCRF7 at 1 h (Fig. 3C). These are some of the first data linking any CRF to ethylene. Expression analysis of 100 μM MeJA treatment showed only a single transcript change, the repression of SlCRF6 at both 1 h and 3 h (Fig. 3D). Expression analysis of 2 mM SA treatment revealed induction of SlCRF1 at 3 h as well as induction of SlCRF4 and SlCRF8 at both 1 h and 3 h (Fig. 3E). Together these results suggest that SlCRF genes can be regulated by factors other than cytokinin.

**SlCRF proteins show nuclear localization in planta**

The cellular localization of specific SlCRF proteins (SlCRF1, SlCRF2, and SlCRF5) was examined by transiently expressing GFP-tagged SlCRF proteins in tobacco leaves via an Agrobacterium infiltration method (Fig. 4A).
Leaves infiltrated with 35S:SiCRF:GFP vectors were examined for expression after 48 h. Each of the SiCRF proteins examined was found localized in the nucleus of leaf mesophyll cells and not other organelles in regions adjacent to infiltration sites as compared with empty transformed vectors or wild-type untransformed plants (Fig. 4A). Although localization of SiCRFs can be seen in the nucleus of cells, it is not obviously absent from the cytoplasm, which is consistent with previous models of AtCRFs that appear to move between the cytoplasm and nucleus. This is also in agreement with the cellular localization of SiCRFs as predicted by PSORT computer protein localization prediction models (data not shown), indicating preferences primarily for nuclear, cytoplasmic, or either nuclear or cytoplasmic protein localization.

**SiCRF proteins interact among themselves**

Protein–protein interactions can be important for functional regulation of proteins. In order to determine if this level of regulation occurs among SiCRFs, potential interactions were examined using the BiFC analysis split-YFP system. SiCRF proteins (SiCRF1, SiCRF2, SiCRF3, and SiCRF5) were placed into specific vectors which enabled their expression linked to either an N- or C-terminal half of a YFP protein, such that fluorescence would not be visible unless proteins containing each YFP half interact. Proteins were examined for interaction by electroporation of tomato leaf mesophyll protoplasts followed by epifluorescence microscopy (Fig. 4B). It was found that homodimers formed between all SiCRFs examined. In addition, heterodimers could also form with all SiCRF combinations examined (Fig. 4). In these experiments, while cytokinin is not required to observe nuclear localization, it is easier to visualize nuclear localization after its addition, so it is routinely added. Overall these findings are consistent with what has been found for AtCRFs and suggest that because there is a pattern for potential of all SiCRF proteins to interact, regulation of SiCRFs at the level of protein dimerization is unlikely to occur (Cutcliffe et al., 2011).

**Discussion**

Cytokinin is involved in various plant growth and developmental processes of great agronomic importance, yet few cytokinin-regulated genes have been studied in crop plants. This study presents the first examination of a complete set of CRF genes in a crop species, tomato (S. lycopersicum). Eleven SiCRF genes (SiCRF1– SiCRF11) were identified in this study as part of a larger group of CRF genes present in all land plants (Rashotte and Goertzen, 2010). SiCRF proteins contain the hallmark domains of this group; a CRF and AP2 DNA-binding domains.
domain, as well as a putative MAPK motif found in many other CRF proteins (Fig. 1; Rashotte and Goertzen, 2010). One SICRF, SICRF3, was found to have a unique protein structure containing two CRF and two AP2 domains (Fig. 1; Supplementary Table S1 at JXB online). While several AP2/ERF proteins contain two AP2 domains, including the founding member of this group, SICRF3 is the only known protein to contain more than a single CRF domain. Despite this, it appears to be actively transcribed, induced by cytokinin, and able to interact with other SICRF proteins.

A phylogenetic analysis of SICRFs shows relationships similar to that seen for AtCRFs and the overall group of CRFs in plants (Rashotte and Goertzen, 2010). Despite overall similarities between tomato and Arabidopsis CRFs, there are several differences that may suggest functional differences between species. An example is the existence of a single SICRF gene where there are two paralogues in Arabidopsis, such as SICRF5 compared with AtCRF5 and AtCRF6 (Fig. 1D). Another difference is that SICRF1 has no direct Arabidopsis orthologue. In fact, most plant species appear to have a SICRF1 orthologue, indicating that the condition in tomato is more common (Rashotte and Goertzen, 2010). It also suggests that the function of SICRF1 is unlikely to be simply determined through studies of CRFs in Arabidopsis.

Expression of SICRF1–SICRF11 in tissues from roots to flowers suggests a broad role for these genes in the plant (Fig. 2). There also appears to be a range of transcript levels of SICRFs potentially indicating different functional roles in different tissues. This is the most complete tissue analysis of a CRF group of genes from any species excluding Arabidopsis where microarray-generated data of AtCRFs reveal a pattern of expression across most tissue types and development, not unlike that seen for the SICRFs in this study, suggesting that CRFs in most plants are likely to be expressed broadly across tissues (data not shown).

Several SICRF genes were found to be induced by cytokinin, mirroring a pattern seen in Arabidopsis where only some CRF genes show strong induction by cytokinin (Rashotte et al., 2006). Interestingly these AtCRF genes parallel the SICRF genes strongly induced in this study. SICRF2, highly similarly to AtCRF2, shows the most rapid induction of tomato CRF genes comparable with very rapid induction of AtCRF2 (Fig. 3A; Rashotte et al., 2006). SICRF5, similar to both AtCRF5 and AtCRF6, is also highly induced by cytokinin (Figs 1D, 3A; Rashotte et al., 2006). SICRF3 is not as rapidly induced as SICRF2, which parallels the slower cytokinin induction of AtCRF6 compared with other CRF genes (Rashotte et al., 2006). SICRF3 is a unique gene, occurring only in tomato, and as such it is difficult to assess its role in cytokinin regulation, although it is clearly induced by cytokinin in a similar fashion to SICRF5. The lack of cytokinin regulation of some highly related pairing of SICRF genes also parallels expression studies of other AtCRF genes, such as SICRF4 and SICRF6 compared with AtCRF3 and AtCRF4. Overall, the pattern of transcriptional cytokinin regulation of SICRF genes is similar to that of AtCRF genes and suggests that there may be similar regulation within specific clades of CRF genes.

Other factors that might transcriptionally affect SICRFs as they had been shown to affect related ERF family members were examined: salt, ethylene, MeJA, and SA (Gu et al., 2000, 2002; Park et al., 2001; Sakuma et al., 2002; Nakano et al., 2006; Zarei et al., 2011). Treatment with salt (NaCl) induced about half of the SICRFs to some degree (Fig. 3B), revealing that CRFs can be induced by abiotic factors. An investigation of related AtCRFs (AtCRF2, AtCRF5, and AtCRF6) also indicated induction by NaCl treatment from an examination of publically available microarray data. Previous examinations of the tobacco stress-induced 1 (TSII) gene (a CRF member) has shown transcript induction during high salt stress in both overexpressing and RNAi (RNA interference) transgenic plants (Park et al., 2001; Han et al., 2006). The present finding that several SICRFs are induced by salt treatment supports the previous finding for TSI1 and suggests that CRFs play a role in salt stress response and may be involved in more general regulation of stress responses. Ethylene treatment resulted in a mixed set of responses from SICRFs, from some induction to repression, with little effect on the majority of SICRFs (Fig. 3C). Previous studies have shown that ethylene had little to no effect on AtCRFs and SICRF1/Pt6, consistent with most SICRFs in this study. The exception, SICRF2 transcript repression, indicates that ethylene may play some role in SICRF function, although a more detailed study is needed to determine further the extent. MeJA treatment showed almost no effect on any SICRFs, suggesting that it plays little role in CRF function, although specific CRFs such as SICRF6 may be exceptions (Fig. 3D). SA treatment resulted in minor induction of three SICRFs similar to MeJA treatments, indicating that SA also appears to have little effect on the transcription of most SICRFs. Together these results suggest that SICRFs can be regulated by factors other than cytokinin and may fall into different groups of regulated genes: some (SICRF3 and SICRF5) regulated primarily by cytokinin, others (SICRF1, SICRF2, SICRF4, SICRF6, SICRF7, and SICRF8) regulated by several factors, and some (SICRF9–SICRF11) showing little response to factors examined in this study. A broader examination of SICRF expression patterns, beyond this study, is needed to determine the functional role of each SICRF.

Previous examinations of non-Arabidopsis CRF genes have shown links to pathogen response when overexpressed for Pt16 from tomato (SICRF1) and Tsi1 from tobacco (Zhou et al., 1997; Park et al., 2001; Gu et al., 2002). While pathogen response was not examined in this study, the finding that SICRF1 is induced by the factors ethylene and SA is linked to this process, and supports this previous reported role for SICRF1 (Zhou et al., 1997; Gu et al., 2002). The finding that several other SICRF genes are affected by these similar treatments may suggest that an effect on pathogen response could be a broader functional characteristic of some SICRF genes.

Cellular localization is often an important factor for determining the function of proteins such as transcription
factor localization to the nucleus required for their mode of action: binding to DNA. AtCRFs in protoplasts were previously shown to be throughout the cytoplasm and localized to the nucleus with the addition of exogenous cytokinin (Rashotte et al., 2006). Protoplasts are good single cell systems to examine cellular localization, but lack several aspects of a true in planta system that may reflect a more accurate result. To overcome this, GFP-tagged SICRF proteins were transiently expressed in tobacco leaves where SICRFs were found to be primarily nuclear localized in the absence of exogenous cytokinin, although some cytoplasmatic localization as well cannot be ruled out (Fig. 4A). SICRF localization to both the nucleus and cytoplasm would be consistent with previous results of AtCRFs and with protein localization prediction data for SICRFs (Rashotte et al., 2006). It may be that CRFs act in a manner similar to the Arabidopsis histo-phospho transfer proteins (AHPs) known to move between the cytoplasm and the nucleus relaying a cytokinin signal in that pathway. Initial work examining AHP localization in protoplasts showed cytoplasmic expression followed by nuclear localization after the addition of exogenous cytokinin, similar to that of the AtCRFs (Hwang and Sheen, 2001). However, a recent in planta examination of AHPs revealed a strong nuclear expression of these proteins in root tissues, where there are high levels of endogenous cytokinin (Punwani et al., 2010). However, AHPs were also found to a lesser degree in the cytosol, consistent with a cycling between nucleus and cytosol needed for these proteins to function as phosphate carriers in cytokinin signalling (Punwani et al., 2010). The identification of SICRFs primarily localized in the nucleus, without the addition of exogenous cytokinin, suggests a similar mechanism, in which intact leaf mesophyll cells contain levels of endogenous cytokinin high enough to focus SICRF to the nucleus. It is contended that protoplasts contain very low levels of endogenous cytokinin, such that CRFs are not routinely found localized within their nucleus until exogenous cytokinin is added, consistent with the findings presented here.

Protein–protein interactions are very common and important in signal transduction, including the regulation of transcription factors by patterns of homo- or heterodimerization with other partners (Pawson and Scott, 1997; Pawson and Nash, 2000; Kasahara et al., 2001). It was found that each of the SICRFs examined was able to form both homodimers and heterodimers with the other SICRFs, suggesting that SICRFs are unlikely to be regulated at this level. Although not all SICRFs were examined in this study, the results of the representative SICRFs examined here are consistent with a larger study of protein–protein interactions among AtCRFs, showing widespread homo- and heterodimerization and indicating that the CRF domain itself is likely to be involved in this interaction (Cutcliffe et al., 2011). Interestingly, the presence of an additional CRF and AP2 DNA-binding domain in SICRF3 does not appear to affect these interactions.

In summary, this work identifies and characterizes 11 CRF genes in tomato (SICRF1–SICRF11). It is shown that SICRF1–SICRF11 are expressed at varying levels over a range of tissues. SICRF proteins appear to show nuclear localization and can interact to form homo- and heterodimers amongst themselves. Several SICRFs show strong induction by cytokinin similar to that previously noted for AtCRFs. Additionally, some SICRFs were found to be regulated by factors other than cytokinin, potentially suggesting a diverse role for CRFs in stress and other hormone regulation in plants. This study indicates that SICRFs appear to have multiple regulatory functions in tomato plants.

Supplementary data
Supplementary data are available at JXB online.

Table S1. SICRF gene and protein sequences. Full-length DNA coding sequences as well as translated amino acid protein sequences for SICRF1–SICRF11 are shown.

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