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ORIGINAL PAPER

Characterization of two tomato AP2/ERF genes, *SlCRF1* and *SlCRF2* in hormone and stress responses

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Abstract
Key message ***SlCRF1*** **and** ***SlCRF2*** **are expressed throughout the plant, prominently in vascular tissue. Each SlCRF has a distinct pattern of cytokinin induction and regulation by abiotic stresses in different organs.**
Abstract Cytokinin is an essential plant hormone involved in the regulation of many growth and developmental processes. While many cytokinin signaling pathway components have been well characterized, the cytokinin response factors (CRFs) that form a branch of this pathway are less well understood. This study examines the tomato (*Solanum lycopersicum* (L.)) *CRF* genes, *SlCRF1* and *SlCRF2* presenting a detailed and novel characterization of their developmental expression patterns, transcriptional regulation by hormones particularly cytokinin, and response to abiotic stresses. Both *SlCRF1* and *SlCRF2* were predominantly expressed in vasculature in tissues throughout the plant, with an overall trend for greater *SlCRF2* expression in younger organs. Hormone regulation of *SlCRF1* and *SlCRF2* transcripts is primarily by cytokinin, which induced both *SlCRF*s in different organs over a range of developmental stages. The strongest cytokinin induction was found in leaves, with *SlCRF2* induced to a higher level than *SlCRF1*. Examination of *SlCRF* transcripts during abiotic stress responses revealed that *SlCRF1* and *SlCRF2* have distinct patterns of regulation from each other and between leaves and roots. Novel connections between *SlCRF*s and stresses were found in particular including a strong induction of *SlCRF1* by cold stress and a strong induction of *SlCRF2* by oxidative stress in roots and unique patterns of induction/repression linking both SlCRFs to drought stress and response during recovery. Overall, this study provides a clear picture of *SlCRF1* and *SlCRF2* expression patterns across tissues during development and in response to cytokinin and specific stresses, indicating their importance in plant growth and environmental responses.

Keywords SlCRF · Cytokinin · Tomato · Abiotic stress

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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). In addition to the well-established model of cytokinin signaling (two-component system-like multistep phosphorelay) (To and Kieber 2008; Werner and Schmülling 2009), a branch pathway of cytokinin signaling featured by cytokinin response factors (CRFs) has been proposed (Rashotte et al. 2006).

Cytokinin response factors are a subgroup of the AP2/ERF transcription factor family that is defined as having at the protein level a CRF domain and an AP2 DNA binding domain (Rashotte and Goertzen 2010). CRFs have recently been further classified into five distinct CRF clades (I–V) based on the presence of a clade-specific C-terminal region in their protein sequences (Zwack et al. 2012). Previous

studies have shown that CRFs are involved in numerous aspects of plant life such as regulation by hormones (Rashotte et al. 2006; Schlereth et al. 2010; Shi et al. 2012; Zwack et al. 2013), cotyledon, leaf, and embryo development (Rashotte et al. 2006), responses to biotic and abiotic stresses (Zhou et al. 1997; Park et al. 2001; Gu et al. 2002; Shi et al. 2012; Jeon and Kim 2013), negative regulation of leaf senescence (Zwack et al. 2013), and positive regulation of plastid division (Okazaki et al. 2009).

CRF genes have preferential localization patterns in vascular tissue, especially the phloem, due to an enriched phloem targeting *cis*-element in their promoter regions (Zwack et al. 2012). Arabidopsis *CRF* loss-of-function mutants from cytokinin-regulated clades show an altered patterning of higher order veins, suggesting a link of *CRFs* to the regulation of developmental processes associated with vascular tissue (Zwack et al. 2012).

While much of what is known about *CRFs* comes from studies on Arabidopsis *CRFs* or *AtCRF*s, ongoing research on tomato (*Solanum lycopersicum* (L.)) *CRFs* or *SlCRF*s is also revealing novel aspects regarding these CRF-domain containing genes. Notably, there are two CRF Clade I members in Arabidopsis (*CRF1* and *CRF2*), but only a single Clade I ortholog in tomato (*SlCRF2*). In addition, the sole CRF Clade IV member in tomato, *SlCRF1* has no direct ortholog in Arabidopsis, since that species contains no Clade IV CRFs. These facts compelled us to conduct an in-depth study on these two unique *SlCRF* genes representing two distinct clades within the CRF family. The present study was conducted to characterize both *SlCRF1* and *SlCRF2*, presenting detailed information regarding their transcriptional regulation by cytokinin, auxin, and abscisic acid, and their expression in response to abiotic stresses.

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 or magenta boxes containing MS medium (pH 5.8) supplemented with Gamborg B5 vitamins and 2 % sucrose under a 16 h/8 h light/dark photoperiod at 150 μE m^{-2} s^{-1}, at a 26 °C day, 22 °C night temperature.

RNA isolation, cDNA synthesis, and expression analysis

For expression analysis, tissues were immediately flash-frozen in liquid nitrogen, RNA extracted using Qiagen RNeasy Kit according to the manufacturer's instructions, and then reverse transcribed with Quanta qScript cDNA supermix. qPCR was performed with these cDNA samples using SYBR-Green chemistry in an Eppendorf Mastercycler ep realplex with gene-specific primers: qSlCRF1F 5′-ACGATGTCGCTTTGTCACC-3′; qSlCRF1R 5′-GGGCAAAATCGTCAAAGTCA-3′; qSlCRF2F 5′-ATGCTGCCGGTCCTAGAGTT-3′; qSlCRF2R 5′-GAGCAGTTTCCGACGATGAC-3′; TIP41 Fw 5′-ATGGAGTTTTTGAGTCTTCTGC-3′; TIP41Rv 5′-GCTGCGTTTCTGGCTTAGG-3′; or SlelFFw SlelFRv for stress experiments. Each reaction has a total volume of 20 μL. The qPCR program consists of one cycle at 95 °C, followed by 40 cycles of 15 s at 95 °C, 45 s at 57 °C/56 °C (stress), and 40 s/50 s (stress) at 68 °C. For leaf samples treated by 5 μM cytokinin, another set of gene-specific primers were used: SlCRF1F 5′-GGAAAATTCAGTTCCGGTGA-3′; SlCRF1R 5′-AAAATTGGTAACGGCGTCAG-3′; SlCRF2F 5′-TGCCGGTCCTAGAGTTGTAA-3′; SlCRF2R 5′-CAGTGGCTGCTCTGCTCTAT-3′; and 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 mean ± SE of two biological replicates. All samples excluding those from stress treatments were compared to the control gene *TIP41* (Expósito–Rodríguez et al. 2008) and samples from stress treatments (including ABA treatment) were compared to the control gene *SlelF*. Since we found TIP41 expression was influenced by same stress conditions, thus not serving as a true control, we used *SlelF* as the control for stress experiments.

Hormone and stress treatments

For hormone treatments, plants were grown as described above and then leaves or other tissues were excised from Micro-Tom plants, placed in water, and gently shaken for 2 h prior to treatment. Then hormone treatment or appropriate controls were added to shaking tissue for various times as indicated: 1, 5, and 10 μM cytokinin (N^6-benzyladenine, BA) or 1, 5, and 10 μM auxin (1-naphthaleneacetic acid, NAA), 100 μM ABA for 3 h, with the carrier solvent DMSO in a 0.01 % solution as the control. After designated treatment times, samples were removed from solution, patted dry, and immediately flash-frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

For initial 24 h cold treatment, 25-day-old plants grown in magenta boxes under standard conditions were covered with foil, and placed in 4 °C fridge for 24 h before tissue samples were taken. A time course of cold response (1, 6, and 24 h) was done in the same way but with 25-day-old soil-grown plants.

For osmotic stress (200 mM mannitol) and oxidative stress treatments (10 and 20 mM hydrogen peroxide),

plants were treated for 3 h in the same way as hormone treatments.

For flooding treatment, 25-day-old well-watered plants grown in soil were placed in trays to maintain water-logged conditions for 4 days. For drought stress, 25-day-old well-watered plants were left un-watered for 7 days followed by re-watering that allowed them to recover from wilt conditions. Root treatments were performed in growth pouches. Samples were collected after 7 days of drought stress, and 1, 3, 6, 12 h after re-watering. Control plants were watered normally in all the experiments.

Histochemical analysis

For GUS activity analysis, excised tissues were placed into GUS staining buffer (Weigel and Glazebrook 2002) and vacuum infiltrated for 20 min followed by additional incubation overnight. Stained tissue was then cleared in 70 % ethanol and examined with a dissecting microscope. 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.

Generation of transgenic plants

SlCRF expression (destination) vectors used in this study were generated through the Invitrogen Gateway cloning technology according to the manufacturer's instructions. *SlCRF1* promoter::GUS destination vector was generated as in Zwack et al. (2012). *SlCRF2* promoter (2 kb upstream of ATG) was placed in a GUS expression vector pKGWFS7 (Karimi et al. 2002) and transformed into *Agrobacterium tumefaciens* C58 and sent to the Plant Transformation Research Center (PTRC) at University of California at Riverside for transformation of Micro-Tom plants as a service as in Zwack et al. (2012).

Results

SlCRF1 and *SlCRF2* expression is strong in vascular tissues of various organs

Previous studies using promoter::GUS reporter lines have shown that *SlCRF1* is predominantly expressed in the vasculature of different plant organs, although its expression can also be seen in epidermal cells, mesophyll of young leaves, and the pericarp of unripe fruits (Zwack et al. 2012). We further detailed the expression of *SlCRF1* across a greater range of tissues and developmental stages from seed through fruit production, revealing previously unreported expression patterns of *SlCRF1* as well as confirming previous findings of strong vascular expression (Fig. 1). Novel expression of *SlCRF1* promoter::GUS reporter lines was found strongly in hypocotyls of young seedlings (Fig. 1a, b) and flower sepals (Fig. 1i), in addition to vascular expression in leaves of different ages (13-, 24-, and 35-day-old) (Fig. 1c–e). Weaker *SlCRF1* expression can also be seen in the stamens of flowers (Fig. 1h, j, k). The strong expression of *SlCRF1* in leaves and unripe fruits is further supported by microarray data of tomato organs obtained through tomato eFP browser at bar.utoronto.ca (Online Resource 1).

SlCRF2 promoter::GUS lines were also generated to determine the expression pattern of this gene. Analyses of these lines revealed that *SlCRF2* has a strong pattern of vascular expression in many tissues throughout development, similar to other Arabidopsis *CRFs* (*AtCRF*s) and *SlCRF1* (Figs. 1, 2). Vascular expression patterning of *SlCRF2* can be seen in cotyledon, leaf, stem, root, and immature green fruit (Fig. 2c–f, j). Importantly, *SlCRF2* expression is not solely limited to vascular tissues, as it can also been strongly seen in leaf primordia, root tips, and flower stamens (Fig. 2a, b, g–i, k, l, n). This suggests that the function of *SlCRF2*, like *SlCRF1*, is probably in a vascular-related process, but not limited to roles only in that tissue. The broad expression of *SlCRF2* in leaves and roots is also supported by microarray data obtained through tomato eFP browser at bar.utoronto.ca (Online Resource 2).

SlCRF2 expression is higher in younger organs, while *SlCRF1* is higher in older organs

To better understand the spatial and temporal expression patterns of *SlCRF1* and *SlCRF2*, qPCR was performed. RNA was taken from leaves, roots, and stems across development: seedling stage (13 days), young plant (24 days), and mature flowering plant (35 days). In addition, at 18 days, expression levels in all developed leaves were examined based on order of emergence (1st emerged = oldest to 4th emerged = youngest). The highest levels of *SlCRF2* within the plant were found primarily in young organs (13- and 24-day-old leaves, 13-day-old stem, and root, and the younger third and fourth leaves; Fig. 3), with much lower or unchanged levels of expression in older organs or roots. This pattern of transcript expression in these organs generally matches the promoter::GUS reporter gene analysis observed (Fig. 2). In contrast, *SlCRF1* expression levels were at their lowest in the plant among many of the youngest samples of organs examined (13-day-old leaf and root, the youngest fourth leaf; Fig. 3a, c, d), but are higher in older tissues. A spatial examination of different root tissues for *SlCRF1* also showed highly reduced expression levels in the youngest parts of the root (Fig. 3e): the lateral roots and root tips relative to whole root. Again, these transcription expression patterns are

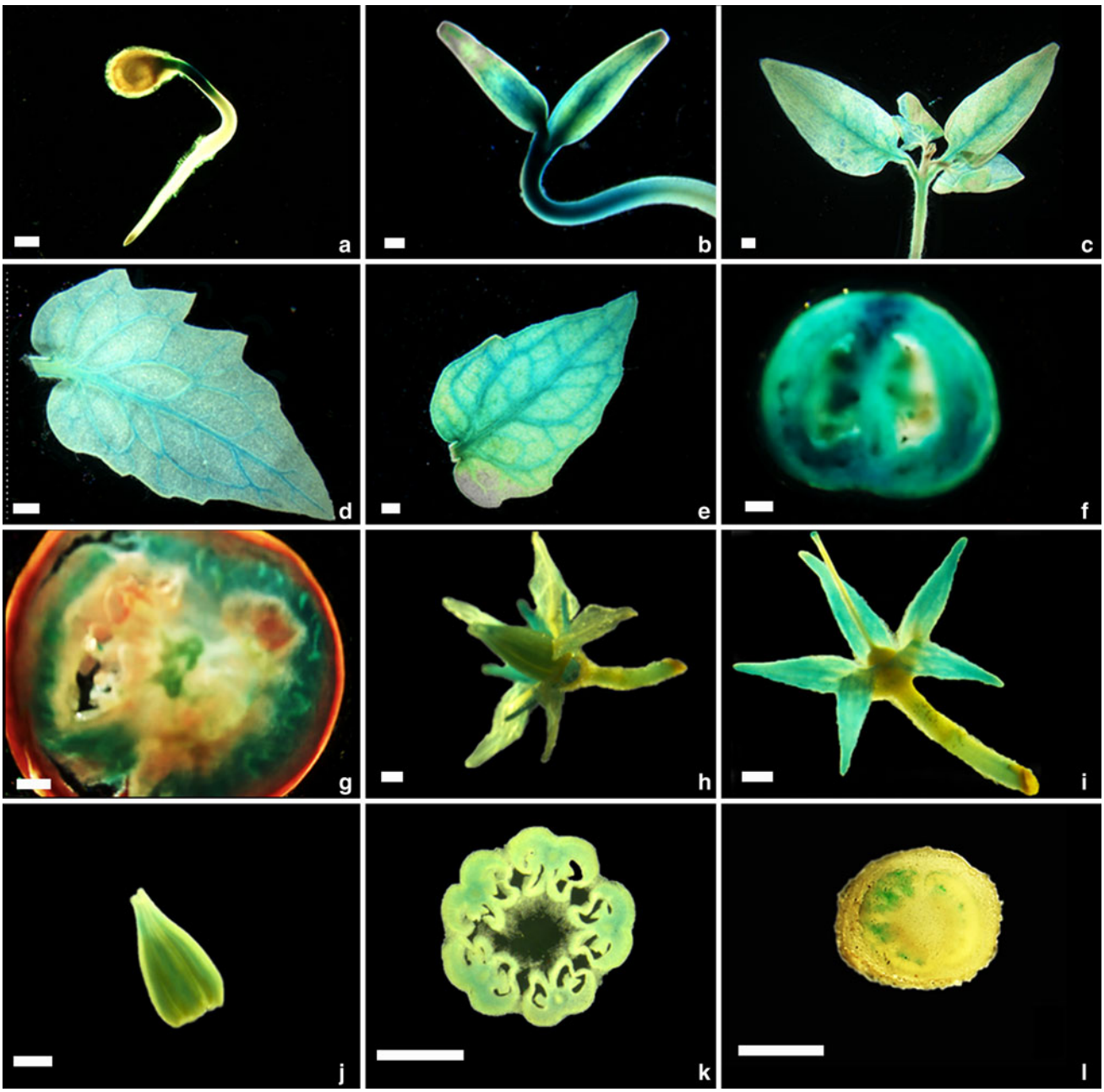


Fig. 1 *SlCRF1* promoter-driven GUS reporter gene expression: **a** 4-day-old seedling, **b** 7-day-old seedling, **c** 13-day-old seedling, **d** 24-day-old leaflet, **e** 35-day-old leaflet, **f** unripe fruit, **g** ripe fruit, **h** whole flower, **i** sepals and pistils, **j** stamens. **k** Free-hand cross section of stamens shown in **j**. **l** Pedicle of the flower. *Scale bar* 1 mm

consistent with the results of the promoter::GUS reporter gene analysis (Fig. 2). Together these profiles along with the promoter analyses suggest distinct spatial and temporal patterns for *SlCRF1* and *SlCRF2*.

SlCRF1 and *SlCRF2* are transcriptionally regulated by cytokinin primarily in leaves

Subsets of *CRF* genes have been shown to be inducible by cytokinin to varying degrees as summarized in Zwack et al. (2012). *SlCRF1* and *SlCRF2* have previously been examined for cytokinin regulation, but in a limited fashion: only in young leaf tissue (15 days), at a single cytokinin concentration (5 μM BA) at early time points (1 and 3 h) (Shi et al. 2012). To provide a greater understanding of how cytokinin regulates these *SlCRF* transcripts, qPCR was performed on cDNA made from RNA extracted of both leaves and stems across development (13, 24, and 35 days). Tissues were treated for multiple lengths of time (2, 7, and 24 h) with different levels of cytokinin (1, 5, and 10 μM BA) and

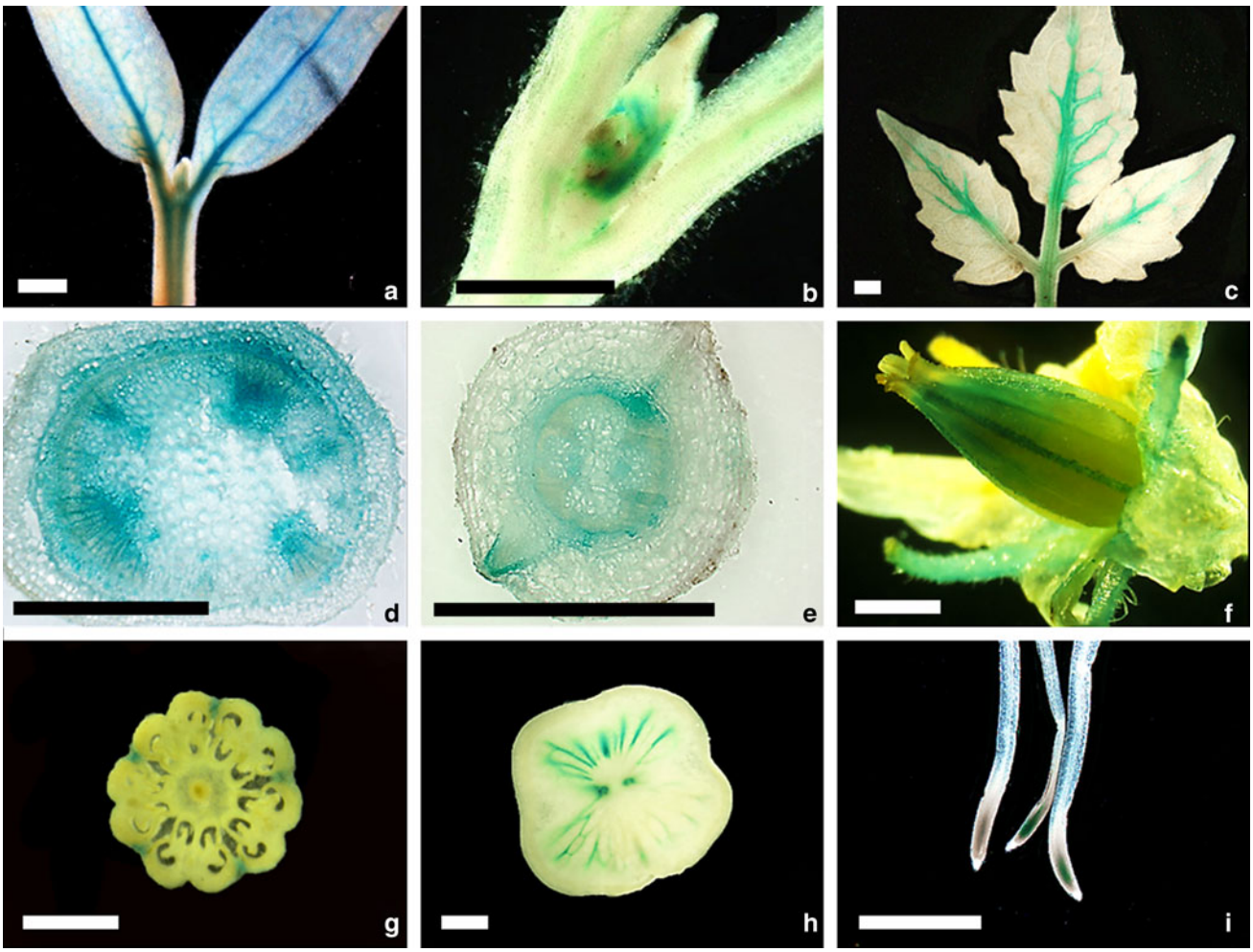


Fig. 2 *SlCRF2* promoter-driven GUS reporter gene expression: **a** 13-day-old seedling, **b** close-up of 13-day-old seedling showing staining in leaf primordium, **c** fully expanded leaf, **d** free-hand stem cross section, **e** free-hand root cross section with emerging lateral root, **f** flower showing stained stamens, **g** free-hand cross section of stained stamens in **f**, **h** free-hand unripe fruit cross section, **i** 13-day-old roots showing staining in the root tip. *Scale bar* 1 mm

compared to the solvent vehicle DMSO for these analyses. Additional qPCR examinations were performed on different root tissues [whole roots (WR), lateral roots (LR), and root tips (RT)] of 14-day-old plants treated with a range of cytokinin (1, 5, and 10 μM BA) or DMSO for 24 h. Both *SlCRF1* and *SlCRF2* were shown to be inducible by cytokinin treatment over a range of treatment times in different organs at different developmental stages beyond what was previously shown, suggesting an active role for cytokinin in the regulation of these genes throughout the lifetime of the plant. Both *SlCRF1* and *SlCRF2* showed their strongest relative induction by the highest level of cytokinin (10 μM) in 13-day-old leaves, with *SlCRF1* induced to 4.0-fold at 2 h after treatment and *SlCRF2* induced to 12.8-fold at 7 h (Fig. 4a, b). *SlCRF1* also showed more than twofold induction by cytokinin in 24-day-old leaves at 2 and 7 h of treatment as well as in 13-day-old stems at 2 h (Fig. 4a, c). *SlCRF2* showed a wide range of cytokinin induction (more than 2-fold) in leaves at each age of plant examined, at multiple time points, and at different cytokinin concentrations (Fig. 4b). *SlCRF2* was also induced by cytokinin in stems at different ages as well (Fig. 4d). It is interesting to note clear differences in cytokinin induction between *SlCRF1* and *SlCRF2*, such as the strong cytokinin induction of *SlCRF2* in 35-day-old leaves at each of the time points examined, whereas *SlCRF1* appears to be not regulated by cytokinin at this developmental stage, despite induction in leaves at earlier stages (Fig. 4a, b).

For the most part, cytokinin regulation of *SlCRF1* and *SlCRF2* transcripts appears to be lacking in whole roots and specific root parts, except possibly for root tips in *SlCRF1* at the highest cytokinin concentration (Fig. 4e). This suggests that a cytokinin-regulated role for these *SlCRF*s may be primarily in aerial tissues, likely the leaves where *SlCRF1* and *SlCRF2* transcript levels are induced to the highest levels.

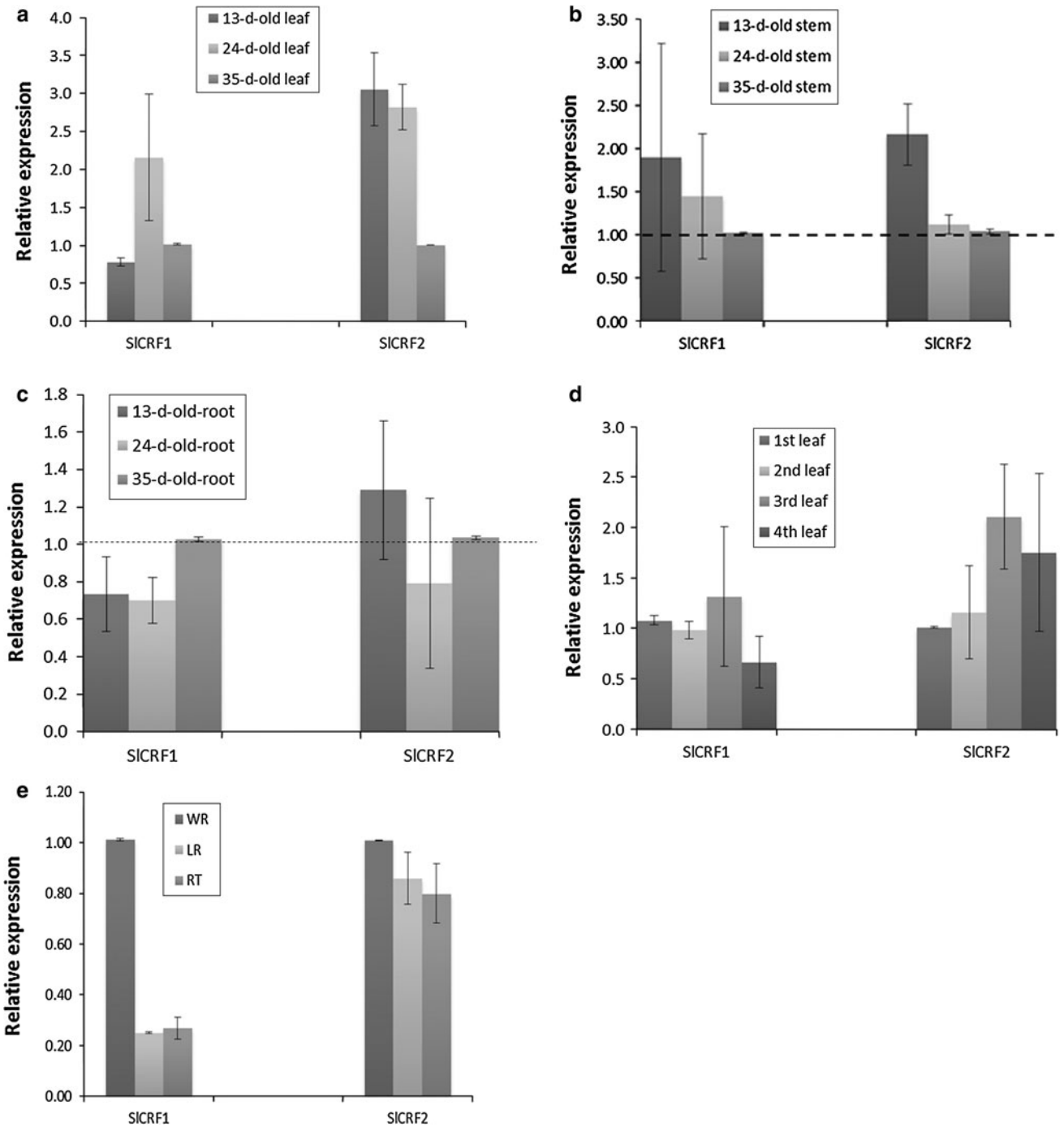


Fig. 3 qPCR analysis of *SlCRF1* and *SlCRF2* expression in various organs of 13-, 24-, and 35-day-old tomato plants. Relative expression of *SlCRF1* and *SlCRF2* in: **a** leaves, **b** stems, **c** roots. **d** True leaves relative to the 1st true leaf in 18-day-old plants, where leaves are based on emergence: 1st oldest, 4th youngest or last to emerge. **e** In different parts of the root: WR (whole root), LR (later root), RT (root tip) collected from 15-day-old plants grown in pouches. *Error bars* represent SE of two biological replicates

Hormone regulation of *SlCRF1* and *SlCRF2* is predominantly by cytokinin

To determine if the expression levels of *SlCRF1* and *SlCRF2* might be affected by treatment of other hormones, qPCR was performed on samples prepared from tissues treated by auxin and ABA for 24 h compared to a solvent-carrier control, DMSO. *SlCRF1* expression was slightly decreased in different root tissues at the highest concentrations of auxin (5 and 10 μM NAA), while *SlCRF2* showed little effect except at the lowest auxin concentration (1 μM NAA) in RT (Fig. 4f). ABA treatment resulted

in only a minor decrease in either *SlCRF1* or *SlCRF2* transcript levels in leaves and roots (Fig. 4g).

SlCRF1 and *SlCRF2* are regulated by abiotic stresses

In order to more thoroughly understand if *SlCRF1* and *SlCRF2* might be regulated by other factors, the transcript level of these genes in leaves and roots was examined in response to a range of different abiotic stresses: temperature, osmotic, oxidative, flooding, and drought followed by recovery (Fig. 5). Both *SlCRF*s responded to different stress treatments in a unique manner, suggesting potential novel and distinct roles of each gene in stress response.

For temperature stress, both cold (4 °C for 24 h) and heat (45 °C for 1 h) stresses were examined. Neither cold nor heat stress resulted in much change in the response of *SlCRF2* transcript (Fig. 5a), suggesting it may not be temperature regulated. In contrast, *SlCRF1* was highly induced by cold in leaves (3.0-fold) and roots (3.0-fold), and was repressed to 0.3-fold untreated levels by heat in roots (Fig. 5a). Further examination of *SlCRF1* in response to cold over 24 h showed that cold induction, more than twofold occurs by 1 h and is maintained through the 24 h treatment in roots; however, induction in leaves after 24 h (3.2-fold) did not occur at the earlier time points examined (Fig. 5b). This illustrates a clear difference in temperature responsiveness between these two *SlCRF*s.

To determine whether *SlCRF1* and *SlCRF2* are involved in osmotic stress response, plants were treated by 200 mM mannitol for 3 h. Neither *SlCRF1* nor *SlCRF2* showed much change in transcript level in response to osmotic stress, with all leaf and root expression levels being 0.7- to 1.4-fold of a normal 1.0 level (Fig. 5c).

Response to oxidative stress was examined by treating plants with hydrogen peroxide (10 and 20 mM) for 3 h. A minor induction in leaves and roots was seen for *SlCRF1* at the lower, but not higher, H_2O_2 concentration. In contrast, *SlCRF2* was strongly induced by both H_2O_2 levels only in roots (Fig. 5d). These findings again indicate a clear difference in stress response between these *SlCRF*s and potential organ specificity in response.

Flooding response was examined by determining *SlCRF1* and *SlCRF2* transcript levels in plants kept in water-logged conditions for 4 days. A similar minor response was noted for both *SlCRF1* and *SlCRF2* revealing a slight 1.4- to 1.6-fold increase in roots transcripts and a 1.4- to 1.7-fold reduction in leaves (Fig. 5e).

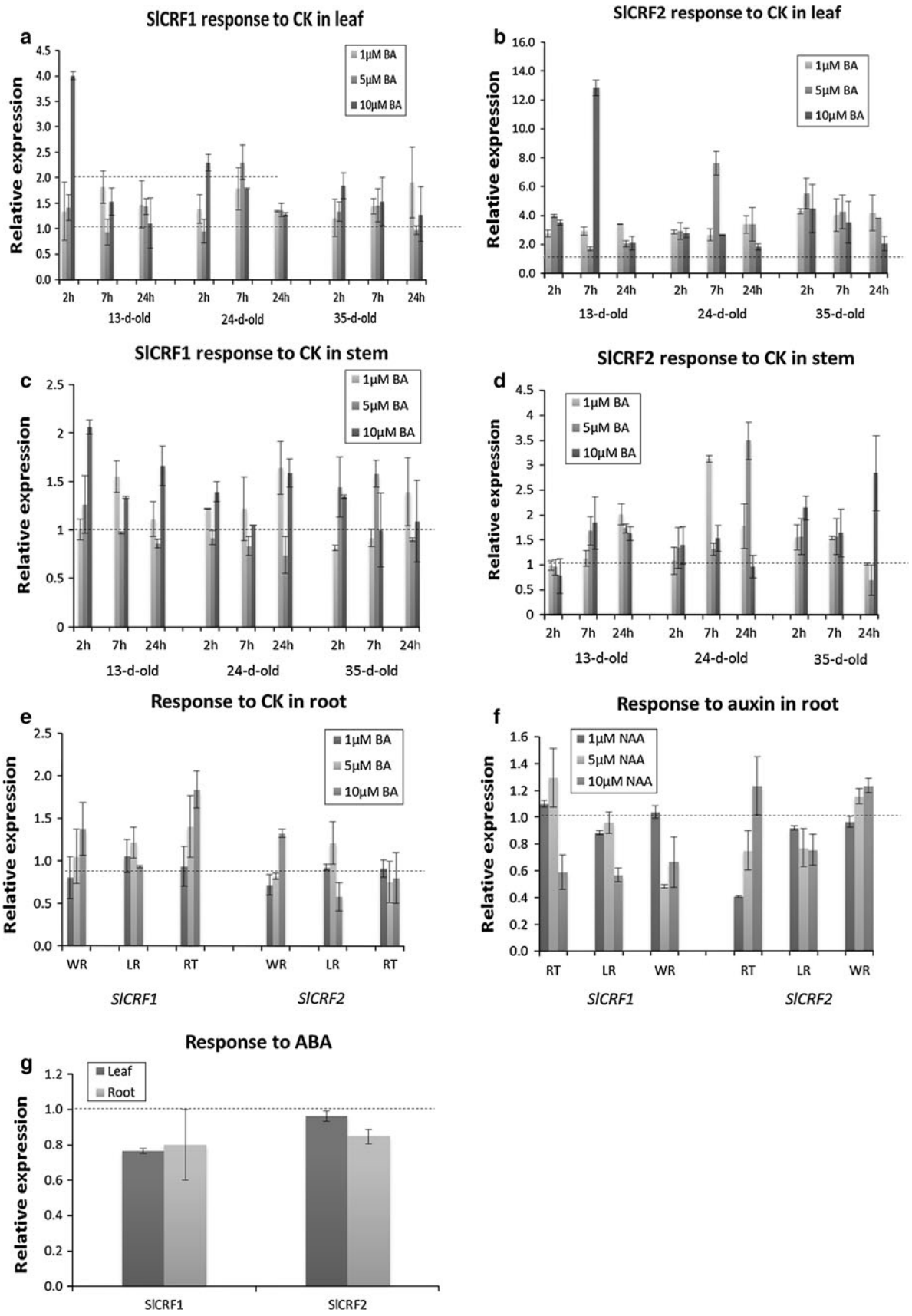
Expression levels of *SlCRF1* and *SlCRF2* were examined in drought-stressed plants that were not watered for 7 days, as well as during the first 12 h of recovery after watering (Fig. 5f, g). In leaves, *SlCRF1* expression was slightly reduced due to drought stress, but during recovery level became highly reduced at 1 h (5.6-fold), 3 h (5.0-fold), and 6 h (3.7-fold) before returning to a normal level at 12 h (Fig. 5f). A different pattern was seen for the root transcript level of *SlCRF1* that was reduced by twofold due to drought and continued to decrease to a higher degree over time during the recovery period examined (Fig. 5g). This suggests that *SlCRF1* is likely involved in drought stress, particularly during the immediate recovery period after re-watering.

A distinct pattern of transcript regulation was seen for *SlCRF2* for drought stress and recovery (Fig. 5f, g). In response to drought, *SlCRF2* expression was increased by 1.8-fold in leaves, but reduced about a similar amount in roots. During recovery in leaves, *SlCRF2* was at its lowest level at 1 h (2.9-fold), reaching a normal level at 3 and 6 h, then increasing to (2-fold) at 12 h, which was similar to drought stress levels (Fig. 5f). In roots, *SlCRF2* was at its highest level at 1 h followed by a steady decline to fivefold reduced levels by 12 h (Fig. 5g). This also suggests a potential role for *SlCRF2* during drought stress and recovery, although based on distinct expression patterns likely different from that of *SlCRF1*.

Discussion

Cytokinin response factors are known to be important AP2/ERF transcription factor members linked to the cytokinin signaling pathway and cytokinin responses, with much of the work on this group having been conducted in *Arabidopsis* (Rashotte et al. 2006; Cutcliffe et al. 2011). However, recent reports have shown that CRFs are found in all land plants in similar numbers ($\sim$12) per species and can be further divided within each species into five distinct subgroups or clades (I–V), which likely have distinct plant functions (Zwack et al. 2012). We previously published the initial report, broadly describing CRFs in tomato (*Solanum lycopersicum* (L.)), known as SlCRFs (Shi et al. 2012). We provide a detailed examination of two *SlCRF*s, *SlCRF1* and *SlCRF2* revealing novel expression patterns across development and in response to hormones and abiotic stresses.

A recent study has shown that most *Arabidopsis CRFs* and *SlCRF1* are preferentially localized to vascular tissues, especially phloem, across the plant likely due to an enrichment of phloem targeting *cis*-elements in the promoters of CRFs (Zwack et al. 2012). We examined if there were a similar vascular localization pattern in tomato by expanding on previous *SlCRF1* work across development and presenting novel *SlCRF1* expression using CRF promoter:GUS reporter lines. From these lines, we determined that vascular expression in *SlCRF1* is present from the seedling stage, in leaves throughout development, as well as occurring in sepals and fruits (Fig. 1). While *SlCRF1* appears to be predominantly in vasculature, expression is


a
SlCRF1 response to CK in leaf
Relative expression
1μM BA
5μM BA
10μM BA
2h 7h 24h 2h 7h 24h 2h 7h 24h
13-d-old 24-d-old 35-d-old
b
SlCRF2 response to CK in leaf
Relative expression
1μM BA
5μM BA
10μM BA
2h 7h 24h 2h 7h 24h 2h 7h 24h
13-d-old 24-d-old 35-d-old
c
SlCRF1 response to CK in stem
Relative expression
1μM BA
5μM BA
10μM BA
2h 7h 24h 2h 7h 24h 2h 7h 24h
13-d-old 24-d-old 35-d-old
d
SlCRF2 response to CK in stem
Relative expression
1μM BA
5μM BA
10μM BA
2h 7h 24h 2h 7h 24h 2h 7h 24h
13-d-old 24-d-old 35-d-old
e
Response to CK in root
Relative expression
1μM BA
5μM BA
10μM BA
WR LR RT WR LR RT
SlCRF1 SlCRF2
f
Response to auxin in root
Relative expression
1μM NAA
5μM NAA
10μM NAA
RT LR WR RT LR WR
SlCRF1 SlCRF2
g
Response to ABA
Relative expression
Leaf
Root
SlCRF1 SlCRF2

◀**Fig. 4** qPCR analysis of *SlCRF1* and *SlCRF2* expression in response to hormones. Expression response to cytokinin (1, 5, and 10 μM BA vs. a DMSO control) for 2, 7, and 24 h in 13, 24, and 35-day-old plants. In leaves: **a** *SlCRF1*. **b** *SlCRF2*. In stems: **c** *SlCRF1*. **d** *SlCRF2*. *SlCRF1* and *SlCRF2* expression in different parts of the root: WR (whole root), LR (lateral root), RT (root tip) collected from 15-day-old plants grown in pouches. **e** In response to cytokinin (1, 5, and 10 μM BA vs. a DMSO control). **f** In response to auxin (1, 5, and 10 μM NAA vs. a DMSO control). **g** Expression of *SlCRF1* and *SlCRF2* in response to ABA in leaves and roots from 25-day-old plants. *Error bars* represent SE of two biological replicates

also present in stamens and other tissues such as leaf mesophyll and fruit pericarp (Fig. 1).

SlCRF2 was also shown for the first time to be predominantly expressed in vascular tissues throughout the plant including cotyledons, leaves, roots, and fruits (Fig. 2a, c–e, h). *SlCRF2* expression could also be found in stamens and non-vascular tissues such as leaf primordia and root tips as well (Fig. 2b, f, g, i, k). The expression pattern of *SlCRF2* is comparable to its orthologous *Arabidopsis* Clade I members *AtCRF1* and *AtCRF2*: having vascular expression in leaves, cotyledons, hypocotyls/stems, and roots, and expression in young leaf primordia. Interestingly, there are differences including expression of *SlCRF2* in root tips (Fig. 2i, k), which is lacking in *AtCRF1*, although not in *AtCRF2* and notably *SlCRF2* expression in reproductive organs such as stamens (Fig. 2f–h), which has not been seen in any other Clade I CRF (Zwack et al. 2012). As *SlCRF1* is a Clade IV CRF member, of which there is no direct ortholog in *Arabidopsis*, a similar comparison of this gene expression to *Arabidopsis* studies cannot be made.

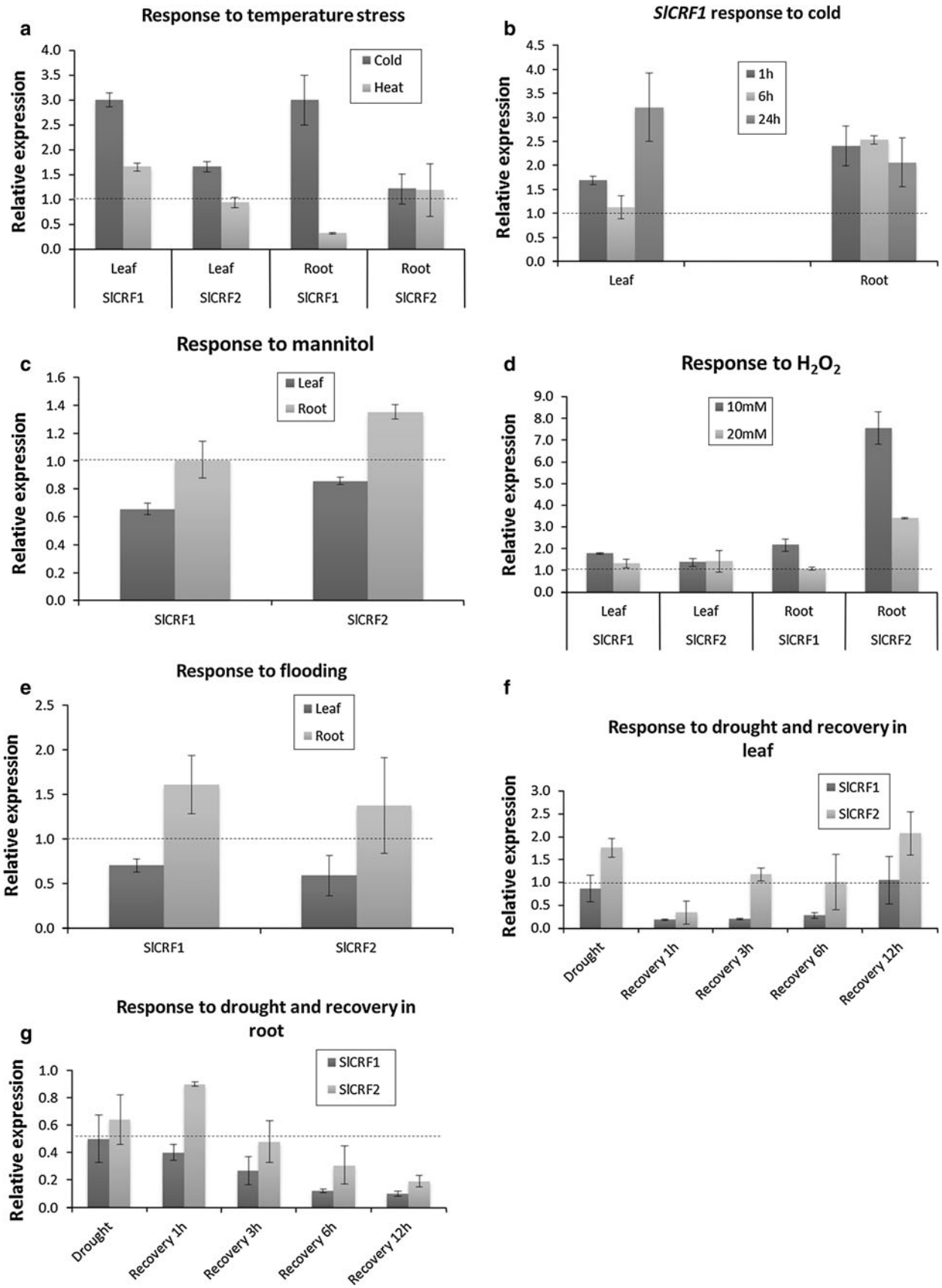
A transcription analysis of *SlCRF1* and *SlCRF2* expression by qPCR in different organs throughout development generally supports the promoter:reporter line expression found for each gene (Fig. 3). In addition, there is a pattern of differential expression as the plant ages, with *SlCRF2* showing higher expression levels in younger tissues and leaves, whereas *SlCRF1* has a slight trend in the opposite direction (Fig. 3).

Transcription induction of CRFs by cytokinin appears to occur only in specific CRF clades, which include Clades I and IV containing *SlCRF1* and *SlCRF2* (Zwack et al. 2012). Previous findings of *SlCRF* cytokinin induction have been limited to leaf tissues in young plants, while here we present a broader examination of cytokinin responsiveness for *SlCRF1* and *SlCRF2* in different tissues and developmental stages using a range of cytokinin concentrations and treatment durations (Rashotte and Goertzen 2010; Shi et al. 2012). While both *SlCRF*s were found to be induced (2+ fold) in leaves and stems under different cytokinin concentrations at different ages after different treatment times, each has a unique induction pattern (Fig. 4). *SlCRF1* showed induction by cytokinin, generally at the highest concentrations in young plants after a short treatment exposure. The highest levels of cytokinin induction of *SlCRF1* were in leaves (Fig. 4). In contrast, *SlCRF2* showed induction by cytokinin at every developmental stage and a wide range of concentrations and treatment lengths in both leaves and stems. *SlCRF2* was induced to higher levels in leaves and generally showed higher induction levels than *SlCRF1* (Fig. 4), indicating that it may be the more cytokinin responsive of these *SlCRF*s. Neither gene was greatly affected by cytokinin in roots; however, *SlCRF1* was moderately induced in root tips at the highest cytokinin level.

Examination of *SlCRF1* and *SlCRF2* for regulation by auxin and ABA showed only minor changes in the transcripts of these genes, with the largest effect being moderate reductions for *SlCRF1* with auxin (Fig. 4f, g). These finding are consistent with previous examination of *SlCRF*s to other hormones (ethylene, methyl jasmonate, and salicylic acid), revealing no change of transcript level in *SlCRF2* and only slight regulation for *SlCRF1* by ethylene and salicylic acid (Shi et al. 2012). Taken together, these results suggest that hormone responsiveness of *SlCRF1* and *SlCRF2* appears to be primarily from cytokinin.

Previous studies have shown that *CRF* genes can be involved in both abiotic and biotic stress responses in addition to cytokinin regulation (Zhou et al. 1997; Park et al. 2001; Gu et al. 2002; Shi et al. 2012; Zwack et al. 2013). An examination of *SlCRF1* and *SlCRF2* to determine if they might similarly respond to stress indicated that both *SlCRF*s are regulated by abiotic stresses with overall unique expression patterns (Fig. 5). *SlCRF1* was induced by cold treatment in both leaves and roots by cold (Fig. 5a, b), suggesting a novel role for *SlCRF1* in cold stress response. Several *Arabidopsis CRFs* have also been implicated in cold response: *CRF3* and *CRF4* (Compton 2012), *CRF6* (Zwack et al. 2013), and *CRF2* (Jeon and Kim 2013). In fact, several specific genes of the cytokinin signaling pathway have been implicated as mediators of cold response, including *ARR1*, *AHP2*, *AHP3*, and *AHP5*, and the cytokinin receptors *AHK2* and *AHK3* (Jeon and Kim 2013). Since cytokinin signaling two-component systems appear to be conserved across plant species, it is possible that *SlCRF1* is also induced by cold through similar mechanism in tomato. It is interesting to note that despite *AtCRF2* being linked to cold response (Jeon and Kim 2013), its tomato ortholog *SlCRF2* was not affected by cold treatment in this study, suggesting that there may not be strict function orthology for cold response between *Arabidopsis* and tomato. This may not be too surprising since *SlCRF1* for which there is no *Arabidopsis* ortholog appears to be regulated by cold stress.

In contrast, both *SlCRF1* and *SlCRF2* are induced by oxidative stress in roots, but not leaves, although *SlCRF2* is


a
Response to temperature stress
Relative expression
Cold
Heat
Leaf SlCRF1
Leaf SlCRF2
Root SlCRF1
Root SlCRF2
b
SlCRF1 response to cold
Relative expression
1h
6h
24h
Leaf
Root
c
Response to mannitol
Relative expression
Leaf
Root
SlCRF1
SlCRF2
d
Response to H2O2
Relative expression
10mM
20mM
Leaf SlCRF1
Leaf SlCRF2
Root SlCRF1
Root SlCRF2
e
Response to flooding
Relative expression
Leaf
Root
SlCRF1
SlCRF2
f
Response to drought and recovery in leaf
Relative expression
SlCRF1
SlCRF2
Drought
Recovery 1h
Recovery 3h
Recovery 6h
Recovery 12h
g
Response to drought and recovery in root
Relative expression
SlCRF1
SlCRF2
Drought
Recovery 1h
Recovery 3h
Recovery 6h
Recovery 12h

◀**Fig. 5** qPCR analysis of *SlCRF1* and *SlCRF2* expression in response to various abiotic stresses in leaf and root of 25-day-old plants. Magenta box-grown plants: **a** cold (4 °C) for 24 h and heat (45 °C) for 1 h. Soil-grown plants: **b** time course of *SlCRF1* expression in response to cold (4 °C). **c** Osmotic stress (200 mM mannitol for 3 h). **d** Oxidative stress (10 and 20 mM hydrogen peroxide for 3 h). **e** Flooding (water-logged conditions for 4 days). Drought (7 days without watering) and recovery (1–12 h after re-watering) in: **f** leaf, **g** root. *Error bars* represent SE of two biological replicates

induced to much higher levels than *SlCRF1* (Fig. 5d). Reactive oxygen species (ROS) such as H_2O_2 are produced in plants as byproducts of aerobic metabolism or in response to abiotic stresses and it has been proposed that H_2O_2 promotes adaptive responses to various stresses such as cold by serving as a stress signal in plants (Apel and Hirt 2004; Desikan et al. 2001; Zhou et al. 2012). A recent study has implicated another CRF; *AtCRF6* in oxidative stress (H_2O_2) response in *Arabidopsis* leaves (Zwack et al. 2013). It is not clear what this induction of *SlCRF1*and *SlCRF2* means in stress-related processes, except to provide a novel connection to oxidative stress and a possible link to various stress pathways.

Both *SlCRF1* and *SlCRF2* show complicated patterns of transcript regulation in response to drought stress and recovery that differ from each other within leaves and roots. Much of the change in regulation of *SlCRF1* occurs during recovery, which could explain why a previous study indicated that *Tsi1*, a tobacco clade IV CRF ortholog of *SlCRF1*, was unresponsive to drought stress (Park et al. 2001). Although it is unclear exactly how *SlCRF1* and *SlCRF2* are involved in these responses, these findings provide a novel link between SlCRFs and drought stress and processes that occur during recovery.

Overall, the examinations of *SlCRF1* and *SlCRF2* during abiotic stress have provided novel links between these genes and different stress processes. *SlCRF1* was revealed to be linked to cold, oxidative, and drought stresses from this study in addition to previous work connecting it to salt stress and biotic defense response (Gu et al. 2002; Shi et al. 2012). This work presents the first link between *SlCRF2* and any stress response, indicating an involvement in oxidative and drought stress processes.

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