Characterization of CRF domain containing ERF genes- *Solanum lycopersicum* Cytokinin Response Factors *SlCRF3* and *SlCRF5* in tomato development.

by

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Abstract

Cytokinin is an important hormone involved in numerous aspects of plant growth and development. A newly identified group of transcription factors- Cytokinin Response Factors (*CRF*s) has been included as a side branch to cytokinin signaling pathway. CRFs constitute a subset of the AP2/ERF family of transcription factor proteins found in all land plants. Within the ethylene response factor (ERF) subfamily, CRFs are defined by the presence of a group-specific domain, known as the CRF domain, several of which were originally identified in Arabidopsis microarray experiments as induced by cytokinin. Initial studies of CRF mutants have also linked these genes to normal leaf/cotyledon development in Arabidopsis. In this study, two transcription factor genes *SlCRF3* and *SlCRF5* (*Solanum lycopersicum* *C*ytokinin *R*esponse *F*actors) were identified and characterized to determine their involvement in regulating cytokinin responses in tomato development. Additionally, the first transcriptome analysis of cytokinin and auxin response in tomato roots was conducted and several novel hormone regulated genes were identified through Illumina RNA-seq technology.

This project was initiated with the following objectives: 1) To characterize *SlCRF3* and *SlCRF5* and their pattern of regulation by cytokinin and abiotic stresses, 2) To determine the roles of *SlCRF5* in tomato development, 3) To characterize novel genes involved in cytokinin and auxin regulation in tomato root. For the first two objectives, previous unknown full length DNA sequences for *SlCRF3* and *SlCRF5* were determined, cloned into different expression vectors, then transformed into plants to study where and when these genes are expressed as well as the effects these genes produced in tomato development. Expression analysis using GUS reporter transgenic lines revealed that these genes are targeted to the vascular tissue, more specifically in the phloem of leaf, stem, root, and floral parts. Knockdown studies, using antisense lines indicated the potential involvement of *SlCRF5* in various aspects of tomato development, including leaf size and primary root length as well as the number of lateral roots, and number of flowers, fruits, and seeds produced.

SlCRF3 and SlCRF5 were also examined at the protein level. Bimolecular florescence (BiFC) experiments using a split YFP system in protoplasts revealed the ability of these two proteins to form both homo and hetero dimers. In addition cellular localization experiments indicated that these proteins were nuclear localized in planta.

In order to investigate the responses of SlCRF3 and SlCRF5 to hormones and stress, tomato plants were treated with cytokinin and other stress hormones and were exposed to abiotic stress conditions. These results revealed that *SlCRF3* and *SlCRF5* are regulated by cytokinin and also by some abiotic stresses.

For the third objective, Illumina RNA-sequencing was utilized to analyze the transcriptome of tomato roots with the main focus on the spatial patterning and regulation of genes by the hormones cytokinin and auxin. The analysis revealed that a number of genes involved in mechanisms such as defense, stress response, cytokinin signaling, hormonal crosstalk, and metabolism were regulated.

Together, these findings indicate that *SlCRF3* and *SlCRF5* are potential regulators of tomato developmental processes associated with cytokinin and abiotic stresses and these results set a foundation for future research directed towards tomato development.

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Chapter I. Introduction and Literature Review

Cytokinins

Cytokinins are an important class of plant hormones that have been implicated in numerous growth and developmental processes such as stem-cell control in roots and shoots, vascular differentiation, plastid division and abundance, root, shoot and inflorescence growth, nutrient balance, leaf senescence, responses to environmental stress, seed development and fruit yield (Mizuno 2004; Ferreira and Kieber, 2005; Müller and Sheen, 2007; Sakakibara, 2006; Werner et al., 2003; Okazaki et al., 2009). Understanding the regulation of gene expression is important in realizing different activities in a developmental- and tissue-specific context, and in this connection, several findings from early efforts to identify cytokinin regulated genes were summarized by Schmülling et al., 1997. Since then, several studies towards a comprehensive understanding of the early and late events of gene regulation by cytokinin (Kiba et al., 2004; Brenner et al., 2005; Shi et al., 2013), and those linking specific transcriptional responses to the biological activities of cytokinin have been performed (Dello Ioio et al., 2008; Müller and Sheen, 2008; Argueso et al., 2010; Zhao et al., 2010; Bishopp et al., 2011; Köllmer et al., 2011). These findings reveal a fine-tuned regulation of gene expression to carry out many different biological activities of cytokinin in plant development.

Natural cytokinins are adenine derivatives comprising an N^6- isoprenoid or aromatic side chain, which in plants, gets hydroxylated (Takei et al., 2004) to produce zeatins. Two isomers of zeatin are trans-zeatin and cis-zeatin of which trans-zeatin is believed to be the most widespread in plants. Other natural cytokinins are dihydrozeatin (with a saturated side chain), N^6 benzyladenine (BA), and topolin (an aromatic cytokinin from poplar plants) (Strnad et al., 1997).

Artificial cytokinins can also be produced by utilizing simple chemical synthesis and among them the most commonly used forms are kinetin, BA and Isopentenyladenine.

History of Cytokinin discovery

Cytokinins were first discovered as a plant hormone in the laboratory of F. Skoog more than a half–century ago, as a cell division factor “kinetin” (Miller et al., 1955). However, the clue that this compound could stimulate cell division came well before this in 1913 from Gottlieb Haberlandt who found that phloem diffusates from plants were able to initiate cell division in wounded potato parenchyma cells (*Solanum tuberosum*). In fact, the first naturally occurring cytokinin named zeatin was isolated from maize (*Zea mays*) embryos (Letham et al., 1963). Because of their ability to promote cytokinesis, they were termed cytokinins (Mok and Mok, 1994) and also suggested to be designated as “phytokinins” indicating both their function and belonging to the plant kingdom (Köhler and Conrad 1996). Subsequent finding led to the identification of many similar compounds which occur naturally in plants.

Cytokinin cross-talk

An important discovery about the hormone cytokinin is its interaction with auxin to coordinate various developmental stages of the plants. It was revealed that interaction between cytokinin and auxin stimulates cell division in cultured plant cells (Miller et al., 1955, 1956). Subsequent finding by Skoog and Miller (1957) showed that it is the ratio of cytokinin to auxin that determines the organ regeneration from undifferentiated callus tissue with a high cytokinin to auxin ratio favoring shoot formation and a low cytokinin to auxin ratio favoring roots. Later, several findings unveiled the involvement of auxin and cytokinin in various processes such as apical dominance, cell cycle control, lateral root initiation, regulation of balance between shoot and root growth, and vascular tissue development (Coenen and Lomax 1997; Bangerth et al., 2000; Swarup et al., 2002). More detailed studies uncovered possible relationships between these two hormones. It was observed that elevated levels of auxin have negative effects on cytokinin abundance in transgenic tobacco (Eklöf et al., 1997) and auxin is also involved in cytokinin breakdown by stimulation of cytokinin oxidase activity (Palni et al., 1988; Zhang et al., 1995). On the contrary, the effect of cytokinin on auxin abundance has been observed to be positive in some cases (Bertell and Eliasson 1992; Werner et al., 2001) while it is still negative in others (Eklöf et al., 1997). Moreover, cytokinin and auxin were also observed to mutually regulate the elements of the other hormones signaling pathway components from work of various transcriptome analyses (Rashotte et al., 2003; Goda et al., 2004).

To further understand the mechanism underlying these interactions, global transcriptional expression profiles of plants treated with auxin, cytokinin, and both auxin and cytokinin were examined (Rashotte et al., 2005) revealing that very few synergistic or antagonistic interactions occurred which suggests that interaction between the two hormones occurs downstream of their individual signaling pathways. Cytokinin-auxin antagonistic interactions were also shown to be fundamental in controlling root development (Dello et al., 2008) with cytokinin promoting cell differentiation by repressing auxin signaling and transport, and auxin favoring cell division and hence sustaining root meristem activity (Blilou et al., 2005; Dello et al., 2007).

Additionally, antagonistic interactions between cytokinin and auxin, where cytokinin has negative effect on auxin distribution, have been found responsible for auxin induced organogenesis (Pernisova et al., 2009), lateral root formation (Laplaze et al., 2007) and determination of leaf positioning (Lee et al., 2009; Shimizu-Sato et al., 2009). Cytokinin-auxin interactions were also found by Muller and Sheen (2008) to be crucial in the establishment of the first embryonic root stem cell niche during Arabidopsis embryogenesis. In this study the authors showed that auxin antagonizes cytokinin output in the basal cell by transcriptional activation of two type-A negative regulators of cytokinin signaling, *ARR7* and *ARR15*. These results suggest that auxin gains control of cytokinin output by controlling its negative regulators. However, in the shoot apical meristem (SAM), auxin represses *ARR7* and *ARR15* although, in part, through AUXIN RESPONSE FACTOR5/MONOPTEROS (MP) transcription factor and hence controls SAM development by integrating with cytokinin signaling elements (Zhao et al., 2010). These results are also consistent with the general roles of auxin and cytokinin in the root and shoot development suggesting that proper interactions between cytokinin and auxin are required for a complex plant developmental program.

Cytokinin signaling and two-component elements in plants

After cytokinins discovery, various researches were focused on how this hormone is perceived and its signal is relayed in a cell. Based on several findings, a model of cytokinin perception and signal transduction has emerged which is similar to bacterial two-component phosphorelays (Kieber, 2001; Hutchison and Kieber, 2002; Sheen, 2002; Heyl and Schmülling, 2003; Kakimoto, 2003; Grefen and Harter, 2004; Mizuno, 2004; Ferreira and Kieber, 2005). The binding of the ligand cytokinin to a membrane bound receptors leads to their autophosphorylation on a conserved His amino acid residue. This phosphate is then passed on to an Asp in the receiver domain of the cytokinin receptor. The signaling cascade continues its multi-step phosphorelay by phosphorylating His residues on another set of proteins called His-containing phosphotransfer (HP) proteins. (Miyata et al., 1998; Hutchison and Kieber, 2007)**.** Upon phosphorylation, the HPs move from the cytosol into the nucleus to transmit the signal by further phosphorylation of response regulators (RRs). One group of RRs are the type-B RRs, which are transcription factors primarily functioning as positive regulators of cytokinin signaling by binding and activating downstream cytokinin regulated genes (Hutchison et al., 2006). Another category of RRs are the type-A RRs, which have similar protein sequences to the type-B RRs, but lack a DNA binding domain to function as transcription factors. As such they compete with type-B RRs for phosphorylation by HPs (Brandstatter and Kieber, 1998) and act as negative regulators of the cytokinin signaling pathway (To et al., 2004). These two types of *RR*s regulate a variety of growth and developmental processes by mediating the transcriptional response to cytokinin (Reviewed in Gupta and Rashotte, 2012).

Cytokinin Response Factors (CRFs)

The cytokinin response factors (*CRF*s) are a family of transcription factor proteins that form a side branch of the cytokinin signaling pathway and are found in all land plants. These proteins were originally identified in microarray experiments of cytokinin response in Arabidopsis as APETALA2/Ethylene Response Factor (AP2/ERF) transcription factor family genes that were highly induced at multiple time points after exogenous application of cytokinin (Rashotte et al., 2003). The AP2/ERF proteins comprise one of the largest families of transcription factors in plants and are defined by the presence of an AP2 DNA binding domain of ~ 68 amino acids. Currently, there are 147 known gene loci in Arabidopsis (Nakano et al., 2006), 200 in poplar (Zhuang et al., 2008), 132 in grapevine (Zhuang et al., 2009), 131 in cucumber (Hu and Liu, 2011), and 163 loci in the rice genome (Sharoni et al., 2011) that code for members of this family. These proteins were classified into AP2 (APETALA2), DREB (dehydration-responsive-element-binding), RAV (related to ABI3/VP), ERF (ethylene-responsive-element-binding-factor), and other proteins (Sakuma et al.2002). Nakano et al. (2006) later presented the modified classification of AP2/ERF superfamily with all of the members in the DREB subfamily included in the ERF family, hence dividing AP2/ERF superfamily into three families, ERF, AP2, RAV, and a soloist gene.

The DNA binding domain of AP2/ERF proteins has been shown to interact with specific DNA sequences depending upon their subfamily membership, e.g. DREB subfamily members have been shown to bind DRE/CRT cis-acting elements and ERF subfamily members bind GCC box cis-elements (Sakuma et al., 2002; Fujimoto et al., 2000). These interactions result in the regulation of a variety of developmental processes in plants.

The ERF subfamily comprises the majority of all AP2/ERF proteins in Arabidopsis (83%), rice and other sequenced plant genomes (Zhuang et al., 2008; Nakano et al., 2006). Through whole-genome analysis of Arabidopsis, Populus and rice, the ERF subfamily has been divided into 12 subgroups with CRFs belonging to subgroup VI and VI-L (Sakuma et al., 2002; Zhuang et al., 2008; Nakano et al., 2006; Xu et al., 2008). Because of their cytokinin regulation and clade placement in the ERF subfamily, these genes were designated as cytokinin response factors (CRFs) (Rashotte et al., 2006). Further examination of CRFs revealed the presence of a group specific domain, the CRF domain and a putative mitogen-activated protein kinase (MAPK) phosphorylation motif (Rashotte and Goertzen 2010; Zwack et al., 2012). Phylogenetic analysis of CRFs proteins further resulted into their placement in five distinct clades (I-V) represented across all major angiosperm lineages, with each clade containing a clade-specific C-terminal region distinct from other CRFs (Zwack et al., 2012). Within these C-terminal regions, the novel conserved motifs -FQDI of clades I and II, the SGY[D/E]S of I, II and III, and the PX[D/E]XF[F] present in all except clade IV proteins, are likely related to the specific function or regulation of these proteins (Zwack et al., 2012).

Interestingly, one of the clade specific features is their upregulation by cytokinin. It was found that members of clades I, III, and IV are cytokinin inducible compared to clades II and V that show little to no transcriptional induction by cytokinin (Zwack et al., 2012). This clade specific response of CRFs to cytokinin was originally found from the analysis of six *At*CRF genes (1-6) by northern blot experiments in Arabidopsis and additional experiments in tomato described later in this dissertation (Rashotte et al., 2006).

Examination of CRF cellular localization via GFP protein constructs in Arabidopsis protoplast revealed that CRFs are ubiquitously present in the cell, and upon treatment with cytokinin, these proteins move to the nucleus, revealing that CRF proteins are regulated by cytokinin (Rashotte et al., 2006). Furthermore, by making use of multiple cytokinin signaling knockouts of various parts of pathway it was possible to determine that cytokinin regulated CRF nuclear localization is dependent on the early steps of the pathway. This included dependence on the AHKs and the AHPs, but not the ARRs and indicated a link between CRFs and other members of the signaling pathway.

Recent examination of CRF proteins interactions demonstrated their ability to form both homo- and hetero-dimers with other CRFs (Cutcliffe *et al.*, 2011). In order to further examine if a specific region or domain of the CRF proteins might be involved in protein–protein interactions, both natural variation among CRF proteins and partial version of a CRF protein were created. It was observed that neither the C-terminal region nor the AP2/ERF DNA binding domain is necessary for protein dimerization and that the CRF domain alone is sufficient for CRF–CRF protein interactions. This study further revealed that CRFs not only interact with each other but also with AHPs again linking CRFs with the cytokinin signaling pathway. However, CRFs neither interacted with AHKs, nor with the type-A or type-B RR proteins. This indicates the possible importance of CRF-CRF and CRF-AHP interactions in the cytokinin regulated developmental processes.

Single loss-of-function mutation analysis for CRF genes in Arabidopsis showed almost no phenotypic change except for minor defects in cotyledon development, although the severity of this phenotype increases with the additional numbers of disrupted genes in double and triple CRF mutants (Rashotte et al., 2006). Beyond the notch-like phenotype that appeared in the cotyledons of CRF mutants, development of the cotyledons in the triple mutants was more severely affected resulting in a reduced size and translucent or white color. Another role for CRF genes is in the development of embryo, as observed in the *crf5*, *crf6* double mutant that displayed embryo-lethal phenotype, where the embryo arrests at the heart stage of development. Recently, through promoter-driven reporter gene expression analysis of transgenic lines in Arabidopsis and tomato, it has been shown that CRFs belonging to the clades I-IV are expressed in the vasculature of various aerial organs, with the strongest expression in phloem (Zwack et al., 2012). This suggests a potential phloem-related role of these genes in possibly mediating cytokinin regulated growth and developmental processes such as sink/source regulation, senescence, and responses to abiotic and biotic stresses (Mok and Mok, 2001; Werner and Schmülling, 2009). Some of the Arabidopsis CRFs have recently been shown to be involved in normal leaf vasculature patterning, plant growth and development and senescence (Zwack et al., 2012; 2013). Since these affected

CRF genes are also cytokinin inducible, this suggests a link between cytokinin regulation of CRFs and these developmental processes.

Cytokinin regulation of plant growth and development

The role of cytokinin in various organ developmental processes is crucial. Either cytokinin or cytokinin interactions with other signaling pathways regulates the growth and development of essential plant organs including the root, stem, leaf, and flower. For the details of regulatory mechanisms and a more detailed discussion of this topic please refer to chapter 3: Down-stream components of cytokinin signaling and the role of cytokinin throughout the plant.

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Chapter II. Transcriptome Profiling of Cytokinin and Auxin Regulation in Tomato Root

Abstract

Tomato is a model and economically important crop plant with little information available about gene expression in roots. Currently, there have only been a few studies that examine hormonal responses in tomato roots and none at a genome-wide level. In this study we examine the transcriptome atlas of tomato root regions (root tip, lateral roots, and whole roots) and the transcriptional regulation of each root region in response to the plant hormones cytokinin and auxin using Illumina RNA sequencing. More than 165 million 1x54 base pair reads were mapped onto the *Solanum lycopersicum* reference genome and differential expression (DE) patterns in each root region in response to each hormone were assessed. Many novel cytokinin and auxin induced and repressed genes were identified as significantly differentially expressed and the expression levels of several were confirmed by qPCR. A number of these regulated genes represent tomato orthologs of cytokinin or auxin regulated genes identified in other species including: *CKX*s, type-A RRs, *Aux*/*IAA*s, and *ARF*s. Additionally, our data confirms some of the hormone regulation studies for recently examined genes in tomato such as: *SlIAA*s and *SlGH3*s. Moreover, genes expressed abundantly in each root region were identified which provide a spatial distribution of many classes of genes, including plant defense, secondary metabolite production, and general metabolism across the root. Overall this study presents the first global expression patterns of hormone regulated transcripts in tomato roots which will be functionally relevant for future studies directed towards tomato root growth and development.

Introduction

Tomato (*Solanum lycopersicum*) is one of the main crops grown throughout the world. Many studies toward the improvement and increase in yield of this plant have focused on flower (Goméz *et al*., 1999; Molinero-Rosales *et al*., 2003) and fruit development (Pattison and Catalá, 2012; de Freitas *et al*., 2012). Roots in tomato, on the other hand, which constitute an important system to carry out water conduction, mineral uptake, anchorage, synthesis of bio-active substances, gravitropic responses, and sensing stress have not been studied in detail. This is particularly true for gene expression patterns in tomato roots that will be essential for any future studies focused on growing, breeding, or engineering plants with more desirable root traits.

The root system is comprised of different regions that are involved in specific functions for the overall plant sustenance. Two essential regions are the root tip and lateral roots. The root tip encompasses the root apical meristem which provides all the cells and base organization for the growing root (Scheres *et al*., 2002). Moreover, this is a region of active cell division, elongation (Dolan *et al*., 1993), and is also the region where synthesis of important hormones (Feldman, 1979; Muller *et al*., 1998) occurs which are transported to different locations in the plant. Lateral roots on the other hand extend from the primary root in horizontal manner to provide support and increased water and nutrient uptake.

Two key hormones that play pivotal roles in root growth and development are cytokinin and auxin. Both of these hormones have been implicated to have roles in several processes such as in root vascular development, initiation of lateral roots, and root gravitropism (Aloni *et al*., 2006; Vanneste and Friml, 2009). Quite often, cytokinin displays crosstalk with auxin during root development, such as in the establishment of root stem-cell niche (Müller and Sheen, 2008), meristem size and root growth, (Dello Ioio *et al*., 2008; Zhang *et al*., 2011), and lateral root organogenesis (Marhavý *et al*., 2011), although sometimes in an antagonistic manner. Through several studies, the regulatory roles of hormones in plant development have been elucidated. This includes several cytokinin induced genes such as, the *CRE1*/*AHK4* cytokinin receptor (Che *et al*., 2002), *CRF*s that are involved in Arabidopsis cotyledon and leaf development (Rashotte *et al*., 2006), and a rice *O*-glucosyltransferase in rice that catalyzes the inactivation of zeatin-type CKs by *O*-glucosylation (Hirose *et al*., 2007). Numerous auxin regulated genes have similar important roles such as, *SAURs* that are strongly expressed in epidermal and cortical cells (Vanneste and Friml, 2009) and *AUF1*that is involved in crosstalk between cytokinin and auxin during Arabidopsis root growth (Zheng *et al*., 2011).

With the availability of the complete tomato genome (The Tomato Genome Consortium, 2012), it has become possible to perform genome-wide transcriptome analysis to study gene expression patterns across different plant tissues under different conditions without *de novo* assembly. Next-generation high-throughput RNA sequencing technology (RNA-seq) using massively parallel sequencing has revolutionized transcriptome analysis and, when compared to microarrays, RNA-seq can detect all expressed genes without the generation of an array of probes, with reduced background noise and large dynamic range. This is particularly important in species such as tomato, where publically available microarrays cover only one third of the complete genome.

Here, we utilized Illumina RNA sequencing on 15 days old hydroponically grown Micro-Tom plants to analyze the transcriptome of tomato roots with the main focus on the spatial patterning and regulation of genes in the root by the hormones cytokinin and auxin. This study is one of only a few tomato genome-wide expression profiles and generates a transcriptome atlas of tomato root regions: root tip, lateral roots, and whole roots. Additionally, this transcriptome analysis of hormone regulation in tomato root reveals novel genes regulated by each of these hormones. This comprehensive analysis of tomato root transcriptome can further be utilized as a reference to conduct future research on tomato roots.

Materials and methods

Plant materials, growth conditions, and hormone treatment

The tomato dwarf cultivar Micro-Tom plants were grown hydroponically in CYG germination pouches from Mega International under a 16:8 h light: dark photoperiod at 150µE, with a 26º C day (light), 22 º C night (dark) temperatures. Seedlings at 14 DAS (days after sowing) were treated with exogenous 5µM cytokinin (N^6 -benzyladenine; BA) or 5µM auxin (Naphthalene acetic acid; NAA) dissolved in Dimethyl sulfoxide (DMSO), for 24 h by directly adding the hormones or control DMSO to the growth pouches, such that 15d old plant tissues were collected.

RNA extraction, Library preparation, and Illumina GAIIX sequencing

Root tips (RT, encompassing the meristem and the elongation zone), Lateral roots (LR), and whole roots (WR, including RT and LR) were collected from 15-day-old Micro-Tom plants from seedlings with or without treatment with cytokinin, auxin, and DMSO and immediately flash-frozen in liquid nitrogen and ground to obtain fine powder. Total RNA was extracted from the tissue using a Qiagen RNeasy Kit as per manufacturer's instructions and used for messenger RNA isolation with polyA selection and subsequent library construction with the TruSeq RNA sample preparation protocol from Illumina (San Diego, CA). Two biological replicates were sequenced and analyzed for each of the 9 tissue-treatment combinations. Single-end sequencing was performed on the 18 samples by the Illumina GAIIX platform, generating 165,894,496 1x54bp reads, totaling 8.99 Gbps. Raw sequence data is available for download at NCBI Sequence Read Archive under the number SRA058709.

Differential expression analysis with Solanum lycopersicum (ITAG2.3) reference

Reads for each of the 18 samples were aligned to the *Solanum lycopersicum* genome reference with GSNAP (Wu and Nacu, 2010). The high throughput sequencing data was managed using the Alpheus^{TM} pipeline and database resource (Miller 2008). Gene expression was quantified as the total number of reads for each sample that uniquely aligned to the reference, binned by gene coordinate. On average, each sample had 7.6 million uniquely aligning reads.

Differential expression analysis was performed with the negative binomial test of DESeq (Anders 2010) on three partitions of the dataset: 3 tissues DMSO-only treatment, 3 tissues DMSO vs cytokinin treatments, and 3 tissues DMSO vs auxin treatments. To perform robust analyses, each set was normalized, filtered, and tested within DESeq separately with recommended settings. The first experiment interrogated tissue specific signals with the DMSO treated samples from root tip, lateral root, and whole root (n=6). The second experiment investigated the effect of cytokinin treatment in each root tissue in reference to the corresponding DMSO control (n=12). The final experiment looked at the effect of auxin treatment in each root tissue in reference to the corresponding DMSO control (n=12). A multiple testing correction (the Benjamini-Hochberg False Discovery Rate method; (Benjamini 1995)) was applied and genes with an adjusted p-value, padj ≤ 0.05 were considered to be significantly differentially expressed. For multiple tissue DE we show comparisons with a $\log_2$ Fold Change (Log_2FC) that were significant (p-adj ≤ 0.05) in one or more of the root regions, indicating padj in all regions.

Verification of mRNA-seq results by qPCR

A 500ng aliquot of the total RNA for each tissue type (see above) was used for reverse transcription using Quanta qScript cDNA supermix and the cDNA was diluted 20 times before it was used for quantitative real-time PCR analysis. Each qPCR reaction consisted of 2µl of a 20-fold cDNA, 9µl of SYBR-Green supermix, 1µl of 6µM forward and reverse primers, and 7µl of sterile water. qPCR was performed in a Eppendorf Mastercycler ep realplex with the following parameters: initial one cycle of 2 min at 95º C, followed by 40 cycles of 15s at 95º C, 45s at 57º C, and 35s at 68ºC. The last step for each reaction was melting curve generation to test the amplicon specificity. All qPCR reactions were performed in two technical and two biological replicates. All samples were compared with the control gene *TIP41* (Expósito-Rodríguez *et al*., 2008). The primer sequences for the genes which were verified through qPCR are presented in Table 3.

Results and Discussion

Tomato Root Transcriptome Analysis

To obtain a global view of genes expressed across the tomato root, single-end Illumina GAIIX RNA sequencing was performed on samples derived from root-tip (RT, encompassing the meristem and elongation zone), lateral root (LR), and whole root tissue (WR, including RT and LRs) after 24 hours of treatment with cytokinin (5µM BA) or auxin (5µM NAA) vs the vehicle control DMSO. This generated a total of 165,894,496 1x54bp reads from all root tissue samples that were aligned to the *Solanum lycopersicum* reference genome. On average, samples generated 7.6 million uniquely aligning reads from which gene expression was quantified as the total number of reads for each sample that uniquely aligned to the reference, binned by gene.

Based on reads and filtering (see methods for details) ~17300 genes were robust enough to be used for differential expression (DE) analysis on three subsets of the data: tissue level expression patterns by utilizing untreated (DMSO) RT, LR, and WR samples; and the hormone expression in these tissues of cytokinin as well as auxin in reference to DMSO. Since a large number of genes were found to be significantly regulated (padj ≤ 0.05) we focused our DE analysis on genes with a high $\text{Log}_2\text{FC} > 2.0$, that we designated as hormone or tissue regulated (Table 1). We have concentrated our discussion of these results on genes regulated commonly across the different tissues examined, as they represent some of the most highly regulated hormone responsive genes with important known gene function in each root region. Furthermore we performed qPCR in order to confirm the results of RNA sequencing on 12 DE regulated genes affected by cytokinin and auxin treatments. This comparison yielded very similar expression directionality and level of regulation for all genes examined across all root tissues, indicating that Log_2FC values obtained from RNA sequencing are accurate (Table 2).

Principal component analysis (PCA) and variance decomposition (both as implemented in SAS JMP Genomics 5.1) of the overall, full transcriptome dataset (n=18), shows distinct differences between the auxin and cytokinin hormone response treatments, responsible for 68.6% of the variance (Fig. 2). With regards to tissue differences, 19.5% of the variance, there was greater similarity between LR and WR samples, while the RT has a more distinct profile.

Cytokinin induced genes

Genes were determined to be cytokinin regulated if they were DE above 2.0 Log_2FC in at least one root region sampled. We identified 89 genes that were cytokinin regulated in all root tissues, while 87, 24, 34 were specifically regulated in RT, LR, and WR tissues respectively. Additionally, there were 24 genes commonly regulated in RT and LR tissues, 23 between LR and WR tissues, and 81 between RT and WR tissues (Fig.1).

DE cytokinin induced genes found in this study appear to be mainly involved in metabolism, development, defense, transport, and tissue specific related processes. One of the commonly found highly cytokinin regulated genes are the cytokinin oxidase/dehydrogenases (*CKX*) (Brenner *et al*., 2012). Three CKX genes (*SlCKX2*; *SlCKX4*; *SlCKX6*) were found to be induced in all root tissues (Table 1), while *SlCKX1* was induced specifically between RT and LR and *SlCKX5* between RT and WR. *CKX* genes across plant genera are involved in the breakdown and catabolism of cytokinin (Frébort *et al*., 2011), so it is not surprising to see several *CKX*s induced with the application of exogenous cytokinin. CKXs have also been linked to a number of cytokinin-related growth and developmental process and recently, it was shown that over-expression of *CKX* genes enhanced root growth, drought tolerance, and leaf mineral enrichment in Arabidopsis and Tobacco (Werner *et al*., 2010). Since we have shown in this study that *CKX*s can be highly induced in roots it would be interesting to see if similar growth and developmental effects could be seen from CKX overexpression in tomato.

Seven type-A Response Regulator (RR) genes were found induced in this study, which we have named as *SlRRA*s: three were induced in all root tissues, *SlRRA2*, *SlRRA6*, *SlRRA7* (Table 1), *SlRRA4* specifically in LR, and *SlRRA1*, *SlRRA3*, *SlRRA5* induced between LR and WR. Type-A RRs are commonly found to be highly induced by cytokinin treatment as they are negative regulators of cytokinin signaling (Brenner *et al*., 2012). It can be proposed that induction of these genes probably occurred to dampen the increased exogenous cytokinin levels added to the roots. Since cytokinin is a negative regulator of root growth, it would be interesting to know how the levels of cytokinin are maintained to appropriate amounts by the genes involved in cytokinin catabolism and the cytokinin signaling pathway components.

Defense genes, including those that produce volatile compounds in response to pathogens or predators were among those induced by cytokinin, including the sesquiterpene synthase gene *TPS14* and (E)-beta-ocimene synthase (Solyc01g105960) (Table 1; Paré and Tumlinson, 1999). Sesquiterpenes such as alpha-humulene are widely distributed in plants and have been shown to function as insect/pathogen repellent in direct defense (Suga *et al*., 1993). In support of our results, *TPS14* has been shown to be highly expressed in tomato roots (Bleeker *et al*., 2011). (E)-beta-ocimene synthase produces a monoterpene released upon herbivory, mechanical wounding, or jasmonic acid treatment (Faldt *et al*., 2003). Cytokinin induction of these defense genes supports previous studies of cytokinin eliciting defense response and defense response linked to the cytokinin signaling pathway (Naseem *et al*., 2012; Choi *et al*., 2010), although the underlying molecular mechanism behind this connection in tomato roots is obscure and needs further investigation.

Genes associated with abiotic environmental stresses were differentially induced, such as Deoxyhypusine synthase *SlDHS* and six Laccase genes: Solyc06g076760 among RT, LR, and WR, Solyc05g052360, Solyc05g052370, and Solyc06g082240 in RT, Solyc05g052400 in LR, and Solyc03g083900 in WR (Table 1). DHS is an enzyme that catalyzes enzymatic reactions leading to the activation of a eukaryotic translation initiation factor-5A (eIF-5A) (Park *et al*., 1993, 1997). In a study it was shown that transcript levels of *SlDHS* increased due to osmotic stress and chilling injury in tomato leaves (Wang *et al*., 2001) and the induction of *SlDHS* by cytokinin as reflected in our data suggests that the hormone possibly has a role in abiotic stress response mediated by DHS.

Laccases are multi-copper containing glycoproteins that have been proposed to be involved in lignin synthesis in plants (O'Malley *et al*., 1993). In maize roots, *ZmLAC1*, was shown to be induced by NaCl (Liang *et al*., 2006). A similar increased transcript level was observed for a tomato laccase gene by salt that were even more abundantly produced when treated with both salt and ABA likely suggesting an involvement in stress response (Wei *et al*., 2000). Interestingly, in maize, it has been proposed that apoplastic laccases may have a role in cytokinin breakdown (Galuszka *et al*., 2005). How this group of genes mediates cytokinin response in responding to stress conditions remains unclear.

Several genes involved in different hormone biosynthesis, degradation, and signaling pathways were also induced. This includes the ethylene biosynthesis enzymes 1-aminocyclopropane-1-carboxylate (ACC) synthase (Solyc12g008740) and ACC oxidase 1 (Solyc06g073580) (Lin *et al*., 2009), an Auxin-regulated protein (Solyc04g010330), and two Gibberellic acid (GA) related genes in involved in GA degradation, Gibberellin 2-beta-dioxygenase 7 (Solyc02g080120) (Thomas *et al*., 1999), and GA signaling (GA), a GAI-like protein 1 (Solyc01g059950) (Table 1; Thomas and Sun, 2004). Induction of these other hormone genes by cytokinin indicates the existence of a complex hormonal crosstalk pathway in tomato roots. Additionally two genes of the *LATERAL ORGAN BOUNDARIES* family (Solyc12g100150; Solyc06g082770) (Table 1) were found to be induced by cytokinin. This gene family has been linked to a range of developmental processes; including lateral root formation, as well as regulation by auxin and even cytokinin in one case, further supporting hormone connections to root development (Majer and Hochholdinger, 2011).

Cytokinin repressed genes

More than 50 genes were repressed by cytokinin treatment and can be broadly categorized into processes related to metabolism, development, and transport. There are a wide range of metabolic processes linked to Cytochrome P450 genes of which four were found to be repressed in the root (Nelson and Werck-Reichhart, 2011). There were several different types of transporters or channel proteins that were repressed including, two aquaporins and proteins associated with potassium, manganese, and malate. While these transporters all facilitate the movement of water or important solutes into the root, it is unclear why they would be repressed in the presence of additional cytokinin. One additionally interesting class of genes was the peroxidases, several of which were found as repressed while others were found to be induced by cytokinin. Peroxidases are known to have a variety of roles in developmental and defense related processes and the altered regulation of different peroxidases indicates a complex role for cytokinin in their regulation (Passardi *et al*., 2005).

Auxin induced genes

There were 107 genes induced among all root tissues over 2.0 Log_2FC by auxin from a total of 17,299 DE genes, whereas, 175, 20, 35 genes were specifically regulated in RT, LR, and WR tissues respectively (Fig. 1). Also, there were 46 genes commonly regulated genes in RT and LR tissues, 18 between LR and WR tissues, and 15 between RT and WR tissues. Overall, the number of auxin induced genes outnumbered the cytokinin induced genes in the root tip. Interestingly, several genes induced by auxin were also induced by cytokinin including the Cytokinin oxidase/dehydrogenase, ACC synthase, Gibberellin 2-oxidase genes (same IDs as mentioned above). This supports a range of findings that indicates there is a complex crosstalk among hormones during root growth and development (Depuydt and Hardtke, 2011).

Among the auxin DE induced genes were four GH3 family genes involved in Indole-3-acetic acid (IAA)-amido synthetase (Solyc01g107400, *SlGH3-3*, *SlGH3-4*, and *SlGH3-9*) with Log_2FC ranging from 2.10-8.22 (Table 1). Of these, *SlGH3-3*, *SlGH3-4*, and *SlGH3-9* genes were recently found as being group III members of the GH3 and were 3 of the 5 strongest auxin induced GH3 family members in tomato etiolated seedlings treated with hormone for 1 or 3 hours (Kumar *et al*., 2012), supporting our finding of the strong auxin induction of these genes. As these genes are involved in controlling auxin homeostasis levels in the plant, conjugating excess active IAA to an inactive form their induction from the addition of exogenous auxin is not surprising, although their endogenous role in the in root is not well known (Kumar *et al*., 2012).

Auxin induction was also seen in a number of genes belonging to two major auxin-responsive transcriptional regulator families: Auxin/indole-3-acetic acid (*Aux/IAA*) and Auxin response factor (*ARF*). These gene family members are essential players in auxin signaling with ARFs acting to active auxin regulated targets and Aux/IAA functioning as transcriptional repressor by binding to ARF and preventing their transcription factor activity, numerous of which has been identified in tomato (Kumar *et al*., 2011; Wu *et al*., 2012). In this study the Aux/IAA genes, *SlIAA2, SlIAA5, SlIAA10, SlIAA11, SlIAA19, SlIAA21, and SlIAA23* were induced among all root tissues (Table 1), while three ARFs were specifically induced in RT: *SlARF2*, *SlARF3*, and *SlARF9*. The induction of these *SlIAA* genes would also be expected in response to the addition of exogenous auxin, in a manner parallel to the induction of *SlRRA*s by cytokinin, as both are acting to block excess hormone pathway signaling. Interestingly a recent report revealed that *SlIAA* genes can also be transcriptionally regulated by ethylene in tomato seedlings (Audran-Delalande *et al*., 2012) suggesting they may function in other signaling pathways and contribute towards complex hormonal crosstalk. Several genetic approaches have been utilized that implicate the importance of *Aux/IAA* and *ARF*s in lateral root formation (De Smet, 2012). While we do find these genes induced by auxin in the root, they are not found uniquely induced in LR tissues; potentially because of LR tissues were not sampled during LR initiation when they are often required.

Auxin also induced *SlAGO7*, a member of the recently described tomato Argonaute (AGO) gene family (Table 1; Bai *et al*., 2012). Argonaute proteins are components of the RNA-induced silencing complex (RISC) involved in small RNA mediated silencing to regulate gene expression. In tomato it was shown that several members of this group were differentially regulated in response to abiotic and viral stresses. Although, *SlAGO7* in particular, was not observed to be differentially regulated by abiotic or viral stress conditions, its induction by auxin provides a new basis for further research. Interestingly miRNAs that are utilized by RISC have been shown to be involved in root cap and LR formation by targeting specific *ARF*s (Wang *et al*., 2005), and can be regulated by auxin in LR development (Yoon *et al*., 2010) suggesting a potential role for *SlAGO7* in this process.

Another gene, *BREVIS RADIX* (*BRX*, Solyc12g096110) that has a role in root development was induced by auxin (Table 1). *BRX* was first implicated as a regulator of cell proliferation and elongation in the growth zone of root tip (Mouchel *et al*., 2004), whose protein function is unknown so far to our knowledge. It has been shown in Arabidopsis that *BRX* has a role in cytokinin mediated inhibition of lateral root initiation which occurred due to loss of auxin response in presumptive founder cells in *brx-2* with indirect involvement of brassinosteroid in the process (Li *et al*., 2009). How auxin regulation of *BRX* is involved in root development needs to be further explored.

Interestingly, two *NPH3* (non-phototropic hypocotyl 3, Solyc03g044090; Solyc07g048000) genes were also induced by auxin (Table 1). The function of most *NPH3* family genes is still being investigated; however, recently this group has been linked to the proper localization of PIN protein auxin transporter (Furutani *et al*., 2011). The addition of exogenous auxin might result in a change in the endogenous auxin transport throughout the root, although this finding does further connect NPHs to auxin regulated processes.

A range of different transcription factor families genes, involved in diverse plant metabolic and developmental roles were induced by auxin in all root tissues. These include two ERFs (Solyc05g050830; Solyc04g072300), *SlCRF4*, a WRKY (Solyc01g079260), and a GRAS family transcription factor (Solyc11g017100) (Table 1). In addition, there was induction by auxin of some transcription factor genes in a root tissue specific manner. Root tip specific transcription factors include six MYB family members, an AP2/ERF (Solyc02g092050), Heat stress transcription factor A3 (Solyc09g082670), and CYCLOIDEA. There were two lateral root specific auxin induced transcription factors: GATA transcription factor 22 (Solyc01g100220) and *SlCRF6*. The transcription factor bHLH151 (Solyc06g009510) was induced in WR, but not in the other specific tissues, suggesting that it may be induced in the non-RT or LR parts of the primary root.

Auxin repressed genes

Auxin was found to repress a larger number of genes, more than 110, compared to the ~50 in cytokinin. Five different 2-oxoglutarate-dependent dioxygenase (ODDs) class of genes with a range of functions from catalyzing the production of alkaloids in roots to regulating GA biosynthesis were repressed. A previous report has shown that auxin can inhibit the production of alkaloid production in *Hyoscyamus niger* and this finding would supports that link (Hashimoto *et al*., 1986). There are also numerous instances connecting auxin and GA in hormone crosstalk, and since ODDs can function to both activate and inactive GAs in the biosynthesis pathway these genes could also support this relationship (Yamaguchi, 2008).

A number of other genes repressed by auxin belong to several of the same gene families or categories that were also repressed by cytokinin such as CYP p450s, peroxidases, hormone regulation (ACC and GA), transporters (aquaporins and solute/nutrient transporters), as well as genes involved in the biosynthesis of secondary metabolites, and other metabolic and developmental processes. Other auxin repressed genes belong to various classes of transcription factors that are involved in protein turnover and stress.

Common genes induced/repressed by both cytokinin and auxin among all root tissues

We identified several genes that were commonly induced (5) or repressed (28) by cytokinin and auxin in all root three tissues that can be broadly categorized into metabolic and developmental processes. One of the commonly auxin and cytokinin induced genes is an expansin (Solyc01g090810) that was further verified as induced by both hormones using qPCR (Table 2). Interestingly this gene is highly related to the beta-expansin *CIM1* from soybean that has previously been shown to be induced by both auxin and cytokinin, indicating that both hormones can be involved in regulating expansin genes and potentially function to control cell growth in the root (Downes *et al*., 2001). We also confirmed by qPCR the common repression by auxin and cytokinin of Ferric reductase oxidase (Solyc01g094910) involved in iron uptake by roots and a Proline rich protein (Solyc12g009650) involved in multiple processes including root nodule formation and response to external stimuli (Sheng *et al*., 1991; Table 2). The joint regulation of these genes by these two hormones indicates that they are likely have vital roles in the root connected to hormone crosstalk.

Transcriptome atlas of tomato root regions: Root-tip, Lateral root and Whole root

In addition to examining the auxin and cytokinin hormone response in root tissues, we used differential expression analysis on non-hormone treated (DMSO) root samples to identify genes expressed in specific regions of the root. We identified RT, LR, or WR abundantly expressed genes using DE comparisons between: RT and WR, LR and WR, and RT and LR with a >2.0 Log_2FC and padj ≤ 0.05 criteria.

Among the DE genes in RT, there were several classes of genes involved in defense response, pectin modification, CYP p450, transcription factors (MYB, GATA, and BHLH), Root cap proteins, an expansin, nodulin, cell division, and genes involved in other metabolic processes. The differential expression of many of these genes in the RT is not surprising as they are involved in active processes that occur in the RT such as expansion, cell division, as well as the encountering of a variety of biotic/abiotic stresses.

DE genes in the LR revealed a number of genes normally play roles in metabolism processes including Decarboxylase family protein, UDP-glucosyltransferases, Alcohol and Aldehyde dehydrogenases, Long-chain-fatty-acid-CoA ligase, Fatty acid elongase 3-ketoacyl-CoA synthase, and ER glycerol-phosphate Acyltransferase. In addition to these metabolic genes, a number of auxin related genes were also DE in LR, such as Aux/IAAs and SAURs that are known to be linked to LRs initiation and development (De Smet, 2012).

Since WR in our study comprised of RT and LRs, there were common genes expressed between these regions that are involved in metabolism and development. Compared to RT, WR and LR displayed similar profile with more than 120 common genes expressed indicating similarity in functionality of these regions. In addition, there was over-representation of genes expressed in WRs related to a range of function such as: ACC oxidases, transport of solute/minerals, CYP p450s, protein turnover, Receptor like kinases, and different transcription factors related to ethylene, auxin, and cytokinin. Other interesting genes expressed in WRs were three class I heat shock proteins, three Water-stress inducible proteins, and two Phloem protein. As these genes cover a wide range of functional classes it is possible that they may work as individuals or have combinatorial effects for the overall root development in tomato.

In conclusion, in order to identify the genes expressed and/or regulated by the hormones cytokinin and auxin in the tomato root, transcriptome analysis was performed using RNA sequencing. These results present the first genome-wide root tissue analysis of gene response to these hormones. We revealed numerous significantly DE novel genes as well as tomato orthologs of auxin and cytokinin regulated transcripts. In addition this RNA sequencing analysis confirmed hormone regulation of some previous identified tomato auxin and cytokinin regulated gene from other studies. Several interactions were identified between different plant hormones and have been discussed in the context of hormonal crosstalk in the root. We also generated an atlas of root tissue specific transcripts and their auxin and cytokinin regulation in whole roots, root tips, and lateral roots. This expression atlas indicates several classes of genes that may have specific roles in different root tissues. Taken together, this study provides a solid foundation of gene expression in tomato roots, both in different tissues and in response to different hormones which should allow future investigations to more easily study and work to improve tomato roots, plant efficiency, and fruit yield.

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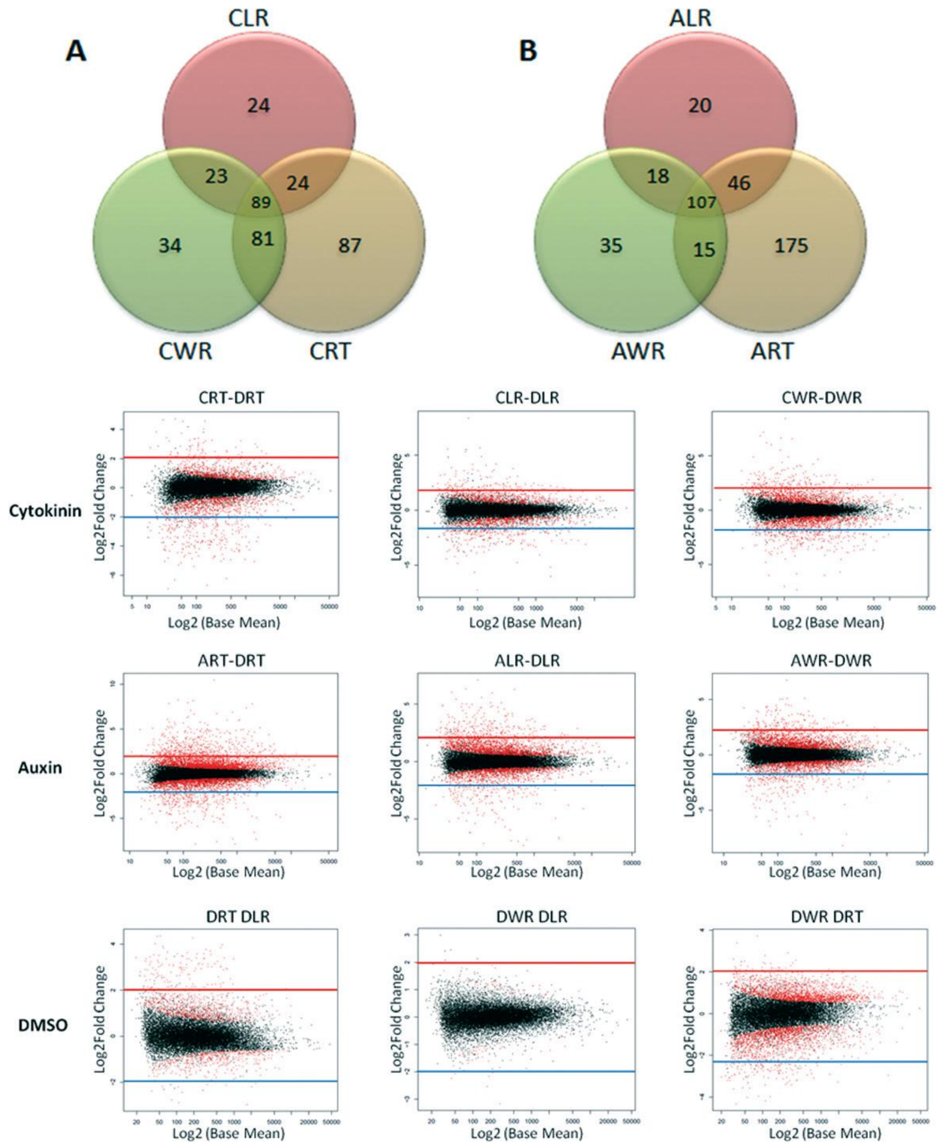

Fig. 1 Differential expression (DE) of genes in tomato root tip (RT), lateral root (LR), and whole root (WR). Treatment of root regions are noted as the prefixes C (for the cytokinin, BA) and A (for the auxin, NAA) or D (for DMSO control) before RT, LR, and WR. (**A**) and (**B**) show venn diagrams illustrating DE and significantly induced genes in tomato root either between or unique to specific tissues. Gene details are presented in Table 1. (**C**) MvA DE analysis plots indicating genes log2 fold change (vertical axis) vs the log2 base mean (horizontal axis) for root tissues treated with cytokinin (top), auxin (middle), and control (DMSO, bottom). Genes with > 2.0 log2 fold change values were identified as differentially regulated as noted in plots by lines (red, above as induced; blue, below as repressed). Significantly (padj≤ 0.05) DE genes are red dots

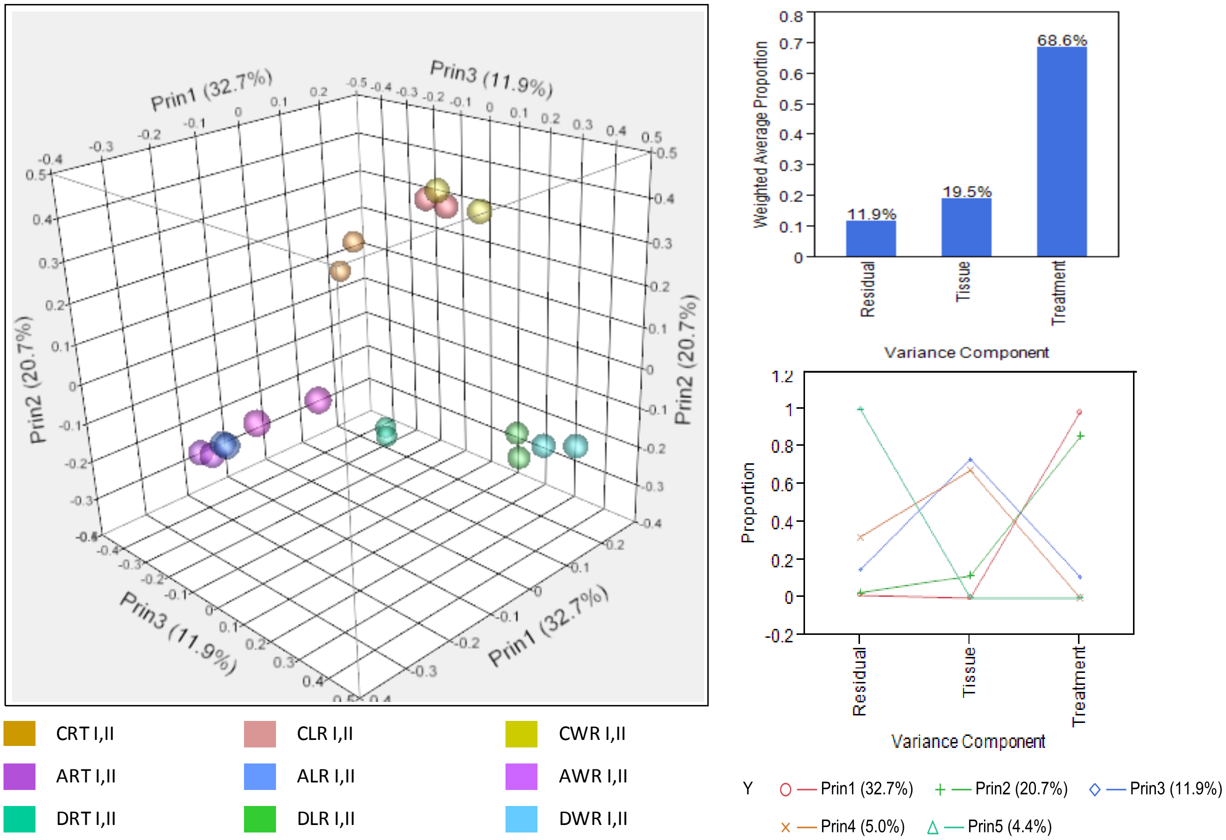

Fig. 2 Principal Component Analysis. Principle component analysis (PCA, JMP Genomics 5.1) depicts hormone treatment as the main variable producing the majority (68.6%) of the transcriptional variance. Treatment of root regions (root tip (RT), lateral root (LR), and whole root (WR)) are noted as the prefixes C (for the cytokinin, BA) and A (for the auxin, NAA) or D (for DMSO control) before RT, LR, and WR. Plots of these component principles in 2D and 3D reveal a strong clustering of individual sample replicates, I and II, as well as distinguishing treatments.

Table 1 Hormone regulated genes commonly induced in all root tissues. Shown are the Log2 Fold Change values for genes commonly induced (> 2.0) by cytokinin (above) and auxin (below) among root tip (RT), lateral root (LR), and whole root (WR) with adjusted p-values ≤ 0.05 in one or more tissue.

	GeneID	**Description**	**RT**		**LR**		**WR**	
			Log2FC	padj	Log2FC	padj	Log2FC	padj
Induced by Cytokinin	Solyc01g088160	*SlCKX2*	2.58	0.046321	3.14	0.00771	3.32	0.003426
	Solyc04g080820	*SlCKX4*	2.90	8.56E-06	3.04	2.12E-06	2.18	0.000725
	Solyc12g008900	*SlCKX6*	5.39	0.027127	7.31	0.00434	7.32	0.002308
	Solyc02g071220	*SlRRA2*	2.31	3.48E-05	3.18	7.98E-09	3.03	2.01E-08
	Solyc06g048930	*SlRRA6*	2.53	0.117332	2.90	0.05133	3.85	0.007115
	Solyc06g048600	*SlRRA7*	2.55	0.000164	2.88	2.00E-05	3.17	2.25E-06
	Solyc09g092470	*TPS14*	6.22	2.07E-26	5.21	1.26E-22	3.85	2.75E-14
	Solyc01g105960	(E)-beta-ocimene synthase	4.42	9.02E-14	3.61	2.66E-11	3.08	3.86E-09
	Solyc02g080790	*SlDHS*	3.45	0.000293	6.05	4.94E-10	5.69	2.61E-09
	Solyc06g076760	Laccase 1a	3.75	8.39E-07	2.27	0.002648	2.63	0.000348
	Solyc06g073580	1-aminocyclopropane-1-carboxylate oxidase 1	2.68	0.016622	2.81	0.014623	2.47	0.022694
	Solyc12g008740	1-aminocyclopropane-1-carboxylate synthase	3.02	8.96E-12	4.26	1.15E-14	3.21	1.38E-08
	Solyc04g010330	Auxin-regulated protein	2.18	0.351047	2.73	0.163185	3.78	0.02978
	Solyc02g080120	Gibberellin 2-beta-dioxygenase 7	3.06	6.76E-05	3.16	5.34E-06	2.37	0.000459
	Solyc01g059950	GAI-like protein 1	4.87	2.53E-27	3.50	2.87E-14	2.37	1.25E-08
	Solyc06g082770	LOB domain family protein	2.60	1.16E-06	3.34	4.00E-18	2.45	7.70E-10
	Solyc12g100150	LOB domain protein 4	4.34	1.24E-05	4.84	2.39E-07	5.99	5.71E-09
Induced by Auxin	Solyc01g107400	Indole-3-acetic acid (IAA)-amido synthetase	3.15	1.24E-06	2.20	0.001216	2.11	0.003209
	Solyc02g064830	*SlGH3-3*	7.31	8.65E-107	6.61	4.49E-98	8.22	1.03E-108
	Solyc02g092820	*SlGH3-4*	6.56	1.56E-55	6.76	2.66E-57	7.16	3.70E-59
	Solyc07g063850	*SlGH3-9*	4.95	7.98E-34	4.93	7.20E-33	4.51	2.70E-28
	Solyc06g084070	*SlIAA2*	2.57	3.34E-11	3.64	7.49E-20	3.31	9.31E-16
	Solyc12g096980	*SlIAA5*	3.99	3.01E-45	3.53	1.50E-38	3.45	1.85E-36
	Solyc06g008590	*SlIAA10*	3.78	7.08E-05	3.64	0.000265	2.99	0.004369
	Solyc03g120380	*SlIAA11*	4.91	1.47E-32	5.69	8.86E-37	5.60	3.68E-29
	Solyc07g008020	*SlIAA19*	7.58	1.41E-50	6.92	7.03E-46	7.17	2.95E-41
	Solyc08g021820	*SlIAA21*	5.47	9.70E-09	5.06	2.01E-07	4.26	1.09E-05
	Solyc09g083280	*SlIAA23*	2.22	2.62E-25	2.50	7.22E-31	2.45	6.08E-29
	Solyc01g010970	*SlAGO7*	2.35	1.05E-16	2.24	5.98E-15	2.02	1.23E-12
	Solyc12g096110	Protein BREVIS RADIX	4.19	9.56E-33	3.67	1.42E-29	3.52	1.92E-20
	Solyc07g048000	Phototropic-responsive NPH3	3.29	4.54E-15	2.97	2.15E-11	2.27	6.34E-07
	Solyc03g044090	Phototropic-responsive NPH3 family protein	2.44	1.70E-10	2.74	3.04E-12	2.43	1.67E-09
	Solyc04g072300	Ethylene-responsive transcription factor 4	3.49	5.57E-05	4.52	9.39E-07	3.72	0.000107
	Solyc05g050830	Ethylene-responsive transcription factor 4	5.99	3.07E-09	6.17	4.36E-09	4.82	3.81E-06
	Solyc03g007460	*SlCRF4*	2.63	5.25E-21	2.70	1.31E-22	2.36	1.04E-15
	Solyc01g079260	WRKY transcription factor 4	2.22	3.11E-23	2.07	5.77E-20	2.21	2.02E-21
	Solyc11g017100	GRAS family transcription factor	3.27	0.000112	3.54	5.35E-05	2.12	0.026739

Table 2 Experimental validation of a subset of cytokinin/auxin regulated transcripts. Shown are the Log2 Fold Change values from qRT-PCR vs RNA sequencing in root tip (RT), lateral root (LR), and whole root (WR) for genes (A) induced by cytokinin, (B) induced by auxin (C) repressed by cytokinin and auxin.

A

Solyc Id	Description		Log2 Fold Change	
			mRNA-seq	qRT-PCR
Solyc04g078900	Cytochrome P450	RT	4.67	4.71
		LR	5.66	3.56
		WR	7.00	2.65
Solyc12g008900	*SlCKX6*	RT	5.39	4.37
		LR	7.30	3.67
		WR	7.31	3.39
Solyc01g111900	O-methyltransferase	RT	6.69	5.54
		LR	4.78	4.88
		WR	4.12	3.83
Solyc01g090810	Expansin	RT	3.88	4.22
		LR	3.76	3.12
		WR	5.97	5.51
Solyc12g100150	LOB domain	RT	4.34	4.61
		LR	4.84	4.28
		WR	5.98	3.99

B

Solyc Id	Description		Log2 Fold Change	
			mRNA-seq	qRT-PCR
Solyc01g090810	Expansin	RT	7.27	6.54
		LR	7.32	6.39
		WR	5.16	4.74
Solyc03g120380	*SlIAA11*	RT	4.91	4.76
		LR	5.68	5.08
		WR	5.60	4.65
Solyc07g008020	*SlIAA19*	RT	7.58	7.41
		LR	6.92	7.62
		WR	7.16	6.87
Solyc03g120060	Cytochrome P450	RT	7.85	7.61
		LR	6.80	7.65
		WR	5.70	5.68
Solyc02g064830	*SlGH3-3*	RT	7.30	8.11
		LR	6.60	8.06
		WR	8.22	6.88

C

			Cytokinin		Auxin	
Solyc Id	Description		Log2 Fold Change			
			RNA-seq	Real-Time	RNA-seq	Real-Time
Solyc12g009650	Proline rich protein	RT	-2.98	-3.69	-7.32	-4.98
		LR	-2.46	-2.17	-4.81	-2.60
		WR	-3.26	-2.75	-3.51	-2.47
Solyc01g094910	Ferric reductase oxidase	RT	-4.17	-2.15	-5.72	-3.24
		LR	-5.50	-2.71	-6.30	-2.83
		WR	-6.54	-3.12	-5.05	-3.50

Table 3 Primers sequences for genes induced by cytokinin or auxin used for the verification of mRNA-seq results by qRT-PCR

		Primer sequences for genes induced by cytokinin	
		Forward	Reverse
Solyc04g078900	Cyt P450	5' AGGCTGTAGCTGATGTGGAA 3'	5' GCATGTACACCACTGCCAAA 3'
Solyc12g008900	*SlCKX6*	5' TTCCATTAGGGGACAAGCCA 3'	5' ACCACCAACGGTAAGGTACA 3'
Solyc01g111900	O-methyltransferase	5' ACACCTGTTTCTCGTCTGCT 3'	5' CAGTGCGTCGTTGATCAGTT 3'
Solyc01g090810	Expansin	5' ACCAACCACCATTCTCGTCT 3'	5' GCTCCAAAAGAAGTGCCACT 3'
Solyc12g100150	LOB domain	5' TCAACGAGGAGATGCAGTGA 3'	5' TGTTGAGATGACGGTGACCA 3'
		Primer sequences for genes induced by auxin	
		Forward	Reverse
Solyc01g090810	Expansin	5' ACCAACCACCATTCTCGTCT 3'	5' GCTCCAAAAGAAGTGCCACT 3'
Solyc03g120380	*SlIAA11*	5' GCAGCAGCAACAACAACAAC 3'	5' CAAAAACGGTGCTCCATCCA 3'
Solyc07g008020	*SlIAA19*	5' AGTGGTGGCAGAGGATCAAT 3'	5' CTCTGCACTGACCCAACAAA 3'
Solyc03g120060	Cytochrome P450	5' TAACTGTCTGTGGAGCACGT 3'	5' TCGTTAGCATCTTGTCCCCA 3'
Solyc02g064830	*SlGH3-3*	5' TACGCATCGTCCGAGTGTTA 3'	5' TTCCCACTTCCACGTTAGCT 3'
		Primer sequences for genes repressed by cytokinin and auxin	
		Forward	Reverse
Solyc12g009650	Proline rich protein	5' TCCCATTCTTGGATGTGGCT 3'	5' GTGGCAATTTGACTGGTGGT 3'
Solyc01g094910	Ferric reductase oxidase	5' TGTTCCAGTCACAAGAGGCT 3'	5' TGCCAATTCACCAGCCAAAT 3'

Chapter III. Down-stream components of cytokinin signaling and the role of cytokinin throughout the plant

Abstract

Cytokinins constitute a class of plant hormones influencing numerous aspects of growth and development. These processes occur through the downstream components of the cytokinin signaling pathway after its perception and signal transduction. The importance of these downstream signaling components has been revealed through the use of both traditional genetic and advanced molecular approaches studying mutants and transgenic lines involving cytokinin and diverse plant growth and developmental processes. Interestingly, these effects are not always directly via cytokinin, but by interactions with other plants hormones or transcription factor cascades, which can involve regulatory loops that affect transcription as well as hormone concentrations. This review covers recent advancements in understanding the role of cytokinin via its signaling components, specifically the downstream responses regulators in controlling vital plant growth and developmental processes.

Introduction

Cytokinins are a group of N6-substituted adenine derivatives that were first discovered in the laboratory of F. Skoog over a half-century ago, as a cell division factor ‘‘kinetin’’ (Miller et al. 1955). Since then, cytokinin has been shown to be involved in numerous developmental processes from meristem specification to cell division and expansion. Much of the work done to understand the complex process of cytokinin regulation of plant development has focused on its two-component system (TCS). This plant system resembles a modified bacterial two component signaling system comprised of receptor histidine kinases (HKs), phosphotransfer proteins (HPts) and response regulators (RRs) (Ferreira and Kieber 2005; Heyl and Schmülling 2003; Kakimoto 2003; Maxwell and Kieber 2005). In the TCS, once cytokinin binds to the receptor it becomes autophosphorylated and then passes this phosphate on to a HPt, which ferries the phosphate signal into the nucleus and on to a RR. The RRs then are involved in translating this signal into cytokinin-regulated growth and developmental processes. This review centers on the downstream cytokinin signaling pathway components, primarily the RRs, and presents the present status of our understanding of the role of these components in plant development.

Response regulators in cytokinin signaling

Response regulators (RRs) are the downstream nuclear localized components of the TCS that are believed to be the major regulators of cytokinin response in plants. TCS cytokinin signaling from the receptors to RR occurs through a conserved phosphorelay receiver protein domain,with required DDK amino acid residues characteristic of similar receiver domains found in prokaryotes and yeast (Fig. 3; Hwang et al. 2002). Through sequence analysis, it was shown that there are 22 RRs in Arabidopsis, which were originally divided into two large classes, type-A and type-B ARRs based on protein sequence and domain structure in addition to transcriptional induction by cytokinin (Brandstatter and Kieber 1998; Kiba et al. 1999; Taniguchi et al. 1998; Imamura et al. 1999). Additional phylogenic analysis on all receiver domain containing proteins has classified RR members into four different groups: type-A, type-B, type-C, and pseudo RRs (Fig. 3; Sakai et al. 1998, 2000, 2001; Mizuno and Nakamichi 2005). The type-A and type-C ARRs contain only a phospho-accepting receiver domain, whereas the type-B ARRs contain both a receiver domain and a C-terminal output domain containing a GARP family myb-like DNA binding and a transactivating region. Type-B ARRs also have a conserved region containing a nuclear localization signal (NLS) responsible for their subcellular localization (Imamura et al. 2001; Hosoda et al. 2002). Five additional proteins in Arabidopsis are related to RRs, but cannot function as phosphate receivers as their required phosphate receiving aspartate is changed to a glutamate. These proteins are called the pseudo RRs (PRR). Similar to type-B

ARRs, PRRs also have a C-terminal extension, however, it contains a CCT plant specific motif (CONSTANS, CONSTANS-like and TOC1) (Makino et al. 2000; Matsushika et al. 2000; Putterill et al. 1995) not a GARP domain. As such, PRRs are not directly involved in TCS and are linked instead to circadian regulation (Mizuno and Nakamichi 2005) in plants which is beyond the scope of this review.

Type-B ARRs

The type-B RR family in Arabidopsis is comprised of 11 members, ARR1, 2, 10, 11, 12, 13, 14, 18, 19, 20 and 21 which can be further divided into three subfamilies based on the phylogenetic analysis of their receiver domains (Mason et al. 2004). It is important to note that none of the type-B RRs are transcriptionally induced by cytokinin. Subfamily I comprises the members: ARR 1, 2, 10, 11, 12, 14, and 18, most of which have been shown to be involved in mediating cytokinin response (Argyros et al. 2008; Hwang and Sheen 2001; Imamura et al. 2003 Ishida et al. 2008; Sakai et al. 2001; Yokoyama et al. 2007). Subfamily I members appear to be the primary players in TCS response, since as yet there is no convincing information that the other subfamily members: ARR 13 and 21 (subfamily II) and ARR 19 and 20 (subfamily III) are involved in cytokinin responses.

Intracellular localization studies, utilizing GFP- or GUS fusion proteins, revealed that localization of all type-B ARRs is within the nucleus, even for truncated protein versions lacking a receiver domain (Lohrmann et al. 1999, 2001; Sakai et al. 2000; Hwang and Sheen 2001; Hosoda et al. 2002; Mason et al. 2004). Additional examination of ARR2-GFP after exogenous cytokinin treatment in Arabidopsis protoplasts showed no change in intracellular position (Hwang and Sheen 2001) suggesting that the type-B ARRs likely are expressed and move to the nucleus to mediate cytokinin responses.

Tissue and organ specific expression patterns of type-B ARRs appear to be overlapping during different developmental stages (Mason et al. 2004; Tajima et al. 2004). Studies based on ARR::GUS fusions for subfamily I members: ARR1, ARR2, ARR10, and ARR12 showed that they are expressed throughout young leaves and as the leaves mature their expression becomes restricted to the vascular tissue and hydathodes. ARR2 and ARR12 are also expressed in trichomes. Similar overlapping patterns were seen in case of roots where these ARRs display variable expression in the root apical meristem (RAM), zone of elongation and in mature root (Mason et al. 2004; Birnbaum et al. 2003). Despite having unique tissue expression patterns subfamily II and III members were observed to overlap with subfamily I in young trichomes (subfamilies I and III) and in aerial tissues (subfamilies I and II) (Mason et al. 2004).

ARR1 and ARR2 were the first RRs to be recognized as transcription factors (TFs) because of the presence of the essential characteristics of a GARP Myb-like transcriptional activator domain (Sakai et al. 2000; Fig. 3). Study of RRs as TFs included examination of direct promoter binding of the TF domain (Lohrmann et al. 2001), and identification of RR DNA binding sequence 50AGATT30 in ARR10 (Hosoda et al. 2002). Further investigations identified specific amino acids in ARR10 responsible for the DNA binding interaction (Hosoda et al. 2002). ARR11 was also shown to receive a phosphoryl group from an AHP in vitro and then bind to the DNA sequence 50GGATT30 (Imamura et al. 2003). Recently it has been proposed that type-B ARRs must also be expressed at more than a minimal threshold level to activate the entire signaling pathway (Müller 2011).

Type-B ARRs appear to act as positive regulators of cytokinin signaling, in contrast to type-A ARRs that act as negative regulators, which together form a negative feedback loop (Hwang and Sheen 2001). Several different groups have utilized transgenic approaches to demonstrate the positive role of type-B ARRs in the pathway, including initially showing that ARR2 overexpressing plants stimulated cell proliferation and shoot formation in vitro in the absence of exogenous cytokinin (Hwang and Sheen 2001). Argyros et al. 2008 observed the arr1, 10, 12 triple mutant had reduced expression of CYCD3;1, a D-type cyclin that regulates cytokinin effects on cell division. Type-B ARRs were also found to regulate the expression of the type-A RRs (Hwang and Sheen 2001; Sakai et al. 2001). Observations also show that the type-B ARRs

(1, 2, and 10) induced ARR6 expression in the absence of cytokinin, in contrast to several type-A ARRs that repressed ARR6 (Hwang and Sheen 2001). Somewhat similar studies examined cytokinin sensitivity of arr1 mutant and ARR1 overexpression lines. These revealed that arr1 displayed reduced sensitivity to cytokinin and reduced expression of ARR6, whereas ARR1 overexpression lines showed increased sensitivity to cytokinin and increased expression of ARR6 (Sakai et al. 2001). Further evidence of type-B RR positive role has come from mutant analyses where the generation of increasing higher order type-B RR multiple mutants resulted in greater reduction in sensitivity to cytokinin (Argyros et al. 2008; Ishida et al. 2008; Mason et al. 2005; Yokoyama et al. 2007).

Several type-B RRs may function in roles outside of the cytokinin signaling. For example, ARR2 has also been shown to mediate ethylene response, in both, analyses of loss-of-function and overexpressing lines (Hass et al. 2004). Another recent finding has shown that type-B ARRs can act by binding with the Salicylic acid (SA) response factor TGA3 to boost resistance against Pseudomonas syringae pv. Tomato (Pst) in Arabidopsis (Choi et al. 2010). These findings indicate that type-B RRs might act as a link between other signaling pathways to coordinate different developmental processes. More investigations on the RRs are needed to fully understand their potential roles in indirect or independent cytokinin-regulated growth and development.

Type-A ARRs

Type-A ARRs were initially observed as members of the TCS pathway whose expression is rapidly induced in response to cytokinin. They are now known to be negative regulators of cytokinin signaling, in direct contrast to type-B RRs. There are generally considered to be 10 type-A RRs in Arabidopsis: ARR3, 4, 5, 6, 7, 8, 9, 15, 16, 17 (To et al. 2004), however, sometimes the type-C RRs are also included in this group. Type-A RRs have the most basic RR protein form containing only a receiver domain, and no transcription factor domain like type-B RRs (Fig. 3). Although all type-A RRs are up-regulated by cytokinin, some type-As, ARR5, 6, 7, and 15 are greatly induced in response to cytokinin, while others, ARR4, 8, and 9 have relatively high basal level of expression and are induced by cytokinin to a lesser degree (Brandstatter and Kieber 1998; D'Agostino et al. 2000; Imamura et al. 1998; Kiba et al. 1999; Rashotte et al. 2003). Similar findings are true for type-A RRs from a range of species beyond Arabidopsis, such as maize and rice (Asakura et al. 2003; Jain et al. 2006).

Type-A ARRs have been shown to have different intracellular localization patterns from studies utilizing GFP-fusion proteins (Hwang and Sheen 2001; Imamura et al. 2001; Kiba et al. 2002). While GFP-fusion proteins for ARR5, ARR6, ARR7 and ARR15 have been detected just in the nucleus, ARR4 and ARR16 have been observed both in the cytoplasm and in nucleus indicating potentially broader roles in TCS signaling.

Type-A ARRs also display overlapping tissue expression patterns in the presence of exogenous cytokinin as seen in the root by promoter::GUS analysis (To et al. 2004). Induction levels of reporter gene activity paralleled previous transcriptional expression induction finding for the type-A RRs noted above. Interestingly, cytokinin treatment caused an expansion of reporter expression to tissues surrounding their normal localization. Type-A ARRs have been shown to respond to exogenous cytokinin across a range of tissues from the root to shoot apical meristem (SAM) in numerous studies (D'Agostino et al. 2000; To et al. 2004). In fact the ARR5 promoter::GUS fusion has been routinely used as the most predominant marker/proxy of cytokinin response in plants.

After activation and prolonged cytokinin signal transduction, there is also a need to mitigate the signaling pathway. This is done through the negative regulation of the TCS by induction of the type-A RRs. Cytokinin induced expression activation of type-A RRs has been observed in a variety of tissues in maize (Zea mays) and rice (Oryza sativa) along with Arabidopsis (Asakura et al. 2003; Jain et al. 2006; D'Agostino et al. 2000; Rashotte et al. 2003). It has also been shown that the transcriptional activation of type-A RRs by cytokinin occurs in the absence of de novo protein synthesis (Brandstatter and Kieber 1998; Sakakibara et al. 1999; D'Agostino et al. 2000) suggesting that type-A ARRs are cytokinin primary response genes. While type-A RRs can be directly activated via TCS signaling through the HKs and HPts, they have also been shown to be direct transcriptional targets of type-B RRs as part of the TCS feedback regulation (Hwang and Sheen 2001; Sakai et al. 2001). Recently, six type-A ARR proteins (ARR5, ARR7, ARR8, ARR15, ARR16 and ARR17) have also been shown to be regulated by a combinatorial mechanism which involves proteasome pathways along with cytokinin (Ren et al. 2009). This indicates that other pathways can interact with downstream components of the TCS to regulate various developmental processes.

There have been a number of studies that have focused on determining the primary function of type-A RRs in addition to those studies initially linking them to the TCS and cytokinin induction. More recent investigations of type-A ARRs as negative regulators of the TCS found that phosphorylation of ARR5 and ARR7 at an aspartate phosphate receiver domain is necessary for them to act as negative regulators and that cytokinin itself regulates their stability (Lee et al. 2008; To et al. 2007). Additional studies also indicate the importance of this phosphorylation. Specifically, as shown for the overexpression of ARR7 that reduces root growth inhibition and callus formation by cytokinin, whereas overexpression of ARR7 mutated to prohibit ASP phosphorylation resulted in no phenotypes (Lee et al. 2007, 2008). Similar findings were shown regarding the importance of phosphorylation for ARR5 including mutation of the conserved ASP residue to prohibit phosphorylation, which was unable to complement phenotype functionality in an arr3, 4, 5, 6 mutant, when an unaltered ARR5 could (To et al. 2007). Furthermore, partial complementation could be seen in an ARR5 ASP mutated to be a phosphor-mimic line. An additional mechanism for type-A RRs to act as negative regulators in the TCS could occur by competitively taking phosphates that would otherwise activate type-B RRs as positive regulators; however, the exact mechanism is currently unknown.

Type-C ARRs

Type-C ARRs, ARR22 and ARR24, have been added as a separate group of RRs that are also basic RRs, like type-A RRs, lacking any domain other than a receiver domain. However, from sequence analysis this domain in type-C RRs has less similarity with other RRs and appears to be more related to the receiver domain found in the hybrid histidine kinase receptors (Kiba et al. 2004; To and Kieber 2008). Additionally, type-C RRs are not induced by cytokinin, making them distinct from the type-A RRs. ARR22 has been shown to be induced transcriptionally as a result of wounding, indicating a possible role of type-Cs in response to other signals (Gattolin et al. 2006). As type-C RRs do not appear to be directly linked to TCS processes, a role in cytokinin mediated growth and developmental processes remains to be found.

Other downstream components: cytokinin response factors (CRFs)

Cytokinin signaling components directly downstream of the receptors and phosphorelay proteins are usually limited to the response regulators as noted above. One other identified group has been sometimes included as a side branch to this signaling pathway is the cytokinin response factors or CRFs. These genes were originally identified as highly related cytokinin induced AP2/ERF-like transcription factors in microarray experiments to identify cytokinin-regulated genes (Rashotte et al. 2003). Additional examination of CRFs in relation to the TCS indicates that they are downstream of the receptors and phosphorelay proteins and form branch in the pathway parallel to the response regulators (Rashotte et al. 2006). More recently CRFs were also shown to directly interact via protein–protein interactions with AHPs, in a pattern that is similar to interactions seen for both type-A and -B ARRs, further supporting the finding of these proteins as a side branch of the TCS pathway (Cutcliffe et al. 2011). Rare specific CRF-RR interactions could be found in that same study for both type-A and -B ARRs potentially suggesting that another complex set of interactions between downstream components could exist to additionally regulate cytokinin signaling, although this will require more study.

CRFs have also been shown to act as transcription factors targeting many cytokinin-regulated genes that type-B RRs also act upon, although CRFs do have cytokinin-regulated targets independent of RR targets (Rashotte et al. 2006). It is not too surprising that each of these groups acts on different targets, since they are entirely different classes of transcription factors with non-overlapping sequences. Phylogenetic analysis has revealed that CRFs are present and broadly represented across all land plants with large radiations of CRFs in angiosperms, similar to other downstream components (Rashotte and Goertzen 2010). Loss-of-function analyses of CRF mutants in Arabidopsis revealed that these genes function in a redundant manner and are likely involved in the normal development of embryos, cotyledons, and leaves (Rashotte et al. 2006). Recently, a complete analysis of CRF genes has also been described in tomato, named Solanum lycopersicum cytokinin response factors (SlCRFs). These 11 SlCRF genes show distinct expression patterns in response to exogenous cytokinin (Shi et al. 2012) similar to some of the Arabidopsis CRFs and possibly have a role in vascular development in leaves (Zwack et al. unpublished results). Although the functional role for CRFs in cytokinin signaling and cytokinin developmental processes is still being determined, it is clear that there are direct connections.

Cytokinin regulation of plant developmental pathways

The role of cytokinin in various organ developmental processes is crucial. Either cytokinin or cytokinin interactions with other signaling pathways regulates the growth and development of essential plant organs including the root, stem, leaf, and flower as discussed in the following sections.

Root development: from stem cell to symbiosis

Beginning from the specification of root stem-cell niche, cytokinin is involved in regulating many different developmental aspects. Work from Friml et al. (2003) and Sabatini et al. (1999) revealed that auxin plays a role in the specification of RAM and this hormone is actively present in the basal cell of the embryo. Additional work by Müller and Sheen (2008) expanded these studies on root stem-cell niche specification, utilizing GFP reporter analysis of TCS genes to track their activity during this process. Cytokinin signaling output via TCS::GFP was first observed in the hypophysis of the 16-cell stage embryo, then after the asymmetric division, its activity becomes restricted to the apical lens shaped daughter cell and repressed in the basal cell. This indicates that cytokinin is likely required to ensure correct divisions needed to form the

RAM. The high levels of endogenous auxin induce the type-A RRs, ARR7 and ARR15 to diminish the presence of cytokinin signal in the basal cell (Müller and Sheen 2008). Hence, the interplay between auxin and cytokinin was found to be important for the establishment of root stem-cell niche.

Along with the establishment of the apical cell in root development, cytokinin has been found to have roles in the RAM size via its signaling components. It was observed that TCS mutant plants ahk3 and arr1, 12 produce a large RAM (Dello Ioio et al. 2007) indicating that cytokinin acts to maintain RAM size (Fig. 4). However, other groups showed that the RAM activity was arrested in the arr1, 10, 12 triple mutant and reduced in ahk 2, 3, 4 triple and ahp1, 2, 3, 4, 5 quintuple mutants (Higuchi et al. 2004; Hutchison et al. 2006; Ishida et al. 2008; Nishimura et al. 2004; Yokoyama et al. 2007). Meristem size and root length were also observed to be decreased in plants with complete absence of cytokinin perception (Higuchi et al. 2004; Nishimura et al. 2004) suggesting a minimum threshold level of cytokinin is necessary; possibly to interact with other signals and have normal RAM growth and development, although the exact relation is unclear.

Crosstalk between cytokinin and auxin also has an influence on meristem size and root growth, as seen in a number of gene regulation linkages. Specifically, the type-B RR, ARR1 was found to be involved in the regulation of RAM size via direct regulation of the SHORT HYPOCOTYL 2 (SHY2/IAA3) gene (Dello Ioio et al. 2008; Taniguchi et al. 2007) that negatively regulates auxin transport by decreasing PIN expression (Fig. 4). Also cytokinin has recently been shown to regulate the levels of PIN efflux carriers via type-A RRs (Zhang et al. 2011). Auxin, in turn, stimulates SHY2 breakdown allowing its PIN-mediated transport/distribution, which ensures cell expansion in the RAM (Mockaitis and Estelle 2008). Cytokinin levels have further been shown to be regulated by SHY2 via down-regulation of the cytokinin biosynthesis gene, isopentenyltransferase5 (IPT5) (Dello Ioio et al. 2008). These results indicate that cytokinin and auxin appear to interact in an antagonistic manner to maintain the level of each other and hence proper RAM development.

Several studies have shown that mutants of CRE1/AHK4 (de Leon et al. 2004; Mähönen et al. 2000; Scheres et al. 1995) or multiple ahk and ahp mutants (Higuchi et al. 2004; Hutchison et al. 2006; Mähönen et al. 2006; Nishimura et al. 2004) displayed an extremely stunted primary root phenotype. Roots of these mutants had fewer vascular cell files with abnormal development, resulting in a root with only protoxylem. This strongly suggests that cytokinin plays a role in vascular initial cell differentiation. Overexpression of ARR22 also led to a similar root phenotype, but the role of this type-C RR in the root vascular process could not be determined (Kiba et al. 2004). These findings reflect the positive role of cytokinin in normal root vascular development.

A variety of transgenic lines with altered cytokinin levels have revealed a negative role for cytokinin in lateral root formation (Lohar et al. 2004; Mason et al. 2005; Riefler et al. 2006; To et al. 2004; Werner et al. 2001, 2003). Exogenous application of cytokinin has also been shown to inhibit lateral root formation (Kuderova et al. 2008; Laplaze et al. 2007; Li et al. 2006). Detailed studies revealed that cytokinin specifically affects the pericycle cell (anticlinal) divisions in the lateral root founder cells (Li et al. 2006; Laplaze et al. 2007); however, later developmental stages are not affected. This indicates that the role of cytokinin in lateral root development may be restricted to the stages of initiation. AHK receptor mutants have also been shown to have enhanced emergence of lateral roots (Dello Ioio et al. 2007, 2008; Li et al. 2006; Nishimura et al. 2004; Riefler et al. 2006) indicating that cytokinin inhibits lateral root formation, although the requirement for a basal level of cytokinin in this process cannot be ruled out. Other hormones such as auxin and ethylene have also been suggested to regulate lateral root development through their respective pathways or interactions with cytokinin (Aloni et al. 2006; Laplaze et al. 2007; Kuderova et al. 2008). Recently, interactions between cytokinin and auxin have been shown to regulate lateral root organogenesis where cytokinin reduces the amount of PIN1 at the plasma membrane indicating that auxin is required in a particular concentration to promote this organogenesis (Marhavý et al. 2011).

In leguminous plants, cytokinin has been observed to play a role in root nodulation. Transgenic plants with either reduced cytokinin level by overexpressing CYTOKININ OXIDASE (CKX) genes or reduced perception by mutating cytokinin receptor genes resulted in decreased numbers or absence of nodulation in Lotus japonicus and Medicago truncatula plants (Lohar et al. 2004; Gonzalez-Rizzo et al. 2006; Murray et al. 2007). Additionally, at least one RR, a type-A, has also been linked to root nodulation in Medicago in connection with an ethylene response factor and the reduction of cytokinin levels (Vernié et al. 2008). Moreover, cytokinin was shown to have opposite effects on nodulation and in lateral root formation, as the plants with reduced cytokinin levels and reduced nodulation developed more lateral roots (Lohar et al. 2004; Gonzalez-Rizzo et al. 2006). From the number of other downstream TCS components that are actively expressed and directly affect other important root functions, it seems likely that more RRs and possibly CRFs may be critical players in this process. More studies directed towards this may help determine cytokinin role here and help to develop better varieties of leguminous species.

Shoot development: the shoot apical meristem and beyond

Skoog and Miller 1957, originally demonstrated that a high cytokinin to auxin ratio promotes shoot formation from callus. Since then continued work in this area, especially over the last decade has led to the finding that cytokinin has a positive role in regulating SAM size and activity (Higuchi et al. 2004; Tucker and Laux 2007; Werner et al. 2001, 2003; Werner and Schmülling 2009). In agreement, studies of ahk triple receptor mutant also displayed decreased SAM activity and as a result a much smaller SAM size with fewer cell layers and fewer cells per layer (Higuchi et al. 2004). Moreover, studies of IPT mutants further strengthened the positive role of cytokinin in the SAM development where ipt1, 3, 5, 7 quadruple mutants and the ipt1, 3, 5, 6, 7 quintuple mutants appeared to havereduced SAM size (Miyawaki et al. 2006).

Several transcription factors have been observed to have a role in the normal SAM function, such as STIMPY (STIP/WOX9) and WUSCHEL (WUS) required for the establishment of both root and shoot meristematic tissue (Wu et al. 2005). STIP was observed to have reduced expression in the SAM of ahk and type-B arr mutants, indicating its expression is dependent on cytokinin (Skylar et al. 2010). Another TF, SHOOT MERISTEMLESS (STM), a class I KNOTTED1-like homeobox is expressed in the central zone (CZ) and peripheral zone (PZ) of the SAM, where it functions to maintain cell division and prevention of cell differentiation. In Arabidopsis, STM and cytokinin are positive regulators of each other (Fig. 5), where STM regulates cytokinin levels via activation of IPT7 indicating that cytokinin is required in the SAM for the maintenance of cell division and prevention of cell differentiation (Jasinski et al. 2005; Rupp et al. 1999; Sablowski 2007; Yanai et al. 2005). Other TF families also regulate STM expression in the SAM, such as the CUP-SHAPED COTYLEDONS (CUC) family involved in positive regulation and ASYMMETRIC EAVES2/LATERAL ORGAN BOUNDARIES (AS2/LOB) involved in negative regulation (Semiarti et al. 2001). CUC2 and CUC3 genes are themselves regulated by cytokinin in the inflorescence meristem indicating positive feedback regulation in the SAM during its development (Li et al. 2010). LONELY GUY (LOG), which was identified in rice is confined to the shoot apical regions and regulates cytokinin levels by the conversion of inactive cytokinin nucleotides to active free bases. The log mutants also have SAMs that are defective in size and activity (Kurakawa et al. 2007) further confirming the role of cytokinin in the SAM development.

Effects of cytokinin in shoot development through type-A RRs have been observed in several plant species including rice where OsRR6 overexpression resulted in complete meristem arrest (Hirose et al. 2007). Effects have also been seen in the alteration of phyllotaxy and shoot organ initiation in both the maize type-A RR mutant abphyl1 (ABERRANT PHYLLOTAXY1) (Giulini et al. 2004; Jackson and Hake. 1999) and in the Arabidopsis septuple type-A ARR mutant, arr3, 4, 5, 6, 7, 8 and 9 (Leibfried et al. 2005). Additionally, it has been shown that WUS, which is required to keep SAM stem cells in an undifferentiated state (Laux et al. 1996; Mayer et al. 1998), is involved in doing so by repressing the expression of several type-A ARRs (Leibfried et al. 2005). Likewise, it has been shown through live imaging in the SAM central zone that cytokinin up-regulates WUS expression (Gordon et al. 2009; Lindsay et al. 2006) possibly for its own maintenance in the SAM. Consistent with this finding, it was observed that WUS positively regulates AHK4 expression and cytokinin abundance and negatively affects the expression of type-A ARRs, ARR5, ARR7 and ARR15 (Gordon et al. 2009). In addition to cytokinin and WUS, auxin also negatively regulates the expression of two type-A ARRs: ARR7 and ARR15 (Zhao et al. 2010) via the auxin response factor MONOPTEROS (MP) (Fig. 5). Cytokinin-auxin interactions have also been shown to be necessary for shoot apex growth as demonstrated in Pisum sativum where PIN mediated flow of auxin basipetally from the shoot apex repressed IPT expression. The reduced level of cytokinin in this system results in increased shoot apical dominance and inhibition of axillary bud formation (Shimizu-Sato et al. 2009).

Leaf development: from expansion to senescence

Findings that cytokinin plays an important role in leaf development date back to the 1950s when it was first shown that kinetin promoted the expansion of excised leaves (Kuraishi and Okumura 1956). Increases in leaf expansion after treatment with cytokinin has also been observed in isolated leaf discs of sweet pepper (Capsicum annuum) (Nielsen and Ulvskov 1992). In tobacco, it was seen that growth under a high gibberellic acid (GA) to cytokinin ratio resulted in narrow leaves, whereas a low ratio produced rounded leaves, further supporting a role of cytokinin in leaf development (Engelke et al. 1973). Examination of the spatial distribution of endogenous cytokinins in pepper leaves revealed that the distribution in young/maturing leaves decreased from the base to tip and was very different from fully matured leaves that had an almost uniform distribution (Ulvskov et al. 1992). A similar finding was observed over a temporal distribution with the highest levels of endogenous cytokinin occurring during leaf expansion compared to other stages of development (Ron’zhina 2003).

Cytokinin receptors have also been utilized to link cytokinin to leaf developmental processes. The study of different combinations of ahk multiple mutants showed that an ahk2, 3 double mutant had smaller leaves, while ahk single mutants and ahk2, 4 and ahk3, 4 double mutants showed no morphological differences from wild type. The greatest effect was seen by completely eliminating cytokinin signaling in the triple receptor mutant ahk2, 3, 4 that showed defective growth and reduced leaf size and number, due to reduced cell numbers and size (Higuchi et al. 2004; Nishimura et al. 2004). One additional cytokinin regulated group that has been linked to leaf and cotyledon development in mutant studies is the CRFs where altered shape was found in these organs (Rashotte et al. 2006).

Cytokinin is also involved in compound leaf development in tomato. This was found by altering cytokinin levels in tomato through the expression of either the cytokinin biosynthesis gene IPT7 or the cytokinin degradation gene CKX3 (Shani et al. 2010). IPT7 expression in normally compound tomato leaves resulted in increased cytokinin levels and the production of super-compound leaves with up to four orders of leaflet complexity compared to wild-type plants with two orders of leaflets. In contrast, CKX3 expression resulted in low cytokinin levels and production of simplified leaves with only primary leaflets. Several additional studies of KNOX1 proteins, which have been linked to cytokinin, showed that these proteins are involved in the regulation of compound leaf development (Hareven et al. 1996; Janssen et al. 1998; Jasinski et al. 2005; Hay and Tsiantis 2006; Shani et al. 2009, 2010; Yanai et al. 2005). KNOX1 genes were found to be expressed in the SAM of simple-leaved species (maize, rice, tobacco, Arabidopsis) and their expression is down-regulated after leaf initiation starts (Vollbrecht et al. 1991; Lincoln et al. 1994; Nishimura et al. 1999; Waites et al. 1998). In contrast, in tomato compound leaves, KNOX1 genes show up-regulation in leaf primordia and as the leaf matures, expression is down-regulated (Hareven et al. 1996; Chen et al. 1997; Janssen et al. 1998). Relation between cytokinin and KNOX1 proteins was shown in tomato where cytokinin was observed to act downstream of KNOX1 proteins hence regulating compound leaf development (Shani et al. 2010). Cytokinin was also shown to suppress the effect of GA (Fleishon et al. 2011) which otherwise results in less complex and non-serrated leaves in tomato (Hay et al. 2002) indicating the interaction between cytokinin and other hormones in compound leaf development.

Leaf senescence is another important area where cytokinin is involved. It was shown that leaf senescence could be manipulated by changing endogenous cytokinin levels (Smart et al. 1991) or delayed by external application of cytokinin to the leaves (Mok and Mok 1994). Involvement of cytokinin in delayed senescence was further strengthened by the expression of IPT driven by a senescence-specific promoter resulting in a later onset of senescence (Gan and Amasino 1995). Attempts to find how TCS components are involved in the process led to the discovery that AHK3 could be the primary cytokinin receptor regulating leaf senescence. AHK3 gain-of-function mutants and overexpression lines were found to delay dark-induced leaf senescence and reduce expression of senescence associated genes, while loss-of-function ahk3 mutants were found to senesce early (Kim et al. 2006). Overexpression of type-B ARR2 also displays delayed leaf senescence. ARR2 has since been found to be phosphorylated by only AHK3 among the three cytokinin receptors, suggesting the initial pathway for cytokinin regulated leaf senescence.

Cytokinins may also have a role in controlling chloroplast numbers in the cell. It was observed in Arabidopsis that CRF2 overexpression results in accelerated division of chloroplasts and increased expression of PLASTID DIVISION 2 (PVD2), that is required for plastid division (Okazaki et al. 2009). This indicates that cytokinin regulation of CRF2 may increase plastid division. Moreover, wild-type Arabidopsis seeds germinated on cytokinin containing medium have increased levels of PVD2. Additionally, it was observed that exogenous cytokinin treatment induces the transcription of 19 out of 26 tested genes responsible for plastid regulation (Zubo et al. 2009) further strengthening the possibility of cytokinin regulation of plastid development.

Reproductive organ development

Cytokinin has a positive role in reproductive organ development. In Arabidopsis, it has been seen that cytokinin levels increase in the SAM after transition to flowering (Corbesier et al. 2003). TCS components have also been utilized to study the role of cytokinin in reproductive organ development. The ahk2, 3, 4 triple mutant as well as plants defective in multiple other TCS components often have sterile or delayed floral transition phenotypes (Higuchi et al. 2004; Nishimura et al. 2004; Werner et al. 2003). Subsequent work manipulating cytokinin levels via the Os-CKX2 gene in rice (Ashikari et al. 2005) and CKX3 and CKX5 genes in Arabidopsis (Bartrina et al. 2011) unveiled the positive role of cytokinin in reproductive organ development. Reduced expression of Os-CKX2 led to an increased amount of cytokinin in the inflorescence meristem that yielded an increased number of reproductive organs. Arabidopsis ckx3,5 double mutant, with approximately fourfold WT cytokinin levels, also produced larger inflorescences and floral meristems, larger sized flowers, that had supernumerary ovules producing increased seed yield. Although this evidence implicates cytokinin in reproductive development more work still needs to be done to understand its full role in this complex process.

Concluding remarks

Based on the findings reviewed here, we can clearly say that cytokinin plays multiple vital roles in plant growth over the course of development. Many of these roles are linked to the cytokinin-signaling pathway, particularly the downstream components known as the Response Regulators (RR). In addition to these roles, there is complex regulation of RR in feedback loops between members of this group, type-A RRs and type-B RRs, as well as with other TFs and hormones such as auxin. Although, we are beginning to understand the role of cytokinin and the TCS in hormone cross-talk that controls developmental processes, more studies are still needed to make these links clearer.

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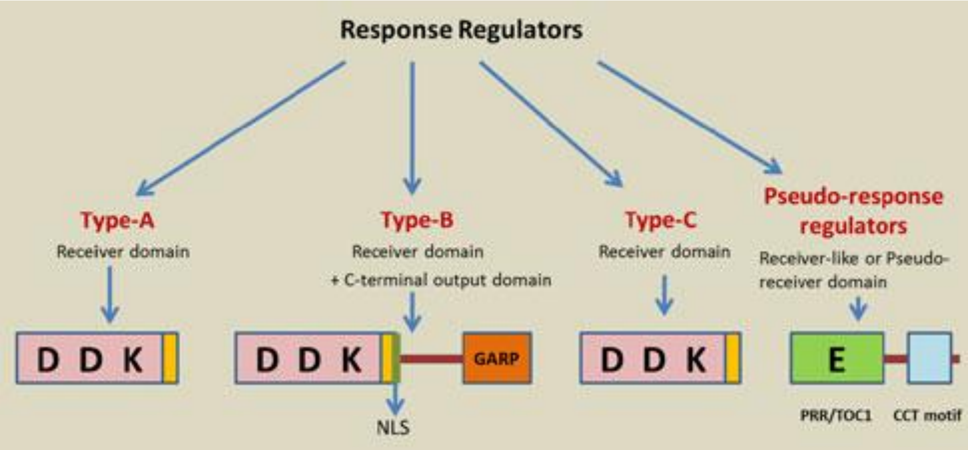

Fig. 3 Schematic representation of Response Regulators: type-A, type-B, type-C and Pseudo Response Regulator proteins showing their specific domain regions. Type-A and type-C ARRs have only Receiver domains, in pink, shown with conserved phosphorylated amino acid residues D, D, and K. Type-B ARRs have a receiver domain, a nuclear localization signal motif (NLS) and a C-terminal output domain, which contains a myb-like DNA binding and transactivating domain, GARP domain, in orange. The Pseudo Response Regulator proteins have the characteristic pseudo-receiver domain, in green, shown with its non-phosphorylated amino acid residue E and a C-terminal CCT-Motif

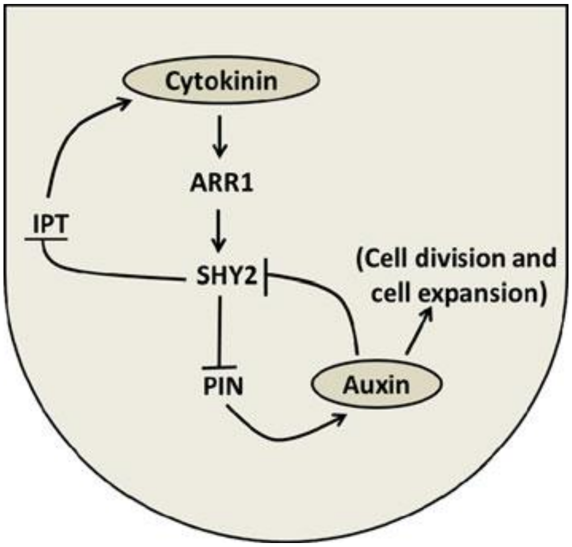

Fig. 4 Interaction between cytokinin and auxin in the root apical meristem. Cytokinin and auxin interact through SHY2 at the signaling level to regulate the level of each other in the process of root apical meristem development. Arrows indicate positive regulation, line with blocks indicate negative regulation. Auxin negatively regulates SHY2 that in turn negatively regulates PIN proteins involved in the transport of auxin that determines this hormones concentration. Cytokinin positively regulates ARR1 that in turn positively regulates SHY2, which negatively regulates IPT proteins involved in cytokinin biosynthesis controlling these hormones' concentration

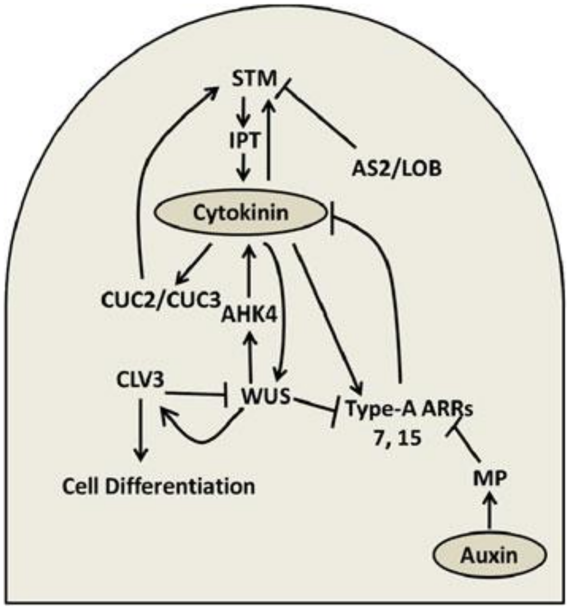

Fig. 5 Interaction between cytokinin and auxin in the shoot apical meristem. Shoot apical meristem development involves interactions among cytokinin signaling pathway components, auxin and different families of transcription factors as further described in the text

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

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 we identify and characterize a tomato family of 11 highly related Cytokinin Response Factor genes designated as *SlCRF1 to 11* (*Solanum lycopersicum Cytokinin Response Factors*). *Sl*CRFs are AP2/ERF transcription factors and generally orthologous to Arabidopsis CRF clade members (*At*CRFs). Some *Sl*CRF genes lack a direct Arabidopsis ortholog and one *Sl*CRF has a unique protein domain arrangement not seen in any other CRF protein. Expression analysis of *Sl*CRF1 to 11 revealed differential patterns and levels across plant tissues examined (leaf, stem, root and flower). Several *Sl*CRFs show induction by cytokinin to various degrees, similar to *At*CRFs. Additionally we show that some *Sl*CRFs can be regulated by other factors, including NaCl, ethylene, MeJA, and SA. Examination of *Sl*CRF proteins in transient Agrobacteria infiltration experiments indicates they can be nuclear localized *in planta*. Using a BiFC (Split-YFP) system we also show that *Sl*CRF proteins can interact to form homo and heterodimers. Overall this work indicates that some *Sl*CRFs resemble previously identified CRFs in terms of structure, expression, and cytokinin regulation. However, *Sl*CRFs 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.

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 signaling 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 multistep 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 (RR) localized in the nucleus. Type-B RR, 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 RR 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 factor genes induced by cytokinin from global expression analyses in Arabidopsis (Brenner et al., 2005; Hirose et al., 2007; Hoth et al., 2003; Kiba et al., 2005; Rashotte et al., 2003; 2006). CRFs appear to form a branch pathway of the cytokinin signaling 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 they may play important roles specific to land plants (Rashotte and Goertzen, 2010). Mutant analyses in Arabidopsis has 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 embryo-lethal 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/*Sl*CRF1 and TSI1, are linked to processes other than cytokinin regulation including disease resistance and stress responses (Gu et al., 2002; Park et al., 2001; Zhou et al., 1997). This study was conducted to completely identify and characterize all CRF genes in tomato *Solanum lycopersicum*, which we have designated as *Sl*CRFs. Eleven *Sl*CRF genes were identified through a combination of existing sequence comparison and RACE-PCR. Once *Sl*CRFs were identified, we examined their expression in different plant tissues, as well as regulation by cytokinin, salt, and other hormones. In addition, we determined the cellular localization of *Sl*CRFs *in planta* and the ability of *Sl*CRF proteins to form homo and heterodimers with each other. 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.

Material 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 150 µE, with a 26°C day(light), 22°C night (dark) temperature.

RNA isolation, cDNA synthesis, and expression analysis

Leaves, stems, flowers, and roots were harvested from 52 d old Micro-Tom plants, and immediately flash-frozen in liquid nitrogen. RNA was extracted using Qiagen RNeasy Kit according to the manufacturer’s instructions. 500 ng 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 RT-PCR. PCR conditions were initiated for 2 min at 95 °C, followed by cycles of 30 sec at 94°C, a 30 sec annealing step, a 35 extension at 72°C, and a 5 min final extension at 72 °C. RT-PCR was conducted for *Sl*CRF1-5, 11 and TIP41 over 29 cycles with a 56°C annealing temperature step and for *Sl*CRF6-10 over 35 cycles with a 54°C annealing temperature step. The *Sl*CRF specific primers used in the RT-PCR are as follows: *Sl*CRF1forward 5’-GGAAAATTCAGTTCCGGTGA-3’

*Sl*CRF1reverse 5’-AAAATTGGTAACGGCGTCAG-3’

*Sl*CRF2 forward 5’-TGCCGGTCCTAGAGTTGTAA-3’

*Sl*CRF2 reverse 5’-CAGTGGCTGCTCTGCTCTAT-3’

*Sl*CRF3 forward 5’-AATGATGCAGTCGAGGAACC-3’

*Sl*CRF3 reverse 5’-CCTGGTCTTCCCATTCTCAA-3’

*Sl*CRF4 forward5’-TGAATCCCTCTGTTCCAAGG-3’

*Sl*CRF4 reverse 5’-GTTTTGCCATTTCCACTGCT-3’

*Sl*CRF5 forward 5’-ACGATGACGACGAGAGGAAT-3’

*Sl*CRF5 reverse 5’-CTGACACCGCGAAACTTTTT-3’

*Sl*CRF6 forward 5’-GGTAATGGGAAGAAGCGAGTA-3’

*Sl*CRF6 reverse 5’- GAAGGAAACGTCTGTGGGTAAG-3’

*Sl*CRF7 forward 5’-GCTTCACGAAAATGAGGTTG-3’

*Sl*CRF7 reverse 5’- GGTTGATGGGGTCGATTTC-3’

*Sl*CRF8 forward 5’-CCACCAAGGATGAGCTAAAG-3’

*Sl*CRF8 reverse 5’- GTGGCACGGTGTTGATGG-3’

*Sl*CRF9 forward 5’- TGAGGAAATGGGGGAAATATG-3’

*Sl*CRF9 reverse 5’- TGTCATCAAAGCCTAGAAGTT-3’

*Sl*CRF10 forward 5’- TGATGATGAAGGGGT TGATGTA-3’

*Sl*CRF10 reverse 5’- TGCTGGAGATGTGTGTGAAGTA-3’

*Sl*CRF11 forward 5’- AAGTGCCTGAGTTGGCTATG-3’

*Sl*CRF11 reverse 5’- TCACCCTCGATCAGATAAAC-3’

All samples are compared to the control gene TIP41 (Expósito-Rodríguez et al., 2008).

*Sl*CRFs 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 to 40 cycles of 30 sec at 94°C, 45 sec at 57°C, and 40 sec 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: *Sl*CRF3 (29 cycles), *Sl*CRF1, 2, 4, 6, 10, 11 (30 cycles), *Sl*CRF5 (30 cycles for salt, 35 for other treatments), SlCRF7 (35 cycles for MeJA, 40 for other treatments), *Sl*CRF8, 9 (40 cycles). Primers used to examine *Sl*CRF3 to 5, TIP41 were as noted above, RT-PCR primers for *Sl*CRF1, 2, 6-11 are as follows:

*Sl*CRF1 forward 5'-AACGATGTCGCTTTGTCACC-3'
*Sl*CRF1 reverse 5'-GGGCAAAATCGTCAAAGTCA-3'
*Sl*CRF2 forward 5'- ATGCTGCCGGTCCTAGAGTT-3'
*Sl*CRF2 reverse 5'- GAGCAGTTTCCGACGATGAC-3'
*Sl*CRF6 forward 5'-AGATGAGCTTTTTGGGCGTA-3'
*Sl*CRF6 reverse 5'-TCGCTTCTTCCCATTACCAC-3'
*Sl*CRF7 forward 5'-ACGTTGGTTGGGAAGTTTTG-3'
*Sl*CRF7 reverse 5'-TAATGGTTGATGGGGTCGAT-3'
*Sl*CRF8 forward5'-ACGTTGGTTGGGAACTTTTG-3'
*Sl*CRF8 reverse 5'-GTGTTGATGGGGTTGATTCC-3'
*Sl*CRF9 forward 5'- GCGTTGCCTAAAGGAGTTAG -3'
*Sl*CRF9 reverse 5'-ACCAGGGCTCAAATTCTTAC -3'
*Sl*CRF10 forward 5'- CTCAGAGTTTGGTCTCACATAC -3'
*Sl*CRF10 reverse 5'- AACATGTCCATCTCCGTATC-3'
*Sl*CRF11 forward 5'- AAGTGCCTGAGTTGGCTATG-3'
*Sl*CRF11 reverse 5'- TCACCCTCGATCAGATAAAC-3'

For characterizing *Sl*CRF7 response to ethephon and *Sl*CRF8 response to MeJA, primers used are the same as those utilized for examining the expression in different organs as noted above.

For qRT-PCR analysis, total RNA was extracted from cytokinin or DMSO control treated leaves using the same reagents and protocol as described for RT-PCR. 500ng of total RNA was converted into cDNA with Qiagen qScript cDNA supermix. 2µL 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 same set of primers used for examining salt or hormone responses except *Sl*CRF1-2. Primers for *Sl*CRF1-2 are the same as used in the first RT-PCR experiment. Each reaction contains 9µL of SYBR-Green supermix, 2µL of cDNA template, 3 µL of 4µM primers, and 3 µL of sterile water. The qPCR program consists of one cycle at 95 °C, followed by 40 cycles of 15 sec at 95°C, 30 sec at 56°C, and 35 sec at 68°C. The relative expression data used in the figure represent means ± SE of two biological replicates. All samples are compared to the control gene TIP41 (Expósito-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 d old Micro-Tom plants, placed in water, and gently shaken for 2h prior to treatment. Then treatments or appropriate controls were added to shaking tissue for various times as indicated: 5µM cytokinin (N^6-benzyladenine, BA), 100µM MeJA (methyl jasmonate), 2mM SA (salicylic acid), each with the carrier solvent DMSO and 200mM NaCl and 1mM Ethephon (of which ethylene is a break down product) with the appropriate level water controls. After designated treatment times (1h or 3h) 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 *Sl*CRFs were originally identified by making use of existing sequence data from the four full length *Sl*CRFs (*Sl*CRF1, 3, 4, 5) that were previously known either through our 3’ RACE-PCR analysis of partial unigene constructs (*Sl*CRF3, 4, 5) or from an existing gene sequence for *Sl*CRF1, also known as PTI6. BLAST analysis of the tomato unigene collection and now fully sequenced tomato genome was conducted using these four *Sl*CRFs 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 Kazusa Full-length Tomato cDNA Database at http://www.pgb.kazusa.or.jp/kaftom. Searches were done primarily using conserved AP2/ERF or CRF specific domains regions of the known SlCRFs 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 *Sl*CRF gene sequences were found they were translated and aligned as proteins in CLC Sequence Viewer v6.5.1 using default parameters. A phylogenic cladogram was generated using Neighbor-Joining method via bootstrap analysis of full length aligned SlCRF proteins again in CLC Sequence Viewer v6.5.1 using default parameters. Arabidopsis genes examined in this paper are designated as follows: CRF9 (At1g49120), CRF10 (At1g68550), CRF11 (At3g25890), CRF12 (At1g25470); and were previously noted as B-clade members of the CRFs in Rashotte and Goertzen 2010, CRF9=CRF-B1, CRF10=CRF-B3, CRF11=CRF-B4, CRF12=CRF-B2.

Protein Examinations

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 *Sl*CRF1, 2, 3, 5 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 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-terminal and C-terminal parts of YFP gene respectively. These destination clones were later used to transform Micro-Tom protoplasts.

To examine cellular localization *in planta*, SlCRF1, 2, 5 were transferred, through LR reaction to 35S:*Sl*CRF:GFP constitutive expression destination vectors 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 d old plants and 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 that which was heated at 55^{o}C for 10 min, then cooled down to room temperature before adding 10 mM $CaCl_2$ and 1% BSA) under vacuum for 30 mins. Next, leaf strips were gently shaken for 4 hours or overnight at 40-60 rpm before increased shaking at 90-100 rpm for 10 mins 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 x g for 2min to pellet the protoplasts. Pelleted protoplasts were resuspended in 2ml of cold wash solution (0.6 M mannitol, 5 mM MES pH 5.7, 20 mM KCl, 10 mM $CaCl_2$) and spun again. After which, the pellet was resuspended in wash solution to obtain the final volume for electroporation and kept on ice until transformation. Electroporation of protoplast was performed as in Rashotte et al., 2006 and left undisturbed in the dark at RT overnight prior to microscopic observation.

Agrobacteria Infiltration and Transformation for in planta Examination of Cellular Location

Tobacco (*Nicotiana tabacum*) plants were grown under a long day 16h light 26^{o} C, 8h dark 22^{o} C cycle. Destination vectors used for transformation (*Sl*CRFs in pMDC84, as described above) were transformed into *Agrobacterium tumefaciens* (C58-C1) in a method similar to 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 $MgCl_2$, 10mM MES, 100µM acetosyringone) and left at room temperature for 3h similar to that of Liu et al., 2002. Agrobacteria was then infiltrated into the abaxial side of 14-21d old plant leaves using a needle-less 2ml syringe. Plants were then examined for transient transformation and GFP expression 48-72h after injection using epifluorescence microscopy as in Cutcliffe et al., 2011.

Epifluorescence Microscopy

Bimolecular fluorescence complementation and Agrobacteria infiltrated tobacco leaves were examined using a Nikon Eclipse 80*i* 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 to initially observe and identify nuclei in intact cells as a measure of the cells viability. A YFP filter that blocks both chlorophyll fluorescence and Hoechst 33342 fluorescence was used to examine localization of any split-YFP fusions that occur due to BiFC between proteins. Cytokinin (2µM 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 Agrobacteria infiltrated tobacco leaves. All photos were taken with a Qimaging Fast 1394 digital camera and presented as composite images using Adobe Photoshop CS3 without altering the original integrity of the picture.

Results

Identification of novel Tomato CRFs (SlCRFs)

We have identified and characterized a family of eleven Cytokinin Response Factor (CRF) genes from tomato, known as *Solanum lycopersicum Cytokinin Response Factors* or *SlCRF1 to 11* (Fig 6; Table 4) 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 CRFs (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 Arabidopsis CRF sequences, and 3'RACE of incomplete EST unigene builds of SlCRFs. Previous work identified transcription of four *Sl*CRF sequences (*Sl*CRF1, 3 to 5), including the existing PTI6 gene, that we have also designated as *Sl*CRF1 (Rashotte and Goertzen, 2010). From this base we have identified ten novel full length expressed CRF genes (*Sl*CRF2 to 11), comprising all proteins in tomato containing a CRF domain, a defining characteristic of CRF proteins (Fig 6; Table 4). 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 BAC contigs have verified the determined sequence we identified from 3'RACE experiments. Full length transcripts for *Sl*CRF1 to 11 are presented (Table S1). *Sl*CRFs at a protein level fall into three classifications (Fig 6A). One is a standard CRF protein (*Sl*CRF1, 2, 4 to 6, 9 to 11), which contains both a CRF and AP2 DNA-binding domain in addition to a putative MAPK phosphorylation motif, as seen in a wide range of plant species (Rashotte and Goertzen, 2010). The second is a shortened CRF protein (*Sl*CRF7, 8), 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 like Arabidopsis (CRF7, 8). The final classification is a unique CRF protein (*Sl*CRF3), 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 over 250 identified CRF proteins examined across all land plants. Interestingly its chromosomal position is very close to the highly related *Sl*CRF8, only 9125 base pairs 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 6B), 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 phylogenic analyses in this study the full length *Sl*CRF3 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 *Sl*CRFs have a paired relation suggesting an ancient duplication as well as most *Sl*CRFs have an Arabidopsis ortholog (Fig 6C; 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 (*Sl*CRF2 with CRF1 and CRF2; *Sl*CRF5 with CRF5 and CRF6). Additionally, *Sl*CRF1 has no orthologous Arabidopsis gene partner (Fig 6D), although it is part of a related subclade of CRF proteins found in a number of other species (Rashotte and Goertzen, 2010).

SlCRFs Are Expressed in Different Plant Tissues

Previous work identified four *Sl*CRFs (*Sl*CRF 1, 3 to 5) as expressed in leaf tissues (Rashotte and Goertzen, 2010). Here we show that *Sl*CRF1 to 11 are expressed in multiple different plant tissues throughout the plant (leaf, stem, root, and flowers) to varying degrees (Figure 7). Generally, SlCRF expression levels were consistent across plant tissues examined. However, some genes showed preferential tissue expression, as seen for roots in *Sl*CRF4, 5 and for stems in *Sl*CRF8, 11 (Fig 7).

SlCRF Transcript Levels Are Regulated by Cytokinin and Salt

Knowing that several CRFs in Arabidopsis have previously been shown as induced by cytokinin we examined the regulation of SlCRF genes by cytokinin. Tomato leaves (15-d old) were treated with cytokinin (5µM BA) or DMSO as a vehicle control for 1h and 3 h and examined using real-time PCR. We found three *Sl*CRFs (*Sl*CRF2, 3, 5) that are strongly (4 to 6 fold) induced by cytokinin (Fig 8A). *Sl*CRF2 showed rapid induction by cytokinin at 1h after treatment to 6 fold over untreated levels and by 3h was still induced, although at this point only about 3.5 fold over control levels. Both *Sl*CRF3 and 5 showed no induction at 1h, but were highly induced (4 to 5 fold) after 3h of cytokinin treatment. A few other *Sl*CRFs showed weaker levels (1.5 to 2 fold) of induction at 3h of cytokinin treatment (*Sl*CRF1, 6, 7, 8, 9), whereas *Sl*CRF4, 10, 11 showed no change in expression (Fig 8A). The results follow a pattern similar to that seen for

Arabidopsis CRFs (*At*CRFs) that some, but not all members of this group are transcriptionally regulated by cytokinin (Rashotte et al., 2006).

We also examined *Sl*CRFs for changes in response to salt and other hormones in leaves treated at 1h and 3h vs. controls using RT-PCR. The results revealed expression changes in several genes although many showed little to no alterations (Fig 8). Expression analysis of salt treatment (200mM NaCl) revealed induction of *Sl*CRF1, 4, 6 at both 1h and 3h as well as a minor induction of *Sl*CRF2, 5 and 7 at 3h (Fig 8B). This suggests a new potential role of *Sl*CRFs in stress regulation. Expression analysis of ethylene treatment (1mM Ethephon) showed some induction of *Sl*CRF1 and 4 at both 1h and 3h, while *Sl*CRF2 was repressed at both 1h and 3h and *Sl*CRF7 at 1h (Fig 8C). This is some of the first data linking any CRF to ethylene. Expression analysis of methyl jasmonate treatment (100µM MeJA) showed only a single transcript change, the repression of *Sl*CRF6 at both 1h and 3h (Fig 8D). Expression analysis of salicylic acid treatment (2mM SA) revealed induction of *Sl*CRF1 at 3h as well as induction of *Sl*CRF4, 8 at both 1h and 3h (Fig 8E). Together these results suggest that *Sl*CRFs can be regulated by factors other than cytokinin.

SlCRF Proteins Show Nuclear Localization in planta

We examined the cellular localization of specific *Sl*CRF proteins (*Sl*CRF1, 2, 5) by transiently expressing GFP tagged SlCRF proteins in tobacco leaves via an Agrobacteria infiltration method (Fig 9A). Leaves infiltrated with 35S:*Sl*CRF:GFP vectors were examined for expression after 48h. Each of the *Sl*CRF proteins examined was found localized in the nucleus of leaf mesophyll cells and not other organelles in regions adjacent to infiltration sites as compared to empty transformed vectors (EV) or WT untransformed plants (Fig 9A). Although localization of *Sl*CRFs can be seen in the nucleus of cells, it is not obviously absent from the cytoplasm, which is consistent with previous models of *At*CRFs that appear to move between the cytoplasm and nucleus. This is also in agreement with the cellular localization of *Sl*CRFs 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.

*Sl*CRF 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 *Sl*CRFs we examined potential interactions using Bimolecular Fluorescence Complementation (BiFC) analysis split YFP system. *Sl*CRF proteins (*Sl*CRF1, 2, 3, 5) 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 9B). We found that homodimers formed between all *Sl*CRFs examined. In addition, heterodimers could also form with all *Sl*CRF combinations examined (Fig 9). 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 *At*CRFs and suggest that because there is a pattern for potential of all *Sl*CRF proteins to interact that regulation of *Sl*CRFs 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 cytokinin response factor (CRF) genes in a crop species, tomato (*Solanum lycopersicum*). Eleven *Sl*CRF genes (*Sl*CRF1 to 11) were identified in this study as part of a larger group of CRF genes present in all land plants (Rashotte and Goertzen, 2010). *Sl*CRF proteins contain the hallmark domains of this group; a CRF and AP2-DNA binding domain, as well as a putative MAPK motif found in many other CRF proteins

(Fig 6; Rashotte and Goertzen, 2010). One *Sl*CRF, *Sl*CRF3, was found to have a unique protein structure containing two CRF and two AP2 domains (Fig 6; Table S1). While several AP2/ERF proteins contain two AP2 domains, including the founding member of this group, *Sl*CRF3 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 *Sl*CRFs proteins.

A phylogenetic analysis of *Sl*CRFs shows relationships similar to that seen for Arabidopsis CRFs (*At*CRFs) 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 *Sl*CRF gene, where there are two paralogs in Arabidopsis, such as *Sl*CRF5 compared to *At*CRF5 and *At*CRF6 (Fig 6D). Another difference is that *Sl*CRF1 has no direct Arabidopsis ortholog. In fact most plant species appear to have a *Sl*CRF1 ortholog, indicating that the condition in tomato is more common (Rashotte and Goertzen, 2010). It also suggests that the function of *Sl*CRF1 is unlikely to be simply determined through studies of CRFs in Arabidopsis.

Expression of *Sl*CRF1 to 11 in tissues from roots to flowers suggests a broad role for these genes in the plant (Fig 7). There also appears to be a range of transcript levels of *Sl*CRFs 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 *At*CRFs reveal a pattern of expression across most tissue types and development, not unlike that seen for the *Sl*CRFs in this study suggesting that CRFs in most plants are likely to be expressed broadly across tissues (data not shown).

Several *Sl*CRFs were found to be induced by cytokinin, mirroring a pattern seen in Arabidopsis where only some CRFs show strong induction by cytokinin (Rashotte et al., 2006). Interestingly these *At*CRF genes parallel the *Sl*CRFs strongly induced in this study. *Sl*CRF2, highly similar to *At*CRF2, shows the most rapid induction of tomato CRFs comparable to very rapid induction of *At*CRF2 (Fig 8A; Rashotte et al., 2006). *Sl*CRF5, similar to both *At*CRF5 and

*At*CRF6, is also highly induced by cytokinin (Fig 1D; 3A; Rashotte et al., 2006). *Sl*CRF5 is not as rapidly induced as *Sl*CRF2, which parallels the slower cytokinin induction of *At*CRF6 compared to other CRFs (Rashotte et al., 2006). *Sl*CRF3 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 *Sl*CRF5. The lack of cytokinin regulation of some highly related pairing of *Sl*CRFs also parallels expression studies of other *At*CRFs, such as *Sl*CRF4 and 6 compared to *At*CRF3 and 4. Overall the pattern of transcriptional cytokinin regulation of *Sl*CRFs is similar to *At*CRFs and suggests that there may be similar regulation within specific clades of CRF genes.

We examined other factors that might transcriptionally affect *Sl*CRFs as they had been shown to affect related ERF family members: salt, ethylene, MeJA, and SA (Gu et al., 2000; 2002; Park et al., 2001; Sakuma et al., 2002; Nakuma et al., 2006; Zarei et al., 2011). Treatment with salt (NaCl) induced about half of the *Sl*CRFs to some degree (Fig 8B), revealing that CRFs can be induced by abiotic factors. An investigation of related *At*CRFs (*At*CRF2, 5, 6) also indicated induction by NaCl treatment from an examination of publically available microarray data. Previous examinations of the Tobacco stress induced 1 (Tsi1) gene (a CRF member) has shown transcript induction during high salt stress in both overexpressing and RNAi transgenic plants (Park et al., 2001; Han et al., 2006). Our finding that several *Sl*CRFs 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 *Sl*CRFs from some induction, to repression, with little effect on the majority of *Sl*CRFs (Fig 8C). Previous studies have shown that ethylene had little to no effect on *At*CRFs and *Sl*CRF1/Pti6 consistent with most *Sl*CRFs in this study. The exception, *Sl*CRF2 transcript repression, indicates that ethylene may play some role in *Sl*CRF function, although a more detailed study is needed to further determine the extent. Methyl jasmonate (MeJA) treatment showed almost no effect on any *Sl*CRFs suggesting that it plays little role in

CRF function, although specific CRFs such as *Sl*CRF6 may be exceptions (Fig 8D). Salicylic acid (SA) treatment resulted in minor induction of three *Sl*CRFs similar to MeJA treatments, indicating that SA also appears to have little effect on the transcription of most *Sl*CRFs. Together these results suggest that *Sl*CRFs can be regulated by factors other than cytokinin and may fall into different groups of regulated genes: some (*Sl*CRF3 and 5) regulated primarily by cytokinin, others (*Sl*CRF1, 2, 4, 6, 7, 8) regulated by several factors, and some (*Sl*CRF9-11) show little response to factors examined in this study. A broader examination of *Sl*CRF expression patterns, beyond this study is needed to determine the functional role of each *Sl*CRF.

Previous examinations of non-Arabidopsis CRF genes have shown links to pathogen response when overexpressed for Pti6 from Tomato (*Sl*CRF1) and Tsi1 from Tobacco (Zhou et al., 1997; Park et al., 2001; Gu et al., 2002). While we did not examine pathogen response in this study our finding that *Sl*CRF1 is induced by factors ethylene and salicylic acid linked to this process, and supports this previous reported role for *Sl*CRF1 (Zhou et al., 1997; Gu et al., 2002). Our finding that several other *Sl*CRFs are affected by these similar treatments may suggest that an effect on pathogen response could be a broader functional characteristic of some *Sl*CRFs.

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. *At*CRFs were previously shown in protoplasts 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 we transiently expressed GFP tagged *Sl*CRF proteins in tobacco leaves where we found *Sl*CRFs to be primarily nuclear localized in the absence of exogenous cytokinin, although we cannot rule out some cytoplasmic localization as well (Fig 9A). *Sl*CRF localization to both the nucleus and cytoplasm would be consistent with previous results of Arabidopsis CRFs and with protein localization prediction data for *Sl*CRFs (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 *At*CRFs (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). Although 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 signaling (Punwani et al., 2010). The identification of *Sl*CRFs 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 *Sl*CRF to the nucleus. We contend 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 our findings 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). We found that each of the *Sl*CRFs examined were able to form both homodimers and heterodimers with the other *Sl*CRFs, suggesting that *Sl*CRFs are unlikely to be regulated at this level. Although not all *Sl*CRFs were examined in this study, the result of the representative *Sl*CRFs examined here are consistent with a larger study of protein-protein interactions among *At*CRFs, showing widespread homo and heterodimerization and indicating that the CRF domain itself is likely involved in this interaction (Cutcliffe et al., 2011). Interestingly, the presence of an additional CRF and AP2 DNA-binding domain in *Sl*CRF3 does not appear to affect these interactions.

In summary, this work identifies and characterizes 11 cytokinin response factors in tomato (*Sl*CRF1 to 11). We show that *Sl*CRF1 to 11 are expressed at varying levels over a range of tissues. *Sl*CRF proteins appear to show nuclear localization and can interact to form homo and heterodimers amongst themselves. Several *Sl*CRFs show strong induction by cytokinin similar to that previously noted for Arabidopsis CRFs. Additionally, some *Sl*CRFs 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 *Sl*CRFs appear to have multiple regulatory functions in tomato plants.

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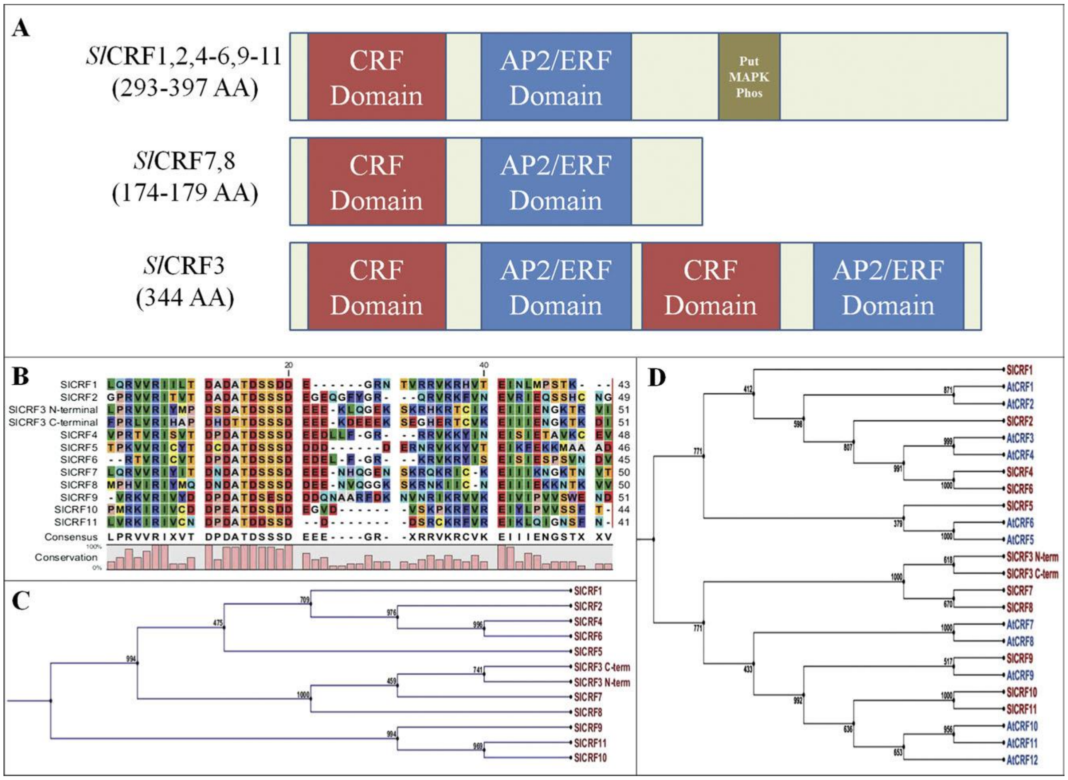


Fig. 6 SlCRF protein form, alignment, and phylogenic relationships. (A) A model of SlCRF protein form including size, domains, and motifs for all 11 SlCRFs. (B) Protein sequence alignment of the CRF domain for SlCRF1– SlCRF11 is shown with a sequence consensus, including both SlCRF3 CRF domains. (C) Neighbor–Joining tree of SlCRF proteins based on alignment of the CRF domain with support values shown out of 1000 bootstrap replicates. (D) Neighbor–Joining tree of SlCRF and Arabidopsis CRF (AtCRF) proteins based on alignment of both the CRF and AP2 DNA-binding domains with support values shown out of 1000 bootstrap replicates.

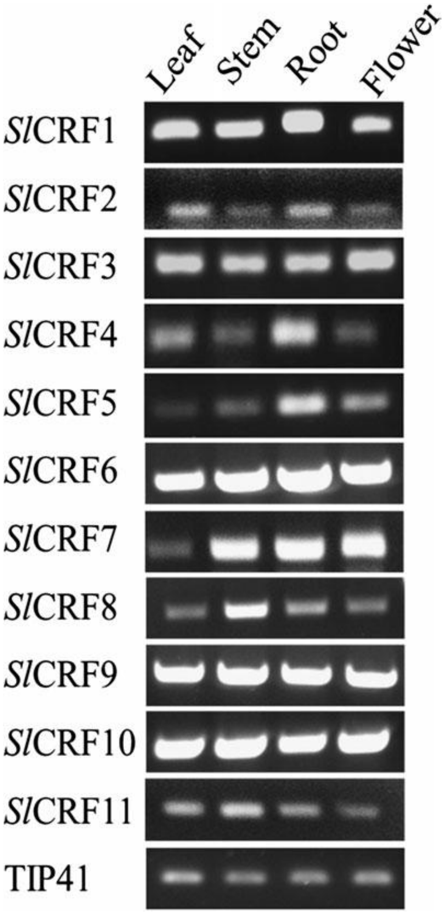


Fig. 7 SlCRF expression patterns in various tomato tissues. RT-PCR analysis of SlCRF1–SlCRF11 in leaf, stem, root, and flower tissues of 52-day-old plants is shown. The TIP41 gene serves as an internal control.

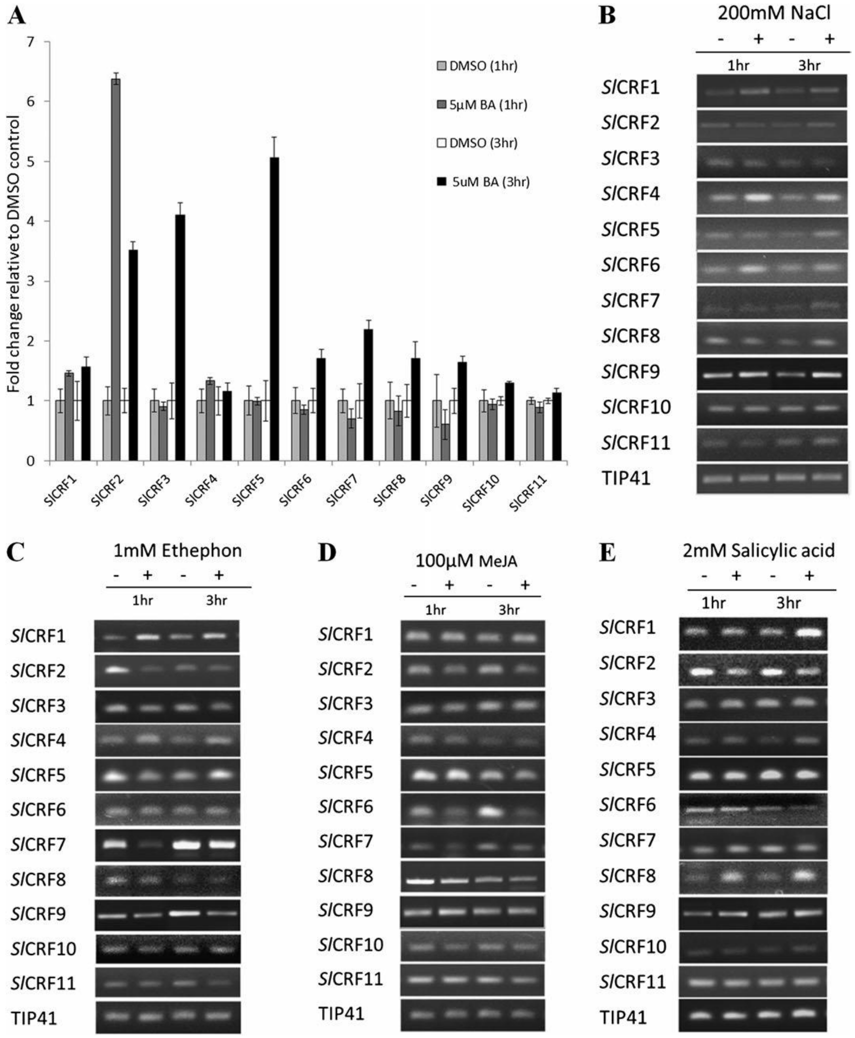
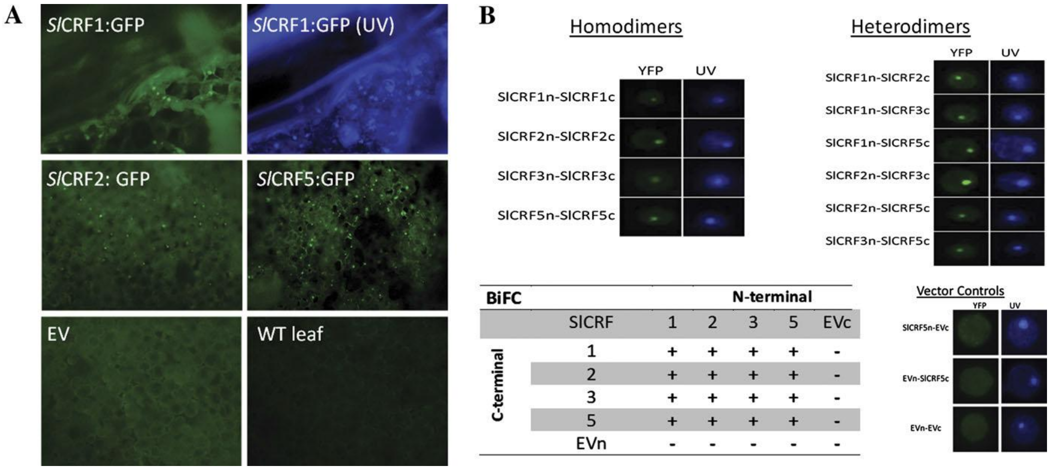


Fig. 8 Expression response of SlCRF genes to hormones and salt. Relative expression in 15-day-old leaves of SlCRF1–SlCRF11 in response to hormone or salt treatment at 1 h and 3 h after treatment versus non-treated controls. (A) qRT-PCR of cytokinin (5 µM BA) treatment. Data presented are a mean6SE (two biological replicates). Light grey bar, 1 h DMSO control; dark grey bar, 1 h BA treatment; white bar, 3 h DMSO control; black bar, 3 h BA treatment. (B) RT-PCR of salt (200 mM NaCl) treatment. (C) RT-PCR of ethylene (1 mM Ethephon) treatment. (D) RT-PCR of methyl jasmonate (100 lM MeJA) treatment. (E) RT-PCR of salicylic acid (2 mM SA) treatment. Data presented for RT-PCR are from a representative sample of experiments, with the TIP41 gene serving as an internal control.



BiFC		**N-terminal**				
	SlCRF	1	2	3	5	EVc
C-terminal	1	+	+	+	+	-
	2	+	+	+	+	-
	3	+	+	+	+	-
	5	+	+	+	+	-
	EVn	-	-	-	-	-

Fig. 9 SlCRF protein localization and protein–protein interactions. (A) Cellular localization of SlCRF1, SlCRF2, and SlCRF5 in tobacco leaves transiently transformed with 35S:SlCRF:GFP vectors via Agrobacterium infiltration. Representative examples of GFP expression from tagged SlCRF proteins indicate a strong nuclear localization in regions of transformed leaves visualized under UV light using a GFP wavelength filter (panels labelled SlCRF:GFP).The panel labelled SlCRF1:GFP (UV) is the same sample as SlCRF1:GFP shown without the GFP filter in the presence of Hoechst 33342 dye denoting the nucleus. EV denotes an empty vector control and WT leaf denotes an untransformed sample. (B) SlCRF proteins (SlCRF1, SlCRF2, SlCRF3, and SlCRF5) were analysed for potential homo- and heterodimerization using BiFC. Representative examples of positive SlCRF dimerizations are shown both under UV light in the presence of Hoechst 33342 dye denoting the nucleus and using a YFP wavelength filter to visualize BiFC interaction. Additionally, representative examples of empty vector (EV) controls for both N- and C-terminal BiFC vectors (EVn and EVc) are shown. A table of SlCRF interactions is shown, with (+) as positive and (–) for non-interactions.

Table 4 SlCRF gene description

Gene name	Chromosome/position (Build 2.40)	Gene model	Size (aminoacids/bp)
SlCRF1/PTI6	Ch 6 (44654446–44653700)	Solyc06g082590	248/747
SlCRF2	Ch 8 (62045738–62046757)	Solyc08g081960	340/1023
SlCRF3	Ch 1 (2911579–2910313)	Solyc01g008890	344/1035
SlCRF4	Ch 3 (2016125–2014935)	Solyc03g007460	396/1191
SlCRF5	Ch 1 (78502891–78503773)	Solyc01g095500	293/882
SlCRF6	Ch 6 (32043471–32044523)	Solyc06g051840	350/1053
SlCRF7	Ch 1 (14595809–14596333)	Solyc01g014720	174/525
SlCRF8	Ch 1 (2901188–2900649)	Solyc01g008880	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

Chapter V. Expression patterns and regulation of *SlCRF3* and *SlCRF5* in response to cytokinin and abiotic stresses in tomato

Abstract

Cytokinin is an influential hormone involved in numerous aspects of plant growth and development. A group of transcription factors- cytokinin response factors (*CRF*s) has been included as a side branch to cytokinin signaling pathway which also constitute a subset of the AP2/ERF family of transcription factor proteins. This study examined the expression patterns of two transcription factor genes *SlCRF3* and *SlCRF5* in tomato to determine their regulation in response to cytokinin and a variety of abiotic stress conditions. Analyses conducted during different developmental stages by RT-PCR or GUS reporter gene expression revealed that these genes are differentially expressed in vegetative and reproductive organs. qRT-PCR experiments were also performed to study regulation by the hormone cytokinin and abiotic stress conditions such as flooding, drought, osmotic, oxidative, and temperature. These showed that *SlCRF3* and *SlCRF5* have different patterns of regulation in leaf, stem, and roots with *SlCRF5* showing greater induction in leaf or root tissue compared to *SlCRF3* in most cases. Additionally, knockdown analysis for *SlCRF5* revealed defects across development including leaf morphology, primary root growth, and lateral root formation. Together, these findings indicate that *SlCRF3* and *SlCRF5* are potential regulators of tomato developmental processes associated with cytokinin or abiotic stresses.

Introduction:

The cytokinin response factors (CRFs) are a subset of the APETALA2/Ethylene Response Factor (AP2/ERF) family of transcription factors found in all land plants. These genes were originally identified in Arabidopsis microarray experiments of cytokinin response as transcription factor family members that were highly induced at multiple time points after exogenous application of cytokinin (Rashotte et al., 2003). Recent works have revealed the presence of 12 *CRF*s in Arabidopsis (*AtCRF*s) and also similar numbers in other plant genomes including 11 (*SlCRF*s) in tomato (Rashotte and Goertzen, 2010; Cutcliffe et al., 2011; Shi et al., 2012). Much of the study of CRFs has focused on the Arabidopsis system (Rashotte et al., 2006; Cutcliffe et al., 2011), although initial examinations of CRFs in tomato has shown that some *SlCRF*s are induced by cytokinin in leaves and that one *SlCRF* gene is expressed in the vasculature of various organs similar to *AtCRF*s (Shi et al., 2012; Zwack et al., 2012). One goal of this study was to conduct a more thorough examination of expression patterns and cytokinin induction for the two *SlCRF* genes, *SlCRF3* and *SlCRF5* in different organs over different stages of tomato development.

While it is clear that a subset of CRFs is cytokinin regulated, little information is known of the roles of *CRF*s in processes other than cytokinin regulation in any plant species. The genes that have been studied in this way in some manner include *PTI6*/*SlCRF1*, other *SlCRF*s *2-11*, and tobacco stress-induced 1 (*TSI1*) gene (a CRF member), which are linked to disease resistance and some stress responses (Zhou et al., 1997; Park et al., 2001; Gu et al., 2002; Shi et al., 2012). Arabidopsis CRF expression data from abiotic stress microarray experiments (Winter et al., 2007) or qPCR/GUS analysis also revealed that several *AtCRF*s appear to be abiotic stress responsive (Zwack et al., 2013; Compton 2012). Abiotic stresses can be highly detrimental to plant growth and can greatly reduce crop yields in plants, including tomato (reviewed in Pandey et al., 2011; Qin et al., 2011; Duque et al., 2013). These stresses cause damage at tissue and cellular levels that can rupture membranes, breakdown photosynthetic machinery components, as well as result in cell death. As such, the regulation of these stresses to generate stress resistant plants is of great importance to plant growers. Cytokinin has been strongly linked to abiotic stress responses in a number of studies examining cytokinin signaling, metabolism and biosynthesis where changes in cytokinin levels, responsive genes, or receptors dramatically altered plant growth under stress conditions (reviewed in Peleg and Blumwald, 2011; Wilkinson et al., 2012; Nishiyama et al., 2011). Since some *SlCRF*s are previously known to be highly induced in response to cytokinin treatment (Shi et al., 2012), we conducted here additional study focusing on abiotic stress responses of these genes to provide insight into the link between cytokinin regulated transcription factors and abiotic stress conditions.

Analysis of loss-of-function CRF mutations in Arabidopsis revealed that the CRFs function redundantly to regulate the development of embryos, cotyledons, and leaves (Rashotte et al., 2006). In order to investigate potentially similar development effects of reduced CRF levels during tomato development, we generate antisense transgenic plants with decreased expression levels of *SlCRF5*. Phenotypic analysis revealed a potentially critical role for *SlCRF5* in overall tomato development. Together, this study presents baseline information on the regulation of *SlCRF3* and *SlCRF5* genes relevant for future research focused on the study of tomato development.

Materials and Methods:

Plant materials and Growth conditions

The tomato dwarf cultivar Micro-Tom plants were grown in Sunshine Mix #8 soil under a 16:8 h light: dark photoperiod at 150 µE, with a 26° C day (light), 22° C night (dark) temperature in controlled-environment chambers. Sterilized seeds were germinated in magenta boxes containing 0.8% agar gel with MS salts (4.8g/L), added Gamborg B5 Vitamins, and 2% sucrose. The pH of the medium was adjusted to a final value of 5.7.

Arabidopsis thaliana (Col-0) plants were germinated on Petri dishes containing 0.8% agar gel with MS salts (4.8g/L) and 1 % sucrose medium of 5.7 pH. Plants were grown under a light:

dark photoperiod at 100 µE, with 22° C day (light), 18° C night (dark) temperature in controlled-environment chambers.

Generation of transgenic plants

Plasmids for expression analysis and antisense were generated using the Invitrogen GATEWAY™ cloning system according to the manufacturer's instructions. For expression analysis, the promoter regions (~2kb upstream of ATG) of *SlCRF3* and *SlCRF5* were amplified from the genomic DNA and cloned into the destination vector pKGWFS7 as described in Zwack et al. (2012). To generate the antisense construct, coding sequence of *SlCRF5* was cloned in the destination vector pK2WG7. All vectors were sent to the Plant Transformation Research Center (PTRC) at the University of California Riverside for transformation of tomato plants. For expression analysis in Arabidopsis, *Agrobacterium tumefaciens* C58 cells were transformed with destination vector pKGWFS7 harboring *SlCRF5* promoter via electroporation, and plants were transformed using the floral dip method (Clough and Bent, 1998). For all transgenics, three independently selected homozygous lines were used for analyses.

Histochemical analysis

GUS activity was analyzed in tomato and Arabidopsis organs of transgenic lines. Tissues and organs from these lines of different developmental stages were vacuum infiltrated for 20-30 minutes with X-gluc buffer (Weigel and Glazebrook 2002) before incubation at 37° for 2- 4 h for Arabidopsis and overnight for tomato followed by clearing of tissues in 70% ethanol at room temperature. Whole tissues or free hand sections were then examined using a Nikon Eclipse 80i microscope and photos were taken with a Qimaging Fast 1394 digital camera.

Cytokinin treatment

Tomato plants were grown in soil as described above, from which leaves and stems of 15, 25, and 35 d old plants were excised, placed in water, and gently shaken for 2 h prior to treatment. Cytokinin (N^6 -benzyladenine; BA) of different concentrations (see text) or the vehicle control DMSO were then added to shaking tissues for various times (see text). For treatment of roots, plants were grown hydroponically in CYG germination pouches from Mega International. Seedlings at 14 DAS (days after sowing) were treated for 24 h by directly adding cytokinin or control DMSO to the growth pouches. After treatment roots (Root tips (RT) - encompassing the meristem and the elongation zone; Lateral roots (LR), and whole roots (WR, including RT and LR) were removed from solutions or germination pouches, patted dry with paper towel and immediately flash-frozen in liquid nitrogen (N_2), and stored at -80° C until RNA extraction. Pro_{SlCRF5}*:GUS* homozygous plants (both tomato and Arabidopsis (grown on agar plates)) were also treated with cytokinin in the same manner as described above.

Stress treatments

Water stress

For flooding stress treatment, 25 d old plants grown under standard conditions in soil were placed in trays to maintain water logged conditions for 1, 4, and 7 d. For drought experiments, 25 d well watered plants were left unwatered for 7 d and rewatered to examine recover from drought conditions. Leaf, stem and root samples were collected after 7 d of wilting, and 1, 3, 6, 12 h after rewatering and compared to control plants grown under standard conditions. Root treatments were performed in CYG germination pouches.

Mannitol, H_2O_2, and ABA treatment

25-d old soil grown plants were treated with 200mM mannitol, 10 and 20 mM H_2O_2, and 50 and 100 µM ABA for 3 h and leaf, stem, and root tissues were collected and immediately flash-frozen in liquid N_2.

Temperature

Plants were grown in magenta boxes and treatments were performed at 25 d age. For cold treatment, magenta boxes were kept in 4° C for 24 h, and heat treatment was performed by moving the plants in magenta boxes from 26° C to a 45° C water bath for 1 h, after which leaves, stems, and roots were collected and immediately flash-frozen in liquid N_2.

RNA isolation, cDNA synthesis, and expression analysis

For expression analysis, tissues were harvested from 15, 25, and 35 d old plants and immediately flash-frozen in liquid N_2. Total RNA was extracted from these samples and other treated plants as described in Shi et al., (2012). For RT-PCR or qRT-PCR analysis, 500 ng of total RNA was converted into cDNA with Quanta qScript cDNA supermix and diluted 20X before use in PCR reactions. *SlCRF3* and *SlCRF5* expression analysis in various organs by RT-PCR was started with a one-step cycle of 2 min at 95° C, followed by 29 cycles of 30 sec at 94° C, 45 sec at 56° C, and 50 sec at 72° C, and a 5 min final extension at 72° C, using gene specific primer as mentioned in Shi et al., (2012).

qPCR was performed using cytokinin or stress treated cDNA samples with SYBR-Green chemistry in an Eppendorf Mastercycler ep realplex using primers as in the RT-PCR. Each reaction contains 9 µL of SYBR-Green supermix, 2 µL of cDNA template, 1 µL of 6 µM forward and reverse primers, and 7 µL of sterile water. The qPCR program consists of 1 cycle at 95° C, followed by 40 cycles of 15 sec at 95° C, 45 sec at 56° C, and 50 sec at 68° C. The last step for each reaction was melting curve generation to test the amplicon specificity. All qPCR reactions were performed in two technical and two biological replicates and the relative expression data used in the figure represent means ± SE of two biological replicates. Samples were compared to the control gene *TIP41* (Expósito-Rodríguez et al., 2008) or *SleIF4A* gene specific primers (for stress experiments):

SleIF4A F 5’ GGCTCATCTCGTGTGCTTAT 3’
SleIF4A R 5’ CATCCTTTCATCATCCTTGG 3’

Results

SlCRF3 and *SlCRF5* show distinct expression patterns with overlapping specificity in vascular tissues

An initial broad examination of *SlCRF*s revealed varying levels of expression in leaf, stem, root, and flower (Shi et al., 2012). To better delineate the expression patterns of *SlCRF3* and *SlCRF5* across different stages of tomato development, RT-PCR was performed on: cotyledon, hypocotyl, leaves; 15 d (1 leaf), 25 d (1-4 leaves, 4th leaf being the oldest, 1st the youngest), 35 d (1-7 leaves, 7th leaf being the oldest, 1st the youngest), 15, 25, and 35 d old stems and roots (root tip (RT), lateral root (LR), and whole root (WR)).

For leaf and cotyledons, *SlCRF3* expression was found to increase in 15 d leaves compared to cotyledons followed by similar expression in 25 d leaves. In 35 d old leaves, *SlCRF3* expression was lower in young leaves, increasing with age as to the highest levels in the 6th and the 7th (oldest) leaves. In comparison, *SlCRF5* expression in cotyledons, 15 and 25 d old leaves was at lower and nearly consistent levels than 35 d leaves with the highest expression in leaf 7 (oldest) of these plants. In stems the expression of *SlCRF3* was highest in the oldest 35 d stems, whereas expression of *SlCRF5* was highest in the young hypocotyl tissue. *SlCRF3* and *SlCRF5* expression was also widely observed in the roots across root tips, lateral roots and whole roots of 15, 25, and 35 d old plants. *SlCRF3* expression appeared consistent in the roots, except for lower levels in root tips of 25 and 35 d old plants, whereas *SlCRF5* was relatively similar in the root over development.

Stably transformed homozygous pro$_{SlCRF}$::GUS (β-glucuronidase) reporter lines for *SlCRF3* and *SlCRF5* were analyzed for *in vivo* spatial and temporal expression patterns in tomato. Independent pro$_{SlCRF3}$::GUS lines showed expression in the emerging radicle during germination, seedling shoot apical meristem and vasculature in: 4, 7, and 9 d old seedlings, 15, 25, 35, 85 d old leaves, and fruit, as well as RT and LR and vasculature in the rest of the root (Fig. 11 A). Expression in pro$_{SlCRF5}$::GUS lines was observed in the embryo, emerging radicle during germination, 4 d old seedling, leaf primordia, RT and LR, flowers, as well as in vasculature of 7 and 9 d old seedlings, 15, 25 and 35 d old leaves, 35 d old stems, roots, and fruits (Fig. 11 B, a-i; l-v). In both *SlCRF3* and *SlCRF5*, the highest GUS expression was observed in 35 d plants compared to 15 and 25 d old leaf stages, consistent with RT-PCR analysis. Pro$_{SlCRF5}$ driven GUS expression was also observed in vasculature of cotyledons and leaves in Arabidopsis plants transformed with pro$_{SlCRF5}$::GUS cassette (Fig. 11 B, j-k). While pro$_{SlCRF5}$::GUS expression was generally the strongest in the vasculature especially in the phloem, its expression could also be observed in other tissues.

Cytokinin induction of *SlCRF3* is primarily in leaves, while *SlCRF5* is broadly induced in different plant organs

While a previous finding indicated a single point of cytokinin induction for *SlCRF3* and *SlCRF5* after 3 h of 5 µM BA cytokinin treatment in 15 d leaves (Shi et al., 2012), here we present a detailed regulation of these genes by a range of cytokinin concentrations, organs/tissues, and developmental stages (Fig. 12). The following were examined for *SlCRF3* and *SlCRF5* expression levels: leaves and stems from 15, 25, and 35 d plants were treated with cytokinin (1, 5, and 10 µM BA) or DMSO as a vehicle control for 3, 6, 12, and 24 h; roots (RT, LR, WR) from 15 d plants after treating 14 d seedlings with cytokinin (1, 5, and 10 µM BA) for 24 h.

SlCRF3 was primarily induced by cytokinin in leaves, with only minor expression changes in stems and roots (Fig. 12 A). The greatest induction of *SlCRF3* was ~4.5 fold by 5 µM BA at 3 h in 15 d old leaves, however, there was also 2-3 fold induction at 12 h in both 15 and 25 d old leaves, suggesting potential cytokinin regulatory roles of *SlCRF3* in leaves at these developmental stages. There were only slight inductions under other conditions in leaves stems and roots.

In contrast, *SlCRF5* was induced by cytokinin 2+ fold in at least one condition in leaves, stems and roots; although similar to *SlCRF3* the highest level of *SlCRF5* induction was ~4.8-fold by 5 µM BA at 3 h in 15 d leaf (Fig. 12 B). Induction of 2.0-2.5 fold was also seen in 25 and 35 d old leaf samples. In stems, *SlCRF5* appeared to be primarily induced at 25 d by 5 µM BA treatment for 3 h, over 2.5 fold. In roots, *SlCRF5* transcript levels were induced more than 2 fold in all tissues (RT, LR, and WR), with the highest induction in RT 2.8 fold by 5 µM BA (Fig. 12 B). To further confirm this wide range of cytokinin induction for *SlCRF5*, pro$_{SlCRF5}$::GUS tomato and Arabidopsis plants were analyzed after treatment with 5 µM BA at 3 h in 25 d plants (Fig. 13). GUS expression of these lines was greatly induced by cytokinin in stem tissue as seen for these similar conditions in transcript analysis and root tissues were also highly induced indicating that the cytokinin transcriptional induction seen in different parts of the root at 15 d continues at this later developmental stage (Fig. 12, 13). In leaves GUS expression was induced by cytokinin, strongly in the vasculature of both tomato and Arabidopsis (Fig. 13), which likely could not be detected from transcript analysis under the same conditions because that analysis was conducted on whole leaves containing a lot of non-vascular tissue (Fig. 12). Overall, these results indicate that both *SlCRF3* and *SlCRF5* expression levels are induced by cytokinin strongly in leaves of 15 and 25 d old plants, with *SlCRF5* also showing strong induction in stems and roots.

***SlCRF3* and *SlCRF5* are regulated by various abiotic stresses**

Flooding stress

In order to investigate the involvement of *SlCRF3* and *SlCRF5* in flooding stress, 25 d leaves, stems, and roots were exposed to water logged conditions for 1, 4, and 7 d. *SlCRF3* showed only slight ~1.5 fold increases in leaf transcript levels at 1 and 7 d, while stem and root levels were unchanged, suggesting that *SlCRF3* is unlikely to be involved in this stress response (Fig. 14 A). In contrast, expression of *SlCRF5* in leaves was induced 2+ fold during flooding stress treatment with a peak of ~5.5 fold at 4 d (Fig. 14 B). *SlCRF5* levels also showed some increase in response to flooding in stems, 2.7 fold at 7 d, and in roots, 1.5-2.0 fold at 1 and 7 d (Fig. 14 B). This suggests that *SlCRF5* is linked to flooding stress, as it is induced in different organs over 7 days of this stress, particularly strongly in leaves.

Drought stress and recovery

Drought stress and response during recovery were examined for *SlCRF3* and *SlCRF5* in plants left unwatered for 7 d and during the 12h after rewatering. *SlCRF5* was found to have a distinct pattern of regulation in different organs (Fig. 14 B). In leaves, *SlCRF5* was highly induced ~2.75 fold during drought, then repressed during the first 6 hours of recovery, returning to normal levels by 12 h. In contrast, *SlCRF5* root transcript was reduced during drought, remaining low during recovery until reaching 2.5+ fold induced levels at 12 h of recovery. *SlCRF5* expression in stems was unchanged during drought stress, but was reduced during 1 and 3 h recovery, reaching normal levels 6 h recovery.

SlCRF3 expression in leaves during drought stress and recovery showed a somewhat similar pattern to *SlCRF5*, with higher expression (1.5 fold) during drought followed by reduced levels until 12 h after recovery when *SlCRF3* levels returned to normal, although overall transcript levels were less effected (Fig. 14 A). In roots, *SlCRF3* expression was consistently reduced during drought as well as recovery (0.4-0.5), whereas in stems expression remained unchanged except for slightly reduced levels around 3 h of recovery (Fig. 14 A). Despite complex patterns of changing expression levels, these results indicate that *SlCRF5* is strongly regulated in leaves and roots during drought and recovery, with *SlCRF3* also involved although to a lesser degree.

Osmotic, oxidative, and ABA hormone stress

To examine *SlCRF3* and *SlCRF5* response to osmotic and oxidative stresses and regulation by the stress hormone ABA, 25 d old plants were treated for 3 h with 200 mM mannitol, 10-20 mM hydrogen peroxide (H_2O_2), and 50-100 µM ABA. Mannitol treatment showed little to no regulation of either *SlCRF3* or *SlCRF5* in leaves, stem or roots indicating that these genes are unlikely to be linked to osmotic stress (Fig. 14).

Both *SlCRF3* and *SlCRF5* were induced by H_2O_2 primarily in roots and to a lesser degree in stems, with similar levels at the different concentrations used. *SlCRF5* transcript levels increased 4 fold in roots and 2 fold in stems, while *SlCRF3* levels increased 2.25 fold in roots and 1.5 fold in stems (Fig. 14). No induction was observed for either *SlCRF3* or *SlCRF5* in the leaves. These findings indicate that these genes may be linked to oxidative stress processes in roots and stems.

In response to exogenous ABA treatment *SlCRF3* expression was mostly unaffected, showing only minor (1.6 fold) induction at 50 µM ABA in roots, with no induction at higher, 100 µM levels (Fig. 14 A). *SlCRF5* expression was unaffected in any tissue at 50 µM, but did show induction to 2.5 fold levels in roots at the higher ABA concentration (Fig. 14 B). This may suggest that high levels of ABA could be involved in the stress regulation of *SlCRF5* in leaves.

Temperature stress

In order to determine *SlCRF3* or *SlCRF5* regulation by cold or heat stress, 25 day old plants were exposed to 4° for 24 h and 45° for 1 h. During cold stress, *SlCRF3* was found to be slightly repressed in leaves (0.57) and stems (0.85) and unchanged in roots. Whereas *SlCRF5* expression was unaffected in leaves and stems, but induced ~2.4 fold in roots, indicating a possible role for *SlCRF5* in cold response in roots. Heat stress treatment resulted in little change in either *SlCRF3* or *SlCRF5* levels in the plant suggesting these genes are unlikely to be connected to this stress.

Reduced *SlCRF5* transcript level results in altered plant development

To gain insight into the role of *SlCRF5*, antisense transgenic plants were generated to determine the effect of reduced a *SlCRF5* level as the plant develops. Three independent homozygous antisense lines *SlCRF5* AS1-3, with reduced *SlCRF5* levels were examined for phenotypic changes from normal plant development (Fig. 15 A, B). Several distinct changes were seen in *SlCRF5* AS plants in different organs throughout development (Fig. 15). Early effects were seen in *SlCRF5* AS seedlings (12d) that showed a highly stunted primary root, a complete lack of lateral roots, and a late emergence of the first true leaves (Fig. 15 A, C, D). *SlCRF5* AS plants continued to have altered effects in roots at later developmental stages, as the primary root never elongates, nor do lateral roots ever emerge. However, plants do produce adventitious roots, which appear to take over as the main root structures (Fig. 15 B, C, D). Continued phenotypic analysis of soil grown *SlCRF5* AS plants over the rest of development revealed a number of aerial differences in *SlCRF5* AS plants including: smaller, poorly developed leaves with thin leaflet laminas and short petioles; thin stems; delayed flowering; and reduced numbers of flowers and fruits containing lower seed number (Fig. 15 E). Despite this number of altered morphological factors, some features such as overall plant height and leaf number remain unchanged. Generally, the organs and regions where *SlCRF5* AS plants have altered morphology correspond to *SlCRF5* transcript and GUS expression patterns. Together these results clearly indicate the importance of *SlCRF5* in tomato developmental stages from seedling through to seed production.

Discussion:

Cytokinin is involved in numerous plant growth and developmental processes, yet only a few cytokinin-regulated genes have been investigated in tomato. This study presents the detailed examination of the tomato CRF genes, *SlCRF3* and *SlCRF5* showing their endogenous expression patterns, regulation by hormones and abiotic stresses, as well as roles for *SlCRF5* in plant development.

Endogenous expression analysis was performed throughout the plant for *SlCRF3* and *SlCRF5* examining both transcript levels by RT-PCR and promoter::GUS reporter analysis. For *SlCRF3*, transcript levels were widely abundant and found to generally increase with age in leaves and stems and with higher levels in organs of mature 35 d old plants, whereas in roots levels were similar across developmental stages except for reduced levels in RT of older plants (Fig. 10). *SlCRF3* expression using pro*SlCRF3*::GUS transgenic lines was consistent with RT-PCR analysis, such as expression in roots, stems and leaves, while additional expression was seen in other tissues including the emerging radicle and fruit. Importantly strong GUS staining was found in vascular tissues in several organs over development, suggesting this is a primary site of *SlCRF3* expressed (Fig. 11). *SlCRF3* vascular expression is consistent with observations for other standard CRFs in Arabidopsis and may be a general feature of the CRF group (Zwack et al., 2012). It is worth noting since *SlCRF3* has a unique protein structure (with two alternating CRF and AP2 DNA-binding domains), which is atypical of other CRFs that contain only a single CRF and AP2 DNA-binding domain (Rashotte and Goertzen, 2010). Possibly because of this unique structure there is no direct ortholog of *SlCRF3* in Arabidopsis with which to compare expression, as *SlCRF3* does not fall into any of the standard clades that most CRFs are subdivided into (Zwack et al., 2012). Despite the differences of *SlCRF3* from other CRFs, it is clearly expressed strongly over a range of organs in the plant across development in many cases preferentially in vascular tissue.

For *SlCRF5*, RT-PCR transcript levels were also found to be widely present across all organs examined at different developmental stages. In leaves expression levels were higher older leaves of mature 35 d plants, whereas in stems levels were higher in younger plants and in the hypocotyl (Fig. 10). GUS expression driven by *SlCRF5* promoter was also found in various organs over development consistent with results from the RT-PCR transcript analysis, in addition to showing expression in the embryo, apical tissues, flowers, and fruit (Fig. 11). Interestingly, in leaves GUS expression became stronger as the plant matured, until being reduced in senescing leaves: a pattern similar to that seen in *AtCRF6*, an Arabidopsis ortholog of *SlCRF5* (Zwack et al., 2013). This could suggest that *SlCRF5* may function like *AtCRF6* as a negative regulator of senescence, although further study is still required to determine this. Tissue level examination of *SlCRF5* GUS stained material revealed vascular expression in a number of organs (leaves, roots, stems, fruit), which appears to be preferentially found in the phloem (Fig. 11), in a manner consistent with general tissue specific CRF patterns previous seen (Zwack et al., 2012). A possible reason for phloem-specific expression is the presence of phloem targeting *cis*-elements present in the *SlCRF5* promoter similar to other tomato and Arabidopsis CRFs (Zwack et al., 2012). Both the vascular and phloem expression of *SlCRF5* suggests a role for *SlCRF5* in phloem and cytokinin regulated processes such as sink/source regulation or senescence (Mok and Mok, 2001; Werner and Schmülling, 2009).

Cytokinin induction of CRFs is not universal and appears to be specific to distinct CRF clades (Zwack et al., 2012). However, this generalization is based primarily on examinations of cytokinin regulation of CRFs in Arabidopsis or from tomato leaves (Rashotte et al., 2003; Rashotte et al., 2006; Rashotte and Goertzen, 2010; Shi et al., 2012). Transcriptional regulation of *SlCRF3* and *SlCRF5* were studied here to generate a detailed profile of regulation by cytokinin over different developmental stages in leaves and stems and different regions in roots. Results confirm previous finding that both *SlCRF3* and *SlCRF5* are induced by cytokinin in young leaves and show that both genes reach maximum induction after 3h by 5µM BA in 15 d old leaves (Fig. 12; Shi et al., 2012) as well as revealing novel organ and stage regulation by cytokinin. *SlCRF3* showed additional cytokinin induction at a later point (12h) in both 15 and 25 d old leaves, also with possibly minor levels of induction in young stems. Roots and older stems showed little to no induction indicating that *SlCRF3* appears to be leaf specific in its cytokinin regulation. Again as *SlCRF3* does not have a direct ortholog in Arabidopsis or fall into any of the five CRF clades (Zwack et al., 2012), the role of this gene in cytokinin regulation or cytokinin regulated plant processes is difficult to predict, although it does seem to function in some cytokinin response.

In contrast, *SlCRF5* is strongly induced by cytokinin in both stems and roots as well as in leaves of 15, 25, and 35 d old leaves (Fig. 12). In stems there was moderate to strong cytokinin induction at 3 h and there was 2+ fold induction in each part of the root with different cytokinin concentrations. In leaves *SlCRF5* showed induction 2+ fold levels at both 3 h in 15 and 35 d old plants with levels approaching 2 fold in 25 d old plants, as well as at 12 h of each developmental stage, with lesser induction at 6 and 24 h that could suggest a bi-phasic regulation by cytokinin. Although *AtCRFs* have variable cytokinin induction levels over the first 24 h of treatment, including the orthologous clade III *AtCRF5* and *AtCRF6*, the pattern seen for *SlCRF5* here is distinct from other CRF genes (Rashotte et al., 2006).

Although there has been limited work examining the role of CRFs in stress response, these findings indicate that particular CRF genes can be regulated by biotic (Zhou et al., 1997; Gu et al., 2002) and abiotic stresses (Park et al., 2001; Shi et al., 2012, Zwack et al., 2013). These studies were not focused on either *SlCRF3* or *SlCRF5* and their only link to stress regulation is a minor induction by salt stress for *SlCRF5* (Shi et al., 2012). Here we found that both *SlCRF3* and *SlCRF5* were transcriptionally regulated by different abiotic stresses. *SlCRF3* transcript levels were largely unaffected by flooding, osmotic, and heat stress or by exogenous ABA and would appear not to be connected to these processes (Fig. 14A). *SlCRF3* did show a strong induction from oxidative stress in roots and a repression from cold stress in both leaves and stems. Expression levels were also affected during drought stress, although larger effects including a strong reduction in roots occurred during the 12 hours of the recovery period. This suggests that *SlCRF3* may be involved in these abiotic stresses or recovery from them to different degrees in a manner that is distinct at the organ level.

SlCRF5 also showed regulation by number of abiotic stresses in a pattern different from that of *SlCRF3* (Fig. 14). *SlCRF5* was induced by different types of water stresses: excess levels of flooding, in leaves, stems and roots over the entire period examined; and lack of water during drought stress in leaves, followed by a complex expression changes in different organs during recovery. This links *SlCRF5* to water stress responses. Similar to *SlCRF3*, *SlCRF5* expression was unaffected by osmotic and heat stress and while S*lCRF5* also was regulated by both oxidative and cold it was to different degrees: *SlCRF5* was induced to higher levels by oxidative stress in roots and stems; in contrast was induced by cold in roots. Interestingly *SlCRF5* also appeared to be induced in leaves at the highest levels of ABA, suggesting either a general stress link or a potential cytokinin-ABA antagonistic response (Tanaka et al., 2006; Polanská et al., 2007).

The induction of both *SlCRF3* and *SlCRF5* by H_2O_2 in roots could suggest an involvement with Reactive Oxygen Species (ROS) with previous links to controlling root meristem size (Tsukagoshi et al., 2010) and other aspects of root growth and development (Passardi et al., 2006; Cheng et al., 1995; Vernoux et al., 2000; Dunand et al., 2007). It is unclear what the differential regulation of *SlCRF3* and *SlCRF5* by cold stress means. However, a similar cold induction pattern as in *SlCRF5* has been seen for other CRFs, including in roots of *AtCRF3* and *AtCRF4* (Compton, 2012). Additionally, *SlCRF5* has been recently shown to be induced by cold in a microarray analysis examining both cold tolerant and -sensitive varieties, suggesting that *SlCRF5* is definitely linked to cold stress response (Liu et. al., 2012).

Further examination of the role *SlCRF5* plays over development was conducted in observing the effects of having reduced levels of *SlCRF5* revealing that in *SlCRF5* AS lines there were profound effects at multiple stages and in multiple organs in plant development (Fig. 15). Previous studies of CRF mutants linked them to roles in embryo, cotyledon, and leaf development, as well as vascular patterning and senescence (Rashotte et al., 2006; Zwack et al., 2012, 2013; Zwack and Rashotte, 2013). In *SlCRF5* AS lines one of the primary developmental alterations was in the root system that had a highly reduced primary root and a complete lack of lateral roots even after many weeks of growth. Eventually these plants produce adventitious roots that appear to take over the role of the major root and allow further plant growth. This phenotype is similar to phenotypes in multiple mutants of the cytokinin signaling pathway in Arabidopsis including the receptor AHKs and the phosphotransfer protein AHPs, suggesting that *SlCRF5* is likely an essential step in a similar cytokinin signaling pathway in tomato regulating root growth (reviewed in Gupta and Rashotte 2012). Additional developmental defects are found in aerial tissues, including reduced leaf size, flower, fruit, and seed number. The reduced fertility corresponds to previous finding of a double knockout *crf5,6* in Arabidopsis that is embryo lethal (Rashotte et al., 2006); as such the reduced seed number in *SlCRF5 AS* might be expected as *SlCRF5* is the tomato ortholog of both *AtCRF5* and *AtCRF6* (Zwack et al., 2012). Regions of the *SlCRF5* AS lines that show developmental defects largely match the GUS reporter expression patters for *SlCRF5*.

Conclusion:

In summary, a detailed examination of two genes from tomato-*SlCRF3* and *SlCRF5* with respect to their expression patterns, cytokinin regulation, abiotic stress response, and the role of *SlCRF5* was performed. Results revealed that *SlCRF3* and *SlCRF5* genes have distinct expression patterns in different organs during plant development. These genes also showed differential regulatory patterns by cytokinin over development and by abiotic stress suggesting a diverse role for *SlCRF*s in hormone regulation and in stress in plants. Additionally, *SlCRF5* antisense knockdown plants produced several defects suggesting its important role in development. Overall, these findings present basic information on the expression and regulation of *SlCRF3* and *SlCRF5* genes relevant for advanced research on tomato development.

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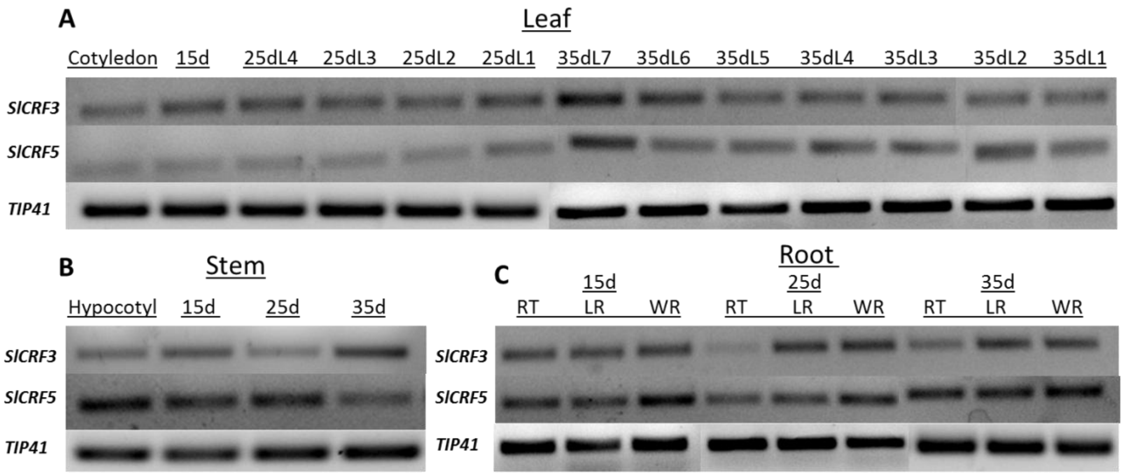


Fig.10 *SlCRF3* and *SlCRF5* expression patterns in three different stages of leaf, stem, and root. RT-PCR analysis of *SlCRF3* and *SlCRF5* in (A) cotyledon, leaf (15 d-1 leaf, 25 d-4 leaves, leaf 4th being the oldest, and 35 d-7 leaves, leaf 7th being the oldest), (B) hypocotyl, stem (15, 25, and 35 d), and (C) root (root tip (RT), lateral roots (LR), whole roots (WR) of 15, 25, and 35 d) is shown. The *TIP41* gene serves as an internal control.

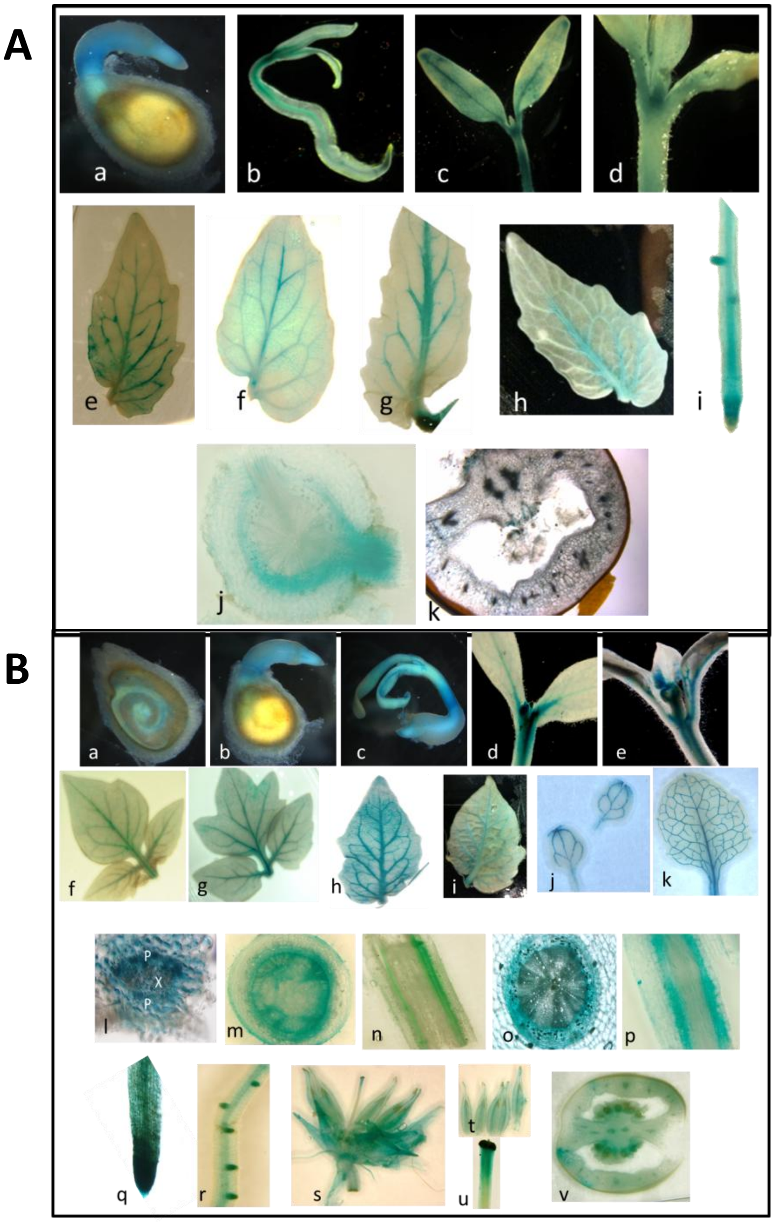


Fig.11 Promoter-driven GUS reporter gene expression for *SlCRF3* and *SlCRF5* is seen in the vascular tissues of various organs. (A) *SlCRF3* expression in the radicle (a), cotyledons of 4-7 d old seedlings after germination (DAG) (b-c), shoot apex of 9 d old seedling (d), leaves of 15, 25, 35, 85 d (e-h), root tip and lateral root (i-j), and mature fruit (k). (B) *SlCRF5* expression in embryo (a), radicle (b), vascular tissue of 4-9 d old seedlings after germination (DAG) (c-e), leaves of 15, 25, 35, 85 d (f-i), Arabidopsis cotyledons (j) and leaf (k), in phloem tissue as seen through free-hand cross-section of mid-vein of 35 d old leaf (l), 35 d old stem (m-n), primary root

(o-p), root tip (q), lateral roots (r) of 35 d old plants, reproductive organs: sepals, petals, anthers, stigma (s-u), and mature fruit. X, xylem; P, phloem.

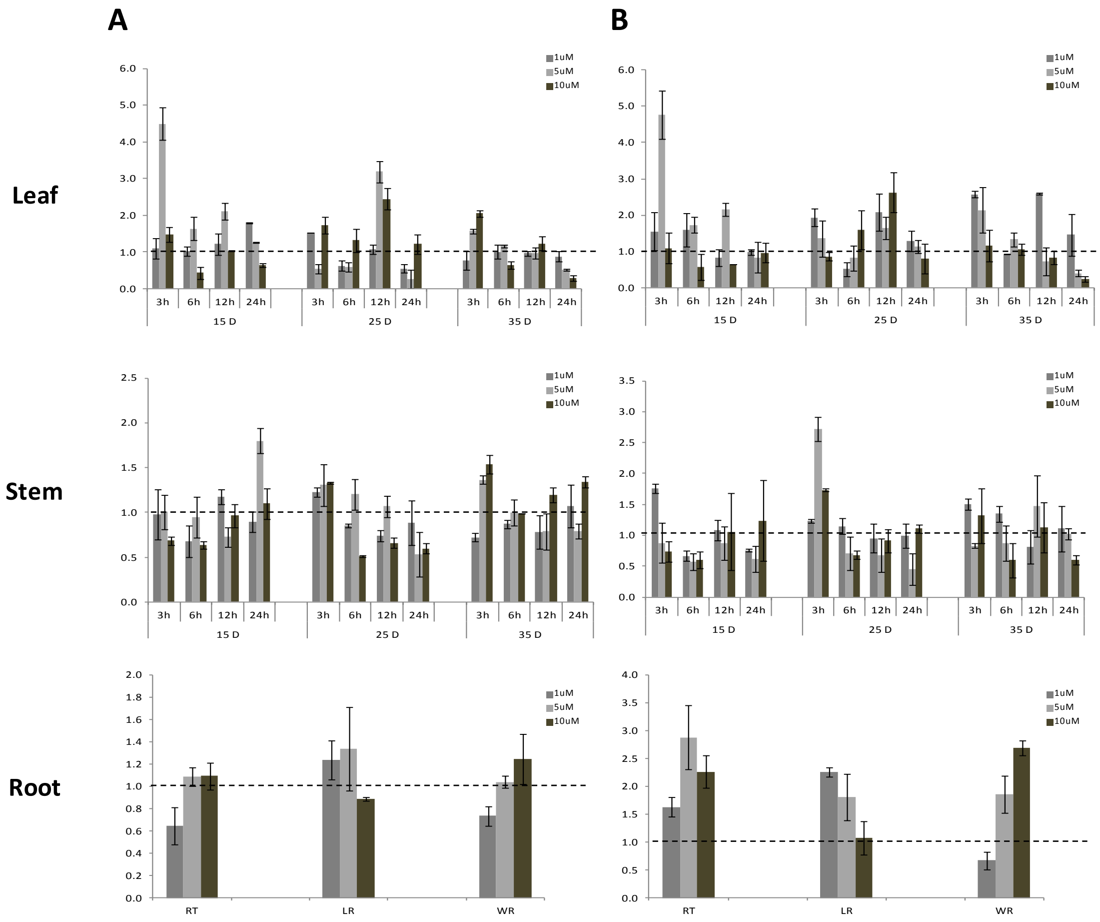


Fig.12 Expression analyses of *SlCRF3* and *SlCRF5* in response to benzyladenine (BA). Expression patterns of *SlCRF3* (A) and *SlCRF5* (B) in response to 1, 5, and 10 µM BA in 15, 25, and 35 d old leaf and stem treated for 3, 6, 12, and 24 h and 15 d old root treated for 24 h is shown. Relative expression was measured after performing qRT-PCR and the data presented are a mean±SE (two biological replicates). *TIP41* was used as an internal control.

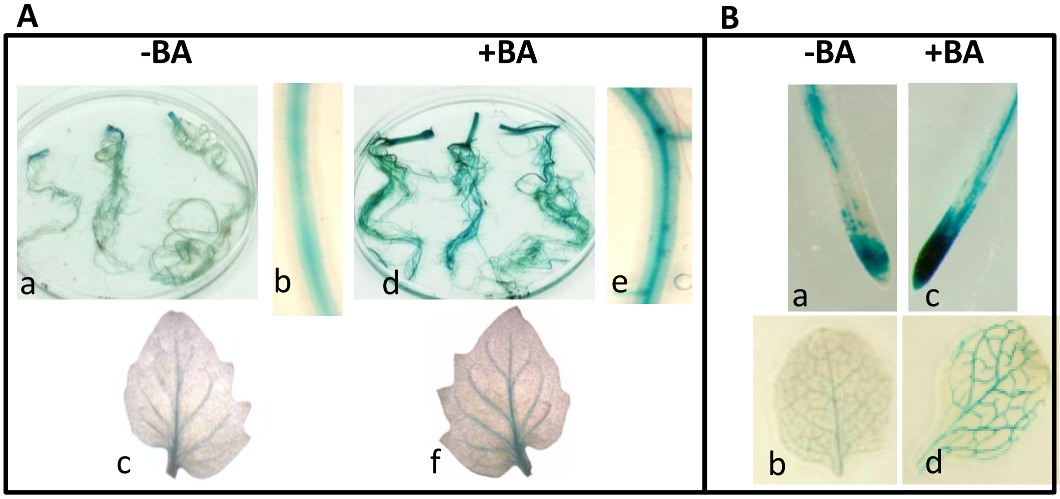


Fig.13 GUS expression is induced in response to BA in proSlCRF5::GUS transgenic plants. (A) Increased GUS expression in 25 d old pro*SlCRF5*::GUS transgenic roots (d-e) and leaf (f) treated with 5 µM BA compared to the roots (a-b) and leaf (c) treated with DMSO as control. (B) Induced GUS expression in root (c) and leaf (d) of 25 d old Arabidopsis plants harboring pro*SlCRF5*::GUS cassette treated with 5 µM BA compared to control root (a) and leaf (b).

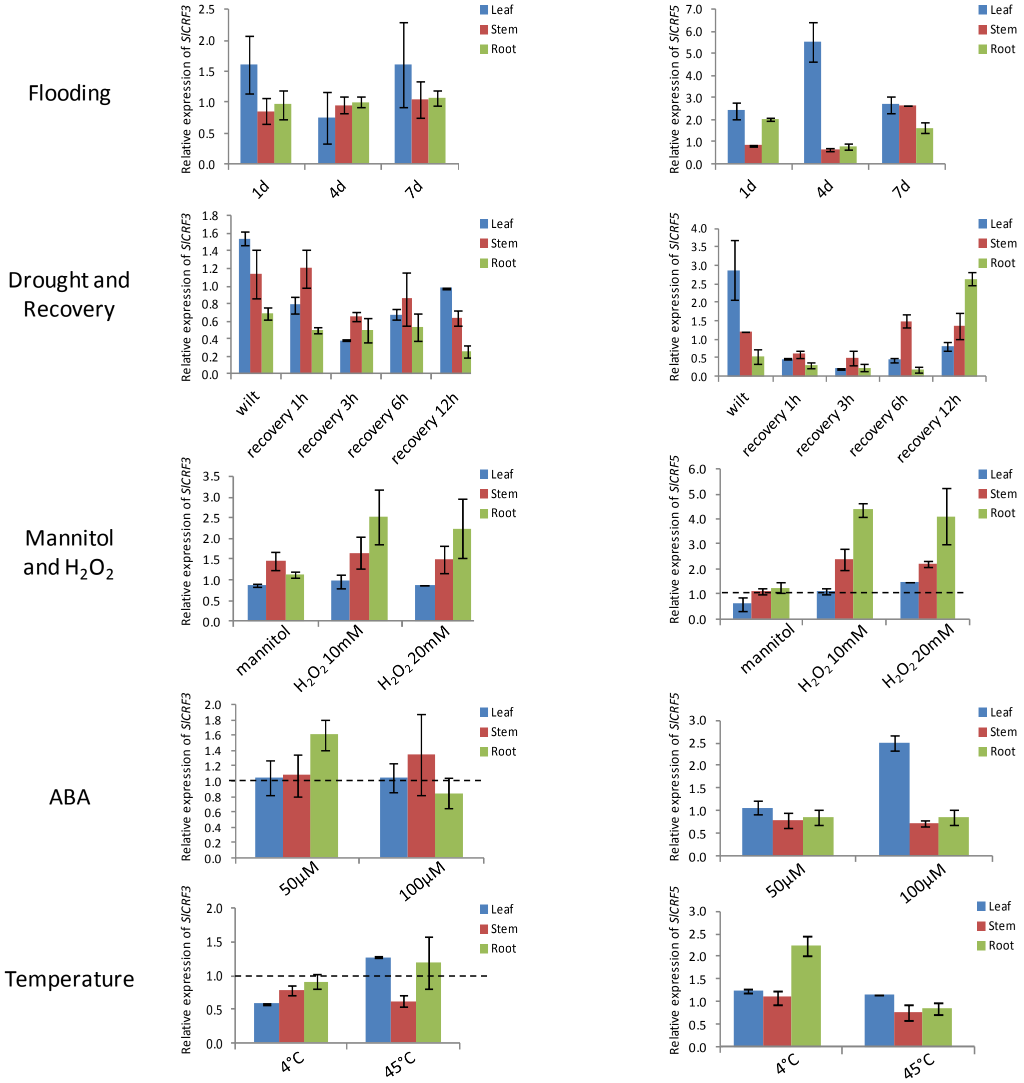
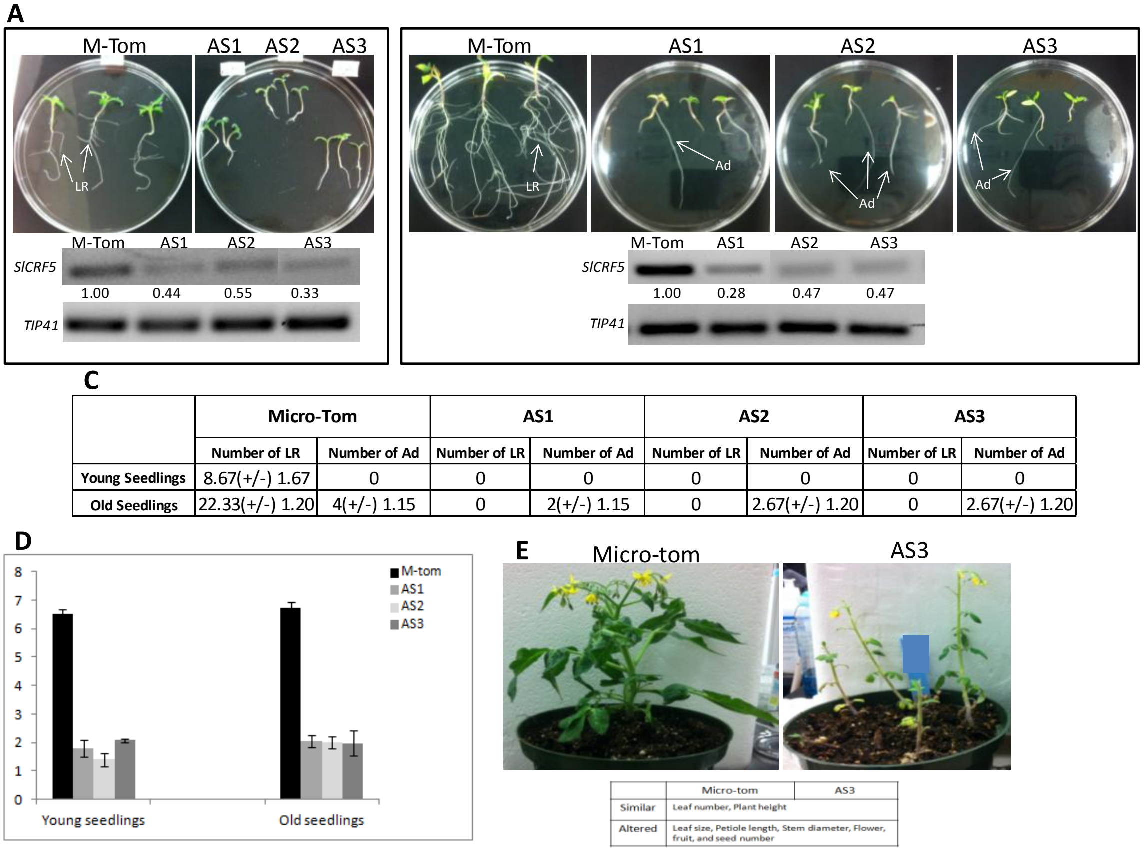


Fig.14 Expression analyses of *SlCRF3* and *SlCRF5* in response to abiotic stresses. Relative expression of *SlCRF3* (A) and *SlCRF5* (B) in 25 d leaf, stem, and root in response to abiotic stress conditions after treatment versus non-treated controls is shown. qRT-PCR was performed on samples exposed to flood conditions for 1, 4, and 7 d (a, b), drought for 7 d and recovery 1, 3, 6, 12 h (c, d), treatment with 200 mM mannitol, and 10 and 20 mM H_2O_2 for 3 h (e, f), treatment with 50 and 100 µM ABA for 3 h (g, h), and exposure to 4° C for 24 hours and 45° C for 1 hr (I, j). Data presented are a mean±SE (two biological replicates). *SleIF4A* was used as an internal control.



	Micro-Tom		AS1		AS2		AS3	
	Number of LR	**Number of Ad**	**Number of LR**	**Number of Ad**	**Number of LR**	**Number of Ad**	**Number of LR**	**Number of Ad**
Young Seedlings	8.67(+/-) 1.67	0	0	0	0	0	0	0
Old Seedlings	22.33(+/-) 1.20	4(+/-) 1.15	0	2(+/-) 1.15	0	2.67(+/-) 1.20	0	2.67(+/-) 1.20

	Micro-tom	AS3
Similar	Leaf number, Plant height	
Altered	Leaf size, Petiole length, Stem diameter, Flower, fruit, and seed number	

Fig.15 *SlCRF5* knockdown leads to stunted primary roots and absence of lateral roots. (A) Roots of 12 DAG and (B) 48 DAG antisense seedlings (AS1- AS3), with three plants each were compared to M-tom seedlings. Lateral roots (LR) and adventitious roots (Ad). Reduced transcript levels were examined in both cases by RT-PCR (C) Table of mean values of LR and Ad in M-tom and AS1-AS3 seedlings ± SE (Three biological replicates). (D) Primary root length of 12 DAG and 48 DAG AS1-AS3 seedlings compared to M-tom seedlings. Data presented are a mean±SE (Three biological replicates). *TIP41* was used as an internal control in all these experiments. (E) M-tom vs AS3 at 65 DAG stage.