Characterization of Tomato Cytokinin Response Factor Genes *SlCRF1* and *SlCRF2* in Tomato Growth and Development

By

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 3, 2013

Key words: tomato; cytokinin; *Solanum lycopersicum* cytokinin response factors (SlCRFs); stress response; leaf; transcriptome analysis

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Abstract

Cytokinin is a plant hormone that plays a myriad of roles in plant growth and development. Cytokinin is perceived in plants by a multiple-step phosphorelay that is similar to bacterial two component system (TCS). In addition to this main cytokinin signaling pathway, a branch pathway has been identified of which Cytokinin Response Factors (CRFs) are important components that act as transcription factors. The Arabidopsis CRFs have been shown to play a role in leaf and embryo development, although almost nothing is known about the role of CRFs in other species. This study addresses this in tomato using Micro-Tom, a miniature model variety to investigate the role of CRFs in this system: known as *Solanum lycopersium* CRFs or SlCRFs. Eleven different *SlCRFs* were identified and examined with particular attention here directed to understanding *SlCRF1* and *SlCRF2*, in growth and development of tomato plants with a focus on the leaf-related processes. Examination of these *SlCRF* responses to hormone (especially cytokinin) and abiotic stress treatments indicates that these genes may play a variety of roles in the regulation of these processes in tomato. qRT-PCR analysis revealed that both *SlCRF1* and *SlCRF2* are inducible by cytokinin in leaves and stems, with *SlCRF2* induced to a much greater level in leaves. Examination of the expression of these two genes in leaf, stem, and roots of different ages indicates that *SlCRF2* transcript levels are higher in younger than older organs while *SlCRF1* shows an opposite expression pattern. Analysis of promoter::GUS reporter lines revealed that both *SlCRF1* and *SlCRF2* are predominantly expressed in the vasculature of leaf, stem, roots, and fruits. To gain a general understanding of the tomato leaf transcriptome and how

it might be regulated by cytokinin, next-generation RNA sequencing has been performed on leaf samples of two ages, 13d and 35d, treated with cytokinin or the solvent vehicle control dimethyl sulfoxide (DMSO). This analysis revealed a large number of novel cytokinin regulated transcripts and provides a solid foundation for the future study of cytokinin and cytokinin regulated genes involved in compound leaf development and other developmental processes in tomato.

Acknowledgments

I'd like to give heartfelt thanks to my advisor, Dr. Aaron M. Rashotte for giving me the opportunity to work with him, and for guiding and helping me through these years. I also want to thank other members of my advisory committee, Dr. Narendra K. Singh, Dr. Joanna Wysocka-Diller, and Dr. Leslie R. Goertzen for their suggestions, time, help, and support. I want to thank Sarika Gupta and Paul Zwack for their help in the lab as well as their friendship and support. I give a thank-you to all Rashotte lab members. I thank my family members for their love, support, and encouragement.

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Chapter 1

Literature review

Introduction

Tomato is one of the main vegetable crops that has gained worldwide popularity with its nutritional fruit. It is not only an important vegetable plant, but also a good model plant in biological research. The tomato plant has some features, such as fleshy fruit, a sympodial shoot, and compound leaves, which make it a good model system for research. Because of its importance as a vegetable crop, a lot of research has been carried out to improve the quality and yield of tomato fruit. Much less emphasis, however, has been placed on the vegetative growth which relies on the leaves to a large extent. Leaves are the major organ conducting photosynthesis, as such are vital to the plant and for the production of tomato fruits as food. Therefore it is of great significance to gain a better understanding of the processes of leaf development which may lead to strategies to help improve crop performance.

The development of the leaf can be classified into three successive and overlapping stages. Initiation is the first stage when leaves emerge from the shoot apical meristem (SAM). Primary morphogenesis is the second stage when the basic leaf form is determined, and lateral structures are formed from the leaf margin. Secondary morphogenesis is the final stage which is characterized by substantial growth of the leaf

and differentiation of cell types typical of a mature leaf (Efroni et al., 2010; Shani et al., 2010). The development of the leaf requires complex cooperated actions of various transcription factors such as Class I *Knotted 1-like homeobox* (*KNOX*) genes, class III HD-ZIP homeodomain proteins, GARP (Golden 2, ARRs, and Psr1 [phosphorous stress response1]) transcription factors, and *YABBY* genes (Fleming 2005, Bryne 2005, and Champagne et al. 2007). These transcription factors regulate various aspects of leaf formation such as SAM growth and maintenance, leaf patterning, and morphogenesis. Some of these transcription factor families including class I KNOX genes have been shown to be regulated by plant hormones such as auxin and cytokinin. For example, cytokinins upregulate homeobox genes *SHOOT MERISTEMLESS* (*STM*) and *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA* (*KNAT1*), which suggests a role for cytokinins in the shoot apical meristem (Rupp et al., 1999).

Cytokinins are a class of plant hormones that are principally N^6-substituted adenine derivatives. Ongoing studies have linked cytokinins to many biological processes such as cell division, seed germination, apical dominance, shoot meristem initiation and maintenance, leaf and root differentiation, stress tolerance, and senescence in plants (Gan and Amasino 1995; Haberer and Kieber 2002; Hwang and Sheen, 2001; Mok and Mok 2001; Werner and Schmülling, 2009). Similar to the two-component systems (TCSs) widely used by bacteria and some fungi (Beier and Gross 2006; Catlett et. al. 2003; Ferreira and Kieber 2005; To and Kieber 2008), a multi-step phosphorelay is responsible for the perception and transduction of the cytokinin signal in plants. In Arabidopsis, cytokinin receptors called Arabidopsis Histidine Kinases (AHKs) recognize and bind to cytokinin. Binding of an AHK to cytokinin results in the autophosphorylation of the

AHK. The phosphoryl group is then transferred to a signal mediator, the Histidine Phosphotransfer protein (AHP), which relays the cytokinin signal from the membrane bound receptor into the nucleus and phosphorylates the downstream Response Regulators (ARRs). There are two major classes of ARRs, type-As and type-Bs, which have been found to function in different aspects of plant growth and development. Both types of response regulators receive a cytokinin signal, but act in different ways. Type-B response regulators are transcription factors that function as positive regulators by acting upon cytokinin regulated target genes, including the type-A ARRs, yet are not transcriptionally indcuced by cytokinin (To et al., 2004; Mason et al., 2005). While it is well known that type-A RRs are cytokinin inducible, they lack the GARP transcription factor binding domain that type-Bs conatin, and instead act as negative regulators of cytokinin signaling (Brandstatter and Kieber, 1998; D'Agostino et al., 2000; Taniguchi et al., 1998). In addition to the primary components of the TCS pathway, a branch pathway of cytokinin signaling has been identified in *Arabidopsis* that includes Cytokinin Response Factors (CRFs) via the AHKs and AHPs (Rashotte et al., 2006).

The TCS components as well as CRFs have been identified in increasing number across a range of species such as maize, rice, *Medicago sativa*, soybeans, and tomato in the past decade (Yonekura-Sakakibara et al., 2004; Ito and Kurata, 2006; Pareek et al., 2006; Gonzalez-Rizzo et al., 2006; Pils and Heyl, 2009; Rashotte and Goertzen 2010; Le et al., 2011; Shi et al., 2013). Given the fact that cytokinin plays a myriad of roles in plant growth and developmental processes, it is not surprising that TCS pathway and CRFs are found conserved across plant species.

Previous studies have shown that *CRF* genes play an important role in *Arabidopsis* leaf and cotyledon development (Rashotte et al., 2006). Recently, 11 tomato *CRFs* (*Solanum lycopersicum CRFs* or *SlCRFs*) have been identified and characterized (Shi et al., 2012). As orthologs of *Arabidopsis CRFs*, *SlCRFs* are likely to be involved in compound leaf development and other growth and developmental processes in tomato. The current study is using the dwarf tomato cultivar "Micro-Tom" as a model to explore the possible roles of *SlCRFs* in the growth and development of tomato plants with a focus on leaf development.

Micro-Tom-the model plant used in this study

Micro-Tom is a dwarf tomato cultivar that was produced by crossing Florida Basket and Ohio 4013-3 cultivars (Martí et al., 2006). The small size (10-20cm tall), rapid life cycle (70-90d), and relatively easy transformability of Micro-Tom make it a convenient model system for studies on many aspects of plant growth and development and advantageous over other tomato varieties that are larger, slower growing and more difficult to transform (Emmanuel and Levy, 2002; Dan et al., 2005; Meissner et al., 1997; Campos et al., 2010). It has also been reported to be very suited for high-throughput mutagenesis (Emmanuel and Levy, 2002). Because of the relatively easy transformability of Micro-Tom, many studies have been conducted to improve its transformation efficiency making it often the choice for researches to generate transgenic lines (Dan et al., 2006; Pino et al., 2010; Sun et al., 2006).

Leaf development

The major classes of transcription factors regulating leaf development include *Class I Knotted 1-like homeobox* (*KNOX*) genes, class III HD-ZIP homeodomain proteins,

GARP transcription factors, and *YABBY* genes (Fleming 2005, Bryne 2005, and Champagne et al. 2007). Many other transcription factors such as *NAM*/*CUC2* (*NO APICAL MERISTEM* and *CUP-SHAPED COTYLEDON*) boundary genes and *IAA9* are also involved in leaf development (Wang et al., 2005; Blein et al., 2008)

The first stage of leaf development is the initiation of the leaf primordium at the flanks of the shoot apical meristem (SAM). The establishment and maintenance of SAM requires the activity of SAM-expressed class I *KNOX* genes (Hake et al., 2004; Hay and Tsiantis, 2009). Class I *KNOX* genes generally found to be downregulated throughout leaf development in simple-leaf species (Blein et al., 2010; Carraro et al., 2006; Scofield and Murray, 2006). However, in compound-leaf species these genes that are transiently repressed during leaf initiation become reactivated in the developing leaf primordium to promote leaflet formation and the complex leaf shape (Hareven et al., 1996; Hay and Tsiantis, 2006). It has been reported that class I *KNOX* overexpression in tomato resulted in excessive leaf compounding and variable leaf phenotypes (Hareven et al., 1996; Janssen et al., 1998). Recent studies provide lines of evidence that tomato class I *KNOX* genes (*Tkn1* and *Tkn2*) act in a spatial- and temporal-specific manner to delay leaf maturation and enable leaflet formation (Shani et al., 2009). Alteration in leaf shape due to *Tkn* overexpression under the control of leaf-specific promoters was only observed during early initiation or primary morphogenesis (PM) stages of leaf development. However, Tkn overexpression caused different effects during these two stages: overexpression during leaf initiation prolonged the initiation stage, while during PM it resulted in a dramatic increase in leaf complexity as indicated by the much higher degree of leaflet reiteration (Shani et al., 2009). Importantly, the tomato mutant *clausa* which

affects the *Tkn2* gene also has altered leaf complexity, further supporting these findings (Avivi et al., 2000). Additionally, class I *KNOX* genes were shown to act through cytokinin to enhance the marginal morphogenetic activity in compound-leaf development (Shani et al., 2010).

The Class III HD–ZIP homeodomain protein family in Arabidopsis includes PHABULOSA (PHAB), PHAVOLUTA (PHAV), REVOLUTA (REV), CORONA (CNA), and ATHB8 that act redundantly to regulate plant growth (McConnell et al., 2001; Prigge et al. 2005). *PHAB*, *PHAV*, and *REV* serve to establish adaxial identity in lateral organs (McConnell et al., 2001; Floyd and Bowman, 2010). Dominat mutations in *PHAB* and *PHAV* resulted in the change of abaxial into adaxial fates in leaves and leaf-like organs (McConnell et al., 2001), and similar mutation in *REV* caused an alteration in the adaxial-abaxial identity of rosette leaves (Zhong and Ye, 2004). Additionally, a recessive *REV* mutant allele caused overgrowth of both rosette and cauline leaves in *Arabidopsis* (Talbert et al., 1995). Mutations in *CNA* and *ATHB8* suppress leaf phenotypes seen in *rev* mutants such as longer, curled and darker leaves (Prigge and Clark, 2006).

KANADI (*KAN*) genes, members of the GARP transcription factor family, specify abaxial cell fate as indicated by leaf phenotypes of higher order *kan* mutants (Byrne 2005). *kan1kan2* mutants showed alterations in leaf polarity, that is, transformation of abaxial cell types to adaxial ones which resulted in narrow leaves (Eshed et al., 2001). *kan1kan2kan3* triple mutant was characterized by radicalized leaves (Eshed et al., 2004).

YABBY genes are predominantly expressed in developing leaves and leaf-derived organs (Floyd and Bowman, 2010). Reduced *YABBY* activity leads to restricted lamina

growth and the severity of phenotype is correlated to the degree to which *YABBY* activity is decreased, indicating an essential role for *YABBY* genes in lamina development (Golz et al. 2004; Siegfried et al. 1999). *YABBY* genes are also known to be involved in the repression of *KNOX* gene expression (Kumaran et al. 2002). They are required for the establishment of abaxial cell fate as well (Eshed et al., 2001).

Leaf development is a complex process that involves the fine-tuned action of many factors. Besides the major classes of transcription factors noted above, there are other genes that are ivovled in leaf growth and development such as *Cytokinin Response Factors* (*CRFs*) and *ABPH1* that determines phyllotactic pattern in maize (Rashotte et al., 2006; Jackson and Hake, 1999; Giulini et al., 2004). The latter is type-A response regulator shown to be inducible by cytokinin (Giulini et al., 2004). In-depth investigation on all these genes will add our knowledge about various aspects of leaf development.

CRFs-components of the TCS branch pathway of cytokinin signaling

Several Arabidopsis *CRFs* were initially identified as putative Apetalla2 (AP2) transcription factor family genes which were inducible by cytokinin in an examination of genome-wide gene expression in response to cytoninin (Rashotte et al., 2003). As members of the AP2/ERF transcription factor family, CRFs form a subgroup of proteins that falls within the ethylene response factor (ERF) subfamily (Rashotte et al., 2006; Rashotte and Goertzen, 2010). Sequence analysis reveals that standard CRF proteins contain a unique N-terminal CRF domain followed by an AP2 DNA binding domain and a putative kinase phosphorylation site (Rashotte and Goertzen, 2010; Shi et al., 2012). The CRF domain is a newly identified conserved motif of about 65 amino acids which defines the CRF family as distinct from other ERF proteins (Rashotte and Goertzen,

2010). This domain was found to be responsible for CRF protein-protein interactions and for interactions between CRF proteins and other components of TCS cytokinin signaling pathway, primarily the AHPs (Cutcliffe et al., 2011). A recent study has classified CRFs into five distinct clades based on the presence of clade-specific C-terminal protein sequence regions (Zwack et al., 2012). Phylogenetic analysis shows that CRF homologues are present in all land plants, indicating the conservation of the CRF branch pathway of cytokinin signaling (Rashotte and Goertzen, 2010).

One way to examine a role for CRFs is through study of their expression patterns and transcript profiles in organs. Prior to this study, work was done primarily in Arabidopsis revealing that *CRF* genes are expressed in a number of different organs throughout development yet appear to be preferentially targeting to phloem tissue within vasculature of these organs (Zwack et al., 2012). Another method to understand the role that CRFs play is through the phenotypic examination of loss-of-function mutants. Analysis of single and multiple *crf* mutants has shown that CRFs possibly play a role in the development of cotelydon, leaves, and embryos (Rashotte et al., 2006). Single *crf* mutations have a minor effect on cotyledon development, but the triple *crf1,2,5* mutant showed a more penetrant and more severe phenotype such as highly reduced and translucent/non-green cotyledons, indicating likely functional overlap among *CRFs* (Rashotte et al., 2006). A similar reduced chlorophyll content could also be observed in cytokinin receptor single and multiple mutants such as *ahk3*, *ahk2 ahk3*, and *ahk2 ahk3 ahk4* with an additive effect (Riefler et al., 2006). Another example of redundancy in *CRFs* occurs between *CRF5* and *CRF6*. Single *crf5* and *crf6* mutants display normal embryo development while the double mutant *crf5,6* exhibits an embryo lethal phenotype

(Rashotte et al., 2006). Although an embryo lethal phenotype has not been reported for loss-of-function mutants of other cytokinin signaling components, a reduced number of seeds as well as a much larger seed/embryo size was observed in *ahk* triple mutant and higher order *AHP* mutants (Riefler et al., 2006; Hutchison et al., 2006), indicating a role of cytokinin in controlling embryo size and a unique role of CRF5 and CRF6 in regulating embryo development. Another clue linking CRFs to a functional process was seen from the overexpression of *CRF2* that showed a higher PLASTID DIVISION 2 (PVD2) protein level and increased chloroplast division rate, indicating a potential role of *CRF2* in the regulation of genes involved in plastid division. Exogenously applied cytokinin had a similar effect on *PVD2* expression and chloroplast division, suggesting that cytokinin may affect this process partly through *CRF2* (Okazaki et al., 2009).

CRFs have also been connected to a few stress related processes. A recent study implicated *CRF2* in an AHK2 and AHK3-mediated cold-responsive signaling pathway. *CRF2* transcript level was increased over 2-fold during cold treatment in wildtype plants and significantly decreased in response to cold in *ahk2 ahk3* mutants. The up-regulation of *CRF2* in response to cold requires the transcription activator ARR1 that can be activated by AHP2, AHP3, or AHP5 once the cold signal sensed by AHKs is passed to AHPs (Jeon and Kim, 2013). There has also been a potential link to biotic stress as it was reported that Arabidopsis plants expressing the Pro35S::CRF5 construct displayed enhanced pathogen resistance and a higher transcript level of some *PR* genes (Liang et al., 2010), suggesting that CRF5 plays a role in plant defense apart from its role in embryo development (Rashotte et al., 2006). Additional recent work has linked *CRF* to senescence. It was found that *CRF6* may function in diverse aspects in plant life such as

cytokinin response, negative regulation of leaf senescence, and response to multiple stresses (Zwack et al., 2013).

As noted above the initial discovery of CRFs in Arabidopsis was expanded into other species with the highest degree of work being conducted in tomato plants where these genes are named *Solanum lycopersicum* Cytokinin Response Factors (SlCRFs). RT-PCR analysis showed that some of the *SlCRFs* were induced by cytokinin (Rashotte and Goertzen, 2010; Shi et al., 2012). SlCRF1 (also called Pti6), like Pti4 and Pti5, was shown to be able to bind specifically, via the AP2 DNA binding domain, like other ERF proteins to the GCC-box found in the promoter regions of various *pathogenesis-related* (*PR*) genes and activate the expression of some *PR* genes when expressed in Arabidopsis (Gu et al., 2002). Overall this suggests that SlCRF1 may play a role in plant defence or biotic stress response. *Tsi1,* a *CRF* homologue in tobacco, which has high sequence similarity to *SlCRF1* and is part of the same *CRF* clade, confers increased resistance to both pathogen and salt stress when overexpressed in tobacco (Park et al., 2001). It is possible that Tsi1 performed its protective roles by binding to the GCC box *cis* element in the promoters of a number of PR genes and inducing their expression as a transcriptional activator (Park et al., 2001). *SlCRF1,* along with a few other *SlCRFs*, have also been shown to be salt inducible in leaf tissue (Shi et al., 2012).

In summary, CRF related proteins seem to widely function in cytokinin signaling, processes of cotyledon, leaf, and embryo development as well as abiotic and biotic stress responses. Studies on many of these SlCRF domain-containing genes are still at an initial stage and intensive work remains to be done to explore their diverse roles and the possible interactions between these genes and other signaling and/or developmental

pathways. The current study undertakes this to further general knowledge of *SlCRFs*, particularily *SlCRF1* and *SlCRF2*.

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*Chapter 2

Characterization of responses of *SlCRF* genes to different hormones

Abstract

Cytokinin is an essential hormone in a myriad of 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*). SlCRFs are AP2/ERF transcription factors and generally orthologous to Arabidopsis CRF clade members (AtCRFs). Some *SlCRF* genes lack a direct Arabidopsis ortholog and one SlCRF has a unique protein domain arrangement not seen in any other CRF protein. Expression analysis of *SlCRF1* to *8* revealed differential patterns and levels across plant tissues examined (leaf, stem, root and flower). Several SlCRFs show induction by cytokinin to various degrees, similar to AtCRFs. Additionally we show that some *SlCRFs* can be regulated by other factors, including NaCl, ethylene, MeJA, and SA. Overall this work indicates that some *SlCRFs* resemble previously identified *CRFs* in terms of structure, expression, and cytokinin regulation. However, *SlCRFs* have novel CRF protein forms and responses to factors suggesting they may have a diverse set of roles in stress and hormone regulation in tomato.

*Published in J of Exp Bot, 63: 973-982, 2012

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 a response regulator (RR) localized in the nucleus. Phosphorylated RRs acting as transcription factors (type-B RR) activate the expression of a set of target genes mediating cytokinin-regulated growth and developmental processes or other aspects of plant life (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*/*SlCRF1* 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 SlCRFs. Eleven *SlCRF* genes were identified through a combination of existing sequence comparison and RACE-PCR. The expression of these *SlCRF* genes was examined in different plant tissues, as well as regulation by cytokinin, salt, and other hormones. Together this study generates a first complete picture of all *CRF* genes in any species suggesting a broader function for *CRF* beyond cytokinin regulation and allowing functional parallels to be made between related clades of CRFs across species.

Materials and Methods

Plant materials and growth conditions

The tomato dwarf cultivar Micro-Tom was used for all experiments. Plants were grown in Sunshine Mix #8 soil under a 16:8 h light:dark photoperiod at 150 μE, with a 26 ℃ day(light), 22 ℃ night (dark) temperature.

RNA isolation, cDNA synthesis, and expression analysis

Leaves, stems, flowers, and roots were harvested from 52-day-old Micro-Tom plants, and immediately flash-frozen in liquid nitrogen. RNA was extracted using 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 Quanta qScript cDNA supermix. The first strand of cDNA was diluted 20 times before it was used in the RT-PCR. PCR was started with a one-step cycle of 2 min at 95 ℃, followed by 29 cycles of 30 sec at 94 ℃, 30 sec at 56 ℃, and 35 sec at 72 ℃, and a 5 min final extension at 72 ℃. The gene specific primers used in the RT-PCR are as follows:

SlCRF1 forward 5'-GGAAAATTCAGTTCCGGTGA-3'

SlCRF1 reverse 5'-AAAATTGGTAACGGCGTCAG-3'

SlCRF2 forward 5'-TGCCGGTCCTAGAGTTGTAA-3'

SlCRF2 reverse 5'-CAGTGGCTGCTCTGCTCTAT-3'

SlCRF11 forward 5'- AAGTGCCTGAGTTGGCTATG-3'

SlCRF11 reverse 5'- TCACCCTCGATCAGATAAAC-3'

All samples are compared to the control gene *TIP41* (Exp ósito-Rodr guez et al 2008).

SlCRFs expression in response to hormone or salt treatment, as described below was examined using RT-PCR started with a one-step cycle of 2 min at 95 ℃, followed by 29 to 40 cycles of 30 sec at 94 ℃, 45 sec at 57 ℃, and 40 sec at 72 ℃, and a 5 min final extension at 72 ℃. RT-PCR at different cycle lengths was performed for genes of varying intensities: *SlCRF3* (29 cycles), *SlCRF1*, *2*, *4*, *6*, *10*, *11* (30 cycles), *SlCRF5* (30 cycles for salt, 35 for other treatments), *SlCRF7* (35 cycles for MeJA, 40 for other treatments), *SlCRF8*, *9* (40 cycles). Primers used for *SlCRF1-11* are as follows:

SlCRF1 forward 5'-AACGATGTCGCTTTGTCACC-3'

SlCRF1 reverse 5'-GGGCAAAATCGTCAAAGTCA-3'

SlCRF2 forward 5'- ATGCTGCCGGTCCTAGAGTT-3'

SlCRF2 reverse 5’- GAGCAGTTTCCGACGATGAC-3’

SlCRF3 forward 5’-AATGATGCAGTCGAGGAACC-3’

SlCRF3 reverse 5’-CCTGGTCTTCCCATTCTCAA-3’

SlCRF4 forward5’-TGAATCCCTCTGTTCCAAGG-3’

SlCRF4 reverse 5’-GTTTTGCCATTTCCACTGCT-3’

SlCRF5 forward 5’-ACGATGACGACGAGAGGAAT-3’

SlCRF5 reverse 5’-CTGACACCGCGAAACTTTTT-3’

SlCRF6 forward 5’-AGATGAGCTTTTTGGGCGTA-3’

SlCRF6 reverse 5’-TCGCTTCTTCCCATTACCAC-3’

SlCRF7 forward 5’-ACGTTGGTTGGGAAGTTTTG-3’

SlCRF7 reverse 5’-TAATGGTTGATGGGGTCGAT-3’

SlCRF8 forward5’-ACGTTGGTTGGGAACTTTTG-3’

SlCRF8 reverse 5’-GTGTTGATGGGGTTGATTCC-3’

SlCRF9 forward 5’- GCGTTGCCTAAAGGAGTTAG -3’

SlCRF9 reverse 5’-ACCAGGGCTCAAATTCTTAC -3’

SlCRF10 forward 5’- CTCAGAGTTTGGTCTCACATAC -3’

SlCRF10 reverse 5’- AACATGTCCATCTCCGTATC-3’

SlCRF11 forward 5’- AAGTGCCTGAGTTGGCTATG-3’

SlCRF11 reverse 5’- TCACCCTCGATCAGATAAAC-3’

For characterizing *SlCRF7* response to ethephon and *SlCRF8* 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 Quanta 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 *SlCRF1-2*. Primers for *SlCRF1-2* 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 ℃, followed by 40 cycles of 15 sec at 95℃, 30 sec at 56℃, and 35 sec at 68℃. 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 plant 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, leaves were removed from solution, patted dry, and immediately flash frozen in liquid nitrogen, and stored at -80℃ until RNA extraction.

Results

SlCRFs Are Expressed in Different Plant Tissues

Previous work identified four *SlCRFs* (*SlCRF 1*, *3* to *5*) as expressed in leaf tissues (Rashotte and Goertzen, 2010). Here we show that *SlCRF1*, *2*, *11* are expressed in multiple different plant tissues throughout the plant (leaf, stem, root, and flowers) to varying degrees (Figure 2.1). Both *SlCRF1* and *SlCRF2* showed more abundant expression in root tissue, and *SlCRF11* in stem (Figure 2.1).

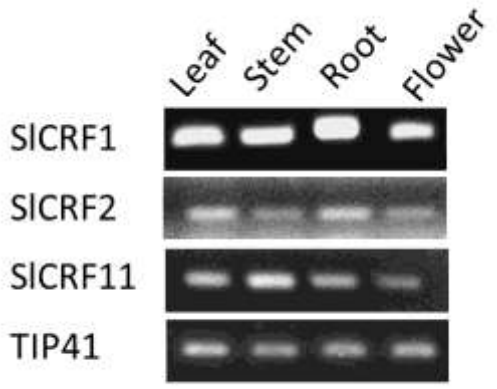


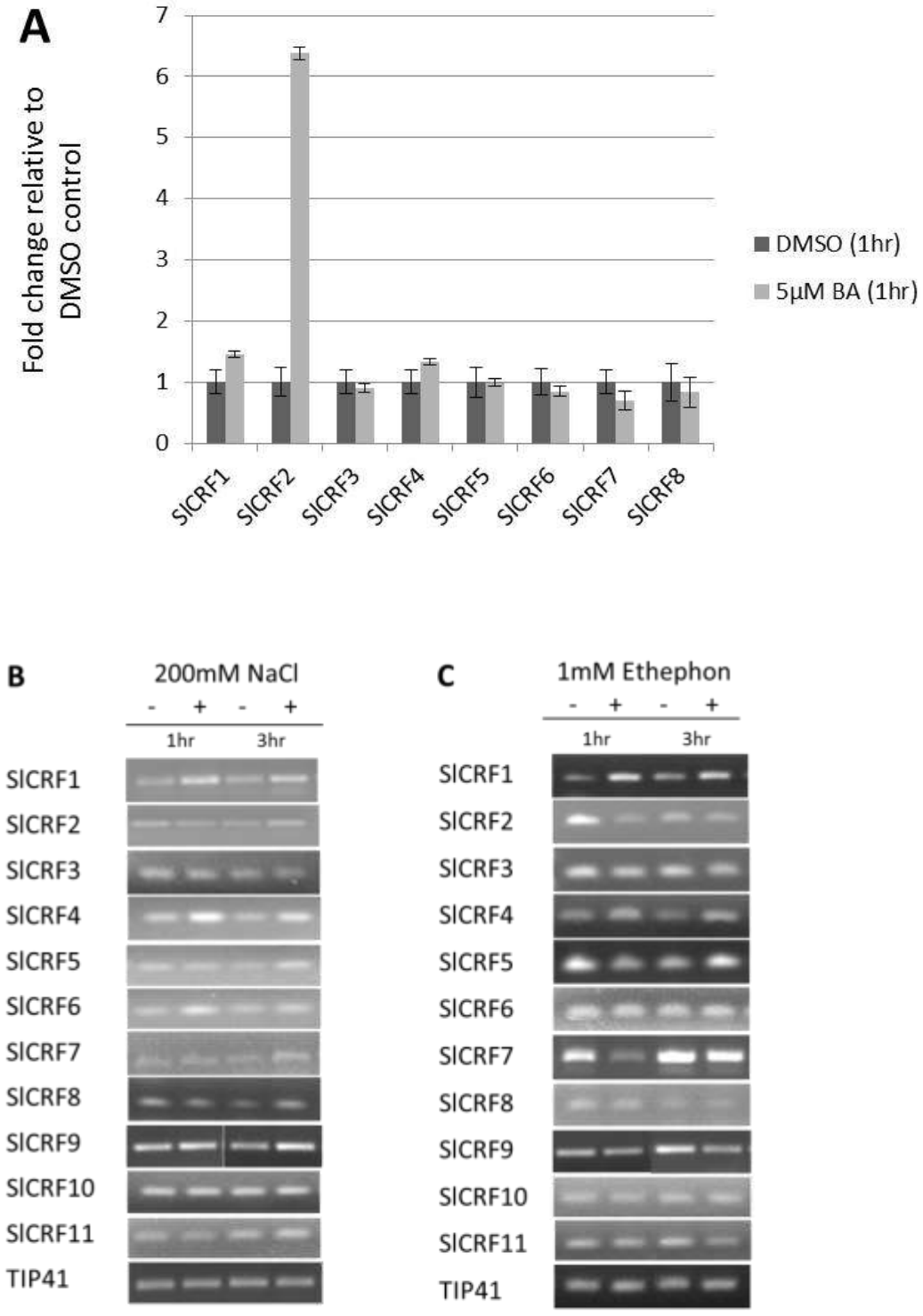
Figure 2.1. *SlCRF* expression patterns in various tomato tissues. RT-PCR analysis of *SlCRF1*, *2*, *11* in leaf, stem, root, and flower tissues of 52-day-old plants is shown. The *TIP41* gene serves as an internal control.

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 (15d) were treated with cytokinin (5 μM BA) or DMSO as a vehicle control for 1 h and examined using real-time PCR. *SlCRF2* showed induction by cytokinin after treatment to 6 fold over untreated levels, whereas none of the other *SlCRFs* showed induction (Figure 2.2A). However, it is possible that some of the *SlCRFs* are late-

responsive genes to cytokinin. Overall, the results follow a pattern similar to that seen for Arabidopsis *CRFs* (*AtCRFs*) that some, but not all members of this group are transcriptionally regulated by cytokinin (Rashotte et al 2006).

We also examined *SlCRFs* for changes in response to salt and other hormones using RT-PCR of leaves treated at 1 and 3 hours vs. controls (Figure 2.2B-E). Expression analysis of salt treatment (200mM NaCl) revealed induction of *SlCRF1, 4, 6* at both 1 and 3h as well as a minor induction of *SlCRF2, 5* at 3h (Figure 2.2B). This suggests a new potential role of *SlCRFs* in stress regulation. Expression analysis of ethylene treatment (1mM Ethephon) showed minor induction of *SlCRF1, 4* at 1h and *SlCRF1, 4, 7* at 3h, while *SlCRF2* was repressed at both 1 and 3h (Figure 2.2C). This is some of the first data linking any *CRF* to ethylene. Expression analysis of methyl jasmonate (100 µM MeJA) showed only a single transcript change, the repression of *SlCRF6* at both 1 and 3h (Figure 2.2D). Expression analysis of salicylic acid (2mM SA) revealed induction of *SlCRF1* at 3h and a slight induction of *SlCRF4* at both 1 and 3h (Figure 2.2E). Together these results suggest that *SlCRFs* may be regulated by factors other than cytokinin.


A
Fold change relative to DMSO control
7
6
5
4
3
2
1
0
SlCRF1
SlCRF2
SlCRF3
SlCRF4
SlCRF5
SlCRF6
SlCRF7
SlCRF8
DMSO (1hr)
5μM BA (1hr)
B
200mM NaCl
- + - +
1hr 3hr
SlCRF1
SlCRF2
SlCRF3
SlCRF4
SlCRF5
SlCRF6
SlCRF7
SlCRF8
SlCRF9
SlCRF10
SlCRF11
TIP41
C
1mM Ethephon
- + - +
1hr 3hr
SlCRF1
SlCRF2
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SlCRF10
SlCRF11
TIP41

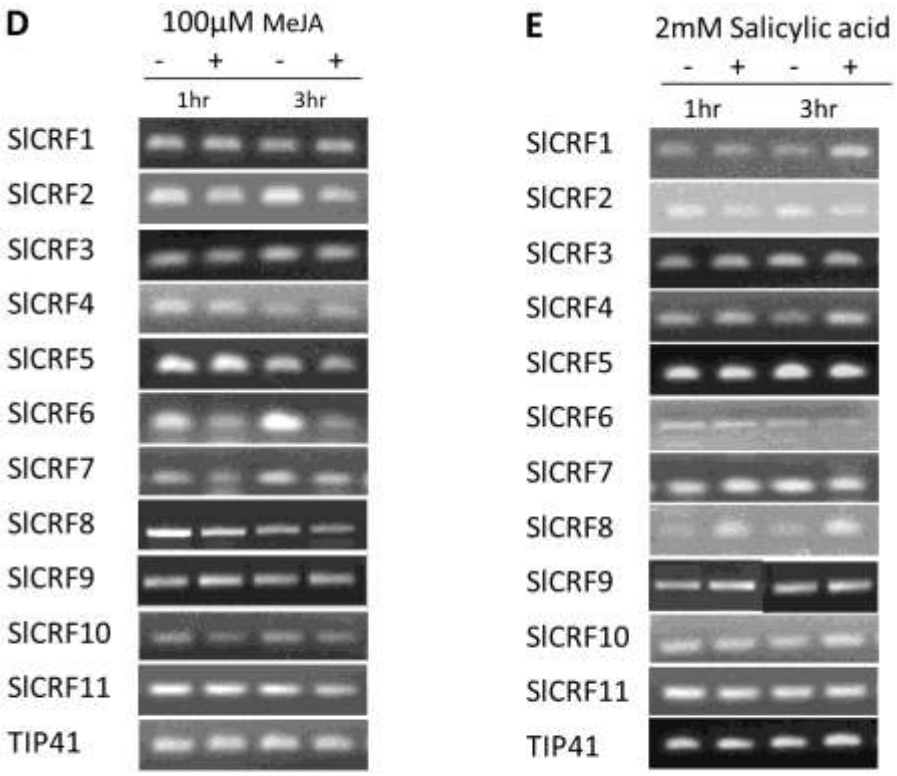

Figure 2.2 Expression of *SlCRF* genes in response to hormones or salt treatment in 15-day-old tomato leaves. (A) qRT-PCR of cytokinin (5 µM BA) treatment for *SlCRF1-SlCRF8*. Data presented are a mean+SE (two biological replicates). Dark grey bar, 1 h DMSO control; light grey bar, 1 h BA treatment. (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 µM 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. (B)-(E) were done for *SlCRF1-SlCRF11*.

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 full set of cytokinin response factor (CRF) genes in a crop species, tomato (*Solanum lycopersicum*). Eleven *SlCRF* genes (*SlCRF1* 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). SlCRF 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 (Rashotte and Goertzen, 2010). One SlCRF, SlCRF3, was found to have a unique protein structure containing two CRF and two AP2 domains. While several AP2/ERF proteins contain two AP2 domains, including the founding member of this group, SlCRF3 is the only known protein to contain more than a single CRF domain. Despite this it appears to be functionally transcribed, induced by cytokinin and able to interact with other SlCRFs proteins.

A phylogenetic analysis of SlCRFs shows relationships similar to that seen for Arabidopsis CRFs (AtCRFs) and the overall group of CRFs in plants (Rashotte and Goertzen, 2010). Despite overall similarities between tomato and Arabidopsis CRFs, there are several differences that may suggest functional differences between species. An example is that *SlCRF1* has no direct Arabidopsis ortholog. In fact most plant species appear to have a *SlCRF1* ortholog, indicating that the condition in tomato is more common (Rashotte and Goertzen, 2010). It also suggests that the function of SlCRF1 is unlikely to be simply determined through studies of CRFs in Arabidopsis.

Expression of *SlCRF1, 2* in tissues from roots to flowers suggests a broad role for these genes in the plant (Figure 2.1). There also appears to be a range of transcript levels of *SlCRFs* potentially indicating different functional roles in different tissues. This is the most complete tissue analysis of a *CRF* group of genes from any species excluding Arabidopsis where microarray generated data of *AtCRFs* reveal a pattern of expression across most tissue types and development, not unlike that seen for the *SlCRFs* in this

study, suggesting that *CRFs* in most plants are likely to be expressed broadly across tissues (data not shown).

Several *SlCRFs* 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 *AtCRF* genes parallel the *SlCRFs* strongly induced in this study. *SlCRF2*, highly similar to *AtCRF2*, shows rapid induction comparable to that of *AtCRF2* (Figure 2.2A; Rashotte et al., 2006). The lack of cytokinin regulation of some highly related pairing of *SlCRFs* also parallels expression studies of other *AtCRFs*, such as *SlCRF4* and *6* compared to *AtCRF3* and *4*. Overall the pattern of transcriptional cytokinin regulation of *SlCRFs* is similar to *AtCRFs* and suggests that there may be similar regulation within specific clades of *CRF* genes.

We examined other factors that might transcriptionally affect *SlCRFs* 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; Nakano et al., 2006; Zarei et al., 2011). Treatment with salt (NaCl) showed induction of nearly half of the *SlCRFs* to some degree (Figure 2.2A), revealing that *CRFs* can be induced by abiotic factors. An investigation of related *AtCRFs* (*AtCRF2*, *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 *SlCRFs* 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 *SlCRFs* from minor induction, to repression, with little effect on most *SlCRFs* (Figure 2.2C). Previous studies have shown that ethylene had little to no effect on *AtCRFs* and *SlCRF1*/*Pti6* consistent with most *SlCRFs* in this study. The exception, *SlCRF2* transcript repression, indicates that ethylene may still play some role in *SlCRF* function, although a more detailed study is needed to further determine the extent. Methyl jasmonate (MeJA) treatment showed no effect on any *SlCRFs* suggesting that it plays little role in CRF function, although specific *CRFs* such as *SlCRF6* may be exceptions (Figure 2.2D). Salicylic acid (SA) treatment resulted in only minor induction of two *SlCRFs* similar to MeJA treatments, indicating that SA also has little effect on the transcription of most *SlCRFs* (Figure 2.2E).Together these results suggest that *SlCRFs* can be regulated by factors other than cytokinin and may fall into different groups of regulated genes: some (*SlCRF3* and *5*) regulated primarily by cytokinin, others (*SlCRF1*, *2*, *4*, *6*) regulated by several factors, and some (*SlCRF7-11*) show little response to factors examined in this study. A broader examination of *SlCRF* expression patterns, beyond this study is needed to determine the functional role of each *SlCRF*.

Previous examinations of non-Arabidopsis *CRF* genes have shown links to pathogen response when overexpressed for Pti6 from Tomato (*SlCRF1*) 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 *SlCRF1* is induced by factors ethylene and salicylic acid linked to this process, and supports this previous reported role for *SlCRF1* (Zhou et al., 1997; Gu et al., 2002). Our finding that several other *SlCRFs* are

affected by these similar treatments may suggest that an effect on pathogen response could be a broader functional characteristic of some *SlCRFs*.

In summary, this work characterizes the cytokinin response factors in tomato (SlCRF1 to 11). We show that *SlCRFs* are expressed at varying levels over a range of tissues. Several *SlCRFs* show strong induction by cytokinin similar to that previously noted for Arabidopsis *CRFs*. Additionally, some *SlCRFs* 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 *SlCRFs* appear to have multiple regulatory functions in tomato plants.

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*Chapter 3

Characterization of Two Tomato AP2/ERF genes, *SlCRF1* and *SlCRF2*, in Hormone and Stress Responses

Abstract

Cytokinin is an essential plant hormone involved in the regulation of many growth and developmental processes and many of the cytokinin signaling pathway components have been well characterized. Cytokinin Response Factors (CRFs) that form a branch of this pathway are less well understood, though previous studies have linked them to developmental processes and some stress responses. This study examines the tomato *CRF* genes, *SlCRF1* and *SlCRF2* presenting a detailed characterization of their developmental expression patterns, transcriptional regulation by hormones particularly cytokinin, and response to abiotic stresses. Both *SlCRF1* and *SlCRF2* were predominantly expressed in vasculature in tissues throughout the plant, with an overall trend for greater *SlCRF2* expression in younger organs and the hormone regulation of *SlCRF1* and *SlCRF2* transcripts is primarily by cytokinin. Cytokinin induced both *SlCRFs* in different organs over a range of developmental stages; yielding the strongest induction in leaves and revealing that *SlCRF2* is induced to higher levels than *SlCRF1*. Examination of SlCRF transcripts during abiotic stress responses revealed that *SlCRF1* and *SlCRF2* have distinct

*Submitted to Plant Cell Reports.

patterns of regulation from each other and between leaves and roots. This includes a strong induction of *SlCRF1* by cold stress in leaves and roots, a strong induction of *SlCRF2* by oxidative stress in roots and unique patterns of induction/repression in response to drought and during recovery. Overall this study reveals a clear picture of *SlCRF1*and *SlCRF2* expression patterns across tissues during development and in response to cytokinin and specific stresses, indicating their importance in plant growth and environmental responses.

Introduction

Cytokinin is an essential plant hormone known to be involved in numerous plant growth and developmental processes (Mok and Mok 2001; Werner and Schmülling, 2009). In addition to the well-established model of cytokinin signaling (two-component system-like multistep phosphorelay) (To and Kieber, 2008; Werner and Schmülling, 2009), a branch pathway of cytokinin signaling featured by Cytokinin Response Factors (CRFs) has been proposed (Rashotte et al., 2006).

CRFs are a subgroup of the AP2/ERF transcription factor family that is defined as having at the protein level a CRF domain and an AP2 DNA binding domain (Rashotte and Goertzen, 2010). CRFs have recently been further classified into five distinct CRF clades (I-V) based on the presence of a clade-specific C-terminal region in their protein sequences (Zwack et al., 2012). Previous studies have shown that CRFs are involved in numerous aspects of plant life such as regulation by hormones (Rashotte et al., 2006; Schlereth et al., 2010; Shi et al., 2012; Zwack et al., 2013), cotyledon, leaf, and embryo development (Rashotte et al., 2006), responses to biotic and abiotic stresses (Zhou et al., 1997; Park et al., 2001; Gu et al., 2002; Shi et al., 2012; Jeon and Kim, 2013), negative

regulation of leaf senescence (Zwack et al., 2013), and positive regulation of plastid division (Okazaki et al., 2009).

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

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

Materials and Methods

Plant materials and growth conditions

The tomato dwarf cultivar Micro-Tom was used for all experiments. Plants were grown in Sunshine Mix #8 soil or magenta boxes containing MS medium (pH 5.8)

supplemented with Gamborg B5 vitamins and 2% sucrose under a 16h/8h light/dark photoperiod at 150 $\mu E \cdot m^{-2} \cdot s^{-1}$, with a 26 ℃ day, 22 ℃ night temperature.

Arabidopsis thaliana (Col-0): Sterilized seeds were germinated on petri dishes containing MS medium (pH 5.7) plus 1% sucrose. Plants were grown in controlled environment chambers under a 16h/8h light/dark photoperiod at100 $\mu E \cdot m^{-2} \cdot s^{-1}$, with a 22 ℃/18 ℃ day/night temperature.

RNA isolation, cDNA synthesis, and expression analysis

Leaves, stems, and roots harvested from Micro-Tom plants or patted dry after treatments were immediately flash-frozen in liquid nitrogen. RNA was then extracted using Qiagen RNeasy Kit according to the manufacturer's instructions. 500 ng of the total RNA was used for each sample in the subsequent reverse transcription with Quanta qScript cDNA supermix. The resulting cDNA samples were diluted prior to qPCR. qPCR was performed with the SYBR-Green chemistry in an Eppendorf Mastercycler ep realplex with gene specific primers: qSlCRF1F 5'-AACGATGTCGCTTTGTCACC-3'; qSlCRF1R 5'-GGGCAAAATCGTCAAAGTCA-3'; qSlCRF2F 5'-ATGCTGCCGGTCCTAGAGTT-3'; qSlCRF2R 5'- GAGCAGTTTCCGACGATGAC-3'; TIP41 Fw 5'-ATGGAGTTTTTGAGTCTTCTGC-3'; TIP41Rv 5'-GCTGCGTTTCTGGCTTAGG-3'; or SlelFFw SlelFRv for stress experiments. Each reaction has a total volume of 20 μL. The qPCR program consists of one cycle at 95 ℃, followed by 40 cycles of 15 sec at 95 ℃, 45 sec at 57 ℃/56 ℃ (stress), and 40 sec/50 sec (stress) at 68 ℃. For leaf samples treated by CK, another set of gene specific primers were used: SlCRF1F 5'-GGAAAATTCAGTTCCGGTGA-3'; SlCRF1R 5'-AAAATTGGTAACGGCGTCAG-3'; SlCRF2F 5'-TGCCGGTCCTAGAGTTGTAA-3';

SlCRF2R 5'-CAGTGGCTGCTCTGCTCTAT-3'; and the qPCR program consists of one cycle at 95 ℃, followed by 40 cycles of 15 sec at 95 ℃, 30 sec at 56 ℃, and 35 sec at 68 ℃. The relative expression data used in the figure represent means ± SE of two biological replicates. All samples excluding those from stress treatments were compared to the control gene *TIP41* (Expósito-Rodríguez et al., 2008) and samples from stress treatments (including ABA treatment) were compared to the control gene *SlelF*. Because we found TIP41 expression was influenced by same stress conditions, thus not serving as a true control, we used *SlelF* as the control for stress experiments.

Hormone and Stress Treatments

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

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

For osmotic stress (200mM mannitol) and oxidative stress treatments (10mM and 20mM hydrogen peroxide), plants were treated in the same way as hormone treatments.

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

Histochemical analysis

For GUS activity analysis, excised tissues were placed into GUS staining buffer (Weigel and Glazebrook 2002) and vacuum infiltrated for 20 min followed by additional incubation: overnight for tomato or 2–3 h for Arabidopsis. Stained tissue was then cleared in 70% ethanol, and examined with a dissecting microscope. Photos were taken with a Qimaging Fast 1394 digital camera and are presented as composite images using Adobe Photoshop CS3 without altering the original integrity.

Generation of transgenic plants

SlCRF expression (destination) vectors used in this study were generated through the Invitrogen Gateway cloning technology according to the manufacturer's instructions. *SlCRF1*promoter::GUS destination vector was generated as in Zwack et al. 2012. The promoter (2 kb upstream of ATG) sequence and coding sequence of *SlCRF2* were amplified using sequence-specific primers with att-B sites. Purified PCR products were introduced into the pDONR221 entry vector (Invitrogen) through a BP reaction. The resulting *SlCRF* entry vectors were sequenced to determine proper insertion and DNA sequence. Then *SlCRF* entry vector products were introduced to destination vector pKGWFS7 (Karimi et al., 2002) or pK2WG7 via a LR reaction to create *SlCRF*

expression vectors. The expression vectors were then transformed into *Agrobacterium tumefaciens* C58. *Agrobacteria* containing *SlCRF2* promoter or coding sequence were streaked onto LB agar medium and sent to the Plant Transformation Research Center (PTRC) at University of California at Riverside for transformation of Micro-Tom plants as a service as in Zwack et al., 2012. *Agrobacteria* containing *SlCRF1* or *SlCRF2* promoter were also used to transform *Arabidopsis* plants using the floral dip method (Clough and Bent 1998).

Results

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

Previous studies using promoter::GUS reporter lines have shown that *SlCRF1* is predominantly expressed in the vasculature of different plant organs, although its expression can also be seen in epidermal cells, mesophyll of young leaves, and the pericarp of unripe fruits (Zwack et al., 2012). Here we further detailed the expression of *SlCRF1* across a greater range of tissues and developmental stages from seed through fruit production, revealing previously unreported expression patterns of *SlCRF1* as well as confirming previous findings of strong vascular expression (Figure 3.1). Novel expression of *SlCRF1* promoter::GUS reporter lines was found strongly in hypocotyls of young seedlings (Figure 3.1a, b) and flower sepals (Figure 3.1i), in addition to vascular expression in leaves of different age (13, 24, and 35d old) (Figure 3.1c-e). Weaker *SlCRF1*expression can also be seen in the stamens of flowers (Figure 3.1h, j, k). The strong expression of *SlCRF1* in leaves and unripe fruits is further supported by microarray data of tomato organs obtained through tomato eFP browser at bar.utoronto.ca (Figure 3.2).

Figure 3.1 *SlCRF1* promoter-driven GUS reporter gene expression in tomato. **a** 4d-old seedling. **b** 7d-old seedling. **c** 13d-old seedling. **d** 24d-old leaflet. **e** 35d-old leaflet. **f** Unripe fruit. **g** Ripe fruit. **h** Whole flower. **i** Sepals and pistils. **j** Stamens. **k** Free-hand cross section of stamens shown in **j**. **l** Pedicle of the flower

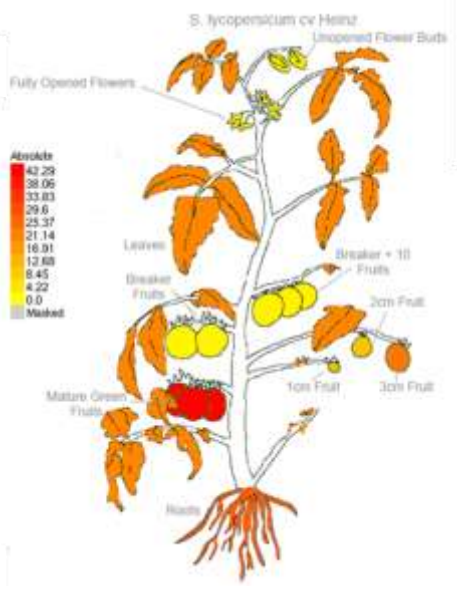

Figure 3.2 Microarray data for *SlCRF1* expression from the tomato eFP browser at bar.utoronto.ca. The strong expression of *SlCRF1* in leaves and unripe fruits supports the

spatial expression of *SlCRF1observed through SlCRF1* promoter::GUS reporter line analysis

SlCRF2 promoter::GUS lines were also generated to determine the expression pattern of this gene. Analyses of these lines revealed that *SlCRF2* has a strong pattern of vascular expression in many tissues throughout development, similar to other Arabidopsis *CRFs* (*AtCRFs*) and *SlCRF1* (Figures 3.1, 3.3). Vascular expression patterning of *SlCRF2* can be seen in cotyledon, leaf, stem, root, and immature green fruit (Figure 3.3c-f, j). A similar pattern was also observed for the same *SlCRF2* promoter::GUS construct transformed into Arabidopsis, as shown in vascular tissues of seedling tissues (Figure 3.3m-o). Importantly *SlCRF2* expression is not solely limited to vascular tissues, as it can also been strongly seen in leaf primordia, root tips, and flower stamens (Figure 3.3a, b, k, l, n, g-i). This suggests that the function of *SlCRF2*, like *SlCRF1*, is probably in a vascular related process, but not limited to roles only in that tissue. The broad expression of *SlCRF2* in leaves and roots is also supported by microarray data obtained through tomato eFP browser at bar.utoronto.ca (Online Resource 3.2).

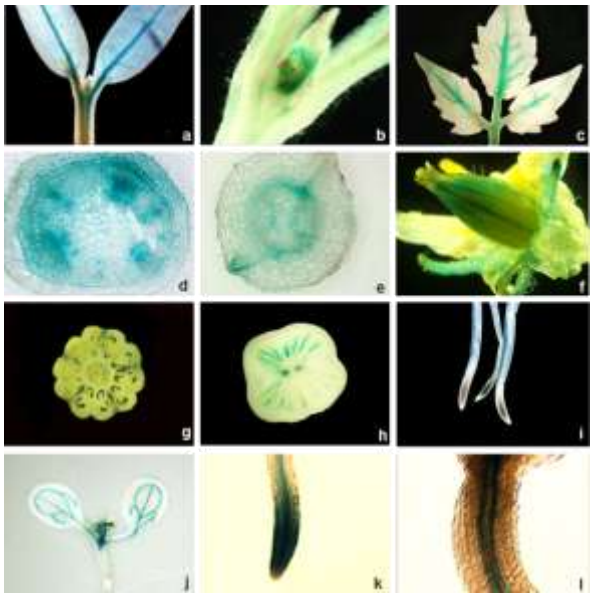

Figure 3.3 *SlCRF2* promoter-driven GUS reporter gene expression in tomato and *Arabidopsis*. Tomato: **a** 13d-old seedling. **b** Close-up of 13d-old seedling showing staining in leaf primordium. **c** Fully expanded leaf. **d** Free-hand stem cross section. **e** Free-hand root cross section with emerging lateral root. **f** Flower showing stained stamens. **g** Free-hand cross section of stained stamens in **f**. **h** Free-hand unripe fruit cross section. **i** 13d-old roots showing staining in the root tip. *Arabidopsis* 13d-old seedling: **j** Apical tissue showing stained cotyledon vasculature and shoot apex. **k** Primary root tip. **j** Hypocotyl

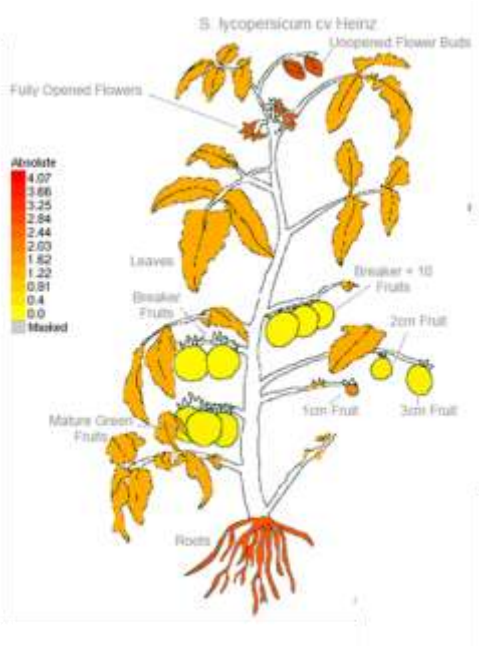

Figure 3.4 Microarray data for *SlCRF2* expression from the tomato eFP browser at bar.utoronto.ca. The expression of *SlCRF2* in leaves and roots supports the spatial expression of *SlCRF2 observed through SlCRF2* promoter::GUS reporter line analysis

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

To better understand the spatial and temporal expression pattern of *SlCRF1* and *SlCRF2*, qPCR was performed. RNA was taken from leaves, roots, and stems across development: seedling stage (13d), young plant (24d), and mature flowering plant (35d). Additionally at 18d expression levels in all developed leaves were examined based on order of emergence (1st emerged = oldest to 4th emerged = youngest). The highest levels

of *SlCRF2* were found primarily in young organs (13d and 24d-old leaves, 13d old stem, and root, and the younger third and fourth leaves; Figure 3.5), with much lower or unchanged levels of expression in older organs or roots. This pattern of transcript expression in these organs generally matches the promoter::GUS reporter gene analysis observed (Figure 3.3). In direct contrast *SlCRF1*expression levels were at their lowest in most of the youngest samples of the organs examined (13d-old leaf and root, the youngest fourth leaf; Figure 3.5a, c, d), thus are higher in older tissues. A spatial examination of different root tissues for *SlCRF1* also showed highly reduced expression levels in the youngest parts of the root (Figure 3.5e): the lateral roots and root tips relative to whole root. Again these transcription expression patterns are consistent with the results of the promoter::GUS reporter gene analysis (Figure 3.1). Together these profiles along with the promoter analyses reveal distinct spatial and temporal patterns for *SlCRF1* and *SlCRF2*.

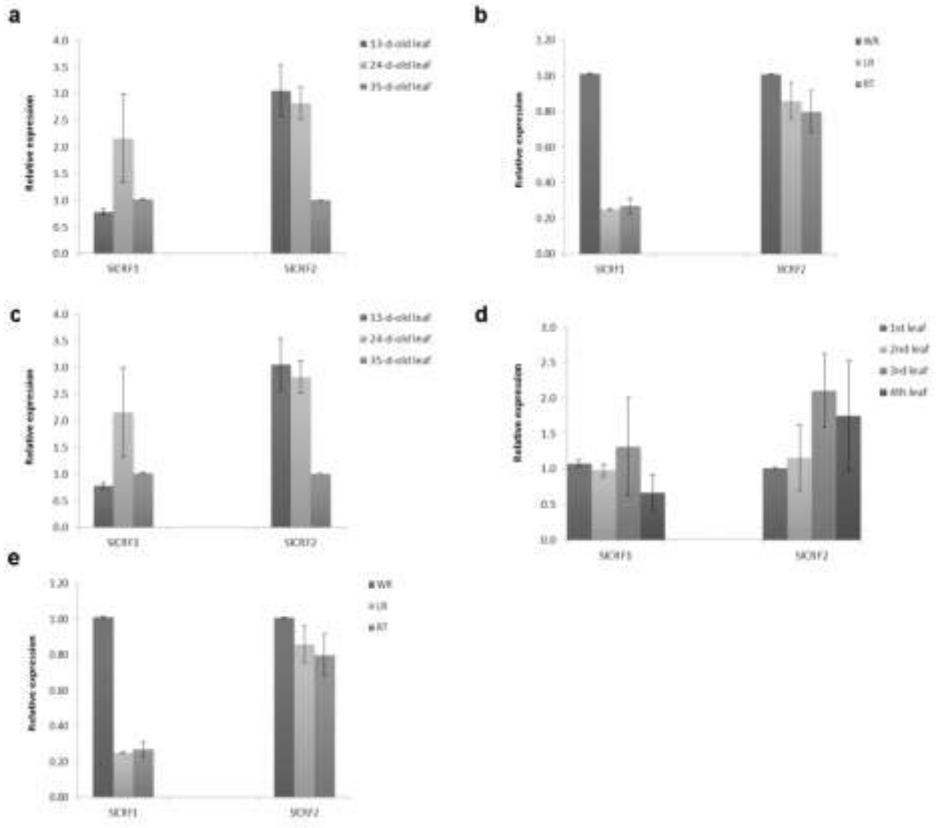

Figure 3.5 qPCR analysis of *SlCRF1* and *SlCRF2* expression in various organs of 13, 24, and 35d-old tomato plants. Relative expression of *SlCRF1* and *SlCRF2* in: **a** leaves. **b** stems. **c** roots. **d** True leaves relative to the 1st true leaf in 18d-old plants, where leaves are based on emergence: 1st oldest, 4th youngest or last to emerge. **e** In different parts of the root: WR (whole root), LR (later root), RT (root tip) collected from 15d-old plants grown in pouches. Error bars represent SE of two biological replicates

SlCRF1 and *SlCRF2* are transcriptionally regulated by CK mainly in leaves

Subsets of *CRF* genes have been shown to be inducible by cytokinin to varying degrees as summarized in Zwack et al. 2012. *SlCRF1* and *SlCRF2* have previously been examined for cytokinin regulation, but in a limited fashion: only in young leaf tissue (15d), at a single cytokinin concentration (5μM BA) at early time points (1 and 3h) (Shi et al., 2011). To provide a greater understanding of how cytokinin regulates the level of these *SlCRF* transcripts, both leaves and stems across development (13, 24, and 35 days) were treated for multiple lengths of time (2, 7, and 24h) with different levels of cytokinin

(1, 5, and 10 μM BA). Treated samples were compared to the solvent vehicle DMSO and from extracted RNA, qPCR analyses were conducted. Additional qPCR examinations were performed in different root tissues (whole roots (WR), lateral roots (LR), and root tips (RT)) in 14d old plants treated with a range of cytokinin (1, 5, and 10 μM BA) or DMSO for 24h. Both *SlCRF1* and *SlCRF2* were shown to be inducible by cytokinin treatment over a range of treatment times in different organs at different developmental stages beyond what was previously shown, suggesting an active role for cytokinin in the regulation of these genes throughout the lifetime of the plant. Both *SlCRF1* and *SlCRF2* showed their strongest induction by the highest level of cytokinin (10 μM) in 13d-old leaves, with *SlCRF1* induced to 4.0 fold at 2h after treatment and *SlCRF2* induced 12.8 fold at 7h (Figure 3.6a, b). *SlCRF1* also showed more than 2 fold induction by cytokinin in 24d old leaves at 2h and 7h of treatment as well as in 13d old stems at 2h (Figure 3.6a, c). *SlCRF2* showed a wide range of cytokinin induction (more than 2 fold) in leaves at each age of plant examined, at multiple time points, and at different cytokinin concentrations (Figure 3.6b). *SlCRF2* was also induced by cytokinin in stems at different ages as well (Figure 3.6d). It is interesting to note clear differences in cytokinin induction between *SlCRF1* and *SlCRF2*, such as the strong cytokinin induction of *SlCRF2* in 35d-old leaves at each of the time points examined, whereas *SlCRF1* appears to be not regulated by cytokinin at this developmental stage, despite induction in leaves at earlier stages (Figure 3.6a, b).

For the most part cytokinin regulation of *SlCRF1* and *SlCRF2* transcripts appears to be lacking in whole roots and specific root parts, except possibly for root tips in *SlCRF1* at the highest cytokinin concentration (Figure 3.6e). This suggests that a

cytokinin regulated role for these *SlCRFs* seems to be primarily in aerial tissues, likely the leaves where *SlCRF1*and *SlCRF2* transcript levels are induced to the highest levels.

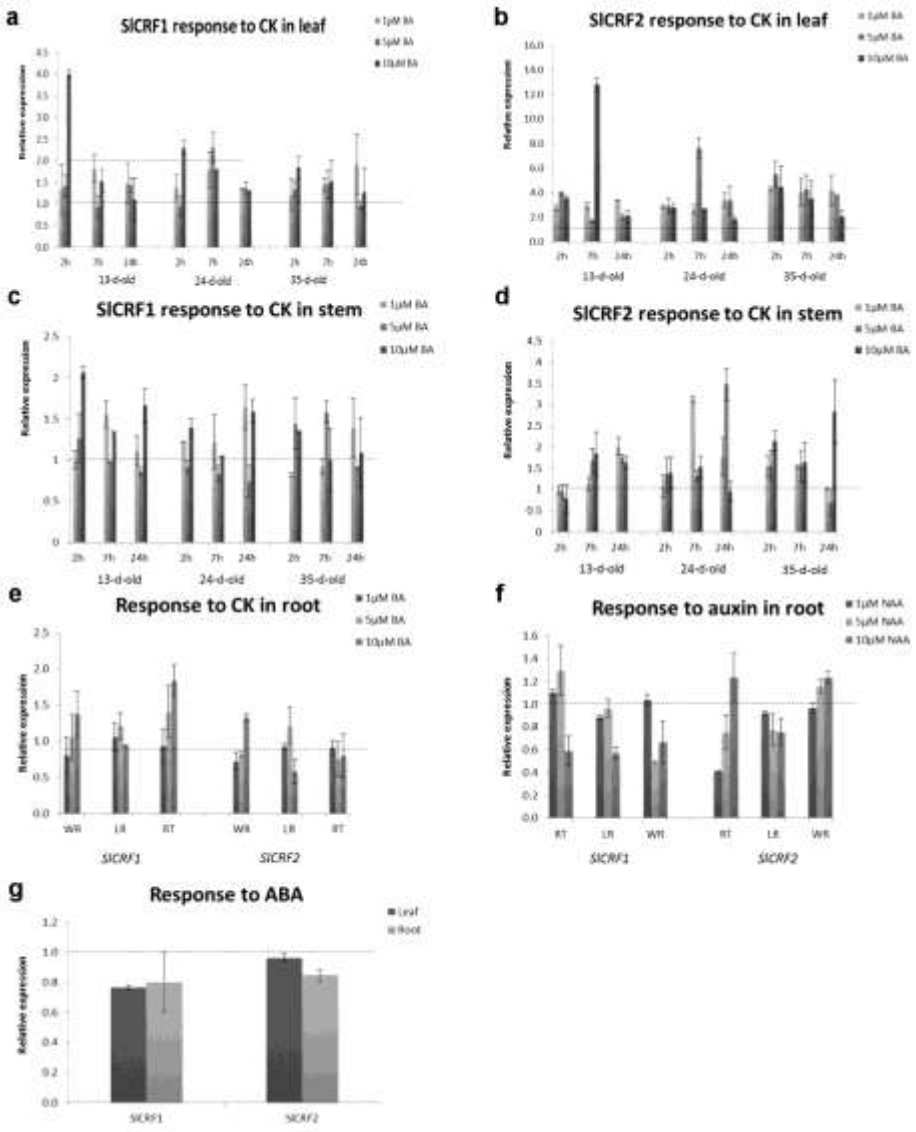

Figure 3.6 qPCR analysis of *SlCRF1* and *SlCRF2* expression in response to hormones. Expression response to cytokinin (1, 5, and 10μM BA vs. a DMSO control) for 2, 7, and 24h in 13, 24, and 35d-old plants. In leaves: **a** *SlCRF1*. **b** *SlCRF2*. In stems: **c** *SlCRF1*. **d** *SlCRF2*. *SlCRF1* and *SlCRF2* expression in different parts of the root: WR (whole root), LR (lateral root), RT (root tip) collected from 15d-old plants grown in pouches: **e** In response to cytokinin (1, 5, and 10μM BA vs. a DMSO control). **f** In response to auxin (1, 5, and 10μM NAA vs. a DMSO control). **g** Expression of *SlCRF1* and *SlCRF2* in response to ABA in leaves and roots from 25-d-old plants. Error bars represent SE of two biological replicates

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

To determine if the expression levels of *SlCRF1* and *SlCRF2* might be affected by treatment of other hormones, qPCR was performed from samples prepared from tissues treated by auxin and ABA for 24h compared to a solvent carrier control, DMSO. *SlCRF1* expression was slightly decreased in different root tissues at the highest concentrations of auxin (5 and 10 μM NAA), while *SlCRF2* showed little effect except at the lowest auxin concentration (1 μM NAA) in RT (Figure 3.6f). ABA treatment resulted in only a minor decrease in either *SlCRF1* or *SlCRF2* transcript levels in leaves and roots (Figure 3.6g).

SlCRF1 and _SlCRF2_ are regulated by abiotic stresses

In order to more thoroughly understand if *SlCRF1* and *SlCRF2* might be regulated by other factors, the transcriptional response of these genes in leaves and roots was examined in response to a range of different abiotic stresses: temperature, osmotic, oxidative, flooding, and drought followed by recovery (Figure 3.7). Both *SlCRFs* responded to different stress treatments in a unique manner, suggesting potential distinct roles of reach gene in stress response.

For temperature stress both cold (4 ℃ for 24h), and heat (45 ℃ for 1h) stresses were examined. Neither cold nor heat stress resulted in much change in response of *SlCRF2* transcript (Figure 3.7a), suggesting it may not be temperature regulated. In contrast, *SlCRF1* was highly induced by cold in leaves (3.0 fold) and roots (3.0 fold), and was repressed to 0.3 fold untreated levels by heat in roots (Figure 3.7a). Further examination of *SlCRF1* in response to cold over 24 hours showed that cold induction, over 2 fold occurs by 1h and is maintained through the 24h treatment in roots, however, induction in leaves after 24h (3.2 fold) did not occur at the earlier time points examined

(Figure 3.7b). This illustrates a clear difference in temperature responsiveness between these two *SlCRFs*.

To determine whether *SlCRF1* and *SlCRF2* are involved in osmotic stress response, plants were treated by 200mM mannitol for 3h. Neither *SlCRF1* nor *SlCRF2* showed much change in transcript level in response to osmotic stress, with all leaf and root expression levels being 0.7-1.4 fold of a normal 1.0 level (Figure 3.7c).

Response to oxidative stress was examined by treating plants with hydrogen peroxide (10mM and 20mM) for 3h. A minor induction in leaves and roots was seen for *SlCRF1* at the lower, but not higher H_2O_2 concentration. In contrast, *SlCRF2* was strongly induced by both H_2O_2 levels only in roots. (Figure 3.7d), again indicating a clear difference in stress response between these *SlCRFs* and potential organ specificity in response.

Flooding response was examined by determining *SlCRF1* and *SlCRF2* transcript levels in plants kept in water logged conditions for four days. A similar minor response was noted for both *SlCRF1* and *SlCRF2* revealing a slight 1.4-1.6 fold increase in roots transcripts and a 1.4-1.7 fold reduction in leaves (Figure 3.7e).

Expression levels of *SlCRF1* and *SlCRF2* were examined in drought stressed plants that were not watered for 7 days, as well as during the first 12 hours of recovery after watering (Figure 3.7f, g). In leaves, *SlCRF1* expression was slightly reduced due to drought stress, but during recovery level became highly reduced at 1h (5.6 fold), 3h (5.0 fold), and 6h (3.7 fold) before returning to a normal level at 12h (Figure 3.7f). A different pattern was seen for the root transcript level of *SlCRF1* that were reduced by 2 fold due to drought and continued to decrease to a higher degree over time during the recovery

period examined (Figure 3.7g). This suggests that *SlCRF1* is likely involved in drought stress, particularly during the immediate recovery period after re-watering.

A distinct pattern of transcript regulation was seen for *SlCRF2* for drought stress and recovery (Figure 3.7f, g). In response to drought *SlCRF2* expression was increased by 1.8 fold in leaves, yet reduced about a similar amount in roots. During recovery in leaves *SlCRF2* was at its lowest level at 1h (2.9 fold) , reaching a normal level at 3h and 6h, then increasing to (2 fold) at 12h, which was similar to drought stress levels (Figure 3.7f). In roots *SlCRF2* was at its highest level at 1h followed by a steady decline to 5 fold reduced levels by 12h (Figure 3.7g). This also suggests a potential role for *SlCRF2* during drought stress and recovery, although based on distinct expression patterns likely different from that of *SlCRF1*.

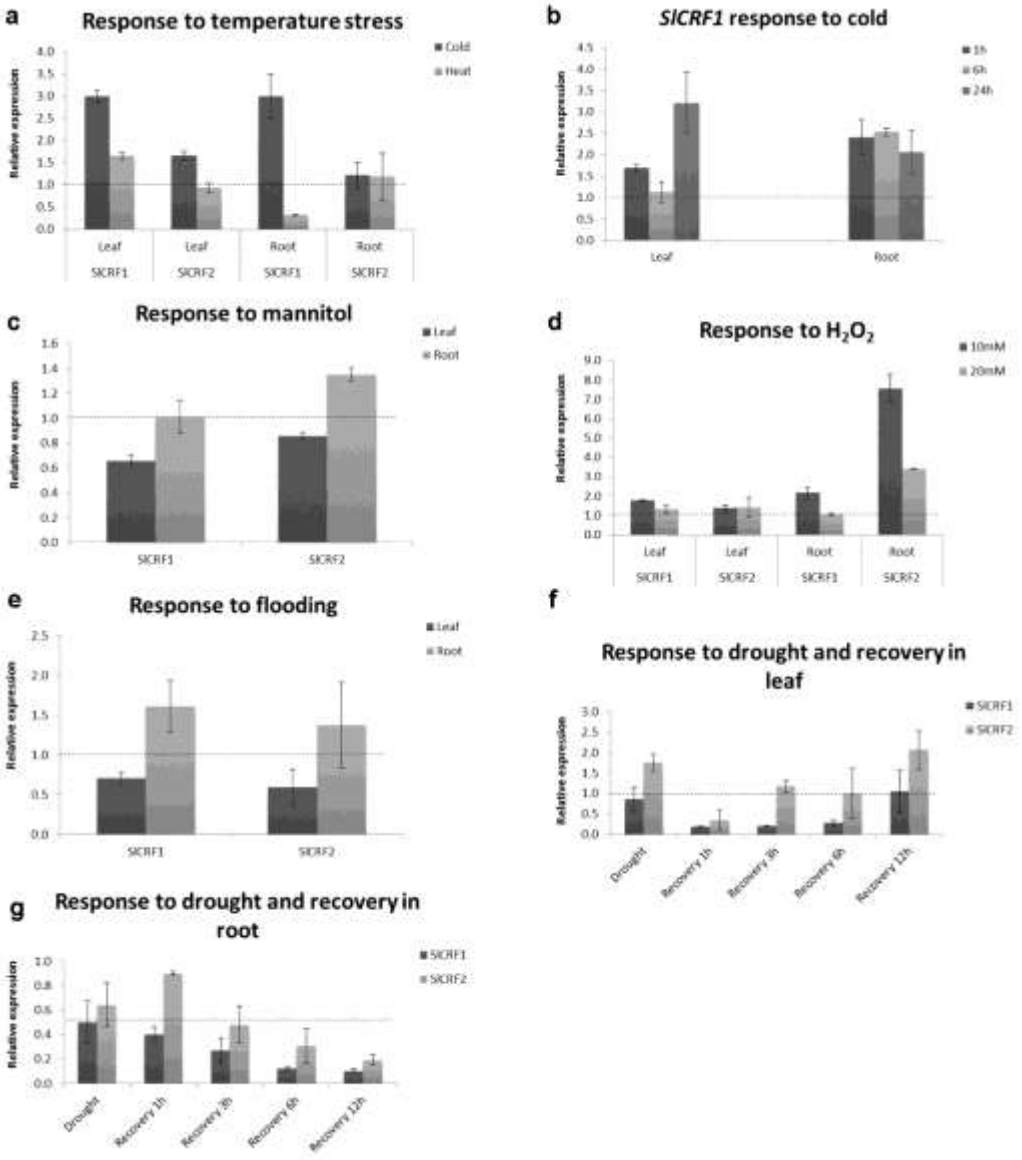

Figure 3.7 qPCR analysis of *SlCRF1* and *SlCRF2* expression in response to various abiotic stresses in leaf and root of 25d-old plants. Magenta box grown plants: **a** Cold (4 ℃) for 24h and heat (45 ℃) for 1h. Soil grown plants: **b** Time course of *SlCRF1* expression in response to cold (4 ℃). **c** Osmotic stress (200mM mannitol for 3h). **d** Oxidative stress (10 and 20mM hydrogen peroxide for 3h). **e** Flooding (waterlogged conditions for 4d). Drought (7d without watering) and recovery (1-12h after re-watering) in: **f** leaf. **g** root. Error bars represent SE of two biological replicates

Discussion

CRFs are known to be important AP2/ERF transcriptions factors members linked to the cytokinin signaling pathway and cytokinin responses, with much of the work on

this group having been conducted in *Arabidopsis* (Rashotte et al., 2006; Cutcliffe et al., 2011). However, recent reports have shown that CRFs are found in all land plants in similar numbers (~12) and can be further divided in each species into five distinct subgroups or clades (I to V), which likely have distinct plant functions (Zwack et al., 2012). We previously published the initial report, broadly describing CRFs in tomato, known as SlCRFs (Shi et al., 2011). Here we provide a detailed examination of two *SlCRFs*, *SlCRF1* and *SlCRF2* expression patterns across development and in response to hormones and abiotic stresses.

A recent study has shown that most *Arabidopsis CRFs* and *SlCRF1* are preferentially localized to vascular tissues, especially phloem, across the plant likely due to an enrichment of phloem targeting *cis*-elements in the promoters of CRFs (Zwack et al., 2012). Here we examined if there were a similar vascular localization pattern in tomato by expanding on previous *SlCRF1* work across development and presenting novel *SlCRF1* expression using CRF promoter:GUS reporter lines. From these lines we determined that vascular expression in *SlCRF1* is present from the seedling stage, in leaves throughout development, as well as occurring in sepals and fruits (Figure 3.1). While *SlCRF1* appears to be predominantly in vasculature, expression is also present in stamens and other tissues such as leaf mesophyll and fruit pericarp (Figure 3.1).

SlCRF2 was also found to be predominantly expressed in vascular tissues throughout the plant including cotyledons, leaves, roots, and fruits (Figure 3.3a, c-e, h). This could also be seen when the *SlCRF2* promoter:GUS reporter construct was stably transformed into *Arabidopsis* (Figure 3.3j-l). In a manner similar to *SlCRF1*, *SlCRF2* expression could also be seen in stamens and non-vascular tissues such as leaf primordia

and root tips (Figure 3.3b, f, g, I, k). *SlCRF2* also shows comparable expression patterns to the orthologous *Arabidopsis* Clade I members *AtCRF1* and *AtCRF2*: having vascular expression in leaves, cotyledons, hypocotyls/stems, and roots, and expression in young leaf primordia. Interestingly, there are differences including expression of *SlCRF2* in root tips (Figure 3.3i, k), which is lacking in *AtCRF1*, although not in *AtCRF2* and notably *SlCRF2* expression in reproductive organs such as stamens (Figure 3.3f-h), which has not been seen in any other Clade I CRF (Zwack et al., 2012). As *SlCRF1* is a Clade IV CRF member, of which there is no direct ortholog in *Arabidopsis* a similar comparison of this gene expression to *Arabidopsis* studies cannot be made.

A transcription analysis of *SlCRF1* and *SlCRF2* expression by qPCR in different organs throughout development generally supports the promoter:reporter line expression found for each gene (Figure 3.5). In addition, there is a pattern of differential expression as the plant ages, with *SlCRF2* showing higher expression levels in younger (13d) tissues and leaves, whereas *SlCRF1* has a slight trend in the opposite direction (Figure 3.5).

Transcription induction of CRFs by cytokinin appears to occur only in specific CRF clades, which include Clades I and IV containing *SlCRF1* and *SlCRF2* (Zwack et al., 2012). Previous findings of *SlCRF* cytokinin induction have been limited to leaf tissues in young plants, while here we present a broader examination of cytokinin-responsiveness for *SlCRF1* and *SlCRF2* in different tissues and developmental stages using a range of cytokinin concentrations and treatment durations (Rashotte and Goertzen, 2010; Shi et al., 2012). While both *SlCRFs* were found to be induced (2+ fold) in leaves and stems under different cytokinin concentrations at different ages after different treatment times, each has a unique induction pattern (Figure 3.6). *SlCRF1*

showed induction by cytokinin, generally at the highest concentrations (10μM BA), in young plants (13d), with a short treatment exposure (2h). The highest levels of cytokinin induction of *SlCRF1* were over 4 fold in leaves (Figure 3.6). In contrast, *SlCRF2* showed induction by cytokinin at every developmental stage and a wide range of concentrations and treatment lengths in both leaves and stems. *SlCRF2* was induced to higher levels in leaves and generally showed higher induction levels than *SlCRF1* (Figure 3.6), indicating that it may be the more cytokinin responsive of these *SlCRFs*. Neither gene was greatly affected by cytokinin in roots, however, *SlCRF1* was moderately induced (between 1.5-2.0 fold) in root tips at the highest cytokinin level.

Examination of *SlCRF1* and *SlCRF2* for regulation by auxin and ABA showed only minor changes in the transcripts of these genes, with the largest effect being moderate reductions (between 1.5 and 2.0 fold) for *SlCRF1* with auxin (Figure 3.6f, g). These finding are consistent with previous examination of *SlCRFs* to other hormones (ethylene, methyl jasmonate, and salicylic acid) revealing no change of transcript level in *SlCRF2* and only slight regulation for *SlCRF1* by ethylene and salicylic acid (Shi et al., 2012). Taken together, these results suggest that hormone responsiveness of *SlCRF1* and *SlCRF2* appears to be primarily from cytokinin.

Previous studies have shown that *CRF* genes can be involved in both abiotic and biotic stress responses in addition to cytokinin regulation (Zhou et al., 1997; Park et al., 2001; Gu et al., 2002; Shi et al., 2012; Zwack et al., 2013). An examination of *SlCRF1* and *SlCRF2* to determine if they might similarly respond to stress indicated that both *SlCRFs* are regulated by abiotic stresses with overall unique expression patterns (Figure 3.7). *SlCRF1* was induced by cold treatment in both leaves and roots by cold (Figure

3.7a, b), suggesting a role for *SlCRF1* in cold stress response. Several *Arabidopsis CRFs* have also been implicated in cold response: *CRF3* and *CRF4* (Compton 2012), *CRF6* (Zwack et al., 2013), and *CRF2* (Jeon and Kim, 2013). In fact, several specific genes of the cytokinin signaling pathway have been implicated as mediators of cold response, including ARR1, AHP2, AHP3, and AHP5, and the cytokinin receptors AHK2 and AHK3 (Jeon and Kim, 2013). Since cytokinin signaling two-component systems appear to be conserved across plant species, it is possible that *SlCRF1*is also induced by cold through similar mechanism in tomato. It is interesting to note that despite *AtCRF2* being linked to cold response (Jeon and Kim, 2013), its tomato ortholog *SlCRF2* was not affected by cold treatment in this study, suggesting that there may not be strict function orthology for cold response between *Arabidopsis* and tomato. This may not be too surprising since *SlCRF1* of which there is not *Arabidopsis* ortholog appears to be regulated by cold stress.

In contrast, both *SlCRF1*and *SlCRF2* are induced by oxidative stress in roots, but not leaves, although *SlCRF2* is induced to much higher levels than *SlCRF1* (Figure 3.7d). Reactive oxygen species (ROS) such as H_2O_2 are produced in plants as byproducts of aerobic metabolism or in response to abiotic stresses and it has been proposed that H_2O_2 promotes adaptive responses to various stresses such as cold by serving as a stress signal in plants (Apel and Hirt, 2004; Desikan et al., 2001; Zhou et al., 2012). A recent study has implicated another CRF; *AtCRF6* in oxidative stress (H_2O_2) response in *Arabidopsis* leaves (Zwack et al., 2013). It is not clear what the induction of *SlCRF1*and *SlCRF2* means in stress related processes, except to connect them to various stress pathways.

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

Overall the examinations of *SlCRF1*and *SlCRF2* during abiotic stress have linked both genes to different processes. *SlCRF1* appears to be linked to cold, oxidative, and drought stresses from this study in addition to previous work connecting it to salt stress and biotic defense response (Gu et al., 2002; Shi et al., 2012). While this is the first report linking *SlCRF2* to any stress response, this gene appears to be linked to oxidative, and drought stresses.

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*Chapter 4

Transcriptome Analysis of Cytokinin Response in Tomato Leaves

Abstract

Tomato is one of the most economically and agriculturally important Solanaceous species and vegetable crops. It serves as a model for examination of fruit biology as well as compound leaf development. Cytokinin is a plant hormone linked to the control of leaf development that is known to regulate a wide range of genes including many transcription factors. To date there is little known of the leaf transcriptome in tomato and how it might be regulated by cytokinin. We employ high throughput mRNA sequencing technology and bioinformatic methodologies to robustly analyze cytokinin regulated tomato leaf transcriptomes. To examine cytokinin regulated gene expression at a transcriptome level in tomato leaves we shotgun sequenced messenger RNA with Illumina technology and assembled *de novo* the tomato leaf transcriptome. Leaf samples of two ages, 13d and 35d were treated with cytokinin or the solvent vehicle control dimethyl sulfoxide (DMSO) for 2h or 24h, after which RNA was extracted for sequencing. To confirm the accuracy of RNA sequencing results, we performed qPCR analysis of select transcripts identified as cytokinin regulated by the RNA sequencing approach. The resulting data provide the first hormone transcriptome analysis of leaves in

*Published in PLoS ONE 8(1): e55090.

tomato. Specifically we identified several previously untested tomato orthologs of cytokinin-related genes found as well as numerous novel cytokinin-regulated transcriptin tomato leaves. Principal component analysis of the data indicates that length of cytokinin treatment and plant age are the major factors responsible for changes in transcripts observed in this study. Two hour cytokinin treatment showed a more robust transcript response as indicated by both greater fold change of induced transcripts and much higher number of those transcripts that were cytokinin responsive in young compared to older leaves. Specifically the number of genes in known the cytokinin-related processes of signaling, metabolism, and transport, found to be induced in young leaves is twice as many as that in older leaves. This difference in transcriptome response in younger vs. older leaves was also found with an extended (24h) cytokinin treatment although to a lesser extent. Overall data presented here provides a solid foundation for future study of cytokinin and cytokinin regulated genes involved in compound leaf development or other developmental processes in tomato.

Introduction

Cytokinins are plant hormones that occur naturally as N6-substituted adenine derivatives. Over 50 years of study has implicated this class of hormones in many aspects of plant growth and development, including de-etiolation, chloroplast differentiation, apical dominance, and leaf senescence [1, 2]. They have also been shown to regulate leaf development and stress response [3-5]. The cytokinin signaling pathway has been determined to be composed of cytokinin receptors (histidine kinases; HKs), signaling mediator histidine containing phosphotransfer proteins (HPts), and response regulators (RRs). It has been established along with a branch pathway that requires the HKs, HPts,

and cytokinin response factors (CRFs) [5-7]. There are two major classes of response regulators- type-A RRs and type-B RRs. Type-A RRs are primary cytokinin response genes that are rapidly induced by cytokinin and are negative regulators of cytokinin signaling which can be activated by transcriptional activator, type-B RRs [5, 8-11]. In addition to the cytokinin signaling components, major cytokinin metabolic genes have been identified, including isopentenyltransferases (IPTs) responsible for cytokinin biosynthesis and cytokinin oxidases/dehydrogenases (CKXs) involved in oxidative degradation of cytokinin [12-14]. Some *CKX* genes are up-regulated by cytokinin whereas *IPT* genes are repressed [3, 9, 12, 15].

The various roles played by cytokinin in plant growth and development have led to efforts of genome-wide analyses of cytokinin regulated gene expression in several species like Arabidopsis and rice and clearly show that a wide range of genes are transcriptionally regulated by cytokinin [3, 6, 9, 10, 15-18]. One class of genes regulated by cytokinins encodes transcription factors that play vital roles in plant growth and development [3, 6, 9, 16, 17]. These findings were widely supported by genetic and molecular studies. In Arabidopsis, cytokinin was shown to up-regulate *SHOOT MERISTEMLESS* (*STM*), a member of the class I KNOX transcription factors [19]; overexpression of *STM* dramatically activate cytokinin biosynthesis gene *AtIPT7*, indicating that KNOXI function in meristem maintenance is mediated by activation of cytokinin biosynthesis [20]. Cytokinin is also known to induce *Cytokinin Response Factor* (*CRF*) genes that have been shown to be involved in or expressed during cotyledon and leaf development [6, 7].

Although some transcriptome data are available for tomato, most of it is focused on fruit biology, defense response, or other aspects not related to cytokinin or leaves [21-25]. In fact, very little is known about the cytokinin regulation of genes in tomato. The advent of next-generation sequencing technologies has provided powerful means to perform effective and accurate analyses of transcriptomes and genomes [26-29]. RNA-sequencing (RNA-Seq) has been proven to be a simpler and more powerful approach to quantifying expression at a transcriptome level, especially in species like tomato where existing microarrays cover only about a third of all genes in the genome [27-29]. Here we used RNA-seq to perform the first transcriptome analysis of cytokinin response in tomato leaves and one of the few conducted in species other than Arabidopsis and rice. We examined genome-wide gene expression in response to cytokinin in 13d and 35d old tomato leaves detecting 28,606 unique transcripts and more than one thousand that showed a response (at least 2.5 log2 fold change) to cytokinin in various samples. Among these cytokinin responsive transcripts were previously un-examined tomato orthologs of cytokinin regulated genes from other species, such as cytokinin oxidases, type-A response regulators, and cytokinin receptors. Additionally, we identified several novel cytokinin regulated genes, including a Xanthine/uracil permease family protein and a Cytochrome P450 with abscisic acid 8'-hydroxylase activity that are both highly induced by cytokinin. This study generated the first complete transcriptome analysis of cytokinin in tomato leaves providing valuable data for identifying cytokinin regulated genes that are involved in leaf developmental processes.

Results and Discussion

Transcriptome Analysis

In order to conduct a full transcriptome analysis of cytokinin response in tomato leaves of different ages we assembled a custom transcriptome reference and assessed differential expression from paired-end (2x50bp) and singleton (1x54bp) Illumina RNA sequences. To obtain a broader sampling of transcripts found in leaf tissue at a single plant age, tomato leaves of two different ages, 13d and 35d, were treated with exogenous cytokinin, 5μM Benzyl Adenine (BA) or the solvent vehicle control dimethyl sulfoxide (DMSO) for 2h or 24h, after which RNA was isolated for sequencing. The messenger RNA was isolated via polyA selection and constructed into paired-end sequencing libraries with the TruSeq RNA sample preparation protocol from Illumina (San Diego, CA). RNA sequencing was performed on the Illumina HiSeq 2000 platform and Illumina GAIIX platform yielding an average of 18 million high-quality reads per sample (Table 4.1). In total, 131,158,386 2x50bp and 60,180,592 1x54bp reads were sequenced, resulting in over 16.4 Gbps of data. Paired-end sequences from all samples were pooled together to construct a de novo tomato leaf transcriptome assembly (see methods for details). The final assembly contained 28,606 synthetic ESTs and was used as a tomato leaf reference transcriptome for subsequent gene expression analyses.

Age	13 DAYS								35 DAYS							
Treatment	(B) +CYTOKININ treatment				(D) -CYTOKININ treatment				(B) +CYTOKININ treatment				(D) -CYTOKININ treatment			
Treatment Length	2 HOURS		24 HOURS		2 HOURS		24 HOURS		2 HOURS		24 HOURS		2 HOURS		24 HOURS	
Replicate	A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
High Quality Paired-End Reads	4,470,442	15,698,582	5,775,868	15,146,942	4,513,670	14,379,784	6,874,016	8,323,882	13,729,084	22,085,930	15,413,056	23,175,630	13,558,856	25,291,538	15,560,492	23,684,340
High Quality Single-End Reads	15,211,523	--	16,280,156	--	15,225,454	--	11,884,089	--	--	--	--	--	--	--	--	--
Uniquely aligning reads	8,567,251	7,492,978	9,388,806	8,216,386	9,090,012	7,629,681	6,903,736	6,489,199	7,346,044	13,925,649	8,027,102	13,904,662	7,871,216	14,547,760	8,324,948	14,322,386

Table 4.1. Transcriptome alignment and assembly statistics. The number of paired end and single end sequence reads and uniquely aligning reads analyzed from Illumina

sequencing runs of all 16 samples are described in the table by plant age, treatment type and length. Individual sample replicates, A and B, are a pool of leaf tissues from multiple plants treated under similar conditions. The 131,158,386 2x50bp paired-end reads were pooled and de novo assembled into a transcriptome assembly of 28,606 synthetic ESTs.

Gene expression was quantified as the total number of reads (paired-end and singleton reads) from each sample that uniquely aligned to the final *de novo* transcriptome reference assembly, binned by transcript using the aligner BWA (v0.5.9) [30]. An average of 9.5 million reads, from a combination of paired-end sequencing and single-end sequencing, uniquely aligned to the reference in each sample (Table 4.1).

An initial examination of the overall dataset, normalized with the TMM strategy [31] using principal component analysis (PCA) as implemented in SAS JMP Genomics 5.1, revealed that individual replicates used in this study, A and B, clustered together indicating relatively low biological variability within sample type (Figure 4.1). Variance decomposition (JMP Genomics 5.1) was used to estimate the proportion of total variance attributable to the experimental variables of age, treatment and length of treatment. Together the variables plant age, cytokinin treatment, and length of treatment account for about 73% of the variance in this study, with the major factors being length of cytokinin treatment (31.0%) and plant age (29.4%) (Figure 4.1). Although cytokinin treatment by itself accounts for a smaller amount of the variance in this study (12.3%), together with length of treatment cytokinin clearly plays a large role in the transcript changes seen in this study.

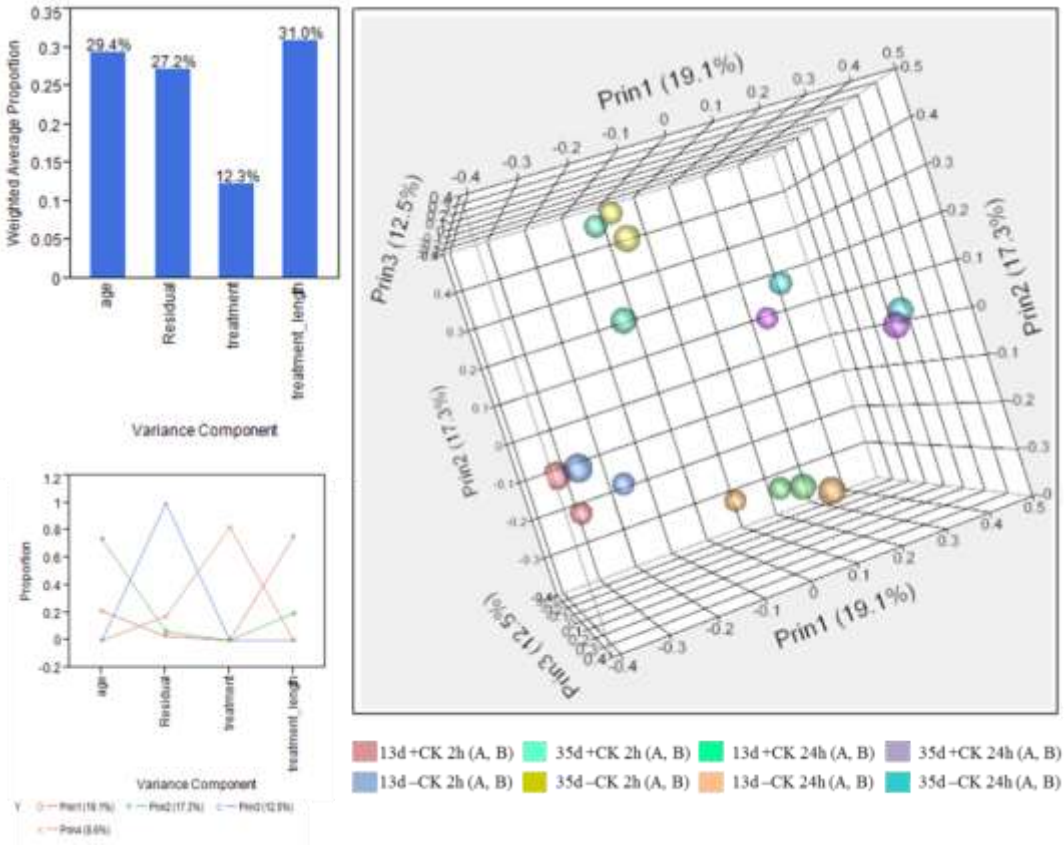

Figure 4.1. Principal Component Analysis and Variance Decomposition of Leaf Sample Variables. Principal component analysis (PCA) and variance decomposition (both as implemented in JMP Genomics 5.1) identify age of plant and cytokinin treatment length as the variables responsible for the majority of transcriptional variance, with cytokinin treatment playing a lessor role. Plots of these component principals in 2D and 3D reveal a strong clustering of individual sample replicates, A and B, as well as distinguishing age and treatment length groupings.

Cytokinin regulation of leaf genes in tomato

In order to determine the regulation of transcripts by cytokinin, differential expression analysis (see methods for details) was performed between treated and untreated samples. This revealed only a small number of different genes (8) as positively regulated by cytokinin across all treatments at a significant level ($p \leq 0.1$), although these same genes were regulated across different treatments. This includes 4 type-A cytokinin response regulators, a cytokinin receptor, a cytochrome p450 – ABA oxidase, a gag polyprotein,

and an unknown protein. Because this represents a small sample of the cytokinin regulated transcripts that have been identified in other species and this is the first study of cytokinin effects on tomato at a transcript level, we further investigated transcripts with high fold changes in response to cytokinin treatment that did not reach significance with DESeq. We define the transcripts that show a change of more than 2.5 log2 fold expression in response to cytokinin as cytokinin responsive genes (See Table 4.2, Table 4.3, 4.4). This is more than double the fold change for genes that have been identified as cytokinin regulated in other species, such as Arabidopsis and Rice using microarray analyses (set at 2 fold) [10]. With the same criteria, we also identified transcripts that are more abundant in young or older leaves (Table 4.5).

In order to confirm the accuracy of the RNA-seq expression results, qPCR was performed to quantify the expression of select transcripts. Four DE genes and four genes identified as cytokinin responsive in 35d plants after 24h cytokinin treatment vs DMSO were examined with qPCR (Table 4.6). Our qPCR analysis revealed similar induction levels and trends for all these genes as was seen from RNA-seq analyses, indicating that changes in expression found by RNA-seq appear to be accurate.

Categories	Early response 2h (BA vs. DMSO)				Late response 24h (BA vs. DMSO)				2h (DMSO)		24h (DMSO)	
Leaf age	13d		35d		13d		35d		13d	35d	13d	35d
Transcript changes	Induced	Repressed	Induced	Repressed	Induced	Repressed	Induced	Repressed	More abundant	More abundant	More abundant	More abundant
# of genes	60	669	14	279	97	95	91	73	926	168	198	123

Table 4.2. Summary of overall transcript changes seen in major compared categories. The number of genes identified as cytokinin responsive (showed at least a transcript change of 2.5 log2 fold) for each of the shown comparisons is listed from the sample reads shown in Table1. Induced (up-regulated 2.5 log2 fold vs control). Repressed (down-regulated 2.5 log2 fold vs control). More abundant (2.5 log2 fold greater than the other age sample at that treatment time).

Cytokinin induced transcripts in young plants (2h)			Cytokinin repressed transcripts in young plants (2h)		
Solyc_ID	Solyc_Description	log2FC*	Solyc_ID	Solyc_Description	log2FC
Solyc07g006630	CONSTANS-like protein	7.34	SGN-U568332	Unknown	-11.04
Solyc12g009930	UDP-glucuronosyltransferase 1-6	5.53	Solyc07g007700	Isoprenylcysteine carboxyl methyltransferase	-9.80
Solyc07g008520	Peptide transporter	5.49	Solyc07g062500	Cytochrome P450	-9.75
Solyc04g016430	SlCKX5	5.41	Solyc10g076250	Aminotransferase like protein	-9.65
Solyc06g069730	Chlorophyll a/b binding protein 4 chloroplastic	4.74	Solyc11g069050	4-coumarate-CoA ligase	-9.65
Solyc09g064910	tRNA dimethylallyltransferase	4.57	Solyc08g006420	Myosin-like protein (Fragment)	-9.40
Solyc07g043420	2-oxoglutarate-dependent dioxygenase	4.35	Solyc04g025650	Monooxygenase FAD-binding IPR003042 Aromatic-ring hydroxylase-like	-9.19
Solyc01g100440	Transcription regulatory protein SNF5	4.18	Solyc11g064960	Unknown protein	-8.93
Solyc09g074490	unknown protein	4.16	Solyc10g047910	Unknown protein	-8.89
Solyc04g015750	Magnesium chelatase H subunit	4.14	SGN-U592967	Unknown	-8.85
Solyc06g048930	SlRRA6	4.12	Solyc05g016230	Breast cancer type 1 susceptibility protein homolog (	-8.71
Solyc07g007560	Vesicular glutamate transporter 3	3.98	Solyc11g066080	Genomic DNA chromosome 5 BAC clone F2O15	-8.66
Solyc01g108210	Cytochrome P450	3.75	Solyc06g005520	ATP binding / serine-threonine kinase	-8.31
Solyc12g011450	Chlorophyll a/b binding protein 13 chloroplastic	3.74	Solyc02g089870	Ring figure protein 5	-8.12
Solyc01g088160	SlCKX2	3.71	Solyc11g017280	Receptor like kinase RLK	-8.03
Cytokinin induced transcripts in young plants (24h)			Cytokinin repressed transcripts in young plants (24h)		
Solyc_ID	Solyc_Description	log2FC	Solyc_ID	Solyc_Description	Log2 FC
Solyc12g008900	SlCKX6	7.88	Solyc10g080980	Nodulin MtN21 family protein (Fragment)	-6.34
SGN-U581385	hypothetical protein VITISV_002825	7.32	Solyc05g006870	Thioredoxin H	-6.23
Solyc01g100440	Transcription regulatory protein SNF5	7.24	Solyc03g033410	Ubiquitin-conjugating enzyme E2 10	-5.50
Solyc06g048930	SlRRA6	5.94	Solyc07g043390	Cellulose synthase family protein expressed	-5.27
Solyc01g060350	Unknown protein	5.60	Solyc00g058890	Unknown Protein	-5.11
Solyc01g088160	SlCKX2	5.39	Solyc01g100920	Nodulin-like protein	-5.08
Solyc06g034410	unknown protein	5.30	Solyc07g008210	TPR domain protein	-5.02
Solyc09g074480	unkown protein	5.16	Solyc07g007250	Metallocarboxypeptidase inhibitor	-4.88
SGN-U583032	unknown	5.06	Solyc12g040790	Menaquinone biosynthesis methyltransferase ubiE	-4.83
Solyc10g079600	SlRRA3	5.02	Solyc03g098790	Kunitz-type protease inhibitor	-4.67
Solyc07g062140	Alpha alpha-trehalose-phosphate synthase	4.91	Solyc02g069490	FAD linked oxidase domain protein	-4.67
Solyc07g008540	CONSTANS-like zinc finger protein	4.77	Solyc07g049150	Ribonuclease III family protein	-4.57
Solyc07g056610	Carboxyl-terminal proteinase	4.75	Solyc04g071790	Cytochrome P450	-4.53
Solyc04g011970	Gag-Pol polyprotein	4.69	Solyc07g043460	Cytochrome P450	-4.32
Solyc12g068060	Unknown Protein	4.63	Solyc07g043420	2-oxoglutarate-dependent dioxygenase	-4.29

Table 4.3 Transcripts identified as up-regulated or repressed 2.5 log2 fold by cytokinin in young leaves. Only the top 15 transcripts identified as most highly up-regulated or repressed by cytokinin were listed here. FC = fold change.

Cytokinin induced transcripts in older plants (2h)			Cytokinin repressed transcripts in older plants (2h)		
Solyc_ID	Solyc_Description	Log2FC*	Solyc_ID	Solyc_Description	log2FC
Solyc09g082080	Plant-specific domain TIGR01568 family protein	4.90	Solyc09g084490	Proteinase inhibitor I	-12.40
Solyc02g071080	Purine permease family protein	4.09	Solyc03g020060	Proteinase inhibitor II	-11.96
Solyc07g054580	SlGH3-8	3.71	Solyc03g098790	Kunitz-type protease inhibitor	-11.69
Solyc02g071090	Purine permease family protein	3.51	Solyc09g084470	Proteinase inhibitor I	-11.60
Solyc02g071100	Purine permease family protein	3.46	Solyc11g021060	Proteinase inhibitor	-11.55
Solyc09g074490	Unknown Protein	3.34	Solyc07g007250	Metallocarboxypeptidase inhibitor	-10.71
Solyc06g082620	RNA-binding protein PNO1-like protein	3.13	Solyc09g089510	Proteinase inhibitor I	-10.39
Solyc12g013830	Unknown Protein	3.00	Solyc09g083440	Proteinase inhibitor I	-9.30
Solyc01g108210	Cytochrome P450	2.87	Solyc03g098780	Kunitz-type protease inhibitor	-8.19
Solyc04g078460	N(4)-(Beta-N-acetylglucosaminyl)-L-asparaginase	2.84	Solyc02g078150	Plant-specific domain TIGR01615 family protein	-7.52
Solyc06g060560	Pentatricopeptide repeat-containing protein	2.72	Solyc09g084440	Proteinase inhibitor I	-7.37
Solyc06g063200	Glutamate-gated kainate-type ion channel receptor subunit GluR5	2.57	Solyc09g089530	Proteinase inhibitor I	-7.18
Solyc10g079600	SlRRA3	2.51	Solyc10g050510	Unknown Protein	-6.84
Solyc05g010010	Plant-specific domain TIGR01570 family protein	2.50	Solyc07g041900	Cathepsin L-like cysteine proteinase	-6.78
			Solyc08g023660	Major latex-like protein	-6.07
			Solyc06g005100	Phosphatidylinositol transfer protein expressed	-5.91
			Solyc03g096540	Wound/stress protein	-5.59
			Solyc03g013440	Amino acid transporter family protein	-5.23
			Solyc08g074640	Polyphenol oxidase	-5.19
			Solyc12g006760	Unknown protein	-5.10
Cytokinin induced transcripts in older plants (24h)			Cytokinin repressed transcripts in older plants (24h)		
Solyc_ID	Solyc_Description	Log2 FC	Solyc_ID	Solyc_Description	log2 FC
Solyc12g008900	SlCKX6	8.79	Solyc12g056670	Unknown Protein	-6.49
Solyc06g034410	unknown protein	7.99	Solyc06g072840	Seed specific protein Bn15D1B	-6.43
Solyc01g100440	Transcription regulatory protein SNF5	7.04	Solyc03g033410	Ubiquitin-conjugating enzyme E2 10	-5.80
Solyc06g048930	SlRRA6	6.38	Solyc01g060350	Unknown protein	-5.78
Solyc05g006420	SlRRA1	5.66	Solyc08g061250	ATP binding / serine-threonine kinase	-5.10
Solyc10g079600	SlRRA3	5.16	Solyc09g059800	Unknown Protein	-5.06
Solyc10g009620	SlGH3-13	5.06	Solyc02g068000	F-box family protein	-4.89
Solyc04g007750	Major latex-like protein	5.00	Solyc05g006870	Thioredoxin H	-4.84
Solyc01g108210	Cytochrome P450	4.92	Solyc12g042680	Mutator-like transposase	-4.78
Solyc04g011970	Gag-Pol polyprotein	4.69	Solyc06g006100	Anthranilate synthase component I family protein expressed	-4.66
Solyc09g082080	Plant-specific domain TIGR01568 family protein	4.67	Solyc01g008150	Unknown Protein	-4.65
Solyc07g054580	SlGH3-8	4.64	Solyc10g080980	Nodulin MtN21 family protein (Fragment)	-4.64
Solyc04g077540	Kinesin-like protein	4.63	Solyc12g041880	Homology to unknown gene	-4.42
Solyc05g014590	Transcription Factor	4.52	Solyc12g044200	Cc-nbs-lrrresistance protein	-4.15
Solyc00g075030	Gag polyprotein	4.52	Solyc03g111820	Sieve element-occluding protein 3	-4.03
Solyc12g068060	Unknown Protein	4.48	Solyc07g017770	Homogentisate phytyltransferase	-4.00
SGN-U583032	unknown	4.46	Solyc12g040790	Menaquinone biosynthesis methyltransferase ubiE	-3.96
Solyc12g005480	Targeting protein for Xklp2 containing protein expressed	4.44	Solyc04g079480	Serpin (Serine protease inhibitor)	-3.94
Solyc10g085380	Alcohol dehydrogenase (Fragment)	4.33	Solyc08g082120	Methanol inducible protein	-3.90
Solyc02g071220	SlRRA2	4.32	Solyc10g006900	Protochlorophyllide reductase	-3.87

Table 4.4 Transcripts identified as up-regulated or repressed 2.5 log2 fold by cytokinin in older leaves. Only the top 20 transcripts identified as most highly up-regulated or repressed by cytokinin were listed here. FC = fold change.

Transcripts more abundant in young plants (2h DMSO)			Transcripts more abundant in older plants (2h DMSO)		
Solyc_ID	Solyc_Description	log2FC*	Solyc_ID	Solyc_Description	log2FC
Solyc07g007700	Isoprenylcysteine carboxyl methyltransferase	11.01	Solyc07g008520	Peptide transporter	7.64
SGN-U568332	Unknown	10.88	Solyc07g006630	CONSTANS-like protein	7.35
Solyc05g053610	ATP-binding cassette transporter	10.58	Solyc09g007010	Pathogenesis related protein PR-1	6.94
Solyc08g006420	Myosin-like protein (Fragment)	9.69	Solyc09g064910	tRNA dimethylallyltransferase	6.93
Solyc10g047910	Unknown	9.56	Solyc07g044900	Unknown Protein	6.87
Solyc10g008070	Bromodomain-containing RNA-binding protein 2	9.39	Solyc00g174340	Pathogenesis-related protein 1b	6.77
SGN-U570157	Unknown	9.36	Solyc01g099990	F-box protein PP2-B1	6.01
Solyc03g113630	Pentatricopeptide repeat-containing protein	9.27	Solyc04g077990	LOB domain protein 38	5.89
SGN-U592967	Unknown	9.13	Solyc03g098790	Kunitz-type protease inhibitor	5.72
SGN-U293475	Unknown	9.07	Solyc04g082120	Prolyl endopeptidase	5.29
Solyc04g077030	Xylulose kinase	8.77	Solyc07g017880	Peroxidase	5.16
Solyc02g089720	Endo-beta-1 3-glucanase	8.68	Solyc12g005380	Diacylglycerol kinase	5.10
Solyc02g081480	Receptor kinase	8.55	Solyc07g044910	Unknown Protein	5.05
Solyc03g064010	Receptor like kinase RLK	8.38	Solyc12g006730	Unknown Protein	5.05
Solyc11g072930	LRR receptor-like serine/threonine-protein kinase, RLP	8.24	Solyc07g043130	Os12g0117400 protein (Fragment)	4.85
Solyc07g062500	Cytochrome P450	8.07	Solyc04g016430	SlCKX5	4.83
Solyc05g032660	Dehydrogenase/ reductase 3	8.05	Solyc07g055990	Xyloglucan endotransglucosylase/hydrolase 7	4.82
Solyc10g076250	Aminotransferase like protein	8.03	Solyc09g090210	Serine/threonine protein kinase	4.76
Solyc11g066080	Genomic DNA chromosome 5 BAC clone F2O15	8.01	Solyc03g006030	Receptor like kinase RLK	4.63
Solyc05g007960	Os01g0841200 protein (Fragment)	7.99	Solyc07g007250	Metallocarboxypeptidase inhibitor	4.59
Solyc08g028970	Unknown protein	7.98	Solyc03g098780	Kunitz-type protease inhibitor	4.55
Solyc08g082250	Endoglucanase 1	7.98	Solyc03g020060	Proteinase inhibitor II	4.42
Solyc04g011680	Cytochrome P450	7.93	Solyc10g050510	Unknown Protein	4.31
Solyc09g061420	Os02g0515000 protein	7.83	Solyc11g021060	Proteinase inhibitor	4.29
Transcripts more abundant in young plants (24h DMSO)			Transcripts more abundant in older plants (24h DMSO)		
Solyc_ID	Solyc_Description	log2FC	Solyc_ID	Solyc_Description	log2FC
Solyc07g014730	Phospholipase A2	6.23	Solyc01g109320	Multidrug resistance protein mdtK	6.60
Solyc08g023660	Major latex-like protein	5.88	Solyc00g174340	Pathogenesis-related protein 1b	6.00
Solyc06g083720	Subtilisin-like protease	5.74	Solyc01g060350	Unknown protein	5.39
Solyc08g008480	Myb transcription factor	5.43	Solyc09g007010	Pathogenesis related protein PR-1	5.38
Solyc04g015620	Os01g0611000 protein	5.36	Solyc09g090210	Serine/threonine protein kinase	5.35
Solyc06g036130	Multidrug resistance protein mdtK	5.30	Solyc10g075150	Non-specific lipid-transfer protein	5.31
Solyc01g014290	Unknown Protein	5.18	Solyc02g072070	Receptor-like kinase	4.83
Solyc01g014280	Unknown Protein	5.16	Solyc07g017880	Peroxidase	4.63
Solyc12g005480	Targeting protein for Xklp2 containing protein expressed	5.12	Solyc06g082620	RNA-binding protein PNO1-like protein	4.62
Solyc02g070970	Chlorophyll a/b binding protein	5.09	Solyc02g062710	Hydroxycinnamoyl transferase	4.61
Solyc07g043390	Cellulose synthase family protein expressed	5.04	Solyc05g053610	ATP-binding cassette transporter	4.59
Solyc02g081120	Knotted-1-like homeobox protein H1	4.97	Solyc12g006570	Germacrene-D synthase	4.52
Solyc04g077540	Kinesin-like protein	4.62	Solyc07g052140	(-)-germacrene D synthase	4.46
Solyc02g070950	Chlorophyll a/b binding protein	4.62	Solyc10g050970	Ethylene responsive transcription factor 2b	4.44
Solyc08g013950	Unknown	4.56	Solyc07g014640	Galactokinase-like protein	4.27
Solyc09g082660	Caffeoyl-CoA O-methyltransferase	4.56	Solyc03g113430	Peptide transporter	4.20
Solyc06g069730	Chlorophyll a/b binding protein chloroplastic	4.43	Solyc07g051940	Alpha-humulene/(-)-(E)-beta-caryophyllene synthase	4.07
Solyc03g033260	Maltose excess protein 1	4.43	Solyc07g008080	WD repeat-containing protein 11	3.98
Solyc11g071200	Unknown Protein	4.36	Solyc02g020990	Unknown protein	3.95
Solyc03g026040	Receptor like kinaseRLK	4.36	Solyc07g026650	1-aminocyclopropane-1-carboxylate oxidase	3.95
Solyc02g069490	FAD linked oxidase domain protein	4.33	Solyc06g007180	Asparagine synthase	3.93
Solyc06g006000	Unknown Protein	4.32	Solyc00g052530	Unknown protein	3.91
Solyc03g026280	CRT binding factor 2	4.24	Solyc12g056820	Laccase	3.87
Solyc07g055950	Meiosis 5	4.23	Solyc01g100380	Calreticulin 2 calcium-binding protein	3.80

Table 4.5 Transcripts identified as more abundant (2.5 log2 fold greater than the other age sample at that treatment time) in control leaf samples. Only the top 24 transcripts were listed in the table. FC = fold change.

Gene ID	Annotations	Log2 FC*-RNA seq	Log2 PCR	FC-qRT-
Solyc04g078460	N(4)-(Beta-N-acetylglucosaminyl)-L-asparaginase	4.17	4.77	
Solyc03g111400	Xanthine/uracil permease family protein	3.17	3.04	
Solyc05g006420	SlRRA1	5.66	3.73	
Solyc12g044200	Cc-nbs-lrr resistance protein	-1.97	-0.63	
Solyc04g008110	SlHK4	3.87	3.42	
Solyc01g108210	Cytochrome P450	4.92	3.86	
Solyc02g071220	SlRRA2	4.32	4.24	
Solyc12g008900	SlCKX6	8.79	4.08	

Table 4.6. qPCR confirmation of select transcripts identified by RNA-sequencing. Transcripts that were identified as cytokinin responsive in 35d leaf samples treated with cytokinin vs. DMSO for 24h using RNAseq were examined using qPCR. Shown is the log2 fold change calculated from cytokinin vs DMSO for RNAseq and qPCR analyses. FC = fold change.

Overall, using the criteria mentioned above a large number of transcripts was shown to be responsive to the application of exogenous cytokinin (5μM BA) vs. the solvent vehicle DMSO in both young and older leaves (Table 4.2). Because of the large number of transcripts that show transcript changes more than 2.5 log2 fold for the different length cytokinin treatments examined, early (2h) and late (24h) in leaves of two ages, we present and discuss here a subset of these (Figure 3.3, Table 4.2, Table 4.3, 4.4, 4.5) with the rest shown in supplemental data. Since most prior studies of cytokinin response at a transcriptome level in other species like Arabidopsis and rice have focused on and shown a small, but consistent set of transcripts that are induced by cytokinin [10], we have concentrated on reporting the positively cytokinin responsive or induced transcripts here.

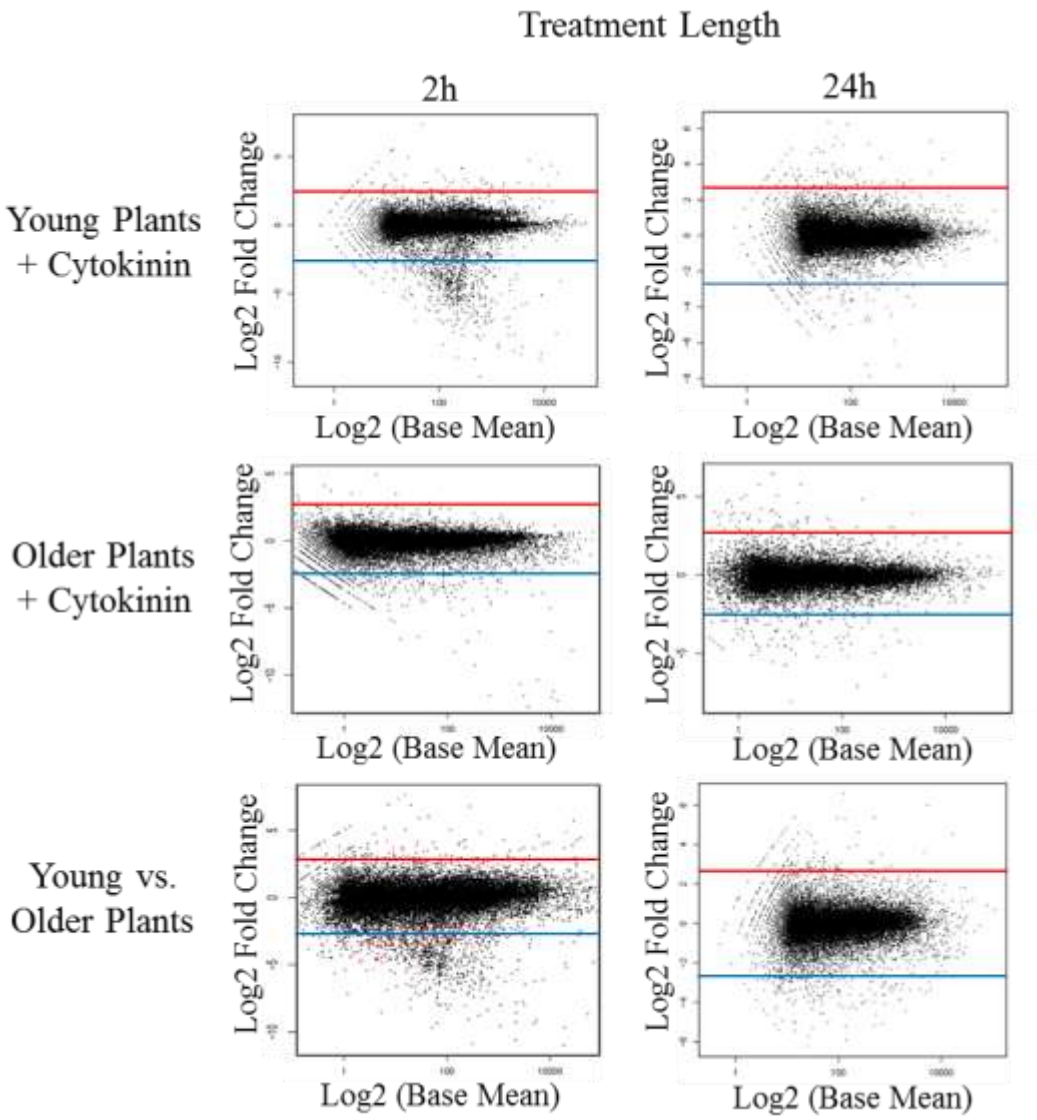

Figure 4.2. MvA Plots of Leaf Expression Analysis. MvA plots are presented as log2 fold change vs. the log2 base mean for either 2h or 24h of treatment. Top shows plots of young (13d) plants treated with cytokinin (5μM BA) compared to a vehicle control (DMSO). Middle shows plots of older (35d) plants treated with cytokinin (5μM BA) compared to a vehicle control (DMSO). Bottom shows plots of comparisons between young (13d) and older (35d) plants after only vehicle control (DMSO) treatment. Lines in each graph indicate 2.5 log2 fold change levels, above which transcripts were primarily examined. Dots colored in red represent genes that were identified as differentially expressed by DESeq [90] with BH (Benjamini-Hochberg) adjusted p-values of 0.1 or less in each of the given comparisons.

In order to have an overall picture of how cytokinin affects gene expression in tomato leaves, we performed gene ontology analysis on the genes identified as cytokinin induced and repressed (Figure 4.3). Within the biological process class, a large number of cytokinin responsive genes fall into the categories of metabolic process, cellular process, response to stimulus, biological regulation, and developmental process, indicating that cytokinin plays a role in the regulation of cellular metabolism, dealing with external stimulus, and development in plants. Within the molecular function class, many cytokinin responsive genes show binding activity (binding to ions, small molecules, nucleic acids, and proteins), enzyme activity, transporter activity, and transcription factor activity. This demonstrates that cytokinin affects genes that encode proteins with diverse functions such as transcription factor genes that can regulate plant growth and development by activating or repressing their specific target genes. Many of these cytokinin responsive genes encode proteins that are localized in intracellular membrane bounded organelles, plastids, mitochondria, cytosol, and vacuole. The plastid thylakoid localization indicates that a number of cytokinin responsive genes are involved in photosynthesis-related processes.

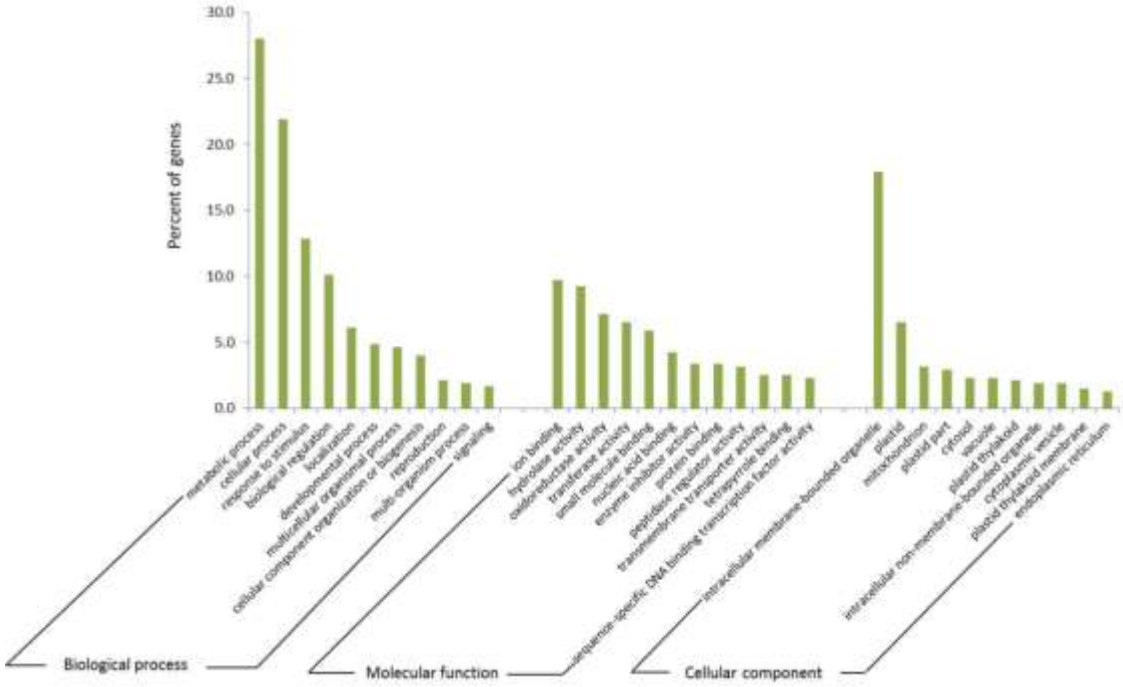

Figure 4.3. Gene ontology analysis of cytokinin regulated genes in both young and older leaves. The percent of cytokinin regulated genes which belong to each of the major GO categories identified is shown.

The gene ontology analysis indicates that a number of cytokinin responsive genes are involved in signaling (Figure 4.3). A close look at the overall RNA seq data shows that some components of cytokinin signaling pathway such as the cytokinin receptor SlHK4 and the type-A response regulators (SlRRAs) were induced by cytokinin, whereas the type-B RRs were not (Table 4.7, 4.8). Several *SlCKX* genes encoding cytokinin oxidases were also induced by cytokinin (Table 4.7, 4.8). It seems that cytokinin treatment has little effect (<2.5 log2 fold) on the expression of histidine phosphotransfer protein encoding genes (Table 4.7, 4.8). Since hormone crosstalk often occurs, we also looked at whether cytokinin treatment has an effect on the biosynthetic genes of other plant hormones such as auxin and ABA. A number of aldehyde oxidases and nitrilases that are thought to be involved in auxin biosynthesis were detectable but not affected much by cytokinin (<2.5 log2 fold, Table 4.7, 4.8). An ABA biosynthetic enzyme, the 9-cis-epoxycarotenoid dioxygenase, does not seem to be affected much by cytokinin either, although it might be slightly repressed by cytokinin since the fold change is near or above two fold (Table 4.7, 4.8).

Young leaves					
Early response			Late response		
Solyc ID	Solyc description	Log2 FC	Solyc ID	Solyc description	Log2 FC
Solyc04g008110	SlHK4	2.69	Solyc04g008110	SlHK4	4.56
Solyc05g015610	SlHK3	-0.67	Solyc05g015610	SlHK3	-0.45
Solyc01g080540	Histidine phosphotransfer protein	0.10	Solyc01g080540	Histidine phosphotransfer protein	0.08
Solyc01g098400	Histidine phosphotransfer protein	0.69	Solyc01g098400	Histidine phosphotransfer protein	1.75
Solyc06g084410	Histidine phosphotransfer protein	-0.01	Solyc06g084410	Histidine phosphotransfer protein	0.25
Solyc08g066350	Histidine phosphotransfer protein	-1.29	Solyc08g066350	Histidine phosphotransfer protein	-0.72
Solyc11g070150	Histidine phosphotransfer protein	-0.67	Solyc11g070150	Histidine phosphotransfer protein	-1.48
Solyc01g065540	Response regulator 8	-0.57	Solyc01g065540	Response regulator 8	-0.34
Solyc03g113720	SlRRA5	2.24	Solyc03g113720	SlRRA5	2.99
Solyc04g008050	Response regulator 8	-0.05	Solyc04g008050	Response regulator 8	0.18
Solyc05g006420	SlRRA1	4.93	Solyc05g006420	SlRRA1	5.05
Solyc05g014260	Response regulator 11	-0.84	Solyc02g071220	SlRRA2	3.40
Solyc05g054390	Response regulator 8	-0.68	Solyc05g054390	Response regulator 8	0.18
Solyc06g048930	SlRRA6	4.12	Solyc06g048930	SlRRA6	-0.33
Solyc07g053630	Response regulator	0.76	Solyc07g053630	Response regulator	5.94
Solyc08g077230	response regulator ARR11	0.35	Solyc10g078310	response regulator ARR11	-0.22
Solyc10g078310	response regulator ARR11	-0.05	Solyc10g079600	SlRRA3	4.36
Solyc10g079600	SlRRA3	3.21	Solyc10g079700	SlRRA4	3.15
Solyc10g079700	SlRRA4	2.36	Solyc11g072330	Response regulator 8	0.01
Solyc11g072330	Response regulator 8	-3.14	Solyc12g010330	Response regulator 9	0.35
Solyc12g010330	Response regulator 9	0.31	Solyc08g061930	SlCKX1	0.30
Solyc04g016430	SlCKX5	5.41	Solyc04g016430	SlCKX5	2.41
Solyc04g080820	SlCKX4	0.59	Solyc04g080820	SlCKX4	3.45
Solyc08g061930	SlCKX1	0.55	Solyc12g008900	SlCKX6	7.88
Solyc01g088160	SlCKX2	3.71	Solyc01g088160	SlCKX2	5.39
Solyc01g088170	Aldehyde oxidase	1.19	Solyc11g071620	Aldehyde oxidase	-0.59
Solyc11g071580	Aldehyde oxidase	-0.08	Solyc01g088170	Aldehyde oxidase	0.51
Solyc11g071620	Aldehyde oxidase	-0.40	Solyc11g071580	Aldehyde oxidase	0.13
Solyc07g041280	Nitrilase 2	0.54	Solyc07g041280	Nitrilase 2	-1.16
Solyc06g064880	Nitrilase 2	0.49	Solyc06g064880	Nitrilase 2	0.99
Solyc08g062190	Nitrilase 2	0.11	Solyc08g062190	Nitrilase 2	-0.04
Solyc07g056570	9-cis-epoxycarotenoid dioxygenase	-0.47	Solyc07g056570	9-cis-epoxycarotenoid dioxygenase	-0.93

Table 4.7 Response to cytokinin of transcripts that are involved in hormone signaling and metabolism in young leaves. These transcripts listed include those that are involved in cytokinin signaling and metabolism, auxin biosynthesis and ABA biosynthesis.

Older leaves					
Early response			Late response		
Solyc ID	Solyc description	Log2 FC	Solyc ID	Solyc description	Log2 FC
Solyc04g008110	SlHK4	1.69	Solyc04g008110	SlHK4	3.87
Solyc05g015610	SlHK3	-0.51	Solyc05g015610	Histidine kinase cytokinin receptor	-0.29
Solyc01g080540	Histidine-containi	0.31	Solyc01g080540	Histidine-containing phosphotransfer protein	0.27
Solyc01g098400	Histidine phosphc	-1.15	Solyc01g098400	Histidine phosphotransfer protein	1.36
Solyc06g084410	Histidine phosphc	-0.14	Solyc06g084410	Histidine phosphotransfer protein	0.54
Solyc08g066350	Histidine phosphc	0.36	Solyc08g066350	Histidine phosphotransfer protein	-1.18
Solyc11g070150	Histidine phosphc	0.48	Solyc11g070150	Histidine phosphotransfer protein	-0.54
Solyc01g065540	Response regulato	-0.57	Solyc01g065540	Response regulator 8	0.03
Solyc03g113720	SlRRA5	0.74	Solyc03g113720	SlRRA5	2.66
Solyc04g008050	Response regulato	-0.07	Solyc04g008050	Response regulator 8	-0.26
Solyc05g006420	SlRRA1	1.77	Solyc05g006420	SlRRA1	5.66
Solyc02g071220	SlRRA2	1.26	Solyc02g071220	SlRRA2	4.32
Solyc05g054390	Response regulato	-0.40	Solyc05g054390	Response regulator 8	-0.31
Solyc06g048930	SlRRA6	1.07	Solyc06g048930	SlRRA6	6.38
Solyc07g053630	Response regulato	0.87	Solyc07g053630	Response regulator	0.19
Solyc10g078310	response regulato	-0.24	Solyc10g078310	response regulator ARR11	-0.06
Solyc10g079600	SlRRA3	1.26	Solyc10g079600	SlRRA3	4.48
Solyc10g079700	SlRRA4	0.97	Solyc10g079700	SlRRA4	2.11
Solyc11g072330	Response regulato	-0.13	Solyc11g072330	Response regulator 8	-0.14
Solyc12g010330	Response regulato	0.61	Solyc12g010330	Response regulator 9	0.77
Solyc08g077230	response regulato	-0.11	Solyc08g077230	response regulator ARR11	-0.25
Solyc08g061930	SlCKX1	-0.20	Solyc05g014260	response regulator 11	-0.20
Solyc04g016430	SlCKX5	0.81	Solyc08g061930	SlCKX1	0.58
Solyc04g080820	SlCKX4	-0.42	Solyc04g016430	SlCKX5	1.11
Solyc12g008900	SlCKX6	-0.03	Solyc04g080820	SlCKX4	3.58
Solyc01g088160	SlCKX2	1.42	Solyc12g008900	SlCKX6	8.79
Solyc11g071620	Aldehyde oxidase	0.96	Solyc01g088160	SlCKX2	2.87
Solyc01g088170	Aldehyde oxidase	0.65	Solyc11g071620	Aldehyde oxidase	1.39
Solyc07g041280	Nitrilase 2	-0.10	Solyc01g088170	Aldehyde oxidase	1.11
Solyc06g064880	Nitrilase 2	0.36	Solyc11g071580	Aldehyde oxidase	0.16
Solyc08g062190	Nitrilase 2	0.03	Solyc07g041280	Nitrilase 2	-0.74
Solyc07g056570	9-cis-epoxycarote	0.65	Solyc06g064880	Nitrilase 2	0.16
			Solyc08g062190	Nitrilase 2	0.00
			Solyc07g056570	9-cis-epoxycarotenoid dioxygenase	-1.21

Table 4.8 Response to cytokinin of transcripts that are involved in hormone signaling and metabolism in older leaves. These transcripts listed include those that are involved in cytokinin signaling and metabolism, auxin biosynthesis and ABA biosynthesis.

Young leaves early cytokinin response

We identified more than 700 transcripts that showed transcript change due to an early (2h) cytokinin treatment in young (13d) tomato leaves (Table 4.2, Table 4.3). From this we found 60 genes that were induced at least 2.5 log2 fold by cytokinin 2h after treatment (Table 4.3). These genes have diverse functions such as signal transduction, transcriptional regulation, metabolism, transport, and photosynthesis, although several have unknown functions. Within this group of genes there are several that are linked to induction by cytokinin in other species. One of these classes of genes is the type-A response regulators, which have been previously shown to be rapidly induced by cytokinin through different approaches and are almost always in the top set of cytokinin induced genes in transcriptome analyses [3, 8, 9, 10, 32]. We identified four different type-A response regulators that are highly induced, from 3.12-4.12 log2 fold (Table 4.3). We have designated these as *Solanum lycopersicum* Response Regulator type-As: *Sl*RRA1 to 3, and A6 (Solyc05g006420-SlRRA1, Solyc02g071220-*Sl*RRA2, Solyc10g079600-*Sl*RRA3, and Solyc06g048930-*Sl*RRA6). Two other classes of commonly found cytokinin induced genes were also identified in this sample: two cytokinin oxidases and a cytokinin receptor. The transcripts Solyc01g088160.2 and Solyc04g016430 encoding a cytokinin oxidase were induced 3.7 and 5.4 log2 fold, respectively. Cytokinin oxidase (CKX) is an enzyme which catalyzes the degradation of cytokinin, and it is not surprising to see it induced since if the plant is exposed to excess levels of cytokinin there would be an attempt to break it down using this enzyme [33, 34]. Interestingly it has been reported that reduced expression of the rice cytokinin oxidase gene OsCKX2 can result in increased grain yield, indicating the potential of this gene in

crop improvement [35].The transcript Solyc04g008110, a histidine kinase was also induced 2.7 log2 fold, which we verified by qRT-PCR as induced to a similar level (Table 4.6). This gene, which we have designated *Solanum lycopersicum* Histidine Kinase 4 (*Sl*HK4) encodes the cytokinin receptor most similar to AHK4 in Arabidopsis that has been noted to be induced by cytokinin in several studies.

The four genes that were identified as the most highly induced from 7.4-5.4 log2 fold by cytokinin in young leaves were a CONSTANS-like protein (Solyc07g006630), a UDP-glucuronosyltransferase gene (Solyc12g009930), a peptide transporter gene (Solyc07g008520), and a cytokinin oxidase gene (Solyc04g016430) already discussed. The CONSTANS-like protein (Solyc07g006630) identified has not been assigned any particular function to our knowledge, however, CONSTANS-like proteins (COLs) are known as a group of plant-unique transcription factors which contain a CCT (CONSTANS, CONSTANS-LIKE, and TIMING OF CAB1) domain [36, 37]. Arabidopsis CONSTANS protein was shown to control flowering in response to photoperiod [36, 37]. Tomato is not a photoperiodic plant, and little is known about the tomato COL proteins. Although an Arabidopsis COL gene (At4g39070) was also found up-regulated by cytokinin in CKX1 overexpressing plants [9], how these genes are involved in cytokinin regulated processes remain unknown.

A gene encoding UDP-glucuronosyltransferase (Solyc12g009930) was highly induced by cytokinin as well. Glycosylation is known to play an important role in the regulation of cellular metabolism by altering activity, solubility, and transport of aglycones like plant hormones, secondary metabolites, and xenobiotics [38, 39]. UDP-glucuronosyl-transferases are multi-family enzymes which catalyze the transfer of a

glucuronosyl group from a UDP-glucuronic acid to various lipophilic aglycones and are mainly found in insects, fish, and mammals [40]. Glucuronidation enhances polarity and excretability of aglycones and is considered an important mechanism in detoxifying and eliminating lipophilic wastes in the body [40, 41]. Interestingly, overexpression of a pea UDP-glucuronosyltransferase-encoding gene, *PsUGT1*, resulted in early senescence phenotype in Arabidopsis and reduction of the expression of this gene in alfalfa delayed root emergence and enhanced lateral root development [42]. Since *PsUGT1was* found to be expressed in regions with active cell division such as root apical meristems [42], leaf primordial and tips of older leaves, it would be interesting to examine whether the cytokinin inducible tomato UDP-glucuronosyltransferase encoding gene plays a role in leaf development.

The third highly induced cytokinin induced gene is a peptide transporter (PTR) gene. Although PTRs have not been previously linked to cytokinin in tomato, s recent study has identified a Medicago gene, *LATD*/*NIP* as cytokinin up-regulated in roots which encodes a member of the NRT1/PTR transporter family [43]; it is not known yet whether this gene encodes a nitrate or peptide transporter [43, 44]. The cytokinin induction of the peptide transporter indicates the involvement of cytokinin in the regulation of peptide transport in young tomato leaves; the specific function of this transporter in relation to cytokinin remains to be examined.

A few other interesting genes were also seen as induced by cytokinin in young plants after 2h of treatment. This includes a few that have some connections to cytokinin or hormone signaling. One of these was surprisingly, a gene encoding a tRNA dimethylallyltransferase (Solyc09g064910), which was induced 4.6 log2 fold. This

enzyme catalyzes the isopentenylation of certain tRNAs in bacteria, animals, and plants [45, 46]. In Arabidopsis two genes encoding the tRNA dimethylallyltransferase, *AtIPT2 and AtIPT9* have been identified [45, 47]. Similar to the bacterial *miaA* gene which isopentenylates some tRNAs to synthesize low-level cytokinins [48, 49], these two genes play an indispensable role in the production of cis-zeatin-type cytokinins in plants [46]. Given the fact that the tomato tRNA dimethylallyltransferase was highly induced by cytokinin only in young expanding leaves and that *AtIPT2 and AtIPT9* were more abundant in proliferating tissues [47], it would be interesting to examine the roles of cis-zeatin-type cytokinins in shoot and root apical meristems, leaf primordia, and growing leaves, although no role for cis-zeatin is currently known in Eudicots.

Two more genes which are involved in hormone signaling or hormonal homeostasis were up-regulated by cytokinin as well. *BES1-INTERACTING MYC-LIKE PROTEIN 2* (*BIM2*, Solyc03g114720), a gene encoding a transcription factor has been shown to positively regulate brassinosteroid (BR) signaling along with *BIM1* and *BIM3* [50]. The induction of *BIM2* by cytokinin suggests that there could be crosstalk between cytokinin and BR signaling. The second gene encodes a GH3 family protein which has jasmonate (JA)-amino synthetase activity and adenylyltransferase activity according to the Sol Genomics Network (http://solgenomics.net/). This gene was also induced by cytokinin in older leaves (Table 4.4). A homolog of this gene in Arabidopsis is JAR1 which has been demonstrated to act as a JA-amino synthetase necessary for the activation of JA for optimal signaling [51, 52]. JAR1 produces JA-Ile which is a key signal for the major jasmonate signaling pathway involving *CORONATINE INSENSITIVE 1* (*COI1*) [53, 54]. The cytokinin responsiveness of the tomato JA-amino synthetase encoding gene

in both young and old leaves suggests a link of cytokinin signaling to jasmonate signaling pathway.

Interestingly, four genes involved in photosynthesis were also highly induced by cytokinin (Table 4.2). Three of them are *LHCB* genes (Solyc10g007690, Solyc06g069730, and Solyc12g011450) which encode chlorophyll a/b binding proteins and the fourth is a photosystem II polypeptide (Solyc07g066310). The induction of these *LHCB* genes supports previous findings that cytokinin can dramatically activate *CAB* promoter activity [55]. Although the role of cytokinin in photosynthesis related processes have been extensively studied [55-58], how cytokinin acts in these processes remains unclear. Notably, the photosynthesis-related tomato genes were up-regulated by cytokinin only in young leaves with active cell division, indicating a potential development-dependent regulation of cytokinin on the transcription of these genes. Earlier studies have provided evidence that growing young leaves have a higher content of zeatin-type cytokinins than older leaves [59]. A higher cytokinin level is likely to have a positive effect on photosynthesis by activating *LHCB* genes and other unknown mechanisms, thus provides enough energy sources for fast growing leaves.

We also identified a large number, 669 transcripts that were repressed 2h after cytokinin treatment (Table 4.2). We are unsure why there was such an abundance of negatively cytokinin responsive or repressed transcripts. The 100 most highly repressed of these are shown in Table 4.3 (the rest of these are shown in Table S1) and include an over-representation of genes involved in signaling, defense and stress responses, and protein turnover. Three genes involved in auxin transport and responses (Auxin efflux carrier, ARF4, and SAUR) were down regulated potentially as part of an antagonistic

relationship between cytokinin and auxin. Interestingly two cytokinin signaling genes (cytokinin receptor and HPt protein) were also found to be repressed.

Young leaves late cytokinin response

We identified nearly 200 transcripts that showed transcript change due to a late (24h) cytokinin treatment in young (13d) tomato leaves (Table 4.2, Table 4.3). About half of these cytokinin responsive transcripts were found to be induced by cytokinin after a 24h treatment, which is nearly twice as many compared to the 2h treatment in young tomato leaves (Table 4.2). The majority of cytokinin induced genes in this longer treatment are transcription factors, signaling genes, or genes involved in hormone metabolism (Table 4.3). Not surprisingly, there is overlap between the two sets of cytokinin induced genes (2h and 24h) in young leaves, which includes several type-A response regulators, the *Sl*HK4 cytokinin receptor, a cytokinin oxidase, and a xanthine/uracil permease family protein. In agreement with the increased number of cytokinin induced genes, several other genes directly linked to cytokinin were also found to be induced. This includes two more type-A response regulators (Solyc03113720 and Solyc10g079700: that we have designated *Sl*RRA5 and *Sl*RRA4, respectively) induced 3.0-3.1 log2 fold and an additional cytokinin oxidase (Solyc12g008900) gene induced 7.9 log2 fold (Table 4.3).

Several other interesting genes were induced by cytokinin in young plants after the 24h treatment that may have some connections to cytokinin or hormone signaling. Among these are some transcription factor genes including two *NAC* (*NAM*) genes induced 2.8-2.9 log2 fold (Solyc08g077110 and Solyc06g061080), a *LOB* induced 3.7 log2 fold (Solyc12g100150), an *ERF2b* induced 3.5 log2 fold (Solyc10g050970), and

two *WRKY* members induced 2.9-3.0 log2 fold (Solyc04g07270 and Solyc08g067360) (Table 4.3). It has been previously shown that some *NAM*, such as At4g27410, and LOB domain genes were up-regulated by cytokinin in Arabidopsis [9, 60, 61]. Additionally transient silencing of a tomato *SlNAM* gene resulted in smooth leaflet margins and highly reduced numbers of secondary and intercalary leaflets [62, 63], a feature whose regulation has been linked to cytokinin [4]. Previous work has also shown that a LOB domain gene, *ASYMMETRIC LEAVES 2 LIKE 9* (*ASL9/LBD3*) has cytokinin-dependent expression in both Arabidopsis roots and aerial parts especially leaves as well as being identified as a primary target of the cytokinin signaling pathway [64]. Some LOB domain genes have also been linked to the establishment of leaf polarity [65] and boundary delimitation [66, 67]. Here the two NAM proteins and the LOB domain protein identified as cytokinin inducible are worth further examination to determine if they play a role in cytokinin regulated leaf development in tomato.

It is well known that cytokinin is involved in crosstalk with many other hormones like ethylene, ABA, and gibberellin in a diverse range of processes [68-71]. Here we find evidence to further support this with three genes encoding enzymes involved in hormone metabolism that were induced 2.9-3.5 log2 fold by cytokinin. These enzymes include a 1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) OXIDASE-like protein (Solyc11g045520) which catalyzes the final step of ethylene biosynthesis [72, 73], a Cytochrome P450 (Solyc01g108210) with ABA 8'-hydroxylase activity which is a key enzyme involved in ABA catabolism [74], and a Gibberellin 2-oxidase 2 (Solyc07g056670) involved in gibberellin degradation [75]. Previous microarray data from other species identified several genes controlling protein turnover as induced by

cytokinin [9]. In our study, two genes regulating protein turnover, which were not responsive to cytokinin after a 2h treatment, were up-regulated by cytokinin after a 24h treatment. One encodes a ring finger protein (Solyc06g049030), the other codes for a U-box domain-containing protein (Solyc07g020870). This indicates a possible involvement of cytokinin in regulating protein turnover via these induced genes. Cytokinin has been recently linked to the vacuolar targeting of PIN1, an auxin efflux carrier, for lytic degradation [76], linking cytokinin in the regulation of protein turnover affecting auxin transport if not other processes. There were also a few transcripts that appear connected to stress or defense response that were induced. Three genes encoding LRR receptor-like serine/threonine-protein kinases were induced 2.8-3.0 log2 fold by extended cytokinin treatment. These protein kinases are known to have a link to signaling and defense responses in plants [77].

The 24h-cytokinin treatment repressed many fewer genes (95) than the short cytokinin treatment, but this number of repressed genes is close to the number (73) found for 35d plants (Table 4.3). Most genes down-regulated by cytokinin in young leaves seem to be involved in metabolic processes. Interestingly, five genes encoding nodulin-like proteins were repressed as well. In contrast, a gene encoding nodulin-like protein was induced to 3.0 log2 fold by cytokinin 24h after treatment in older leaves (Table 4.4). These results suggest a potential differential regulation of these nodulin-like genes by cytokinin in an age-dependent manner.

Older leaves early cytokinin response

Only a small number of genes (14; Table 4.4) were found induced by cytokinin 2h after treatment in older 35d leaves. The transcript Solyc07g054580 encoding a GH3

family protein and the transcript Solyc04g078460 encoding an asparaginase were induced 3.4-2.9 log2 fold and 2.7-2.8 log2 fold respectively, by cytokinin 2h after treatment in both young and older tomato leaves. We also identified three purine permease encoding genes (Solyc02g071090, Solyc02g071100, and Solyc02g071080) which were highly induced by cytokinin 2h after treatment in older tomato leaves. It is known that Arabidopsis purine permeases (AtPUP1 and 2) mediate transport of adenine and possibly cytokinins as well [78, 79]. If purine permeases do function as cytokinin transporters, it could be that exogenous application of cytokinin activates these transporters which in turn transport the extra cytokinin to other parts of the plant.

The 2h cytokinin treatment resulted in the repression of a large number of transcripts in older leaves (279; Table 4.2) as seen in young leaves at the early time point. However, the absolute number of induced genes in older leaves (14) is fewer than that of young leaves (60) and the ratio of repressed to induced of older leaves (19:1) is much greater than that of young leaves (11:1), indicating that cytokinin may have a greater ability to induce genes in young vs. old tissues. A majority of these genes down-regulated by cytokinin in older leaves are involved in signaling, metabolism, stress and defense responses. We listed only the top 20 most highly repressed transcripts in Table 4.4.

Older leaves late cytokinin response

After a 24h cytokinin treatment, the number of genes (91; Table 4.4) that showed highly increased transcript level in older leaves is very close to that seen in young leaves (97; Table 4.3). Six type-A response regulator genes were found highly induced by cytokinin (*Sl*RRA1-6) as seen in young plants. Among the cytokinin induced transcripts are several genes encoding proteins involved in hormone signaling and metabolism.

These proteins include the cytokinin receptor (*Sl*HK4), three cytokinin oxidases, two cytochrome P450s (Solyc01g108210 and Solyc04g078900) with abscisic acid 8'-hydroxylase activity, a cytochrome P450 (Solyc02g094860) with steroid hydroxylase activity, a Gibberellin 2-oxidase (Solyc07g061720), two GH3 family proteins, and an adenine phosphoribosyltransferase (APT/APRT)-like protein (Solyc08g079020), that has not been previously linked to cytokinin regulation. APRT (EC 2.4.2.7) catalyzes the conversion of adenine to AMP and has been shown to be able to convert N6-benzyladenine to its nucleotide form in young Arabidopsis plants [80, 81]. If the proposed role of APRTs in the inter-conversion of cytokinins is true, induction of the APRT-like gene by cytokinin shown in the present study may result in the conversion of the active cytokinin nucleobase that was exogenously added to its inactive nucleotide in the leaf, thus regulating the level of active cytokinin.

A few other interesting genes that were induced have potential links to either cytokinin or leaf/cell morphology (Table 4.4). This includes some transcription factors linked to stress and defense responses that encode a dehydration-responsive family protein, ERF4, and a Heat stress transcription factor. The induction of stress- and defense-related genes by cytokinin has been reported in earlier studies as well [3, 9, 82]. A transcript (Solyc04g080780) coding for BEL1-like homeodomain protein 11 was also induced by cytokinin. A few members of the BEL1-like protein family in Arabidopsis were shown to play roles in leaf morphogenesis by interacting with KNOX homeodomain proteins [83], but little is known about other BEL1-like proteins such as the one identified here. Additionally, two transcripts encoding cell wall-related proteins

(Expansin protein, Solyc03g093390 and Pectinesterase, Solyc01g099950) were also found induced by cytokinin, in agreement with previous findings [9, 84].

The extended cytokinin treatment in older leaves repressed around 75genes (Table 4.2). The most repressed genes encode a seed specific protein (Solyc06g072840), an ubiquitin-conjugating enzyme E2 10 (Solyc03g033410), an F-box family protein (Solyc02g068000), and a thioredoxin H protein (Solyc05g006870). Several nodulin-like protein encoding genes were repressed as well, as seen in young leaves treated by cytokinin for 24h.

Comparison of transcriptome response to cytokinin in young and older leaves

Five genes (Solyc01g108210, Solyc04g078460, Solyc07g054580, Solyc09g074490, Solyc10g079600) were induced by the 2h cytokinin treatment in both young and older leaves (Table 4.3, 4.4, and Figure 4.4A). This treatment resulted in much more robust response in young leaves compared to older leaves. First, the number of genes induced by cytokinin in young leaves (60) is more than four times that (14) in older leaves (Table 4.2, Figure 4.4A). Second, the log2 fold change of young leaves ranges from 2.50 up to 7.34, while that of older leaves ranges from 2.50 to 4.9. Third, more genes know to be involved in cytokinin-related processes of signaling, metabolism, and transport were induced in young leaves (8) compared to older leaves (4).

The 24h cytokinin treatment induced 36 genes (mainly cytokinin-related genes) in both young and older leaves (Table 4.3, 4.4, and Figure 4.4B) that are more than half of the genes induced either in young or older leaves. Both the number and the range of log2 fold change of cytokinin induced genes in young leaves are comparable to those in older

leaves (Table 4.2, Table 4.3, 4.4). However, the number of receptor-like (protein) kinases (7) induced by cytokinin in young leaves is more than three times that (2) in older leaves, indicating a stronger ability of cytokinin to trigger signaling transduction in young leaves. Importantly, the 24h data indicates that cytokinin is able to induce different genes which fall into the same gene families in young and older leaves.

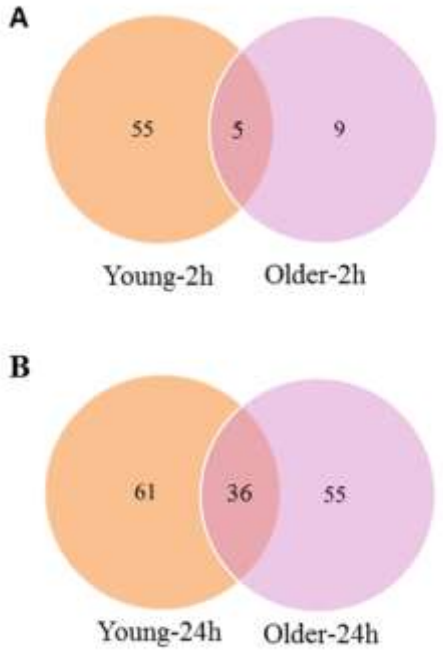

Figure 4.4. Venn diagram of cytokinin induced genes in young and older leaves. (A) Venn diagram showing number of genes induced by 2h cytokinin treatment in young and older tomato leaves. (B) Venn diagram showing number of genes induced by 24h cytokinin treatment in young and older tomato leaves. In both diagrams the number of common genes are shown in the overlapping segment.

Genes expressed more abundantly in Young and Older leaves

Using untreated (DMSO) 2h data, the number of transcripts (926; Table 4.2) identified as expressed more abundantly in young leaves is five times as many that (168; Table 4.2) in older leaves, indicating development-dependent expression of these transcripts. The expression levels of the more abundant transcripts in young leaves ranges from 2.5 to 11.0 log2 fold relative to that in older leaves, in contrast to the range of 2.5 to 7.6 log2 fold for more abundant transcripts in older leaves relative to that in young leaves (Table 4.5). The abundant transcripts in young leaves include at least three which are cytokinin-related genes, among which one is a type-A response regulator (Solyc11g072330:*Sl*RRA8), one is involved in cytokinin transport (Solyc02g071080: purine permease family protein), and one is cytokinin inducible (Solyc03g115900: chlorophyll a-b binding protein). In older leaves, at least seven cytokinin-related genes were found, among which three encode type-A response regulators (Solyc06g048930: *Sl*RRA6, Solyc02g071220: *Sl*RRA2, Solyc05g006420: *Sl*RRA1), two are cytokinin inducible (Solyc12g011450: chlorophyll a-b binding protein 13, Solyc07g006630: CONSTANS-like protein), and two are involved in cytokinin metabolism (Solyc04g016430: *Sl*CKX5, Solyc09g064910: tRNA dimethylallyltransferase). None of the cytokinin related genes found in young leaves were identified as DE genes, while two (Solyc07g00663 and Solyc09g064910) out of the seven cytokinin related genes found in older leaves were identified as DE genes. Among the top 100 abundant transcripts (only the top 24 were listed in the table) in young leaves are several genes encoding proteins which function in transcription, translation, cell division, and signal transduction (Table 4.5). In contrast, the majority of the highly expressed transcripts in older leaves have

functions in various metabolic processes. Interestingly, both young and older leaves showed high expression levels of several different signaling genes, such as receptor like kinases indicating that differential types of signaling play vital roles across development.

From the 24h DMSO treatment data, we also identified a large number of transcripts more abundant in young leaves (198 genes; Table 4.2) or in older leaves (123 genes; Table 4.5). In the top 100 abundant transcripts in young leaves, there were six chlorophyll a/b binding proteins, four receptor-like kinases, and three UDP-glucosyltransferases. In the top 100 highly expressed transcripts in older leaves there were four cytochrome P450s, five different receptor like kinases, six genes functioning in defense or stress response, and three genes involved in protein degradation.

We also examined the abundant transcripts that were present at 2h and 24h of DMSO treatment in each age sample. Although there was not much overlap between lists of abundant transcripts using a 2.5 log2 fold cutoff, a reduction in the cutoff to 1.5 log2 fold revealed that all abundant transcripts seen at 24h were also present as abundant transcripts in the 2h list. Additionally, it is important to note that all 18383 filtered genes used for comparisons were found in both 2 and 24 hour treatment samples in both young and older leaf tissue sample, indicating that these samples are largely similar.

Materials and Methods

Plant materials and growth conditions

The tomato cultivar Micro-Tom was used for all experiments. Plants were grown in Sunshine Mix #8 soil under a 16h light/8h dark photoperiod at 150 μE, with a 26 ℃ day(light), 22 ℃ night (dark) temperature.

Cytokinin treatment and RNA extraction

In each sample treatment six leaves each from different individual plants were excised. For both 13d and 35d old plants only the apical most fully expanded leaves were collected in this manner. In 13d plants these were the only true leaves that were fully expanded and present. The excised leaves were placed in water, and gently shaken for 2h prior to treatment with cytokinin 5μM benzyladenine (BA) and the solvent control DMSO for 2h or 24h. At the end of treatment leaves were patted dry then immediately flash-frozen in liquid nitrogen [7, 85]. RNA was subsequently extracted using Qiagen RNeasy Kit according to the manufacturer's instructions.

Library preparation and Sequencing

Messenger RNA was isolated with polyA selection and constructed into paired end sequencing libraries with an insert size of 180bp with the TruSeq RNA sample preparation protocol from Illumina (San Diego, CA).

Paired-end sequencing was performed on 16 samples on the Illumina HiSeq 2000 platform, generating 131,158,386 2x50bp read pairs. Additionally, 60,180,592 1x54bp single-end reads were generated on the Illumina GAIIX platform to attain adequate read counts for each sample for assessing differential expression. In total, over 16.4 Gbp were sequenced for *de novo* assembly and differential expression analysis. Raw sequence data is available for download at NCBI Sequence Read Archive under the accession (currently awaiting SRP # assignment).

Assembly

Paired-end sequences from 16 samples were pooled together to construct a *de novo* tomato leaf transcriptome assembly. Reads passing initial Illumina filters were further trimmed with the FASTX-Toolkit [86] at the 3' end with a quality score threshold

of Q15. Reads were first assembled with ABySS (v1.2.6) [87] with a kmer sweep of select kmers from 25 to 50 and scaffolding enabled. Gaps in the assembly were closed with GapCloser (v1.10, SOAP package) [88]. Contigs from the kmer-sweep were pooled and deredundified with CD-HIT-EST (v4.5.4) [89]. An overlap-layout-consensus assembly from these contigs, or synthetic ESTs, was created with MIRA (v3.2.1) [90] operated in Sanger EST mode. The final assembly contained 28,606 synthetic ESTs and was used as a reference for subsequent gene expression analysis.

Expression analysis with custom transcriptome reference

The 3'-trimmed reads used in *de novo* assembly and additional single-end sequences were aligned to the final assembly with BWA with default settings (v0.5.9) [30]. Gene expression was quantified as the total number of reads for each sample that uniquely aligned to the reference, binned by transcript. Twelve comparisons wherein one variable changed were performed to elucidate the transcripts differentially expressed with age (13 and 35 days), treatment (cytokinin and control vector), and treatment length (2 h and 24 h). To perform robust analyses, we only considered transcripts that were covered by at least 2 reads per million in at least 2 samples in any given comparison; this reduced the number of transcripts assessed from 28,606 to 18,838. Differential expression analysis of these, per-sample read counts was performed with the negative binomial test in DESeq [91]. Genes were identified as differentially expressed if they had an adjusted (Benjamini-Hochberg False Discovery Rate (FDR) method for multiple testing corrections) p-value of 0.1 or less. These transcripts were annotated against the International Tomato Annotation Group (ITAG) Solanum lycopersicum protein reference version 2.3 reference with Blastx [92].

Gene ontology analysis

The functional annotation software Blast2go (http://www.blast2go.com/b2ghome) was used to do gene ontology analysis of the cytokinin responsive genes. The major GO categories to which the cytokinin responsive genes belong were determined after the genes were subject to blast, mapping, and annotation. Results were presented as a bar chart showing the percent of genes belonging to each GO category identified.

qPCR analysis

To synthesize cDNA, 500 ng of the total RNA, the same as isolated for RNA-seq analysis, was used for each sample in the reverse transcription with Quanta qScript cDNA supermix. The first strand of cDNA was diluted 50 times before it was used in the qRT-PCR. qRT-PCR was performed with the SYBR-Green chemistry in an Eppendorf Mastercycler ep realplex with gene specific primers (Table S3). Each reaction contains 9 μL of SYBR-Green supermix, 5 μL of cDNA template, and 3 μL of forward and reverse primers (4 μM). The qRT-PCR program consists of one cycle at 95 ℃ for 15 sec, followed by 40 cycles of 15 sec at 95 ℃, 45 sec at 57 ℃, and 25 sec or 40 sec at 68 ℃. The relative expression data used in the table represent means ± SE of two biological replicates. All samples are compared to the control gene TIP41 [93].

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*Chapter 5

Advances in upstream players of cytokinin phosphorelay: receptors and histidine phosphotransfer proteins

Abstract

Cytokinins are a class of plant hormones that have been linked to numerous growth and developmental aspects in plants. The cytokinin signal is perceived by sensor histidine kinase (HK) receptors and transmitted via Histidine phosphotransfer proteins (HPts) to downstream response regulators (RRs). Since their discovery, cytokinin receptors have been a focus of interest for many researchers. Ongoing research on these transmembrane receptors has greatly broadened our knowledge in terms of cytokinin-receptor interaction, receptor specificity, receptor cellular localization, and receptor functions in cytokinin related growth and developmental processes. This review focuses on the recent advances on the cytokinin receptors and HPt proteins in *Arabidopsis.*

Key words: cytokinin, plant hormone, cytokinin receptors, phosphotransfer proteins

Introduction

Cytokinins are a class of plant hormones that are principally N^6-substituted adenine derivatives. Over fifty years of study has shown that cytokinins play a myriad of roles in biological processes including cell division, seed germination, apical dominance,

*Published in Plant Cell Rep 31:789–799.

shoot meristem initiation and maintenance, leaf and root differentiation, stress tolerance, and senescence in plants (Gan and Amasino 1995; Haberer and Kieber 2002; Hwang and Sheen 2001; Mok and Mok 2001; Werner and Schmülling, 2009). In the past decade genetic and molecular studies have led to the establishment of a well-defined cytokinin signaling model which resembles the two-component systems (TCSs) widely used by bacteria and some fungi (Beier and Gross 2006; Catlett et. al. 2003; Ferreira and Kieber 2005; To and Kieber 2008). In Arabidopsis, cytokinin signaling starts with perception of the cytokinin molecule by a sensor Histidine Kinase (AHK). The signal is then relayed by a Histidine Phosphotransfer protein (AHP) through phosphorylation to the Response Regulators (ARRs) in the nucleus (Figure 5.1). A branch pathway has also been identified in Arabidopsis that includes AHKs, AHPs, and Cytokinin Response Factors (CRFs) (Rashotte et al. 2006).

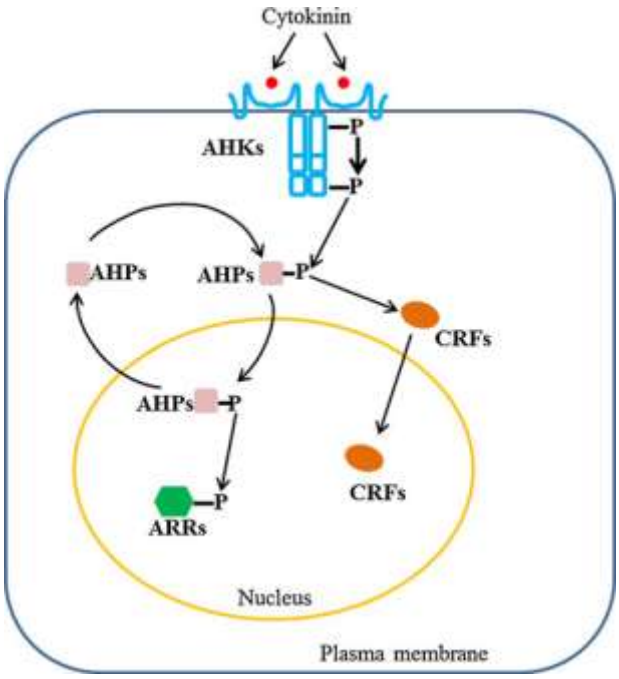

Figure 5.1. A schematic diagram of classical cytokinin signal phosphorelay. The perception of cytokinin by one of the AHKs at the plasma membrane results in the autophosphorylation of the receptor, the phosphoryl group is subsequently transferred to an AHP which passes the phosphoryl group to an ARR in the nucleus. AHKs, AHPs, together with CRFs, form a branch pathway of cytokinin signaling.

The perception and relay of a cytokinin signal through this type of cytokinin signaling pathway appears to be conserved across plant species as Cytokinin receptors (HKs), phosphotransfer proteins (HPts), response regulators (RRs), and even CRFs have been identified in increasing number of species such as maize, rice, *Medicago sativa*, soybeans, and tomato (Yonekura-Sakakibara et al. 2004; Ito and Kurata 2006; Pareek et al. 2006; Gonzalez-Rizzo et al. 2006; Pils and Heyl 2009; Rashotte and Goertzen 2010; Le et al. 2011). This level of conservation indicates the essential role that cytokinin signaling plays in plants. This review focuses on the research advances on the cytokinin receptors and HPt proteins of the cytokinin signaling pathway in *Arabidopsis*.

Cytokinin receptors

Nearly half a century after the discovery of cytokinin, its receptors were first identified in Arabidopsis as ARABIDOPSIS HISTIDINE KINASE 4 (AHK4) /WOODENLEG (WOL) /CYTOKININ RESPONSE 1 (CRE1), ARABIDOPSIS HISTIDINE KINASE 2 (AHK2) and ARABIDOPSIS HISTIDINE KINASE 3 (AHK3) (Mähönen et al. 2000; Inoue et al. 2001; Ueguchi et al. 2001b; Yamada et al. 2001; Suzuki et al. 2001a; Kieber and Schaller 2010). Further studies revealed that these cytokinin receptors play both redundant and specific roles in cytokinin-mediated growth and developmental processes in Arabidopsis (Higuchi et al. 2004; Nishimura et al. 2004;

Riefler et al. 2006). Recent work on cytokinin receptors has shown that each receptor mediates the specificity of different cytokinins in the signaling pathway (Stolz et al. 2011). Perhaps more importantly, new experimental works have demonstrated the localization of cytokinin receptors not to the plasma membrane as originally predicted, but to the ER membrane instead (Caesar et al. 2011; Lomin et al. 2011; Wulfetange et al. 2011). Each of these findings is likely to have profound impacts on our understanding of cytokinin signaling.

Structure of the cytokinin receptors – hybrid histidine kinases

The three cytokinin receptors (AHK2, 3, and 4) belong to a small Arabidopsis histidine kinase family which is comprised of 16 known members with diverse roles. These family members of kinases are involved in ethylene signaling [ETR1, ERS1, ETR2, ERS2, and EIN4] (Chang et al. 1993; Hua and Meyerowitz 1998), cytokinin signaling [AHKs] (Ueguchi et al. 2001b; Yamada et al. 2001; Hwang and Sheen 2001), cytokinin-independent activation of two-component signaling pathway [CKI1] (Kakimoto 1996, 1998; Hejátko et al. 2009; Deng et al. 2010), osmotic stress responses [CKI2/AHK5] (Urao et al. 1999; Tran et al. 2007), and light responses [light receptors PhyA-E] (Quail 2002; Schepens et al. 2004; Strasser et al. 2010).

AHK2, AHK3, and AHK4 are hybrid histidine kinases because they contain both the HK domain and the RR domain also known as a receiver domain (West and Stock 2001; Ueguchi et al. 2001a). The HK domain contains a conserved His residue which is autophosphorylated when a stimulus is perceived (West and Stock 2001). The phosphoryl group is transferred to the receiver domain from the HK domain before it is relayed further down the signaling pathway. Amino acid sequence analysis revealed that

cytokinin receptors are highly homologous to each other with great similarity in overall predicted protein structure (Ueguchi et al. 2001a). AHKs are all transmembrane proteins that possess: transmembrane segments, a typical ligand binding domain, a conserved histidine kinase domain (also called transmitter domain), and a receiver-like domain followed by a receiver domain. AHK2 and AHK3 are three pass transmembrane proteins whereas AHK4 is two pass (. Although this has not been specifically examined it would be interesting to explore how different numbers of transmembrane segments might result in functional differences between these proteins. The ligand binding domain was characterized and named CHASE (cyclases/histidine kinases associated sensing extracellular) domain by two research groups (Anantharaman and Aravind 2001; Mougel and Zhulin 2001). The CHASE domain found in various prokaryotic and eukaryotic receptor-like proteins was shown to directly function as the cytokinin binding region of the receptor with four specific amino acids crucial for ligand-binding (Heyl et al. 2007). Interestingly, one out of five identified ChASE domain containing proteins in rice contains a Ser/Thr protein kinase domain instead of a His kinase domain (Han et al. 2004; Ito and Kurata 2006; Pareek et al. 2006). This protein was named OsCRL4 (Han et al. 2004) or CHARK (CHASE domain Receptor-like serine/threonine Kinase) (Ito and Kurata 2006). OsCRL4 was found to be able to complement *cre1* mutation and was suggested to represent a CRE1-like new member of cytokinin receptors in rice (Han et al. 2004). The histidine kinase domain of the Arabidopsis cytokinin -receptors was found to possess five consensus motifs (H, N, G1, F and G2) as well as a conserved histidine residue. The receiver domain contains three regions that include the conserved D, D and K amino acids residues (Ueguchi et al. 2001a). Although studies have shown that the

conserved Histidine residue in the Histidine kinase domain and the conserved aspartate residue in the receiver domain are necessary for the signaling capacity, autophosphorylation, and phosphorelay (Hwang and Sheen 2001; Inoue 2001), the specific roles of those motifs remain to be determined.Recently, the crystal structures of AHK4 CHASE domain (sensor somain) binding to different cytokinins have been determined (Hothorn et al. 2011). The CHASE domain consists of an N-terminal long helix and two PAS-like domains that are connected by a linker helix. It turns out that AHK4 CHASE domains form homodimers in crystals. The membrane-distal PAS domain of AHK4 recognizes cytokinins. The structure of AHK4 CHASE domain in complex with iP shows that both the adenine moiety of iP and its isopentenyl tail occupy the binding pocket of AHK4. In the lower part of the ligand-binding pocket, the central β-sheet of the PAS domain is lined with small hydrophobic residues such as Ala and Gly, which are believed to be crucial for the receptor activity. Two β-strands in the upper part of the pocket are responsible for hydrophobic interactions as well. Hydrogen bonds formed between Asp262 and the adenine ring within the pocket may be crucial for receptor function. Further examination revealed an additional hydrogen bond between hydroxylated isopentenyl side chain of tZ with Thr294, the only hydrogen-bond acceptor in the tail binding pocket, providing the structural basis for the high affinity of AHK4 to tZ other than cZ. This study by Hothorn et al. (2007) also indicates synthetic cytokinins such as thiadiazuron occupy the binding pocket of AHK4 as natural cytokinins. In summary, by determining the crystal structures of the sensor domain of AHK4 in complex with different cytokinins, the authors revealed how AHK4 interacts with cytokinins and how new useful cytokinins can be potentially designed.

Ligand-receptor interaction – perception is everything

Elucidating the cytokinin-receptor interaction is an important step to understand the cytokinin signaling pathway. To this end, many assays have been developed to study the cytokinin-receptor interaction including the biological activity of cytokinins, the binding activity and ligand-binding specificity of the receptors. These assays include plant-based bioassays, bacterial assays, and even yeast-based assays. Yamada et al. (2001) revealed that three cytokinins (trans-zeatin [tZ], isopentenyladenine [iP], and a synthetic cytokinin thidiazuron [TDZ]) could be perceived by AHK4 receptor expressed in an *E. coli* mutant which lacked the endogenous histidine kinase RcsC. Using live cell hormone binding assays, Romanov et al. (2005) revealed that AHK4 is highly specific for tZ (Table 1), confirming the role of AHK4 as a cytokinin receptor. Examination of the ligand specificity of AHK4 with diverse cytokinin analogues showed the following order of affinity for AHK4: tZ>zeatin riboside (ZR)>dihydrozeatin (DZ)>cis-zeatin (cZ)>zeatin O-glucoside. This affinity order based on live cell binding assay is in agreement with that produced with purified bacterial AHK4-expressing membrane preparations as well as that from a reporter gene assay on the AHK4-expressing *E. coli* clone (Spichal et al. 2004).

Further hormone binding assays provided evidence that AHK2 CHASE-TM (TM represents the two transmembrane segments adjacent to the CHASE domain) has similar ligand preferences to AHK4 CHASE-TM (Stolz et al. 2011). Both CHASE-TM domains displayed a much higher affinity to iP and tZ than to their ribosides and a very low affinity to cZ. A comparison study on the ligand specificity of AHK3 and AHK4 indicated that AHK3 –has a much lower affinity to iP and its ribosides but a higher

affinity to DZ than AHK4(Table 1; Romanov et al. 2006). In addition, cZ could activate AHK3 at 1μM but not AHK4 in bacterial assays (Spichal et al. 2004). What is responsible for these differences in ligand recognition and signaling ability, however, remains unclear. An in-depth study on the structural difference of the receptor binding regions as well as the space-filling models of various cytokinins might provide some insights into this intriguing question.

Expression patterns of the cytokinin receptors – both unique and overlapping

All three Arabidopsis cytokinin receptors have been detected in different organs such as roots, leaves, stems, and flowers at varying levels, although individual specificity can be detected (Ueguchi et al. 2001a). *AHK4* generally seems to be root-specific detected in this tissue at high levels by RT-PCR, Northern, and with *AHK4* promoter::GUS fusion expression (Ueguchi et al. 2001a; Mähönen et al. 2000; Higuchi et al. 2004). This root-specific expression of *AHK4* was confirmed by *in situ* hybridization which also further revealed it to be localized to the vascular cylinder and pericycle in primary roots: a pattern specified fairly early during embryogenesis (Mähönen et al. 2000). Similar vascular expression patterns of *AHK4* have also been observed in sections of roots expressing an *AHK4* promoter::GUS fusion (Mähönen et al. 2006). *AHK2* transcript was found to be the most abundant in leaf tissue and least abundant in stems as shown in RT-PCR (Ueguchi et al. 2001a). *AHK3* also shows its highest expression in rosette leaves although it is also strongly expressed among roots, stems, and flowers (Ueguchi et al. 2001a; Higuchi et al. 2004). Overall, *AHK2*, *AHK3*, and *AHK4* receptors have been shown to have overlapping expression patterns with each other, although specificity such as *AHK4* localization to the root has been reported (Table 5.1). For a

more detailed discussion of these expression patterns see papers by Higuchi et al. (2004), Nishimura et al. (2004), and Mähönen et al. (2006a).

Table 5.1. Cytokinin binding specificity, tissue expression pattern, and predominant roles of cytokinin receptors in Arabidopsis

Cytokinin receptors	Cytokinin binding specificity	Tissue expression pattern	Functional roles
AHK2	iP>tZ>iPR>tZR> DZ[1]	Leaves, roots, flowers[3]	Root branching[7,8]; abiotic stress responses[9,10]; shoot vascular tissue development[11]
AHK3	tZ>tZR>DZ>iP>cZ[2]	Leaves, stems, roots and flowers [3,4]	Retardation of leaf senescence and Root branching[7,8]; abiotic stress responses[9,10]; shoot development[8]; shoot vascular tissue development[11]
AHK4	tZ> iP> tZR> iPR[2]	Roots[3-6]	Vascular morphogenesis[5]; responses to exogenous cytokinins[8,12]

[1] Stolz et al. 2011; [2] Romanov et al. 2006; [3] Ueguchi et al. 2001a; [4]Higuchi et al. 2004; [5]Mähönen et al. 2000; [6] Mähönen et al. 2006; [7]Kim et al. 2006; [8]Riefler et al. 2006; [9]Jeon et al. 2010; [10]Tran et al. 2007; [11]Hejatko et al. 2009; [12]Nishimura et al. 2004

Subcellular localization of the cytokinin receptors – plasma membrane or ER

Cytokinin receptors are predicted to be plasma membrane localized based on domain analysis, sequence similarity to two-component hybrid molecules, and some experimental evidence obtained with receptor-GFP fusion proteins (Inoue et al. 2001; Kim et al. 2006; Mähönen et al. 2000). Several very recent studies, however, have presented evidence supporting the localization of the AHKs to a different membrane: the endoplasmic reticulum (ER) (Caesar et al. 2011; Lomin et al. 2011; Wulfetange et al. 2011). Analysis of separated plasma membrane and endomembrane fractions indicated a predominant endomembrane location of cytokinin binding sites; examination of AHK-GFP fusion proteins expressed in tobacco leaf epidermal cells revealed ER localization of these proteins (Wulfetange et al. 2011). Immunoblots with Myc-tagged cytokinin receptors following fractionation of cell membranes lent further support to the ER localization of

the receptors (Wulfetange et al. 2011). Similarly, another study has demonstrated the ER localization of fluorophor-tagged AHK3 and AHK4 proteins (Caesar et al. 2011). In addition, the maize ZmHK1 cytokinin receptor has recently also been shown to localize to ER (Lomin et al. 2011). Although the above experimental evidence strongly indicate that the cytokinin receptors are mainly localized to ER, partial plasma membrane localization cannot be ruled out (Wulfetange et al. 2011). If both localizations exist, the question arises as to when cytokinin signaling occurs from the plasma membrane and when it occurs from the ER membrane. In addition, how the targeting of cytokinin receptors to these different membrane locations in the cell remains to be elucidated. Given that the ER localization of cytokinin receptors is experimentally supported, a revised model of cytokinin signal phosphorelay is proposed here (Figure 5.2). This recent finding is critically important as it presents a general shift in thinking about how cytokinin signaling functions in the cell. Previously, it was most important to have cytokinin outside or at the edge of the cell for proper perception and then signaling. Now that the receptor is internal, likely requiring cytokinin to be perceived within the lumen of the ER, movement of cytokinin into the cell and cellular organelles could be much more crucial to normal signaling than once thought. Since neither of these transport processes have been well studied their characterization is likely to have profound effects on the field of cytokinin signaling.

Functions of cytokinin receptors in physiological processes – an actor in many roles

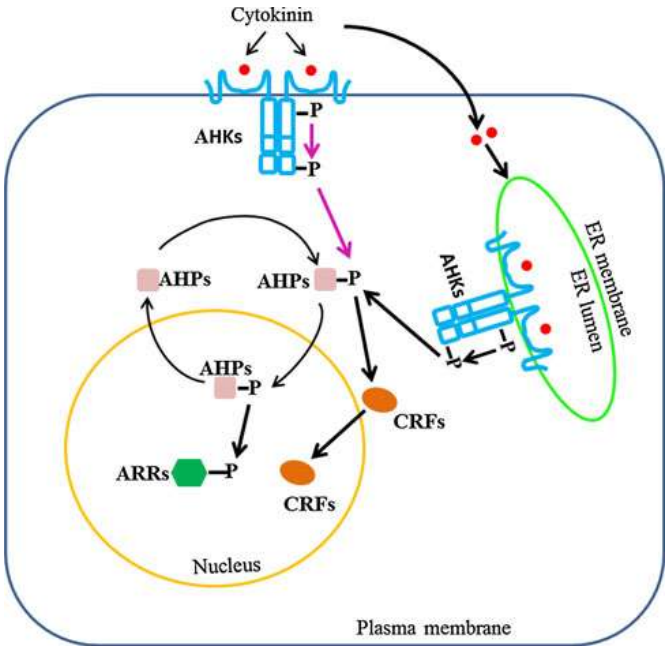

Figure 5.2. A modified cytokinin signal phosphorelay model. Cytokinin receptors are mainly localized at the ER membrane but might also be localized at the plasma membrane. Cytokinin binding to one of the AHKs in the ER lumen or at the plasma membrane results in the autophosphorylation of the receptor, the phosphoryl group is subsequently transferred to an AHP which passes the phosphoryl group to an ARR in the nucleus. AHKs, AHPs, together with CRFs, form a branch pathway of cytokinin signaling.

Ongoing research has provided evidence that cytokinin receptors play multiple roles in cytokinin mediated physiological processes (Table 1), such as regulation of root vascular morphogenesis (Mähönen et al. 2000), retardation of leaf senescence (Kim et al. 2006), regulation of shoot vascular development (Hejatko et al. 2009), and mediation of abiotic stress responses (Merchan et al. 2007; Tran et al. 2007; Coba de la Peña et al. 2008; Jeon et al. 2010).

Cytokinin receptors play roles in both root- and shoot-related processes – three coworkers in roots vs. two coworkers in shoots

Initial studies on *wol* (AHK4) mutants indicated an important role of the *WOL* allele in vascular cell divisions as mutants had fewer vascular initial cells resulting in a root vascular system comprised only of protoxylem (Mähönen et al. 2000; Cano-Delgado et al. 2000). The *wol* mutation is an amino acid substitution in the CHASE domain (T278I) that eliminates the cytokinin binding activity of the WOL Protein (Mähönen et al. 2000; Heyl et al. 2007; Yamada et al. 2001). Unlike *wol* mutants, AHK4 T-DNA insertion null mutations *cre1-2* and *cre1-11* did not show visible defects in root morphology under standard conditions, although they exhibited reduced sensitivity to cytokinin inhibition of root growth and adventitious root formation (Higuchi et al. 2004; Inoue et al. 2001; Mähönen et al. 2006a). Further experiments demonstrated that the mutated WOL protein serves as a constitutive phosphatase in the absence of cytokinin to inhibit cytokinin signaling mediated by AHK2 and AHK3, which results in the *wol* phenotypes (Mahonen et al. 2006a). Notably, single mutants of *AHK2* or *AHK3* responded normally or slightly less to cytokinin in root elongation assay (Higuchi et al. 2004), whereas *cre1-2*, *ahk4-1*, and combinations of *cre1-2* with other *ahk* mutants were resistant to cytokinin, indicating a predominant role of CRE1/AHK4 in responses to exogenous cytokinins (Nishimura et al. 2004; Riefler et al. 2006). Surprisingly, *ahk2ahk3* double mutants displayed a much more branched root system compared with that of wild type, indicating a negative role of AHK2 and AHK3 in regulating root branching (Riefler et al. 2006). Taken together, *AHK2*, *AHK3*, and *WOL/CRE1/AHK4* exert overlapping roles in roots with *WOL/CRE1/AHK4* functioning as a predominant root regulator. This

functional overlap of these three receptors is consistent with their overlapping expression patterns (Mähönen et al. 2006a).

Unlike the root-specific expression of *AHK4*, both *AHK2* and *AHK3* transcripts are abundant in leaves or stems, raising the possibility that these two receptors play roles in leaf- or shoot-related developmental processes (Ueguchi et al. 2001a; Mähönen et al. 2000; Higuchi et al. 2004; Ueguchi et al. 2004). The greatly reduced rosette size in the *ahk2ahk3* double mutant indicates the involvement of these two receptors in shoot development (Riefler et al. 2006). A recent study has implicated *AHK2* and *AHK3* in regulating procambium development, as seen from a reduced number of procambial cell layers found in the mutants of these genes (Hejatko et al. 2009). An additive effect was seen in the *ahk2 ahk3* double mutant that exhibited a stronger phenotype than either single mutant as manifested by a reduction in procambium and vascular bundle size as well as the lack of an interfascicular cambium. In addition, a depletion of endogenous cytokinin in either *CKX1* or *CKX3* overexpressors resulted in similar defects in vascular bundle development, confirming the role of AHK2 and AHK3 mediated cytokinin signaling in vascular bundle development (Hejatko et al. 2009).

Senescence and leaf longevity – receptors for going green

Leaf senescence is a programmed natural process influenced by various internal and external factors and phytohormones such as cytokinins, auxin, ethylene, and ABA are important internal factors involved in that regulation (Lee et al. 2001; Quirino et al. 2000; Smart 1994; Lim et al. 2010). Particularly cytokinins are known to delay leaf senescence as shown in experiments that prolonged the life span of leaves when the cytokinin biosynthetic *IPT* gene was expressed under the control of a senescence-specific

promoter *SAG12* (Gan and Amasino 1995) or a heat shock promoter *HSP18.2* (Merewitz et al. 2010). Identification of an *AHK3* gain-of-function mutant, *ore12-1*, revealed that AHK3, not AHK2 or AHK4, is the primary cytokinin receptor functionally regulating leaf senescence via an ARR2-specific phosphorelay cascade (Kim et al. 2006). This is consistent with the finding that AHK3 is the major contributor to cytokinin-dependent chlorophyll retention in leaves (Riefler et al. 2006). Interestingly it is currently unknown which AHP might specifically be responsible for transferring the phosporyl group from AHK3 to ARR2 to delay leaf senescence.

A cytokinin induced delay in leaf senescence has notably been accompanied by a large increase in extracellular invertase activity, suggesting that changed source-sink relations might also be linked to these processes (Lara et al. 2004). This idea is supported by two worksin which the invertase expression was manipulated in different ways. . One expressing an invertase during senescence in tobacco using a *SAG12*:*Cin1* (an invertase from *Chenopodium rubrum* driven by a senescence induced promoter). A second approach blocked extracellular invertase activity through the expression of a tobacco invertase inhibitor under control of a cytokinin-inducible promoter, thus rendering cytokinin ineffective in delaying leaf senescence (Lara et al. 2004). Together these results suggest that extracellular invertase is involved in the suppression of leaf senescence by cytokinins. Surprisingly little work had been done in Arabidopsis to elucidate how cytokinin signaling and the members of this pathway are linked to senescence and connected to extracellular invertases in this process.

Mediating abiotic response - receptors to keep from stressing out

Cold-induced expression of a subset of type-A ARR genes, including *ARR5*, *ARR6*, *ARR7*, and *ARR15* was shown to be mediated by the receptors AHK2 and AHK3 (Jeon et al. 2010). In fact, the *ahk2 ahk3* double mutant displayed significantly greater freezing tolerance compared with wild type as did *arr5*, *arr6*, and *arr7* loss-of-function mutants, while an ARR7 overexpressing line was hypersensitive to freezing temperatures (Jeon et al. 2010). These results directly link the receptors to cold stress response and suggest a negative role of AHK2 and AHK3 in cold stress tolerance. A similar negative regulatory role has also been suggested for AHK2 and AHK3 in osmotic stress response as evidenced by the increased tolerance of *ahk2*, *ahk3*, and *ahk2 ahk3* double mutants to drought and salt stress (Tran et al., 2007). In addition, *ahk4* mutants also exhibited enhanced salt tolerance compared with wild type in presence of cytokinin, suggesting a similar role for AHK4 as well (Tran et al. 2007).

Additional evidence of cytokinin receptors linked to salt stress regulation has been seen in work in other species such as *Medicago sativa* where *MsHK1*, an *AHK3* homolog showed induction by salt (Coba de la Peña et al. 2008). This has also been seen in *Medicago truncatula* where two of its cytokinin receptors, *MtHK2* and *MtCRE1* were induced in roots both by salt stress and during recovery from this stress (Merchan et al. 2007). These results indicate the conserved roles of cytokinin receptors in mediating stress responses in different plant species likely as negative regulators of abiotic stress signaling (Tran et al. 2007).

Positive regulation of nodulation in legumes

Homologs of AHK4 have been identified in two legumes: MtCRE1 in *Medicago truncatula* (Gonzalez-Rizzo et al. 2006) and LHK1 in *Lotus japonicas* (Tirichine et al.

2007). Both cytokinin receptors were found to play a role in regulation of root nodulation. *MtCRE1*RNAi roots were insensitive to cytokinin and displayed strongly impaired nodulation compared with wide type, *MtHK2* RNAi, and *MtHK3* RNAi roots, indicating that MtCRE1mediated cytokinin signaling is required for normal nodulation in *Medicago* (Gonzalez-Rizzo et al. 2006). A gain-of-function mutation in CHASE domain of LHK1 in lotus resulted in spontaneous formation of root nodules, and conferred cytokinin independent activity on LHK1 (Tirichine et al. 2007). Further examination revealed that cytokinin perception occurs downstream of Nod factor signal transduction but upstream of cortical cell activation. Together, these results indicate that cytokinin signaling is necessary in root nodulation in legumes.

Regulation of the specificity of cytokinin signaling – location matters

Response to cytokinin across plant tissues is regulated by a number of factors including both the ligand binding affinity and the spatial expression pattern of specific cytokinin receptors (Stolz et al. 2011). The sensitivity of AHKs to iP and tZ was examined by -quantitation of cytokinin primary response gene (ARR5, 6) transcripts and reporter gene (pARR5:GUS) expression in receptor double mutants containing only a single functional receptor (Stolz et al. 2011). *ahk2 ahk4* mutants were less sensitive to iP compared with *ahk2 ahk3* mutants (Stolz et al. 2011), consistent with the observed higher affinity of AHK4 and weaker affinity of AHK3 to iP (Romanov et al. 2006). However, *ahk2 ahk4* mutants showed a higher sensitivity to tZ compared with *ahk2 ahk3* mutants (Stolz et al. 2011). Furthermore, AHK2 and AHK4 were able to activate pARR5:GUS reporter gene to a similar extent in the presence of iP or tZ (Stolz et al. 2011).

Overlapping yet distinct expression patterns of the three AHK receptors was seen using the pARR5:GUS reporter gene as a general proxy for cytokinin mediated expression in receptor double mutants (Stolz et al. 2011). All three receptors are strongly active in both the shoot and root tips. AHK2 and AHK3 are actively expressed in parenchyma cells, while AHK3 also stimulates reporter activity in stomata (Stolz et al. 2011). Interestingly, cytokinin has been linked to regulation of stomata opening in concert with other hormones (Acharya and Assmann 2009; Tanaka et al. 2006). Therefore, further examination is needed to determine whether the stomata-specific activity of AHK3 is responsible for cytokinin-mediated stomata opening. Overall, AHK2 and AHK3 display a broader activity domain in the shoot apex than AHK4 and they lead to increased expression of pARR5:GUS in leaves in the presence of cytokinin that is also lacking for AHK4 (Stolz et al. 2011). This indicates a predominant role of AHK2 and AHK3 in leaves and shoots similar to that seen in Higuchi et al. 2004. Strong pARR5:GUS reporter staining of root vasculature after a 5h incubation in *ah2ahk3* mutant other than *ahk2 cre1* and *ahk3 cre1* mutants (Stolz et al. 2011) confirms root vascular specific activity of AHK4 (Mahonen et al. 2000).

Analysis of receptor activity by way of a promoter-swap experiment shows that AHK4, when expressed under the control of an *AHK2* promoter can complement the *ahk2 ahk3* mutant consistent with the similar ligand binding spectrums of AHK2 and AHK4 (Stolz et al. 2011). Interestingly, *ahk2 ahk3* mutant can be partially complemented by expression of CHASE-TM of AHK3 fused with the cytoplasmic domain of AHK4 directed by the AHK3 promoter, suggesting an essential role of CHASE domain in specifying AHK3 function (Stolz et al. 2011). Taken together, these results indicate that

the differing ligand binding affinities and expression patterns of AHKs, possibly along with other yet unknown factors, contribute to the specification of cytokinin signaling in Arabidopsis plants.

Interestingly, both AHK4 and AHK3 seem to be able to mediate cytokinin specificity in the root tissue. AHK3 mediated cytokinin signaling was shown to regulate root meristem size as evidenced by enlarged root meristem in *ahk3* mutants, while AHK4 and AHK2 had little to no effect in this aspect (Dello Ioio et al. 2007). On the other hand, AHK4 is the predominant cytokinin receptor which regulates root vascular development (Mähönen et al. 2000, 2006). This specific activity of AHK3-and AHK4-mediated cytokinin signaling in roots is possibly achieved through differing receptor ligand affinity (Romanov et al. 2006; Bishopp et al. 2011) and expression patterns (Mähönen et al. 2006).

Histidine phosphotransfer proteins – the next step in cytokinin signaling Structure of HPt proteins

HPt proteins are essential players in the His-Asp-His-Asp phosphorelay which transfer the phosphoryl group from hybrid kinase receptors to downstream RRs (West and Stock, 2001). In Arabidopsis there are five HPts (AHP1-5) which carry the conserved phospho-accepting His residue (Heyl and Schmülling 2003; Hutchison et al. 2006). AHP6, also known as APHP1, is a pseudo HPt which does not contain the conserved His residue necessary for phosphotransfer activity (Suzuki et al. 2000; Mähönen et al., 2006a). Since AHPs contain only a short HPt domain their structure is much simpler compared with that of AHKs (Suzuki et al. 2000). Each of the AHPs is about 150 amino acids long except AHP4 which contains 127 amino acids (Suzuki et al. 2000). An EST database

search identified HPts in a variety of plant species which show great similarity in amino acid sequence to AHPs, indicating the conserved nature of these HPt proteins (Suzuki et al. 2000).

In maize, the crystal structure of one HPt protein, ZmHP2, has been determined (Sugawara et al. 2005). ZmHP2 contains four C-terminal helices which form an antiparallel bundle connected to two N-terminal helices by a β-turn. The residue Arg102, close to the phosphor-accepting His80 residue, was predicted to promote the formation of interaction complex between ZmHP2 and receiver domains based on the role of corresponding residues in bacterial and yeast HPt proteins. The His80 phosphorylation site is surrounded by conserved residues of ZmHP2 which are localized on three helices of the four-helix bundle and one N-terminal helix. Notably, the protrusion of the imidazole ring of His80 from ZmHP2 molecule surface is believed to be important for phosphate transfer. The conserved residues surrounding His80 possibly act as a docking interface for receiver domains, while the non-conserved residues seem to be responsible for specific activities of different HPt proteins.

Expression patterns and transcriptional regulation of AHPs by cytokinin

AHP1 is predominantly expressed in the roots; *AHP2* and *AHP3* are detectable across the plant including roots, stems, leaves, flowers, and siliques with the highest expression of *AHP2* in roots/flowers and that of *AHP3* in roots/leaves (Suzuki et al. 1998; Hradilová and Brzobohatý 2007). *AHP5*, similar to *AHP2* and *AHP3*, is expressed in various organs (Hradilová and Brzobohatý 2007), while *AHP4* is hardly detectable in leaves and in roots (Suzuki et al. 2000). *AHP6* is expressed in developing protoxylem and pericycle cells, shoot apex, and young leaves (Mähönen et al. 2006a). In general, these observations are

consistent with those of another study on *AHP* expression profiles through Northern blot hybridization (Tanaka et al. 2004). However, the latter was able to detect *AHP4* transcripts with varying size in aerial parts of plants, indicating the possibility of alternative splicing and/or alternative polyadenylation (Tanaka et al. 2004). The expression of *AHP4* has also been examined by another group that reported *AHP4* expression predominantly in young flowers (Jung et al. 2008). Interestingly, alternative splicing also seems to occur during *AHP5 RNA* processing as shown by two RT-PCR products of different size specific for *AHP5* transcript. Sequence analysis revealed the presence in the longer PCR product AHP5L of the second intron which is absent in the shorter PCR product AHP5, confirming the alternative splicing of AHP5 transcript (Hradilová and Brzobohatý, 2007).

Previous data based on Northern blot and microarray analysis indicate that *AHPs* are not transcriptionally regulated by cytokinin (Suzuki et al. 2000; Rashotte et al. 2003; Brenner et al. 2005), although *AHP5* has been seen to be regulated under specific conditions (Hoth et al. 2003). Hradilová and Brzobohatý, however, demonstrated that AHP1 to 4 transcripts increased in response to both a short-term cytokinin treatment and an increased level of endogenous cytokinin through inducible *IPT* gene expression as shown by qRT-PCR analysis (Hradilová and Brzobohatý 2007). It is not clear what factors are responsible for these differences; the sensitivity of detection methods or growth-stage specific induction by cytokinin might be reasonable explanations. As such, it is currently unclear whether AHPs are transcriptionally affected by cytokinin and if so what that would mean for signaling processes.

Cellular localization of AHPs

Initial studies on cellular localization of AHPs with AHP-GFP fusion proteins showed that most AHPs were generally localized in the cytosol but were then translocated to the nucleus upon exposure to or treatment by cytokinin (Hwang and Sheen 2001; Tanaka et al. 2004). A recent paper revealed that AHP2 and AHP5 show both nuclear and cytoplasmic localization in plants and that contrary to what was previously seen, cytokinin treatment has no effect on their subcellular localization (Punwani et al. 2010). Regardless of the role of cytokinin on AHP localization, it is still believed that AHPs function in relaying the phosphate signal from the membrane localized receptor to the response regulators in the nucleus. As such AHPs are thought to move in a cyclic pattern between the cytoplasm and nucleus and back again and thus can be found distributed between these parts of the cell (Figure 5.1, 5.2).

The role of HPt proteins in cytokinin-mediated processes

It is known that HPt proteins are part of the cytokinin signaling pathway, but how HPts regulate cytokinin-mediated processes? Initial findings using Arabidopsis protoplast and a pARR6: LUC reporter assay indicated that overexpression of AHPs (AHP1, AHP2, or AHP5) had little effect on cytokinin signaling (Hwang and Sheen 2001). However, Arabidopsis AHP2 overexpressors are hypersensitive to cytokinin in the root elongation assay, although no other morphological changes were observed (Suzuki et al. 2002). An opposite effect was found by eliminating AHP in loss-of-function T-DNA insertion mutants. This was most easily seen in *ahp* multiple mutants that have reduced cytokinin sensitivity in various cytokinin bioassays and in greatly reduced induction of type-A *ARR* transcripts by cytokinin in an *ahp1,2,3,4,5* mutant (Hutchison et al., 2006). While single and double AHP mutants responded to cytokinin normally in root elongation assays,

presumably due to functional redundancy, the *ahp1,2,3* triple mutant was less responsive to cytokinin than the wild type. In addition, multiple *ahp* mutants which include *ahp2*, *ahp3*, and *ahp5* mutations (*ahp2,3,5*, *ahp1,2,3,5*, *ahp2,3,4,5*, and *ahp1,2,3,4,5*) displayed a short primary root phenotype which could be rescued by a wild type *AHP5* gene (Hutchison et al. 2006). Further examination of the root of the quintuple mutant *ahp1,2,3,4,5* showed that exclusive protoxylem development occurred in the vascular cylinder reminiscent of *wol* mutant (Mähönen et al. 2000) and of the *ahk2,3,4* triple mutant (Mähönen et al. 2006). In fact, the multiple mutant lines lacking either all receptors (*ahk2,3,4*) or all phosphotransfer proteins (*ahp1,2,3,4,5*) are initially very similar in physiological appearance as small dwarf-like plants with little to no root, indicating a positive role of AHPs in cytokinin signaling. *ahp1,2,3,4,5* mutants, however, can recover through the generation of an adventitious root, that substitutes as a primary root, although plants still produced shorter siliques with fewer, but larger seeds per silique compared with the wild type (Hutchison et al. 2006). Examination of the *ahp1,2,3,4,5* quintuple mutant by another research group also revealed a decrease both in cell size and cell number in leaves (Deng et al. 2010). The greatly reduced cell number in the *ahp1,2,3,4,5* mutant resembles that seen in leaves of *ahk2,3,4* mutant (Nishimura et al. 2004). Taken together, AHPs, as mediators between AHKs and ARRs, are positive regulators with overlapping functions of cytokinin signaling which is essential for various developmental processes.

While most HPts in Arabidopsis function in a similar manner, two of them AHP4 and AHP6 are slightly different. AHP4 is often placed by phylogenetic analysis in the same clade with rice pseudo-HPts (Hutchison et al. 2006), and shows very low transcript

levels in both RT-PCR (Suzuki et al. 2000) and transcriptome analysis (Zimmermann et al. 2004). Analysis of *ahp* loss-of-function mutants suggests that AHP4 may play no role, a slightly positive role, or even a negative role in cytokinin signaling, depending on growth processes examined and the genetic background (Hutchison et al. 2006). Interestingly, a recent study indicates that AHP4 may function in regulating fertility (Jung et al. 2008). However, more work remains to be done to clarify whether the *ahp4* mutant and the AHP4 overexpressor used in this study have altered cytokinin responses and whether AHP4 regulates fertility via cytokinin signaling.

AHP6 is a pseudo-HPt protein in that it does not contain the conserved His residue necessary for phosphotransfer activity and its transcript expression is down-regulated by cytokinin treatment (Mähönen et al., 2006a). It was also found that AHP6 was unable to accept a phosphoryl group from the yeast SLN1 histidine kinase in the *in vitro* phosphotransfer assay, indicating that AHP6 is not functional in phosphotransfer (Mähönen et al., 2006a). Further examination indicates that protoxylem differentiation is negatively regulated by cytokinin but promoted by AHP6 which acts by inhibiting cytokinin signaling (Mähönen et al., 2006a). Thus the balance of the actions of cytokinin and AHP6 is important for the proper specification of protoxylem in the root system.

Interaction of HPt proteins with proteins within and outside the cytokinin signaling pathway-HPts act as a signaling connector

As mediators of a multistep phosphorelay, AHPs were shown to be able to interact with HKs (Urao et al. 2000; Suzuki et al. 2001a; Dortay et al. 2006), RRs (Urao et al. 2000; Lohrmann et al. 2001; Suzuki et al. 2001b; Tanaka et al. 2004; Dortay et al. 2006), and CRFs (Cutcliffe et al. 2011). An extensive analysis of protein interactions within

cytokinin signaling pathway indicated that AHPs act as hubs to interact with members of all other protein families (Dortay et al. 2006). In addition, AHPs have also been shown to interact with proteins outside cytokinin signaling pathway (Dortay et al. 2008). One example is that AHP1 could interact with ETR1as indicated by yeast two-hybrid assays and fluorescence spectroscopy (Urao et al. 2000; Scharein et al. 2008). It has also been shown that phosphorylation at the conserved His residue is important for the interaction of AHPs with its protein partners (Suzuki et al. 1998; Scharein and Groth 2011).

Concluding remarks

Cytokinin signaling is a complex pathway which requires coordinated functions of members from multiple gene families. Although functional redundancy was observed with cytokinin receptors and HPt proteins (as well as with RRs), recent work has been revealing specific roles for these players in cytokinin signaling pathway. More importantly, the very recent finding that cytokinin receptors are localized to ER will not only deepen our understanding about cytokinin signaling, but also raise interesting research questions. These recent advances will lead to more profound findings in cytokinin signaling and provide a new perspective in decoding the complex interaction between cytokinin signaling pathway and other signaling pathways.

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