Expression and Functional Roles of Cytokinin Response Factor Proteins in *Arabidopsis thaliana*

by

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Abstract

Cytokinin response factors (CRFs) are transcription factor proteins from the model plant *Arabidopsis thaliana* (Arabidopsis). CRFs comprise a small, highly conserved subgroup of the AP2/ERF family of plant specific transcription factors. CRF6 is among the first identified members of this group, which were found to be transcriptionally regulated by the plant hormone cytokinin. Cytokinins play key regulatory roles in a wide range of growth and developmental processes from embryogenesis to senescence.

Examination of the evolutionary relationships among CRF proteins in Angiosperms reveals a high degree of conservation within five distinct CRF lineages. Expression analyses of the 12 Arabidopsis CRFs demonstrate that all are expressed predominantly in vascular tissues with differences in organ level and developmental patterns.

Functional characterization of CRF6 revealed that this gene acts downstream of cytokinin signaling to delay leaf senescence, a process classically linked to cytokinin application. Specifically, the inhibition of leaf senescence known to occur as a result of treatment with exogenous cytokinin is greatly decreased in mutant plants lacking a

functional CRF6 gene. In contrast, over-expression of CRF6 leads to delayed leaf senescence without cytokinin treatment.

Finally, transcriptome wide analyses indicate that CRF6 acts to repress a set of cytokinin related genes in response to oxidative stress possibly as an adaptive response to such stress. This proposed function is supported by an enhanced stress tolerance phenotype of transgenic plants over-expressing CRF6.

Additionally, literature concerning cytokinin regulation of leaf senescence and interactions between cytokinin and stress response will be reviewed and discussed.

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This dissertation and the work which has made it possible are dedicated to the memory of my brother, James Carter Zwack.

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List of Abbreviations

ABA	abscisic acid
ABC	ATP-binding cassette
AHK	Arabidopsis HK
AHP	Arabidopsis HPt
AP2	apetala 2
ARR	Arabidopsis RR
BA	benzyl adenine
CaMV	cauliflower mosaic virus
CHK	CHASE domain containing HK
CK	cytokinin
CKX	cytokinin oxidase
CRF	cytokinin response factor
CWINV	cell wall invertase
DAG	days after germination
ER	endoplasmic reticulum
ERF	ethylene response factor

GUS	β-glucuronidase
H_2O_2	hydrogen peroxide
HK	histidine kinase
HPt	histidine containing phosphotransfer protein
HXK	hexokinase
IPT	isopentenyl transferase
LOG	lonely guy
MAPK	mitogen activated protein kinase
MD	mitochondrial dysfunction
PCD	programed cell death
qPCR	quantitative reverse transcription-polymerase chain reaction
ROS	reactive oxygen species
RR	response regulator
RRA	A-type RR
RRB	B-type RR
RRC	C-type RR
SAG	Senescence Associated Gene
SlCRF	*Solanum lycopersicum* cytokinin response factor
TCS	two-component cytokinin signaling pathway
TF	transcription factor
WT	wild type

Chapter 1 Introduction

Cytokinin Response Factors (CRFs) are APETALA2 (AP2)/ Ethylene Response Factor (ERF) family transcription factor (TF) proteins linked to cytokinin signaling and response.

1.1 CRFs are AP2/ERF Transcription Factors

CRFs comprise a monophyletic group within the AP2/ERF family of TFs. This family defined by the presence of a conserved plant lineage specific DNA binding domain is among the largest in number in many species including *Arabidopsis thaliana* (Arabidopsis). CRFs of which there are 12 in Arabidopsis, contain an additional unique domain known as the CRF domain which is involved in protein-protein interactions (Cutcliffe et al., 2011). CRF genes have been identified in genomes of all major groups of land plants including bryophytes, but appear to be absent in more basal taxa (Rashotte and Goertzen, 2010).

1.2 CRFs are Linked to Cytokinin

Cytokinin, as its name implies, is a hormone that was first characterized as a chemical capable of promoting cell division in cultured plant tissues (Miller et al., 1955a; Miller et al., 1955b). It was soon shown that high levels of cytokinin (relative to auxin) in tissue culture medium induced shoot differentiation while repressing roots (Skoog and Miller, 1957). The particular bioactive molecule used in these early experiments, kinetin, was isolated from autoclaved herring sperm, however a number of naturally occurring, plant-synthesized forms of cytokinin have since been identified (Mok and Mok, 2001; Sakakibara, 2006). Cytokinin is now known to regulate numerous developmental processes throughout the plant, spanning the interval from early events in embryogenesis to reproductive development and leaf senescence (Werner and Schmulling, 2009; Gupta and Rashotte, 2012).

The primary pathway of cytokinin signal transduction is fairly well understood as a result of work done over the last 10 to 15 years, primarily in Arabidopsis (Gupta and Rashotte, 2012; Shi and Rashotte, 2012). Upon perception by one of three Hybrid Histidine Kinase Receptors (HKs) the cytokinin signal is relayed via a modified version

of the bacterial two-component system known as the Two-component Cytokinin Signaling pathway (TCS) (Kieber and Schaller, 2010). The binding of the cytokinin molecule to the receptor leads to auto-phosphorylation of conserved a Histidine residue. These hybrid receptors also contain a receiver domain with the Aspartate residue necessary to accept the phosphoryl group from the original Histidine (Mahonen et al., 2000; Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). The signaling cascade continues with the phosphorylation of another His residue on a Histo-Phosphotransfer Protein (HPt) (Suzuki et al., 1998; Suzuki et al., 2002). In Arabidopsis there are five HPts which are largely redundant in function. As a phosphorylated HPt moves into the nucleus (a process which occurs independent of cytokinin and phosphorylation), it transfers the phosphate group to a final receiver domain of a Response Regulator protein (RR) (Hutchison et al., 2006; Shi and Rashotte, 2012). RRs of two distinct types mediate the transcriptional response to cytokinin (Imamura et al., 1998; Taniguchi et al., 1998; Imamura et al., 1999; Kiba et al., 1999; D'Agostino et al., 2000). B-type RRs are transcription factor proteins which become activated by phosphorylation, and direct transcription of primary response genes (Sakai et al., 2000; Hwang and Sheen, 2001; Sakai et al., 2001; Hosoda et al., 2002; Imamura et al., 2003; Yokoyama et al., 2007; Argyros et al., 2008; Ishida et al., 2008). Among these primary response genes are the A-type RRs which do not contain the DNA binding domain necessary to function as transcription factors, but compete with the B-type RRs for phosphorylation by HPts (Brandstatter and Kieber, 1998). As such, A-type RRs act as negative regulators of the cytokinin signaling pathway (To et al., 2004). This signaling pathway has been shown to mediate the cytokinin response in numerous classical and novel cytokinin-regulated processes (Gupta and Rashotte, 2012).

CRFs were initially identified in a screen for cytokinin responsive genes in Arabidopsis where three (CRF2, 5 and 6) were found to be transcriptionally induced by cytokinin treatment (Rashotte et al., 2006). The remaining members of the group (CRF1, 3, 4 and 7-12) were subsequently identified (Rashotte et al., 2006; Rashotte and Goertzen 2010). It is now known that CRFs act in concert with the B-type RRs in the

transcriptional regulation of cytokinin responsive genes, and that CRF proteins interact with TCS components (Rashotte et al., 2006; Cutcliffe et al., 2011).

1.3 Leaf Senescence is a Highly Regulated Process

Leaf senescence is a developmental process ultimately resulting in coordinated cellular apoptosis throughout the organ. The initiation of senescence can be triggered as part of an age-dependent genetic program or in response to environmental stress. In neither case is the senescent state passively acquired nor is it simply an unfortunate consequence. Rather, senescence is a regulated process dependent upon coordination between increased synthesis and activity of some proteins and the degradation or inactivation of others. This regulation is crucial because prior to cellular death, valuable nutrients and energy (in the form of carbohydrates) are released as a result of the breakdown of macromolecules and reallocated to other parts of the plant (Himelblau and Amasino, 2001; Hortensteiner and Feller, 2002). The recycling of resources which occurs as a result of senescence has been described as “altruistic”, and is thought to make the substantial investment of nutrients and energy that is required for the formation of leaves and operation of their photosynthetic machinery more evolutionarily favorable (Noodâen and Leopold, 1988; Lim et al., 2007).

As stated, senescence can be either developmentally or stress induced. Developmental senescence in annual and other monocarpic pant species primarily occurs as vegetative growth and development ceases and reproductive organs begin to form. Nutrients, as well as carbohydrates, are reallocated from leaves to support the growth and development of reproductive organs or developing embryos (Noodâen and Leopold, 1988; Buchanan-Wollaston et al., 2005; Diaz et al., 2008). The leaves of some perennial plant species die as a result of a developmentally programmed or environmentally triggered senescence event. For instance, the leaves of some plant species in preparation for winter undergo senescence in order to remove valuable nutrients from relatively fragile leaves. Following remobilization, these nutrients can be stored in more robust organs for protection from the harsh winter environment, in which photosynthesis would be inhibited by low temperatures and light levels (Matile, 2000). Additionally, leaves of many species emerge sequentially, resulting in leaves of various ages on one plant. As the

older leaves undergo a decrease in photosynthetic efficiency, the resources from these leaves can be reallocated toward the development of new ones. This process is known as sequential senescence (Noodâen and Leopold, 1988; Singh et al., 1992b; Diaz et al., 2008).

In response to physiologically stressful conditions, leaf senescence can serve multiple overlapping functions. Individual leaves may undergo stress induced senescence, for example sun-adapted canopy leaves that become shaded by another plant have a reduced photosynthetic capacity and senescence of such leaves not only facilitates reallocation of resources but also prevents water consumption by the unproductive organ (Munne-Bosch and Alegre, 2004). When an entire plant is stressed, reproduction may be hastened and resources remobilized from senescing leaves, can be allocated to production of, or storage in, seeds. This allows the subsequent generation to potentially establish in a different location or when conditions have improved (Buchanan-Wollaston et al., 2003; Lim et al., 2007; Wingler and Roitsch, 2008). Biotic stress can lead to senescence as a defense particularly against biotrophic pathogens. Often in order to prevent the spread pathogens, export from infected leaves is inhibited resulting in limited nutrient reallocation (Zimmermann and Zentgraf, 2005; Wingler and Roitsch, 2008).

Ultimately, the physiological processes, as well as the end result of developmental and stress-induced senescence, appear to be the same; however transcriptome analyses have indicated that distinct yet overlapping sets of genes are differentially expressed as a result of the two triggers. This seems to suggest that the pathways which regulate the initiation and progression of senescence under different conditions may be only partially redundant (Park et al., 1998; Buchanan-Wollaston et al., 2005; Lim et al., 2007). Whole genome microarray analyses of senescence induced by various stress conditions or age, have revealed some 8000 genes in Arabidopsis that are differentially expressed during senescence (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Lim et al., 2007). Recently, a similar number of genes were identified in a study which examined transcriptional changes over 11 developmental time points from leaf maturity to full senescence (Breeze et al., 2011). Expression analyses specifically targeting transcription factor genes differentially expressed during senescence have implicated numerous candidates as serving in upstream regulation of these global expression pattern changes

(Chen et al., 2002; Balazadeh et al., 2008). Transcriptome-based studies such as those mentioned above have expanded general understanding of the genetic regulation of senescence such that it can now be described as a vast network of interconnected signal transduction and regulatory pathways. This complex system of regulation further emphasizes the notion of leaf senescence as a highly regulated, dynamic process in plant development.

There are a number of signals known to influence the regulation of leaf senescence: exogenous signals such as light quality, nutrient availability, and pathogenic elicitors; and endogenous signals including sugars and phytohormones (Noodâen and Leopold, 1988; Rousseaux et al., 1996; Zimmermann and Zentgraf, 2005; Lim et al., 2007; van Doorn, 2008; Wingler and Roitsch, 2008; Wingler, 2011). The hormones ethylene (Abeles et al., 1988; Jing et al., 2005; van der Graaff et al., 2006), abscisic acid (Gepstein and Thimann, 1980; Zeevaart and Creelman, 1988; Weaver et al., 1998), jasmonic acid (Ueda and Kato, 1980; He et al., 2002; Kong et al., 2006), and salicylic acid (Morris et al., 2000; Rao et al., 2002) are all well known to be involved in stress responses and have also all been implicated in positive regulation of senescence. Auxin has been suggested to have a negative effect on senescence processes, but its precise role remains unclear (Noh and Amasino, 1999; Hong et al., 2000; Ellis et al., 2005; Okushima et al., 2005; van der Graaff et al., 2006). Cytokinin also negatively regulates senescence; its effects on this process, in contrast to auxin, have been reasonably well characterized and will be examined in detail in chapter 2.

1.4 Oxidative Stress is a Symptom and Signal of Stress

Plants utilize the redox potential of molecular oxygen (O_2) and water (H_2O) to drive both reductive (photosynthesis) and oxidative (respiration) energy. During both processes partially reduced/oxidized O_2/ H_2O can form. Such redox intermediates readily oxidize other molecules and are known as reactive oxygen species (ROS) (Apel and Hirt, 2004; Gill and Tuteja, 2010). Oxidation of many biomolecules including nucleic acids, proteins and lipids is detrimental to cellular integrity and can lead to cell death. Because the formation of ROS is an unavoidable aspect of metabolism, plants possess a wide array of scavenging mechanisms utilizing both enzymatic reactions as well as

antioxidants capable of detoxifying free-radicals (Mittler, 2002). Under steady state conditions these mechanisms maintain low, relatively harmless levels of ROS. However, perturbations to cellular homeostasis that can occur as a result of unfavorable environmental conditions, lead to metabolic dysfunction and an increase in ROS production (Gill and Tuteja, 2010). The resulting imbalance between the production and removal of ROS is known as oxidative stress, which if left unchecked can lead to excessive tissue damage.

Cell death resulting from oxidative stress in not solely the result of ROS toxicity, high levels of ROS also stimulate a programmed cell death (PCD) response. Moreover ROS are thought to act as systemic indicators of stress (Dat et al., 2000). Thus these molecules play a role in signal transduction in addition to simply wreaking havoc upon the plant (Apel and Hirt, 2004).

Drought or osmotic stress (for example, as a result of soil salinity or the formation of extracellular ice at sub-freezing temperatures) inhibits transpiration, limiting the availability of water in leaves. Stomatal closure limits water loss under these conditions, however this in turn leads to a decrease in [CO_2], over-reduction of the electron transport chain and an increase in electron transfer to O_2 (Miller et al., 2009). The depletion of CO_2 also leads to elevated photo-respiratory production of the ROS hydrogen peroxide (H_2O_2) in peroxisomes (Noctor et al., 2002).

Temperature extremes can also induce oxidative stress. Cold, non-freezing temperatures reduce membrane fluidity disrupting electron transport chain reactions. The same can occur as a result of membrane destabilization by elevated temperatures, primarily in mitochondria (Suzuki and Mittler, 2006). Conditions of excessive heat are often associated with high light levels that can also lead to ROS production by overwhelming photosystem II and its light harvesting complexes with excitation energy (Gill and Tuteja, 2010).

ROS can also be produced directly through O_2 interactions with heavy metals, UV radiation and pollutants such as ozone and sulfur dioxide (Mittler, 2002; Gill and Tuteja, 2010). Biotic stresses such as pathogen attack and herbivory lead to the production of ROS as well (in part as a defense mechanism) (Grant and Loake, 2000; Apel and Hirt, 2004). In fact many stresses, including those that do not directly cause oxidative stress,

often stimulate a response that includes production of ROS, supporting the notion of these molecules acting signals of general stress response (Apel and Hirt, 2004; Mittler et al., 2004).

In addition to ROS many other general and condition-specific signals function downstream of oxidative and other stress stimuli (Mittler et al., 2004). Phytohormones including abscisic acid, ethylene, salicylic acid, jasmonic acid and cytokinin act as regulators of both local and systemic responses to stress. Interactions among these and other signals are key to an adaptive response that is effective yet minimal negative effects on growth and reproduction (Argueso et al., 2009; O'Brien and Benkova, 2013). Cytokinin is generally considered to have negative regulatory role in stress responses; the current knowledge of the nature of this role and the interactions between cytokinin signaling and stress responses is discussed in detail in Chapter 5.

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Vascular Expression and C-Terminal Sequence Divergence of Cytokinin Response Factors in Flowering Plants

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Cytokinin response factors (CRFs) are important transcription factors that form a side branch of the cytokinin signaling pathway and have been linked to cytokinin-regulated processes during development. CRF proteins are defined as belonging to a specific transcription factor family by the presence of an AP2/ERF DNA-binding domain and can be distinguished within this family by a group-specific CRF domain involved in protein–protein interactions. Here we further delimit CRFs into five distinct clades (I–V) represented across all major angiosperm lineages. Protein sequences within each clade contain a clade-specific C-terminal region distinct from other CRFs, suggesting ancient evolutionary divergence and specialization within this gene family. Conserved patterns of transcriptional regulation support these clade divisions. Despite these important differences, CRFs appear to show preferential localization or targeting to vascular tissue in quantitative real-time PCR and reporter line analyses of *Arabidopsis thaliana* and *Solanum lycopersicum* (tomato). Phloem tissue expression within the vasculature often appears the strongest in CRF reporter lines, and an analysis of CRF promoter sequences revealed conservation and significant enrichment of phloem targeting *cis*-elements, suggesting a potential role for CRFs in this tissue. An examination of CRF loss-of-function mutants from cytokinin-regulated clades revealed alterations in higher order vein patterning. This supports both the general link of CRFs to vascular tissue and clade-specific differences between CRFs, since alterations in vascular patterning appear to be clade specific. Together these findings indicate that CRFs are potential regulators of developmental processes associated with vascular tissues.

Keywords: Arabidopsis • CRF • Cytokinin response factor • Genomic analysis • Phloem • Tomato.

Abbreviations: AHK, Arabidopsis hybrid histidine kinase receptor; AP2, apetela 2; ARR, Arabidopsis response regulator; CRF, cytokinin response factor; ERF, ethylene response factor; GUS, β-glucuronidase; MAPK, mitogen-activated protein kinase; qPCR, quantitative real-time PCR.

Introduction

The cytokinin response factors (CRFs) are a subset of the AP2/ERF family of transcription factor proteins found in all land plants. Within the ethylene response factor (ERF) subfamily, CRFs are defined by the presence of a group-specific domain, known as the CRF domain, several of which were initially identified in Arabidopsis microarray experiments as induced by cytokinin (Rashotte et al. 2003, Rashotte et al. 2006, Rashotte and Goertzen 2010). More recent studies have expanded the number of CRFs genes to 12 in Arabidopsis (*At*CRFs) and identified similar numbers in other plant genomes (Rashotte and Goertzen 2010, Cutcliffe et al. 2011, Shi et al. 2012). Studies to date on CRF genes have primarily focused on their interactions with cytokinin and the cytokinin signaling pathway. A broader analysis of CRF gene family diversity and tissue specificity that might suggest potential functional roles beyond cytokinin linkages has not been performed.

One way to improve understanding of a group of highly related genes is through a genomic approach utilizing the rapidly expanding number of available genome sequences, expressed sequence tag (EST) libraries and other genomic data resources from across the plant kingdom. A similar approach used previously for CRFs helped identify and characterize the conserved CRF domain that now defines this group of proteins as distinct from other ERFs (Rashotte and Goertzen 2010). Patterns of evolutionary change within a gene family may also imply functional differentiation, and the clarification of evolutionary relationships should help in interpretation of the results of functional studies. One goal of this study was to identify further conserved sequence motifs and particular amino acid residues within the understudied C-terminal ends of CRFs potentially to understand the evolution and function of these key transcription factors.

In addition to functional and genomic studies, much can be learned about a group of related genes by studying key similarities and differences in expression patterns. Often genes encoding proteins of highly similar structure and function are differentially expressed either spatially or temporally.

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Expression can also be regulated by myriad different signals including endogenous developmental signals such as hormones, as well as external stress responses. While it has been previously shown that some, but not all CRFs are transcriptionally regulated by cytokinin (Rashotte et al. 2003, Kiba et al. 2005, Rashotte et al. 2006, Shi et al. 2012), this study reveals that several CRFs from at least two species show a high degree of expression in vascular tissue, which appears to be a unifying characteristic of this group of genes. Future investigation of CRF function can now be directed at specific processes related to vasculature development and function.

Results

CRF proteins have distinct lineages within flowering plants

Although CRF genes have been identified across all land plant groups, a significant expansion and radiation of CRFs is evident in the angiosperms. We identified multiple, distinct groups of CRF genes in all major flowering plant lineages including *Amborella*, a representative of the earliest branching or most 'basal' flowering plants.

Phylogenetic analyses of >400 (403) expressed CRF proteins across land plants were performed using only the conserved CRF and AP2 domain sequences due to a lack of homology in C-terminal regions. These analyses repeatedly indicate that CRF genes may have diversified into five distinct groups in flowering plants. Nearly every available angiosperm genome contained at least one CRF gene from each group, whereas gymnosperm and other non-flowering plant genomes contain CRFs of a single type, themselves distinct from any in angiosperms. Despite consistent identification of this pattern, the limited resolution afforded by phylogenetic analyses of short, highly conserved CRF and AP2 domains yields little support for distinct nodes corresponding to these divergences. However, independent analyses of individual groups led to the identification of novel highly similar motifs in the previously CRF-wide unalignable C-terminal regions (**Fig. 1**; **Supplementary Figs. S1–S3**). Importantly, these group-specific C-terminal regions that range in size from 70 to 200 amino acids cannot be aligned with other groups and therefore could not be included in phylogenetic analyses. However, these sequences map out as nearly perfect clade-specific characters on the phylogenetic tree, lending strong support to relationships otherwise based solely on conserved domains.

Phylogenetic analyses of the five angiosperm CRF groups show they are collectively a derived, monophyletic group with respect to all other plant CRF domain-containing proteins, which we have named CRF clades (I–V) (**Fig. 1**; **Supplementary Fig. S1**). Each CRF clade contains 1–2 genes per species, except for clade V which is represented by 2–4 genes in several taxa (e.g. four in Arabidopsis *At*CRF9–*At*CRF12 and three in tomato *Sl*CRF9–*Sl*CRF11). Clade V is sister to all other clades, as was previously found in a study where these genes were collectively referred to as 'B-clade' CRF-related sequences (Rashotte and Goertzen 2010). Clade V CRFs show little to no transcriptional induction by cytokinin (Winter et al. 2007, Shi et al. 2012).

Clades I, II and III appear to be closely related based on loose similarities in the C-terminal and extreme N-terminal regions of these genes, coupled with a low overall sequence divergence. The resolution of clade III as a monophyletic group sister to clades I and II is not always captured in phylogenetic analyses, but is strongly suggested by the presence of conserved C-terminal elements (**Fig. 1**; **Supplementary Figs. S1–S3**). Clade III includes members that are transcriptionally induced by cytokinin: *At*CRF5, *At*CRF6 and *Sl*CRF5 (**Table 1**). Similarly, clade I members are induced by cytokinin: *At*CRF2 and *Sl*CRF2 (**Table 1**). Additionally, our quantitative real-time PCR (qPCR) analyses of the other Arabidopsis clade I member, *At*CRF1, also revealed transcriptional induction to 1.93 ± 0.16-fold the levels in 14-day-old seedlings after cytokinin treatment of 1 μM BA for 2 h. Clade II members, in contrast, are not induced by cytokinin: *At*CRF3, *At*CRF4, *Sl*CRF4 and *Sl*CRF6 (**Table 1**; Rashotte et al. 2006, Shi et al. 2012).

Although the exact relationship between clade IV proteins and those of clades I–III is not clearly resolved by phylogenetic analysis, the overall lack of similarity in C-terminal sequence suggests that they could be sister to the remaining clades. At least one member of this group (*Sl*CRF1) has been shown to be transcriptionally induced by cytokinin (**Table 1**). Interestingly, clade IV sequences are conspicuously absent from Brassicaceae genomes including Arabidopsis, but are well represented by other rosid families and even other Brassicales such as papaya. This appears to be a major exception to the rule of each clade being represented in every genome.

Although some clade-unique features can be identified within the CRF and AP2 domains, it is the C-terminal sequences that show the greatest degree of clade specificity. The beginning of each clade-specific region is marked by a putative mitogen-activated protein kinase (MAPK) phosphorylation site recognizable by the SP[T/S]SVL motif (**Fig. 1**). Even this motif found in all CRFs has some clade-based variation to it: the variable [T/S] residue of this motif in clade V is always a S, while it is primarily a T in the other clades (**Fig. 1**). Despite differences in C-terminal size and sequence composition, some clades do contain shared amino acid motifs, including a strongly conserved FQDI motif in both clades I and II (**Fig. 1**). Additionally, clades I–III all have a PX[D/E]XF[F] motif, as well as a SGY[D/E]S motif.

Clade IV proteins are defined by a short, but broadly conserved C-terminus with an abundance of acidic residues of little similarity to other CRF clades. Clade V proteins have the least C-terminal homology to the other clades; however, a PX[D/E]XF[F] motif can be seen similar to that found in clades I, II and III, although positioned further from the MAPK site.

We further attempted to find support for these CRF clade divisions using a hierarchical cluster analysis of microarray transcriptome experiments, since differences in sequence among clades might also be linked to functional expression differences.

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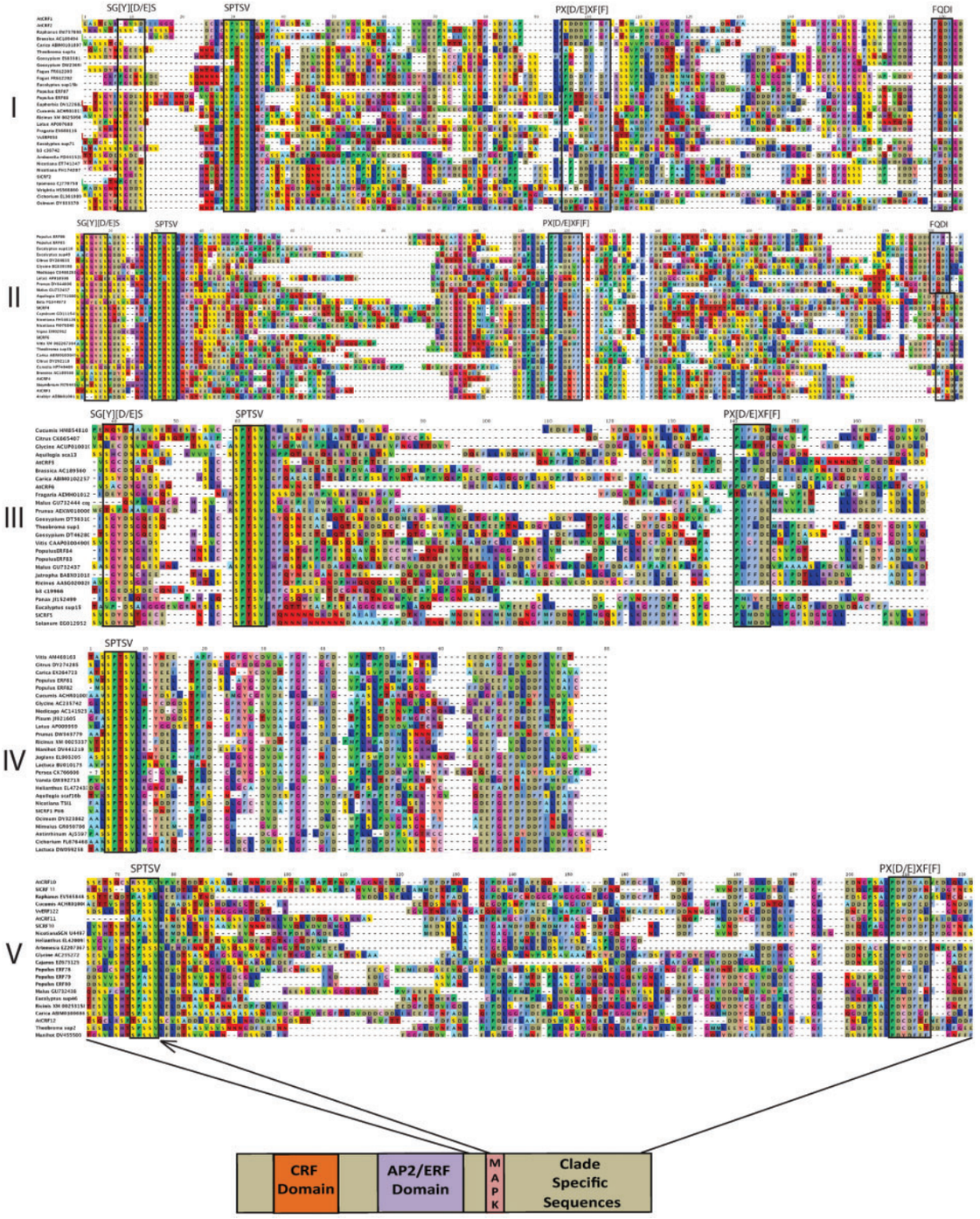

Fig. 1 Five distinct clades of CRF proteins. Alignment of C-terminal sequences of CRF protein clades. Partial sequence alignment from representative members of each CRF protein clade I–V is shown. Conserved motifs are indicated by boxes and are labeled with conserved residue patterns. A model of a CRF protein shows the orientation of conserved domains along with lines expanded to show the relative position of aligned sequences.

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Table 1 Cytokinin induction of specific CRF clades

CRF clade	Cytokinin induction	References
I	+	*AtCRF1*[a], *AtCRF2*[b,c], *SlCRF2*[d]
II	–	
III	+	*AtCRF5*[b,c], *AtCRF6*[b], *SlCRF5*[d]
IV	+	*SlCRF1*[e]
V	–	

CRF clades are indicated as transcriptionally induced by cytokinin (+) or not (–) along with citations of specific CRF genes that have been shown to be induced.
[a] This study.
[b] Rashotte et al. (2006).
[c] Brenner et al. (2012).
[d] Shi et al. (2012).
[e] Rashotte and Goertzen (2010).

Two species that have numerous publicly available microarray experiments as well as distinct CRF clade sequences were analyzed: Arabidopsis and Populus. Analysis of the seven Arabidopsis clade-associated CRFs on ATH1 microarrays from all experiments (1,529 arrays) showed clustering of the genes similar to the sequence-based arrangement of clades (**Supplementary Fig. S4**). Identical results were found from analysis of more specific groups of experiments as well, including perturbation (1,473 arrays), and anatomical/developmental data sets (74 arrays). A similar analysis of 11 CRFs in Populus from all five clades over 384 microarray experiments also yielded clustering that mirrors the clade divisions (**Supplementary Fig. S4**). Together these findings further suggest that the conserved sequence diversity among CRF clades is probably correlated to functional differentiation.

CRFs are expressed in the vasculature

Stably transformed homozygous CRF promoter::GUS (β-glucuronidase) reporter plant lines for clades I–IV (*At*CRF1, 2, 4, 5 and 6, and *Sl*CRF1) were analyzed for spatial and temporal expression patterns in Arabidopsis and tomato. For all genes examined, in multiple independent lines, GUS expression was observed in vascular tissues across the plant, with the strongest expression in the phloem and procambium (**Figs. 2**, **3**), different from other gene promoters expressed using the same vector (Siefers et al. 2009). Clade I genes (*At*CRF1 and 2) showed strong expression in the vasculature of leaves, cotyledons, hypocotyls and roots (**Figs. 2**, **3**). Expression in the first pair of leaves at very early stages of development appears to be strong and widespread; whereas these leaves when older, as well as subsequent leaves, have somewhat attenuated yet highly vascular-specific expression (**Fig. 2**). In roots, both clade I genes were expressed in the stele; *At*CRF1 was restricted to the basal-most region of the primary root closest to the hypocotyl, whereas *At*CRF2 could be found throughout the mature vasculature of primary and lateral roots and the tip in the area of the columella (**Fig. 3**). Clade I expression in the shoot apex was unique compared with genes of other clades in being present in young leaf primordia of the first pair of leaves (**Fig. 2E**).

The clade II gene *At*CRF4 was found broadly expressed in the vasculature across the plant in primary and lateral roots, hypocotyls, cotyledons, rosette and cauline leaves, inflorescence stems, sepals and petals (**Figs. 2**, **3**). Staining was also observed for *At*CRF4 in the root tip in a pattern similar to *At*CRF2 (**Fig. 3**), but no expression was observed in the shoot apex.

Clade III CRFs (*At*CRF5 and 6) were also found to be highly expressed in vascular tissues. Both *At*CRF5 and *At*CRF6 promoters directed expression in veins of mature expanded leaves, while younger non-expanded leaves showed patchy or discontinuous expression (**Fig. 2A**). Leaves undergoing expansion began to show vascular-specific expression at the distal end first, possibly coinciding with maturation of higher order veins or the transition of the leaf to a source state. These genes also have tissue expression patterns distinct from each other. Similar to clade I gene expression, *At*CRF6 expression was found in the shoot apex, although it is absent in leaf primordia (**Fig. 2E**). *At*CRF6 expression in the roots was strongest in the stele, yet it could also be detected in root tip columella and in pre-cortical cells (**Fig. 3**). *At*CRF5 was conspicuously absent from the root vascular column just above the zone of maturation, while found abundantly in root tips around the quiescent center expanding into the columella and developing vascular column (**Fig. 3**). Expression is strong in the majority of the mature stele, with the exception of the region noted above, and can be observed in the phloem (**Fig. 3B**). In lateral root tips, pro*At*CRF5:GUS expression could be detected shortly after the initial periclinal division in the pericycle (stage III or IV) (**Fig. 3**).

Because Arabidopsis lacks a clade IV gene, GUS expression analysis of this clade was carried out in tomato using *Sl*CRF1. *Sl*CRF1 was expressed in the vasculature of roots, stems, leaves and fruit (**Fig. 4**). In leaves, vascular expression was limited to the mid-vein along with the first- and second-order laterals. Examination of secondary growth in both the root and stem also indicated that secondary vascular tissues highly express *Sl*CRF1 (**Figs. 2F**, **4**). No expression was observed in root or shoot apices, or in any floral tissues. Interestingly, the pattern of the clade IV expression showed the least tissue specificity in our analysis. While expression was generally strongest in the vasculature, it was also found in other tissues including epidermal cells, the mesophyll of younger non-expanded leaves and the pericarp of unripe fruits (**Fig. 4**).

CRF promoters contain clade-specific vascular-related *cis*-elements

To investigate possible regulatory mechanisms responsible for CRF vascular expression, 1 kb of upstream CRF promoter sequences was examined for conserved motifs using MEME analysis (Bailey and Elkan 1994). A highly conserved TC-rich 29-mer (Motif 1) was found to be significantly enriched in promoters across all CRFs, and multiple copies occur in nearly every CRF in

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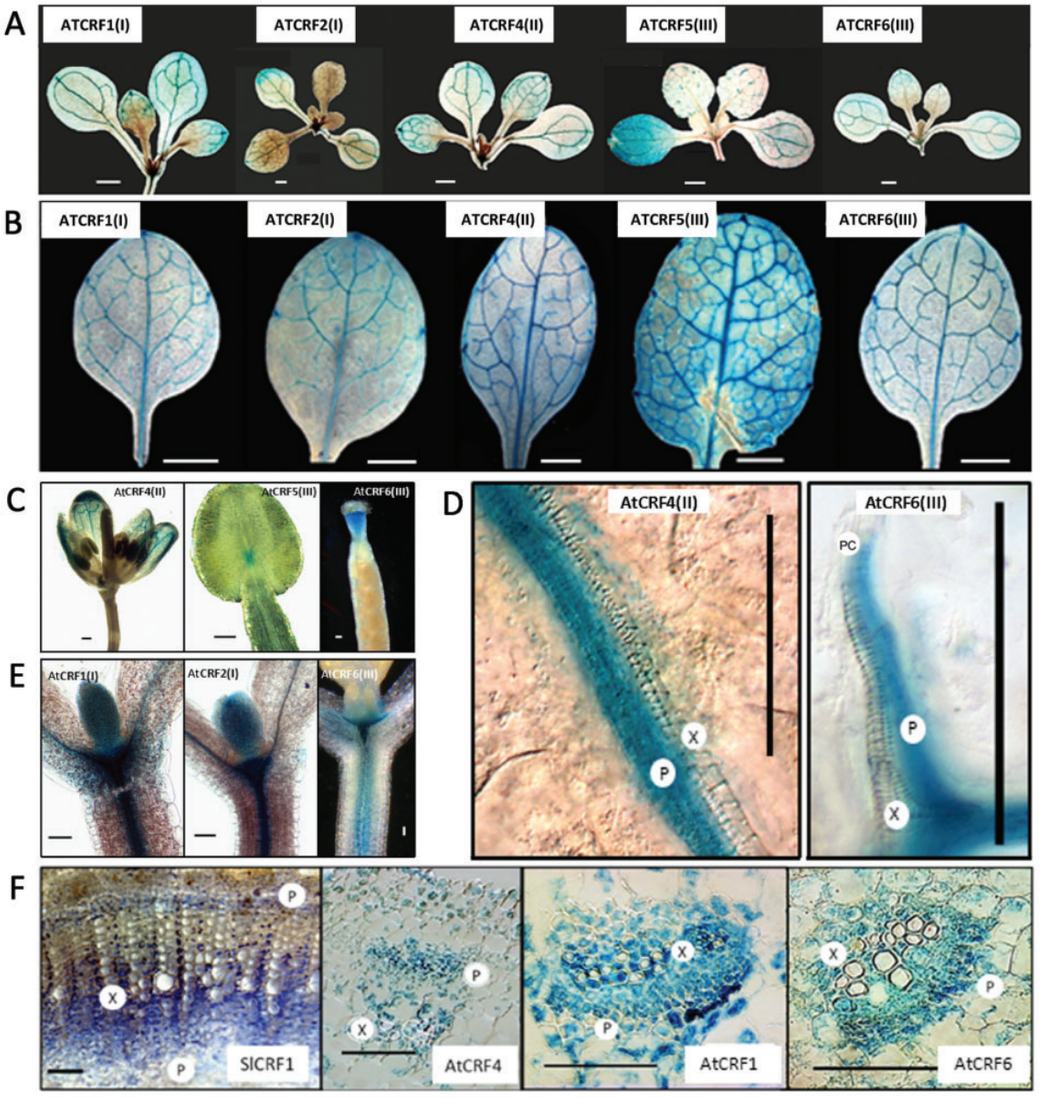

Fig. 2 Promoter-driven GUS reporter gene expression for *At*CRF1, 2, 4, 5 and 6 and *Sl*CRF1 is seen in vascular tissues of various aerial organs. (A) Leaves and cotyledons of young Arabidopsis rosettes [10 days after germination (DAG)]. (B) Fifth or sixth leaves from 18 DAG. (C) Reproductive organs: *At*CRF4 in sepals and petals, *At*CRF5 in anthers, *At*CRF6 in the transmitting tissue of an immature silique. (D) Close-up of minor veins in leaves expressing GUS under the regulation of *At*CRF4 and *At*CRF6 promoters (E) Shoot apices. (F) Free-hand (*Sl*CRF1) and microtome sections from stems (*Sl*CRF1), inflorescence stems (*At*CRF4) and leaves (*At*CRF1 and *At*CRF6). Scale bars are equal in each grouping. X, xylem; P, phloem; PC, procambium.

Arabidopsis and tomato (**Fig. 5A, B**). Several shorter TC-rich motifs were also identified in the promoters of individual CRF clades (from a wide range of species). These clade-specific motifs were even more conserved, in agreement with the evolutionary relationships inferred from the sequence alignments (**Fig. 5A**). Each of the motifs presented was the highest scoring and most abundant motif identified in the specific analysis indicated (i.e. of all CRFs or of a specific clade).

TC repeat motifs similar to those which were identified have been shown to direct expression to the phloem (Ruiz-Medrano et al. 2011). GUS expression was examined more closely to determine if CRF expression could be found in the phloem,

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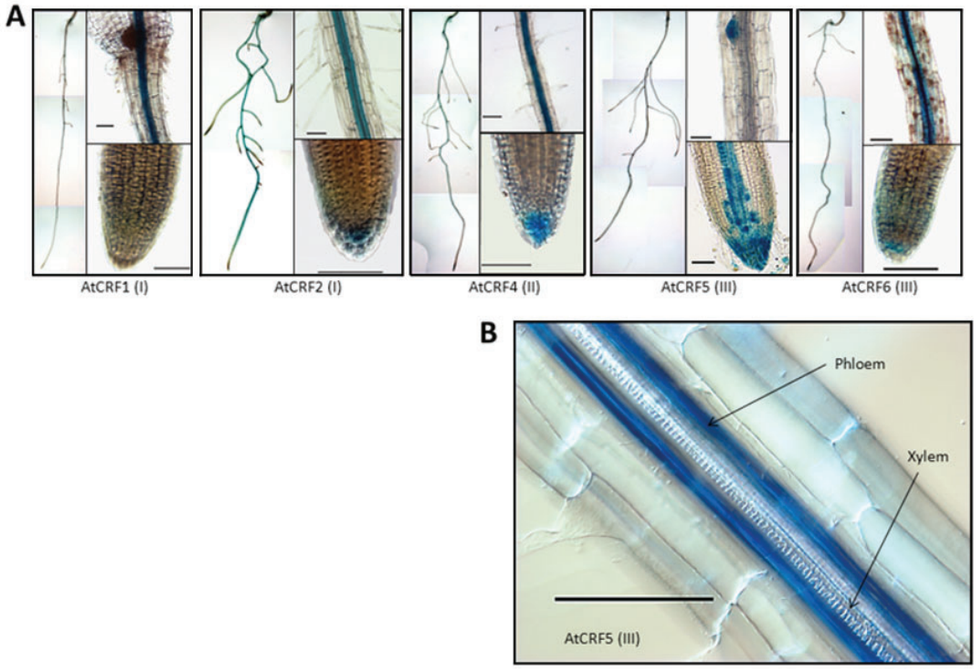

Fig. 3 (A) Promoter-driven GUS reporter gene expression for *At*CRF1, 2, 4, 5 and 6 is seen in root tissues. A whole root, and a close-up of root segments and tips are shown. Note that *At*CRF5 expression is absent from the stele but is strong in the emerging lateral root, and there is a lack of expression of *At*CRF1 in root tips. (B) Detailed close-up of pro*At*CRF5::GUS expression in a primary root using Nomarski optics. Spiral thickening of the primary protoxylem walls can be seen flanked presumably by phloem in a diarchal arrangement.

possibly due to these conserved putative *cis*-elements. Cross-sections cut from roots, stems and leaves from proCRF:GUS lines revealed expression of CRFs in the phloem, as well as other vascular-associated cells (**Figs. 2F**, **4A**). The phloem was (often along with others) the tissue with the strongest GUS expression in the samples examined, supporting the prediction based on promoter motif analysis. Additionally, using Nomarski optics, GUS expression in the veins of cleared intact leaves was found to be associated with the phloem, as well as with procambium (**Fig. 2D**).

We hypothesized that the expanded expression domain of *Sl*CRF1 beyond the vascular tissue could be due to other factors interacting with vascular targeting to modulate its expression. The fact that Arabidopsis lacks a clade IV CRF presented a unique way to examine this possibility, since it may also have lost or lack certain non-vascular-specific targeting enhancers that would bind to clade IV promoters. As such, a clade IV CRF promoter in Arabidopsis should only have the TC-rich repeat motif acted upon to regulate and target expression. This was tested by transforming Arabidopsis with the tomato *Sl*CRF1-promoter::GUS construct. Examination of *Sl*CRF1 promoter::GUS expression in Arabidopsis revealed a highly vascular-specific pattern, lacking expression in the additional non-vascular tissues seen in tomato (**Fig. 4**); this was taken as partial support of the hypothesis.

Examination of CRF vascular transcript expression for all clades in Arabidopsis and tomato leaves was conducted by comparing vascular-enriched or vascular-poor tissues. To accomplish this, RNA was isolated from vascular-enriched tissue (the mid-veins of leaves or leaflets), and relatively vascular-poor tissues (the remaining leaf laminae), and the relative transcript abundance between these two tissue samples collected from a pool of plants was compared using qPCR (**Fig. 5C**). *At*CRF1–*At*CRF6 showed between 55 and 110% greater levels of expression in vascular-enriched tissue. Additionally, tomato clade I–III members, *Sl*CRF2, 4, 5 and 6, showed the same trend, and in some cases nearly 200% greater expression. Analysis of clade V members in both Arabidopsis and tomato, *At*CRF9–*At*CRF12 and *Sl*CRF9–*Sl*CRF11, also revealed a similar vascular-enriched expression pattern, although at slightly lower differentials (**Fig. 5C**). Statistical analysis of the 23 different CRF genes from Arabidopsis and tomato revealed that there was a significant increase in expression in vascular-enriched tissue for 19 genes (at $P < 0.05$) and an increase in all 23 (at a relaxed $P < 0.10$). This indicates that most CRFs are expressed at greater levels in vascular tissue, with the rest of the CRFs showing a strong trend towards that same finding. Overall, these findings are in agreement with GUS expression analyses and suggest that CRFs are transcriptionally targeted to the vasculature.

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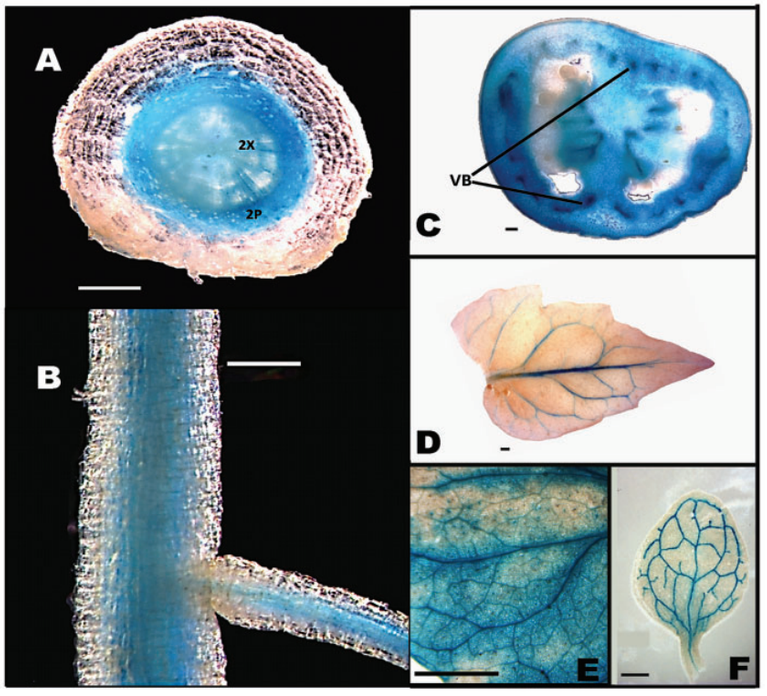

Fig. 4 *Sl*CRF1 promoter-driven GUS reporter gene expression is seen in vascular tissues of various organs. (A) Free-hand section of a primary root. (B) Primary and lateral root. (C) Immature fruit. (D) Leaflet. (E) Close-up of a leaf showing vascular and non-vascular expression. (F) Expression in a transformed Arabidopsis leaf. 2X, secondary xylem; 2P, secondary phloem; VB, vascular bundles.

CRFs are required for normal leaf vasculature patterning

Visual examination of leaves from Arabidopsis *crf* mutants (*crf1*, *crf2*, *crf3*, *crf5*, *crf6* and *crf9*) suggested that vein patterning in clades I and III was altered with respect to the wild type, particularly for secondary and higher order venation. While examination of primary and first-order lateral veins for mutants in all clades showed them to be similar to the wild type, mutants from clades I and III showed alterations in higher order veins and areoles. This was further investigated using LEAF GUI software specifically designed to examine these features (Price et al. 2011). Again analyses of whole leaves revealed only minor vein patterning changes in *crf* mutants due to similarities in primary and first-order vein measurements, as initially noted (**Supplementary Table S1**). However, examinations of higher order veins, from areas bounded by the mid-vein and two first-order lateral veins, revealed significant differences in *crf* mutants (**Fig. 6**).

Analyses of *crf1* and *crf2* (clade I) as well as *crf5* and *crf6* (clade III) leaves revealed distinct clade-specific patterns of higher order veins: the number of areoles and the density of free-ending veins (**Supplementary Table S1**). In contrast, no differences were seen in the clade II mutant (*crf3*) that was examined (**Fig. 6**; **Supplementary Table S1**). Because initial analyses revealed highly similar results for within-clade members, a single representative from each clade (*crf2*, *crf3*, *crf5* and *crf9*) was used for extensive analysis of additional parameters: areole area and vein network density (**Fig. 6**). *crf2* was found to have fewer areoles that were larger in area (**Fig. 6**). This is in direct contrast to *crf5* with an increased number of smaller areoles. Neither difference was due to changes in overall leaf area. Both mutants also had increases in the density of vein networks and the density of free-ending veins (***Fig. 6***). It should be noted that *At*CRF2 and *At*CRF5 (along with their respective clade members) have nearly opposing expression patterns in young developing leaves before transitions into a vascular-specific pattern: ubiquitous for clade I vs. almost absent for clade III. Examination of the clade V mutant (*crf9*) did show differences in areole numbers in initial analyses; however, this was due to a larger overall leaf size rather than alterations in patterning (**Supplementary Table S1**).

Discussion

There are five distinct clades of CRF proteins in flowering plants

We have shown that the monophyletic group of CRF proteins underwent considerable diversification prior to the divergence

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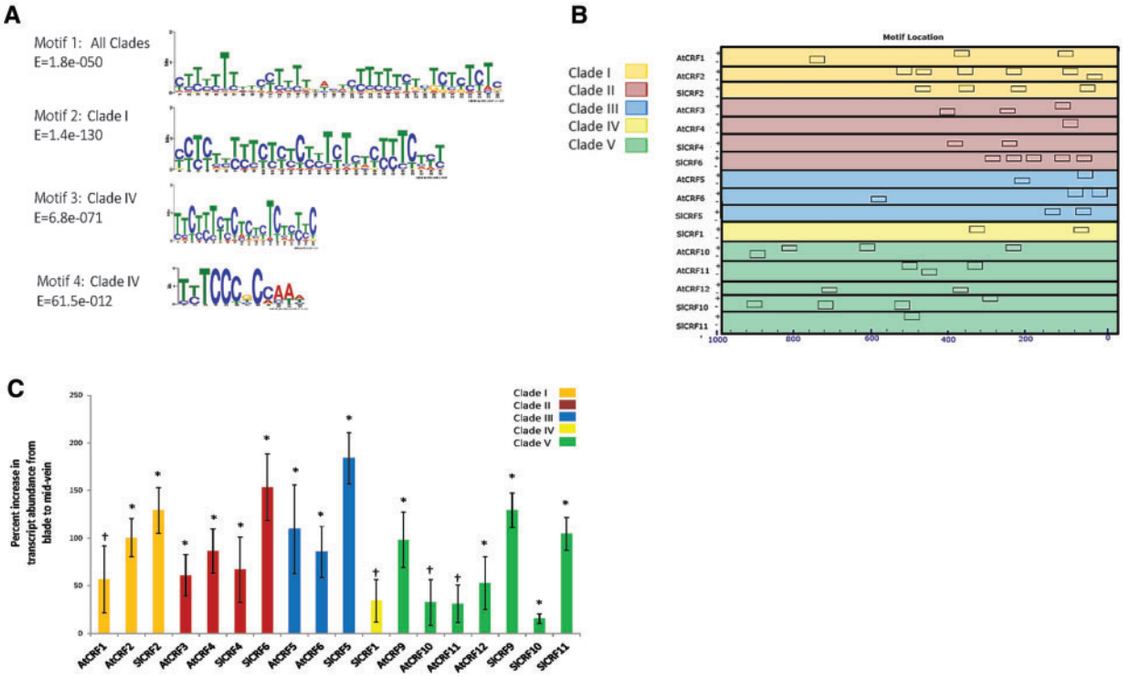
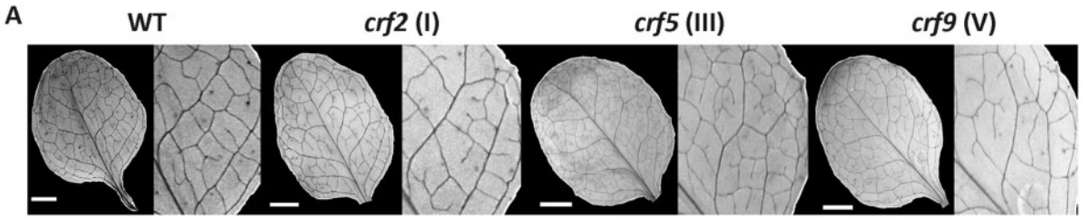


Fig. 5 (A) Over-represented motifs found in angiosperm CRF promoters by MEME analysis. Motif 1, consensus from all clades; motif 2, clade I consensus; motif 3, the TC-rich sequence from clade IV; and motif 4, the CA-rich motif from clade IV. Motifs shown are those with the lowest E (Expectation) values for each group, with the exception of motif 4 which is the second highest to motif 3. (B) Block plot showing the position of motif 1 in Arabidopsis and tomato gene promoters (1 kb upstream of ATG). (C) CRF transcripts in vascular-enriched tissue samples. Expression of CRF genes as indicated was measured in tissue from both the mid-veins and leaf blades or leaflets using qPCR. Values presented are the percentage increase in normalized expression from blade to mid-vein. Data represent the average ± SE of two biological replicates comprised of pooled RNA from at least six individual plants. Each run was performed with at least four technical replicates.



B

	Number of Areoles	Mean Areole Area (mm²)	Density of Vein Network (mm/mm²)	Density of Free Ending Veins (mm⁻²)
WT	9.00(+/-) 0.65	0.677 (+/-) 0 .051	4.72(+/-)0.20	0.507(+/-) 0.053
crf2 (I)	***7.55(+/-) 0.55**	***0.865(+/-) 0.073**	4.33(+/-) 0.16	0.575(+/-) 0.050
crf3 (II)	9.20(+/-) 1.11	0.720(+/-)0.053	4.36(+/-) 0.15	0.563(+/-) 0.041
crf5 (III)	***12.9 (+/-) 1.07**	***0.503(+/-) 0.026**	***5.29(+/-) 0.22**	***0.889(+/-) 0.040**
crf9 (V)	***12.1(+/-) 1.29**	0.606(+/-) 0.054	4.57(+/-) 0.19	***0.755(+/-) 0.062**

Fig. 6 Aberrant vascular patterns in *crf* mutant leaves. (A) Representative examples of wild-type and mutant leaves used for analysis from each clade with magnification of the regions examined showing alterations in the patterns formed by minor veins. (B) Table of mean values of data collected for genotypes representative of mutants for each clade ± SE. An asterisk indicates a significant change from the wild type (Student's *t*-test $P < 0.05$).

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of flowering plants, giving rise to five distinct CRF clades (I–V). Each of these clades is best defined by unique and highly conserved (within clade) C-terminal sequences, and is supported by clade-specific signature sequences in promoter, CRF and AP2 domains as revealed by sequence-based analyses. While CRFs are present in all land plants, it is only within angiosperms that this remarkable diversification can be observed. Even earlier branching angiosperm lineages such as *Amborella* or *Nuphar* have CRF genes from the more derived CRF clades. Almost every fully sequenced angiosperm genome contains at least one representative gene per clade. Interestingly, there are consistently low numbers of CRFs found in each clade: 1–2 in clades I–IV and 1–4 in clade V. From this pattern it may be inferred that a degree of selective pressure has maintained these clades and their low gene numbers, indicating that each group is of functional importance. Phylogenetic analyses using only the conserved CRF and AP2 domains define these clades roughly with moderate to low support, but their definition by conserved C-terminal sequence motifs is nearly perfect. Additionally, hierarchical clustering analyses of CRFs based on large sets of transcriptional data from both Arabidopsis and Populus indicate that expression patterns reflect the clade-specific evolutionary relationships.

A rapidly growing pool of newly available sequences as well as the ability to identify additional CRFs based solely on C-terminal characters has greatly increased our ability to resolve CRFs via genomic analysis. The inclusion of many more CRF sequences with greater diversity has allowed us to find several previously unidentified well-conserved blocks in C-terminal regions (**Fig. 1**). Foremost is the identification of a conserved MAPK phosphorylation motif in all CRF proteins, which was only weakly detectable previously (Rashotte and Goertzen 2010). In addition, the existence of other novel conserved amino acid motifs became evident in specific clades. The FQDI of clades I and II, the SGY[D/E]S of I, II and III, and the PX[D/E]XF[F] element, recognizable in all except clade IV proteins, are all likely to be of significance in the function or potential regulation of these proteins. Aside from these short intraclade conserved sequences, most of the CRF C-terminal regions are uniquely CRF clade specific and indicate divergence from one another potentially during the emergence of angiosperms as a dominant plant group.

There are some additional CRF proteins, including *At*CRF7, *At*CRF8, *Sl*CRF3, *Sl*CRF7 and *Sl*CRF8, that are not easily placed into any clearly defined clade, primarily because they lack C-terminal sequences downstream of the AP2 domain including the conserved MAPK motif. Phylogenetic analyses of these CRFs with just the AP2 and CRF domains does not clearly resolve their clade membership, although detailed examination of Arabidopsis and tomato genomes, including large-scale synteny determination around these loci, suggests that each may have been the result of independent duplications of sequences from different clades (**Supplementary Fig. S1**; data not shown). Our inability to place such sequences clearly into a specific CRF clade emphasizes the significance of C-terminal sequences in these analyses. There are also a group of unplaced monocot sequences, probably belonging to either clade I, II or III (based on the presence of the FQDI motif), that in Neighbor–Joining and maximum likelihood trees group somewhat on their own near the base of clades I, II and III (**Fig. 1**). The inability to place this set of sequences is likely to be the result of the relatively poor availability of monocot sequences other than fast evolving grass sequences.

Sequence divergence among clades probably indicates a similar divergence or specification in gene function, which we found was supported by hierarchical clustering of transcriptome analysis experiments in Arabidopsis and Populus. An example of this specification is immediately evident from differences in transcript induction by cytokinin that occur in some clades (I, III and IV) but not others (II and V) (**Table 1**). There are likely to be additional characteristics pertaining to both expression and function that further delimit the proteins from each clade, some of which are probably related to the clade-specific C-termini. While there has been little study of CRF functionality, two genes have been examined that are now known to be clade IV CRFs: *Pti6*/*SlCRF1* from tomato and *Tsi1* from tobacco, both linked to pathogen resistance and immune responses (Zhou et al. 1997, Park et al., 2001, Gu et al. 2002). Our study would suggest that similar functions might exist for other clade IV members; however, it is unclear what this potentially means for Brassicaceae species, such as Arabidopsis, that lack clade IV CRFs. Interestingly, the final 15 amino acids at the C-terminal end of Tsi1 were shown to be necessary for activation of this transcription factor. Amino acids in this region are well conserved in clade IV proteins; however, a homologous motif is not obvious in other CRF clades. Preliminary data from expression and physiological analyses of other CRFs suggest there may also be clade-specific functionality related to different phloem-based processes; however, additional work is required to determine these connections further and the overall level of functional specification among CRFs.

CRF genes show vascular expression

Despite the observed clade-based sequence divergences of CRF genes, we have shown in Arabidopsis and tomato that CRFs show vascular expression patterns with the strongest expression in phloem tissues based on our in situ and in silico examinations. One possible mechanism for CRF phloem-specific expression is the abundance of phloem targeting *cis*-elements occurring in CRF promoters. Schneidereit et al. (2008) showed that a small region containing a TC-rich motif was necessary for phloem-targeted expression of the Arabidopsis Sucrose Transporter 2 (*At*SUC2) gene. Additionally, it has recently been shown that similar TC sequence repeats along with a minimal promoter are sufficient to target expression of their cognate genes to phloem cells (Ruiz-Medrano et al. 2011). The expression of CRFs in phloem cells makes them good candidates for mediators of a number of developmental and environmental response processes known to be cytokinin regulated,

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such as sink/source regulation, senescence and responses to biotic and abiotic stresses (Mok and Mok 2001, Werner and Schmulling 2009). Additionally, an AC-rich motif was found to be highly enriched in clade IV promoters (**Fig. 5A**). Highly similar motifs have been implicated in specifying xylem expression in *Phaseolus vulgaris* (Hatton et al., 1995) and *Pinus taeda* (Patzlaff et al. 2003). Together, the identification of these vascular-related promoter motifs supports the vascular-specific expression found in the reporter gene analysis and provides a potential mechanism for such specification.

Broad expression patterns of clade I *CRF* genes in young leaves that transition to highly vascular-specific expression as leaves expand is juxtaposed to that of clade III *CRF* genes that lack expression in young leaves, yet are expressed in a vascular-specific manner as they mature. Interestingly, these contrasting expression patterns parallel the findings from CRF mutant examinations indicating that these clades have opposing defects in leaf vein patterning, while other clades do not appear to follow any similar patterns. Together these findings suggest that while clade I and III CRFs may regulate vascular expression in developing leaves, they would appear to do so in differing if not opposing manners.

Root expression could be seen for nearly all genes examined, and at least one member from each clade was expressed in the root vasculature (**Figs. 3**, **4**). Although *At*CRF5 lacked vascular expression just above the zone of maturation in the root, it was the only CRF examined showing expression in pre-vascular meristematic cells. This may imply a role for *At*CRF5 in actively dividing cells, also supported by its expression in emerging lateral roots. Other non-vascular regions of the root tip such as the quiescent center, columella and pre-cortical cells could also be detected in CRF reporter lines, indicating that expression of these genes is not limited to vascular tissue in the root. *Sl*CRF1 expression appeared to be confined to the vasculature in the root and is absent in the root tip; however, more extensive examination will be necessary to determine if this lack of expression is a clade-defining characteristic.

High levels of expression could be seen in the meristematic tip of shoot apices for clade I (*At*CRF1 and 2) along with *At*CRF6 from clade III (**Fig. 2E**). Each of these genes is regulated by cytokinin and could be involved in shoot apical meristem maintenance, known to be dependent upon cytokinin. After transition to flowering, no *AtCRF* expression could be detected in aerial meristems. However, genes from each clade examined were found in either floral or fruit tissues, often in vasculature tissue of those organs.

Cytokinin-regulated CRFs are involved in determining leaf vascular patterns

We have shown that CRFs from clades I and III are necessary for normal higher order vein patterning in leaves. The fact that genes from these clades are cytokinin inducible implies a link between cytokinin-regulated CRFs and leaf vein patterning (**Table 1**). Several CRFs could play a role in vascular development in leaves, similar to the cytokinin response regulator ARR1 shown to function as a mediator of hormone cross-talk between cytokinin and auxin in roots (Dello Ioio et al. 2008). Interestingly CRFs have been shown to interact with ARR1 at the protein level (Cutcliffe et al. 2011). Previous study of the Arabidopsis histidine kinase family of cytokinin receptors has also linked them to vascular development as seen in triple receptor mutants (*ahk2,3,4*) with altered shoot and root vasculature (Nishimura et al. 2004, Hejátko et al. 2009). Although leaf vascular patterning of the *ahk2,3,4* mutant was not quantified, its defect appears qualitatively similar to those found in *crf2*: reduced numbers of larger areoles. Several mutants related to auxin signaling and transport have irregular vascular patterning phenotypes often resulting in increased numbers of free-ending veins; frequently the result of vein discontinuity (Mattson et al. 2003, Dettmer et al. 2009). A significant increase of free-ending veins was observed in *crf5* leaves; however, these do not appear to result from vein discontinuities since that would predict *crf5* to have a reduced number of areoles that are larger in size, whereas the opposite was found. This suggests that the increase of free-ending veins is instead a result of an overall increase in vascular density, which was observed. Because *AtCRF5* and *AtCRF6* transcripts are abundant in the phloem, we have hypothesized that an increased density of veins found in clade III mutants may be an attempt to compensate for a diminished ability either to load or to unload the phloem.

Interestingly the observed areole phenotypes of *crf2* leaves were the opposite of *crf5*: reduced numbers of larger areoles in *crf2* vs. increased numbers of smaller areoles in *crf5*. While this shows that both clade I and III transcripts are necessary for normal areole patterning, they appear to regulate the process differentially. In fact, a similar examination of *crf2,5* double mutants revealed no significant differences from the wild-type (**Supplementary Table S1**), implying that the two genes function in different pathways antagonistically regulating formation of areoles. As this result appears to mirror clade I and III expression differences seen during leaf development, it suggests that these genes play more complex roles than simply the regulation of areole number and size in vein patterning, such that analysis of multiple interclade mutant combinations is likely to be complex and potentially confounding for functional interpretation of individual genes.

Conclusions

In summary, a comparative genomic/transcriptomic examination of CRF protein sequences across land plants revealed that diversification of CRFs resulted in five clearly defined lineages represented in nearly every angiosperm genome. Despite limited phylogenetic resolution of sequences in CRF and AP2 domains, strong support for the presence of distinct CRF clades was found in C-terminal amino acid alignments, revealing novel and highly conserved regions for each clade. Species representing early branching lineages of angiosperms possess sequences

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from each clade, implying maintenance of an ancient functional diversification within this gene family. In contrast, CRF promoters from all clades have a significantly enriched TC-rich *cis*-element similar to motifs linked with phloem-specific expression. Both transcript and reporter gene analyses revealed that CRFs from every clade are expressed in vascular tissues, often the phloem, in agreement with promoter motif analysis. Together this suggests that while each clade may have specific functions including some unique expression patterns, all CRFs appear to be involved in vascular-related processes. Finally, an examination of CRF mutants revealed anomalous patterns in leaf vascular networks. The varying nature of mutant abnormalities found between clades supports the notion of clade-specific functional involvement in vascular patterning probably linked to cytokinin. This study has further elucidated the evolutionary relationships among angiosperm CRFs as well as general patterns of CRF expression. We believe this generates a better picture of the role these proteins play in plant development and hopefully provides the baseline for future work targeted at understanding specific roles of CRFs.

Materials and Methods

Informatics

Novel CRF gene sequences were identified in sequence databases (NCBI NR/NT, EST, GSS, HTGS and WGS), trace archives and several plant genome sequencing project databases with BLAST searches using blastx, tblastn and tblastx. Iterated profile searches were conducted using PSI-BLAST (Position-Specific Iterated BLAST) to target successive rounds of searching. All sequence databases were also queried using HMMER3 software employing hidden Markov probabilistic models capable of detecting remote homologs (Eddy 1998). CRF and AP2 domains from diverse CRF genes (as they were discovered), and variously broad consensus sequences of each domain were used as query sequences in multiple searches. Default settings of the BLAST programs were used except for the low complexity filter.

Sequence analyses

Promoter motif detection was performed with MEME (MEME version 4.1.0, http://meme.sdsc.edu/meme/) using standard settings and 1,000 bp of 20–50 different CRF sequences upstream of their ATG start sites. The most highly significant motifs identified from analysis of specific clades and all CRFs are presented as described in **Fig. 4**. All 403 CRF domain-containing proteins were aligned across their CRF and AP2 domains where possible. Amino acid alignments were performed initially with MAFFT (Katoh, 2008) and adjusted manually where necessary. Phylogenetic analyses were performed in PAUP*v.4.0b11 using the Neighbor–Joining method, and in RAxML v7.3.0 using maximum likelihood as an optimality criterion. Only CRF and AP2 domain sequences were included in phylogenetic analyses (**Supplementary Figs. S1–S3**). The best fitting amino acid substitution model (PROTCATWAGF) was chosen by the RAxML model selection Perl script, which tests the fit of amino acid substitution models by optimizing model parameters and branch lengths on an initial JTT-based phylogenetic hypothesis. Non-parametric bootstrap procedures were used to estimate clade support (Felsenstein 1985).

Hierarchical clustering analysis

Clustering analyses were performed for Arabidopsis and Populus data sets individually. The 11 clade-specific Populus CRF genes (ERF78–87) were used to perform an ExCluster-Heatmap from all 384 microarray experiments available at the Populus Genome Integrative Explorer: PopGenIE v2.0 and are displayed in a Heatmap with the default settings of a Euclidean distance function and Ward hierarchical function. The seven clade-specific Arabidopsis *At*CRF genes found on ATH1 microarrays were used to perform hierarchical clustering analysis of all 1,529 microarray experiments or subsets (anatomical/development and perturbations) available at Genevestigator using default settings and displayed as a Pearson correlation.

Growth conditions

Arabidopsis thaliana (Col-0). Sterilized seeds were germinated on Petri dishes containing 0.8% agar gel with MS salts (4.8 g l^{-1}) plus 1% sucrose medium of pH 5.7. Plants were grown under a 100 μmol m^{-2} s^{-1} light–dark cycle: 16 h, 22°C/8 h, 18°C in controlled-environment chambers. For extended growth, seedlings were transferred to soil (sunshine mix #8) and grown under the same conditions as above, but under ~150 μmol m^{-2} s^{-1} light.

Solanum lycopersicum. Micro-Tom cultivar plants were grown in Sunshine Mix #8 soil under a 150 μmol m^{-2} s^{-1} light–dark cycle: 16 h, 26°C/8 h, 22°C in controlled-environment chambers.

Generation of transgenic plants

The promoter (~2 kb upstream of ATG) sequences of *AtCRF1–AtCRF6* and *SlCRF1*; and promoter plus coding sequence of *AtCRF1*, *AtCRF2*, *AtCRF4* and *AtCRF5* were amplified using sequence-specific primers with att-B sites. PCR products were cloned into the pDONR221 entry vector (Invitrogen) and plasmids were generated using the Invitrogen GATEWAY™ system according to the manufacturer's instructions. Destination vectors pBGWFS7 or pKGWFS7 (Karimi et al. 2002) were used to create transcriptional and translational fusion expression vectors. For Arabidopsis, plasmids were transformed into *Agrobacterium tumefaciens* C58 via electroporation, and plants were transformed by agrobacteria using the floral dip method (Clough and Bent 1998), with marker-selected homozygous lines used for analyses. For tomato, vector pKGWFS7 containing the *SlCRF1* promoter was sent to the Plant Transformation Research Center (PTRC) at University of California Riverside and transformed into Micro-Tom plants as a service. Homozygous plants were used for all analyses. Between six

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and 15 independent transgenic lines of all plants were examined, from which a single representative line is shown.

Histochemical analysis

Analysis of GUS activity from various aged tissues noted in the text were vacuum infiltrated for 20 min with X-gluc buffer (Weigel and Glazebrook 2002) then further incubated at 37°C for 2–6 h for Arabidopsis and overnight for tomato. Tissue was then cleared in 70% ethanol at room temperature or 50°C, and examined. Mounted sections were fixed, embedded in paraffin, cut into 10 μm sections and viewed using a Nikon Eclipse 80i 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.

Extraction of vascular-enriched tissue

Fine-tip forceps slightly bent inward were used to gently grasp and remove the mid-vein from fully expanded Arabidopsis leaves (numbers 5–8 at bolting) and tomato leaflets. Mid-veins and the remaining blades were immediately frozen in liquid nitrogen, after which RNA was extracted, reverse transcribed and used for qPCR analysis. Each sample was composed of pooled RNA from multiple leaves.

RNA isolation, cDNA synthesis and quantitative PCR

RNA was isolated with a Qiagen RNeasy Kit according to the manufacturer's instructions. RNA concentrations were equalized and pooled; reverse transcription was carried out using Qiagen qScript cDNA supermix. The resulting cDNA was diluted prior to qPCR performed with the SYBR-Green chemistry in an Eppendorf Mastercycler ep realplex. Arabidopsis reactions contained 9 μl of SYBR-Green supermix, 10.76 μl of cDNA template and 0.12 μl of 100 μM gene-specific primers. The qPCR program consisted of one cycle at 95°C, followed by 40 cycles of 15 s at 95°C, 45 s at 57°C and 30 s at 68°C. Tomato reactions contained 9 μl of SYBR-Green supermix, 2 μl of cDNA template, 3 μl of 4 μM gene-specific primers and 3 μl of sterile water. The qPCR program consisted of one cycle at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 56°C and 35 s at 68°C. Relative expression data are the mean of two biological replicates each consisting of a pool from at least six individual plants from which at least four technical replicates were performed. Expression levels of reference genes for normalization were confirmed with reverse transcription–PCR as in Rashotte et al. (2006) and Shi et al. (2012).

Measurement of leaf vasculature

The homozygous T-DNA insertion mutant alleles analyzed were obtained from the ABRC stock center or as previously described: *crf1-1*, *crf2-1*, *crf3-3* (salk_138253), *crf5-6* (salk_024228), *crf6-2* and *crf9-1* (sail_770_B09) and compared with the wild type (Col) (Alonso et al. 2003, Rashotte et al. 2006). All lines were confirmed as homozygous T-DNA insertions using PCR with gene-specific and T-DNA-specific left border primers. For analysis, leaves 3–4 were excised at bolting, cleared in 95% ethanol overnight, then rehydrated in a graded ethanol series and stained overnight with 0.25% basic fuchsin. Stain was replaced with slightly alkaline (one drop of 1 N NaOH in 100 ml) water then rinsed until clear of excess stain. Leaves were further cleared of background stain with a 3 : 1 ethanol : HCl solution and photographed as described above. Images were processed and analyzed using LEAF GUI software (Price et al. 2011). For each leaf, the first area bounded by the mid-vein and two first-order lateral veins fully within the right blade was analyzed. Sample sets contained 9–30 individual leaves from ≥2 independently grown repetitions; mean, standard error and *P*-value based on one-tailed Student's *t*-test were computed in Microsoft Excel 2010.

Supplementary data

Supplementary data are available at PCP online.

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MINI-REVIEW

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Cytokinin inhibition of leaf senescence

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Keywords: senescence, cytokinin, leaf, cell wall invertase, carbon allocation, sink/source relationships

The senescence delaying effect of cytokinin is well known, however, the details behind how this process occurs remain unclear. Efforts to improve understanding of this phenomenon have led to the identification in *Arabidopsis* of specific cytokinin signaling components through which senescence signal responses are regulated. These include the cytokinin receptor (AHK3), the type-B response regulator (ARR2) and the recently identified cytokinin response factor (CRF6). At the mechanistic end of this process, it was found that increased cell-wall invertase activity which occurs in response to cytokinin is both necessary and sufficient for the inhibition of senescence. Yet, a direct link between the signaling and mechanistic steps of a cytokinin regulated senescence process has yet to be demonstrated. This may be in part because the relationship between senescence and primary metabolism implied by the key role of cell-wall invertase is the subject of two apparently opposing bodies of evidence. Here we briefly summarize and propose a model in which cytokinin mediated changes in sink/source relationships leads to delayed senescence which is consistent with existing evidence both for and against sugars as a trigger for developmental senescence.

Leaf Senescence: Maximizing Return on Investment

Leaf senescence is a developmental process actively initiated as part of an age-dependent genetic program or in response to environmental stress. Although this may ultimately lead to cellular apoptosis across the organ, it is in no way a simply unintended or unfortunate consequence. In fact, the process of senescence is highly regulated and dependent upon concurrent increases in both synthesis and activity of some proteins as well as degradation or inactivation of others. Precise regulation of senescence is crucial because in preparation for cellular death, the valuable nutrients and energy released by the breakdown of macromolecules during this process are reallocated to the rest of the plant for growth or storage.[1,2] This senescence-based recycling of nutrients and energy that are invested in the production of leaves and the photosynthetic machinery within has been described as "altruistic" and evolutionary advantageous process.[3]

This review focuses on the current understanding of the mechanisms behind the well-known cytokinin inhibition of senescence. The elucidation of the two-component cytokinin signaling (TCS) pathway in *Arabidopsis* has facilitated the identification of the initial signaling components which mediate senescence-specific responses; specifically the cytokinin receptor (AHK3) and the type-B response regulator (ARR2).[4] Recently another transcription factor, cytokinin response factor 6 (CRF6) thought to act as a side branch of the canonical TCS pathway was shown to be involved in senescence delay.[5] While at the other end of this process, increased cell wall invertase (CWINV) activity has been shown to be an integral part of the downstream response mechanism through which cytokinin delays leaf senescence.[6] Despite this knowledge of upstream signaling and downstream mechanistic parts of a cytokinin-regulated senescence process, these portions have not been connected and a unified pathway remains unresolved (**Fig. 1**).

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Cytokinin: The Foliar Fountain of Youth

Richmond and Lang (1957) first showed that cytokinin treatment leads to greater retention of chlorophyll and protein in excised leaves of cocklebur plants (*Xanthium pennsylvanicum*).[7] This cytokinin effect has since been shown in many other species, even resulting in re-greening of yellowing leaves.[8] Experimentation has led to the understanding that cytokinin increases in longevity is specific to leaves and that it can be influenced by other factors such as light and sugars.[9,10]

Much of the evidence supporting the role of cytokinin as an endogenous negative regulator of senescence has come from studies which examined changes in cytokinin content and the expression of cytokinin metabolism genes during senescence. Work in numerous species has indicated a strong correlation between decreased leaf cytokinin content and the onset and progression of senescence.[3,11] Cytokinin synthesized in roots is transported into leaves through the transpiration stream; it has been found that the amount of cytokinin in the xylem of *Glycine max* rapidly decrease at the onset of senescence.[12] Similarly, a sorghum cultivar exhibiting delayed leaf senescence had a greater abundance of cytokinin in its xylem sap as compared with a normally senescing cultivar.[13] Cytokinin in leaves may also be the product of local synthesis. Transcriptome analyses of *Arabidopsis* leaves demonstrate that expression of cytokinin biosynthetic genes greatly decreases during senescence, while transcripts of cytokinin degrading enzymes become more abundant.[14,15] This suggests that cytokinin may delay leaf senescence not only as a result of exogenous treatment, but as part of an endogenous developmental program. Although an antagonistic role of cytokinin in leaf senescence is strongly supported in these studies, many of them rely on correlations and do not clearly demonstrate a causal relationship.

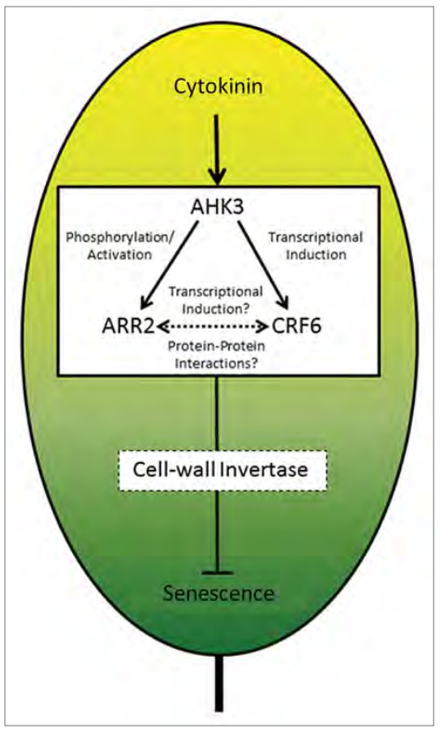

Figure 1. Model of an emerging pathway integrating current knowledge of cytokinin regulation of senescence. In *Arabidopsis*, cytokinin perception by AHK leads to activation of ARR2 and induced expression CRF6. It is unclear whether ARR2 directly regulates CRF6 expression in this process. ARR2 and CRF6 proteins may also interact physically in the regulation of downstream genes. One such gene may be cell-wall invertase which has been shown in tobacco and tomato to be necessary for senescence inhibition.

A new era of investigations in cytokinin physiology began with heterologous expression of the *Agrobacterium tumfaciens* isopentenyl transferase (IPT) gene which is transferred into plant cells during infection by *A. tumfaciens*. The IPT enzyme catalyzes the rate-limiting step of cytokinin biosynthesis in plants; ectopic IPT expression in a wide variety of plant species results in dramatic increases in endogenous cytokinin production.[16,17] Because constitutive expression using the CaMV 35S promoter severely limited regeneration of plants due to cytokinin inhibition of root organogenesis, tissue-specific or inducible promoters allowing for targeted over-production of cytokinin have been employed to varying levels of success in many species as has been extensively reviewed.[9,18]

Given the senescence delaying effects of exogenous cytokinin treatment, it was expected that cytokinin overproducing plants would also display increased leaf longevity. However, actual findings were mixed with some plants showing delayed senescence as expected, but many studies found no change or even accelerated leaf senescence.[9,19] It has been suggested that these unexpected results are due to a cytokinin imposed shift in sink and source identities of organs.[20]

An elegant system was designed to resolve these confounding results (as well as concerns regarding the effects of expression-inducing conditions): the IPT gene was expressed in tobacco plants under the promoter of SAG12 (Senescence Associated Gene12), such that the plants had increased cytokinin production limited to leaves which had begun to senesce. This auto-regulatory loop specifically targeted cytokinin increases to senescing cells, yet prevented over-accumulation. The result was a striking delay of leaf senescence.[21] The extraordinary leaf longevity exhibited by these plants remains among the most convincing lines of evidence for the negative regulation of leaf senescence by cytokinin. This proSAG:IPT system has since been implemented in a number of important crop species including: lettuce, rice, ryegrass, tomato, alfalfa, cauliflower, wheat, cassava, broccoli and cotton; all of which demonstrated delayed leaf senescence.[22-31]

A Specific Subset of Cytokinin Signaling Slows Senescence

The two-component cytokinin signal (TCS) pathway is fairly well understood as a result of work done over the past 15 y. It functions as a multi-step phospho-relay involving hybrid histidine kinase receptors (HKs) and downstream transcription factors, such as the type-B response regulators (RRs) and cytokinin response factors (CRFs) that mediate the cytokinin signal.[32,33] The first direct link between the TCS pathway and senescence regulation came about with the characterization of an *Arabidopsis* mutant with a delayed senescence phenotype, *ore12*, which turned out to be a gain of function allele of the cytokinin receptor AHK3. Further investigation indicated that AHK3 specifically mediates the senescence-delaying response in leaves in a manner partially dependent upon the phosphorylation/activation of the type-B RR ARR2.[4] It has since been shown that plants expressing a proteolytic-resistant version of ARR2 exhibit delayed dark-induced leaf senescence.[34] A similar phenotype was observed in plants overexpressing the CK inducible transcription factor CRF6; and *crf6* mutants were found to have reduced sensitivity to the senescence delaying effects of cytokinin.[5] While CRFs (cytokinin response factors) have been shown to function as a side branch of the TCS, this is one of the first functional roles in a cytokinin regulated process directly linked to a CRF protein—the first involving senescence.[35] Interestingly, the CRF6 protein has been shown to directly interact with type-B ARR proteins.[36] Although interaction with ARR2 was not tested, those which were examined (ARR1, ARR10 and ARR12) are closely related and function in a redundant manner.[37] This suggests that CRF6 and ARR2 could potentially function in complex to regulate transcriptional response to cytokinin during senescence, however further examination is required (**Fig. 1**). Notably, AHK3, ARR2 and CRF6 are all expressed in leaf vascular tissues which, as will be addressed in subsequent sections, serve an important role in senescence and may be crucial in regulating this process.[38-41]

Cell-Wall Invertase: Cytokinin Sweetens the Deal

The downstream mechanism of cytokinin delayed leaf senescence is not fully understood, though it is widely thought to involve the regulation of sink/source relation.[20,42,43] The influence of cytokinin upon sink/source relations is exerted in part by regulation of cell-wall invertase (CWINV) activity.

The CWINV enzyme is secreted and ionically bound to cell walls where it catalyzes the cleavage of sucrose into hexose monomers.[44] Doing so allows sucrose unloaded from the phloem into the apoplasm of sink organs to be rapidly metabolized and taken up by adjacent cells which possess hexose but not sucrose transport proteins. Because sucrose diffuses passively through the phloem, the rate of its metabolism at the site of unloading is a major determinant of sink strength.[45,46]

A cytokinin-induced increase in invertase activity was first demonstrated in calli from *Cichorium intybus*.[47] It was later shown that a similar increase specifically of CWINV in the cultured cells of *Chenopodium rubrum* and leaves of tomato (*Solanum lycopersicum*) was due to induced gene expression.[48,49] Importantly, a coordinated increase was also observed in hexose transporter expression, which is required for uptake of the products of the invertase reaction into cells.[48] A link between cytokinin induced CWINV and delayed leaf senescence was first observed in an analysis of tobacco proSAG12::IPT lines, where it was found that the long-lived leaves of these plants had unusually high levels of CWINV activity. It was further demonstrated that plants expressing a proSAG12::CWINV transgene exhibited delayed leaf senescence, as did specific tissue regions in which an inducible CWINV construct was expressed in a localized manner. Moreover when a CWINV inhibitor protein was expressed under a cytokinin inducible promoter, treatment with the hormone no longer resulted in delayed senescence.[6] Similar results were obtained in a later study where activity of CWINV in tomato leaves was increased by the silencing of its inhibitor.[46] Together these works demonstrate that the induced expression/activity of CWINV which occurs naturally in response to cytokinin is both necessary and sufficient to cause a delay in leaf senescence. This is a highly significant point as it provides both a physiological link between senescence regulation and primary metabolism as well as at least a partial mechanism by which senescence is delayed by cytokinin. Interestingly, it also emphasizes another aspect of senescence regulation which remains poorly understood and in some ways, controversial as discussed below.

Bitter-Sweet Senescence

The implications of CWINV activity in leaves negatively influencing senescence are striking because an accumulation of sugars in leaves, particularly glucose and fructose (the products of the invertase reaction) coincides with the onset of senescence.[50,51] It has even been suggested that this accumulation may serve as an underlying trigger of senescence.[52,53] Toward this point the overexpression of a yeast invertase in the apoplasm of plants resulted in elevated hexose levels leading to premature senescence.[54] However, this finding does not actually conflict with the delayed senescence due to increased CWINV activity reported by Lara et al. (2004) because in that study increased steady-state levels of hexoses were not observed.[6] The authors interpreted this as an indication of rapid subsequent metabolism of the glucose and fructose products.

The debate regarding this point referenced above has come about because in addition to the theory in which sugar accumulation leads to senescence, a strong body of evidence suggests that sugar starvation initiates the onset of senescence.[55] For example, the dark-induction of senescence in detached leaves is generally thought to result from starvation due to a lack of photosynthesis; a hypothesis supported by the finding that treatment with sugar can both delay dark-induced senescence (similar to cytokinin) as well as repress the expression of SAGs.[56-59]

In *Arabidopsis*, hexokinase1 (HXK1), in addition to its catalytic function, serves as both a sensor of hexose levels and regulator of gene expression.[60] Mutants with impaired HXK1 function have reduced sensitivity to glucose and a delayed senescence phenotype, which has been interpreted as a deficiency of the sugar signal causing the delay.[61] However, further experimental support of this interpretation may be necessary as it is based on phenotypic analysis of mutants lacking a key regulatory and metabolic enzyme. When interpreting the results from these experiments, it is crucial to consider that the metabolic relevance of sugars is greatly dependent upon their spatial distribution both among and within cells. For instance, sucrose in the phloem of the leaf would be unlikely to have the same metabolic fate as sucrose present in the vacuole of a mesophyll cell, yet these differences cannot be determined in experiments making sugar measurements from a whole leaf extract. Considering these distinctions is important both when measuring sugar levels and in experiments involving sugar-feeding or supplementation.

Sink/Source Identity Theft

Regardless, both sugar accumulation and starvation appear to serve as triggers for senescence under certain conditions; yet to date neither condition has been irrefutably demonstrated to play such a role. This perhaps suggests that changes in carbon flux rather than steady-state levels of primary metabolites may stimulate the onset of senescence: a mechanism that could also explain the key role of CWINV. The co-induction of CWINV along with a plasma membrane hexose importer would provide a mechanism for increased carbon flux through a so-called "futile cycle."[62,63] Such a "futile cycle" would rely upon processes involved in apoplastic phloem loading (**Fig. 2A**). In apoplastic phloem loading sucrose produced in mesophyll cell cytoplasm is exported into the apoplastic space prior to uptake into specialized phloem companion cells by sucrose transporters.[64] Until recently it was unclear whether sucrose export occurred at the site of synthesis or if the sucrose was symplastically transported to phloem parenchyma and then exported in closer proximity to the companion cells. The identification of the *Arabidopsis* sucrose efflux proteins involved in this process allowed their localization, specifically to phloem parenchyma adjacent to companion cells to be determined.[65] This suggests that the efflux of sucrose directly precedes its uptake into the phloem.

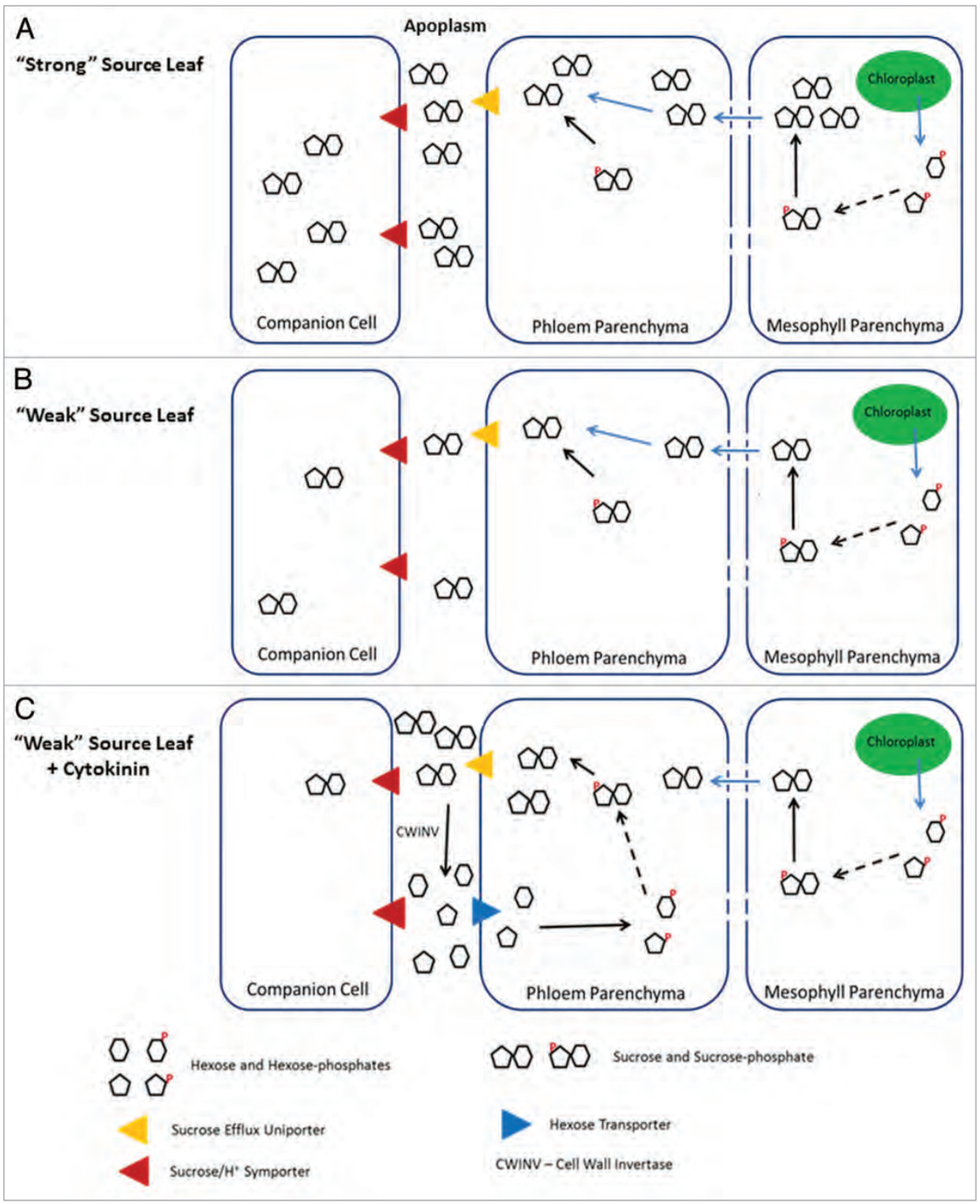

Figure 2. A futile cycle involving CWINV mimics a high rate of carbon export. (**A**) Loading of sucrose into phloem when photosynthesis rate is high involves pumping large amounts of sucrose into the apoplasm. From the apoplasm it is taken up by companion cells via sucrose symporters. (**B**) When photosynthesis rate is decreased, less sucrose is available to be pumped into the apoplasm. (**C**) Cytokinin stimulation of a futile cycle of sucrose export, hydrolyses, uptake and re-synthesis could maintain high rates of sucrose efflux at the expense of long distance transport.

Increased CWINV activity is likely to disrupt apoplastic phloem loading through the hydrolysis of effluxed sucrose (**Fig. 2B and C**). The abundant hexose monomers resulting from apoplastic sucrose cleavage could then be taken back into cytoplasm of parenchyma cells via the co-induced high-affinity hexose transporters. Hexoses that are then phosphorylated by cytosolic hexokinase (as required for subsequent metabolism) result in a high intracellular concentration of glucose-6-phosphate which in turn enhances the activity of sucrose-phosphate synthase.[66] This ultimately results in the regeneration of cytoplasmic sucrose which would again be transported into the apoplasm, generating a "futile cycle" of both the compartmentalization and molecular form of sugars (**Fig. 2C**). The continuous sucrose efflux affected by this cycle partially mimics conditions in the phloem parenchyma similar to those associated with high rates of phloem loading for carbon export that are characteristic of productive source leaves. This cycle could therefore provide even a poorly productive leaf with an artificially strong source identity despite a reduced amount of sugar actually being exported. Importantly at least one key enzyme in this cycle that is not directly induced or activated by cytokinin (sucrose-phosphate synthase) would be activated as a result of the preceding steps of the cycle (increased glucose-6-phosphate).

It is noteworthy that accelerated senescence has been reported in experiments where whole plants are supplemented with sugar, which may be a consequence of changing sink/source relationships in the opposite direction. For instance, a plant fed glucose through its growth medium would have an abundance of sugars available to its roots, which would result in a decreased amount of sugar unloaded from the phloem of these sink tissues. This would cause a reduced phloem turgor differential between source leaves and sink roots and inhibit bulk flow and diminished sugar export from source leaves. As a result, the phloem parenchyma of such leaves would experience decreased sucrose efflux and could thereby acquire a "weak source" identity and as such become candidates for senescence.

Sink regulation of source tissue identity has been extensively described elsewhere including the increases in sink strength during fruit development and root nodulation that are correlated with elevated photosynthetic rates.[67-69] The inverse effect has also been found, where restricting carbon export from leaves (mimicking a lack of a strong sink) reduces photosynthesis in part from the repression of photosynthetic genes.[70,71] One known mechanism by which this may occur is through the accumulation of starch within chloroplasts which inhibits efficiency of the thylakoid-bound photosystems.[72]

It is difficult to interpret experiments where longevity is enhanced in detached leaves supplemented with sugar in terms of sink/source relationships because similar results are not found in intact plants. However, findings appear to suggest that the two seemingly incongruent lines of evidence regarding sugar and senescence actually indicate that it is the loss of a relatively strong source identity which triggers leaf senescence.[42,73] Furthermore, that in plants with apoplastic phloem loading it is the rate of sucrose efflux out of phloem parenchyma which provides a sink/source identity. Unfortunately this model may prove difficult to directly test as it is likely to require: (1) the ability to accurately determine changes in sub-cellular (or extracellular) concentrations of sugars; (2) manipulation or misexpression of enzymes involved in indispensable metabolic processes; and (3) a more complete understanding of the role of sugars and primary metabolism in signal transduction.

Concluding Remarks

Elucidation of the mechanism by which cytokinin enhances leaf longevity is likely to have a significant impact on the basic understanding of numerous areas of research including hormone signal transduction, regulatory mechanisms of primary metabolism and the aging processes. Moreover, novel connections among these diverse areas are likely to emerge as the pathway that links the perception of cytokinin to CWINV activity and the resulting changes in carbon allocation is further elucidated.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cytokinin Response Factor 6 Negatively Regulates Leaf Senescence and is Induced in Response to Cytokinin and Numerous Abiotic Stresses

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Regular Paper

Cytokinin response factor 6 (CRF6) is an Arabidopsis AP2/ERF transcription factor which is transcriptionally induced by cytokinin. Cytokinin is known to delay leaf senescence in wild-type (WT) plants, for example in dark-incubated detached leaves. This response is mediated by the cytokinin receptor Arabidopsis histidine kinase receptor 3 (AHK3). Similar to *ahk3* mutants, *crf6* leaves show decreased sensitivity to this cytokinin effect. Leaves overexpressing CRF6 retain more Chl than those of the WT under these conditions without exogenous cytokinin. It therefore appears that an increase in expression of CRF6 downstream of the perception of cytokinin by AHK3 is involved in the delay of leaf senescence. Intact *crf6* plants also begin to undergo monocarpic senescence sooner than WT plants. Interestingly, plants overexpressing CRF6 display a more extreme acceleration of development than *crf6* mutants, suggesting that a specific expression level or localization of CRF6 is necessary to prevent premature senescence. Expression analyses indicate that CRF6 is highly expressed in the veins of mature leaves and that this expression decreases with age. CRF6 expression is shown to be induced by abiotic stress, in addition to increased cytokinin. Together, these findings suggest that CRF6 functions to regulate developmental senescence negatively and may have a similar role in response to stress. CRF6 may therefore be involved in fine-tuning the timing of developmental and stress-induced senescence. CRF6 functioning in negative regulation of senescence is significant in that it is the first process known to be regulated by cytokinin, in which a CRF can be placed specifically downstream of the cytokinin signaling pathway.

Keywords: Abiotic stress • Arabidopsis • Cytokinin • Cytokinin response factor • Senescence • Transcription factor.

Abbreviations: AHK, Arabidopsis histidine kinase receptor; ARR, Arabidopsis response regulator; BA, benzyl adenine; CK, cytokinin; CRF, cytokinin response factor; DAG, days after germination; GUS, β-glucuronidase; qPCR, quantitative reverse transcription–PCR; TCS, two-component cytokinin signaling pathway; WT, wild type.

Introduction

Leaves are the primary sites of photosynthesis in nearly all higher plants, exporting energy captured from sunlight in the form of carbohydrates to other heterotrophic organs and tissues of the plant. Photosynthetic efficiency begins to decrease at around the time a leaf is fully expanded and continues to decline with age. When the first emerging and therefore oldest leaves of a plant are unable to meet the demand for photosynthate, their senescence provides a means to recycle the valuable nutrient resources within for production of and use by new leaves (Noodâen and Leopold 1988, Singh et al. 1992, Quirino et al. 2000, Lim et al. 2007). Developmental leaf senescence is a process which allows nutrients along with carbohydrates to be released through catabolism of macromolecules for subsequent remobilization to other tissues in the plant (Himelblau and Amasino 2001, Hortensteiner and Feller 2002). In annual and other monocarpic species, the recipient tissues of these remobilized resources are often associated with reproductive growth and development (Noodâen and Leopold 1988, Zimmermann and Zentgraf 2005, Lim et al. 2007). In such cases, leaf senescence is referred to as monocarpic senescence (Noodâen and Leopold 1988, Buchanan-Wollaston et al. 2005). The distinct process of sequential leaf senescence is also developmentally triggered; however, it may not be directly linked to reproductive development (Noodâen and Leopold 1988, Singh et al. 1992, Quirino et al. 2000, Lim et al. 2007).

In contrast to leaf senescence that occurs as part of a developmental program, premature senescence can also be induced by external environmental factors or stresses, of which major sources include osmotic or salt stress, extreme temperatures, shading, herbivory or pathogenesis (Thomas and Stoddart 1980, Noodâen and Leopold 1988). In such cases, the senescence of an individually affected leaf provides a means of minimizing the loss of nutrient resources to the whole organism, whereas

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senescence in response to overall plant stress can provide energy and nutrients for accelerated reproduction. In both instances, some energy and biomass are sacrificed. Transient stress can also result in losses that could be unnecessarily detrimental to the plant, particularly during early stages of development, if not well regulated. It stands to reason, therefore, that the senescence response to environmental cues is regulated in a complex manner, allowing for fine-tuning based on the integration of multiple signals (Weaver et al. 1998, Ono et al. 2001, Chen et al. 2002, Breeze et al. 2011). Interestingly genome-level studies have shown that global changes in expression patterns associated with induced senescence only partially overlap with those that occur during developmental senescence and imply that there are distinct regulatory networks involved for each process (Park et al. 1998, Buchanan-Wollaston et al. 2005).

Phytohormones have been shown to play important roles in these regulatory networks. ABA in particular is known to act as a positive regulator of this process (Gepstein and Thimann 1980, Zeevaart and Creelman 1988, Weaver et al. 1998). Ethylene (Abeles et al. 1988, Jing et al. 2005, van der Graaff et al. 2006), jasmonic acid (Ueda and Kato 1980, He et al. 2002, Kong et al. 2006) and salicylic acid (Morris et al. 2000, Rao et al. 2002) have also been shown to induce senescence. In contrast, cytokinin has long been known to function in delaying senescence, particularly in leaves, a role often described as antagonistic to the promotion of senescence by other hormones or signals (Gan and Amasino 1996, Peleg and Blumwald 2011, Wilkinson et al. 2012).

The senescence-delaying effect of cytokinin has been shown to be dependent upon the canonical two-component cytokinin signaling pathway (TCS) in Arabidopsis. In particular, perception of cytokinin by the receptor kinase AHK3 (Arabidopsis histidine kinase receptor 3) and subsequent phosphorylation of the downstream type-B response regulator ARR2 (Arabidopsis response regulator 2) is required for increased leaf longevity in Arabidopsis (Kim et al. 2006). Further downstream targets in this pathway are unknown, and direct transcriptional regulation of senescence-related ARR2 targets has not been shown. It is also unclear to what extent this pathway is involved in developmental vs. stress-induced senescence.

Some members of another group of transcription factors, the cytokinin response factors or CRFs, have been shown to be transcriptionally regulated by cytokinin and are capable of physical interactions with components of the TCS via a group-specific CRF domain (Rashotte et al. 2006, Cutcliffe et al. 2011). In this study, phenotypic analysis of mutant and overexpressing plants suggests that a particular member of this group, CRF6, may act downstream of the TCS pathway in negatively regulating leaf senescence. Expression analyses indicate that the in planta function of CRF6 may be in the regulation of stress-induced rather than developmental senescence. A proposed model is presented in which endogenous developmental cues and environmental factors influence the expression of CRF6, thereby integrating multiple inputs into the fine-tuning of stress-induced senescence.

Results

CRF6 is involved in the delay of dark-induced leaf senescence by cytokinin

CRF6 has been shown to be transcriptionally regulated by cytokinin as well as to interact physically at the protein level with several downstream members of the TCS (Rashotte et al. 2006, Cutcliffe et al. 2011). These connections to cytokinin as well as the leaf vascular/phloem-localized expression pattern of this transcription factor implicated it in regulating sink/source-related processes such as senescence (Zwack et al. 2012). Quantitative reverse transcription–PCR (qPCR) was used to determine if the cytokinin induction of CRF6 is dependent on the primary cytokinin receptors expressed in leaves (AHK2 and 3) by examining CRF6 transcript levels with and without cytokinin in *ahk2* and *ahk3* single mutant, and the *ahk2,ahk3* double mutant backgrounds relative to the wild type (WT). While both of these cytokinin receptors in Arabidopsis regulate leaf-based cytokinin processes, it is AHK3 which mediates the delay of senescence. The *ahk2,ahk3* double mutant was included in the analysis as the two receptors operate redundantly in many processes (Ueguchi et al. 2001).

After 6 h of treatment with cytokinin, a time period we have found to optimize the induction of CRF6 expression, transcripts had increased about 4-fold (3.96) in the leaves of WT plants (**Fig. 1A**). The level of induction was decreased to around 2-fold (2.35) in *ahk2* mutants; however, this was not found to be significantly different from the WT ($P = 0.052$). In the *ahk3* background, CRF6 was induced to only 1.56-fold by cytokinin, which was found to be significantly less than the WT ($P < 0.01$) and *ahk2* ($P = 0.045$). No induction was found in the double mutant background (fold change = 0.86, $P < 0.01$). The induction levels observed in the *ahk3* single and *ahk2,ahk3* double mutants were not significantly different ($P = 0.29$). Together the data suggest that the transcriptional induction of CRF6 in leaves is mediated primarily by AHK3 but that AHK2 may be involved to a limited degree or be capable of minimal compensation for the loss of AHK3 with respect to the induction of CRF6. Numerous cytokinin-induced genes are likely to act downstream of AHK3; alone, this finding does not necessarily imply a functional role in any particular process.

To determine whether CRF6 could be functionally linked to senescence regulation, possibly downstream of AHK3, the *crf6* knock-out mutant was assayed for altered sensitivity to cytokinin during dark-induced senescence. During a 6 d dark incubation in the presence of cytokinin, detached WT leaves retained 100% of total Chl (initial = 121 ± 2, $final_{+CK}$ = 121 ± 5 nmol total Chl mg^{-1} starting FW). Under the same conditions, leaves from *crf6* plants retained only 76% of initial Chl (initial = 125 ± 6, $final_{+CK}$ = 95 ± 12 nmol mg^{-1} FW). Both WT and mutant leaves retained a greater amount of Chl in the presence of cytokinin (above) than with water alone [WT $final_{-CK}$ = 59 ± 4 (49%), *crf6* $final_{-CK}$ = 42 ± 9 (34%) nmol mg^{-1} FW]. As these findings suggested that CRF6 does have a role in the

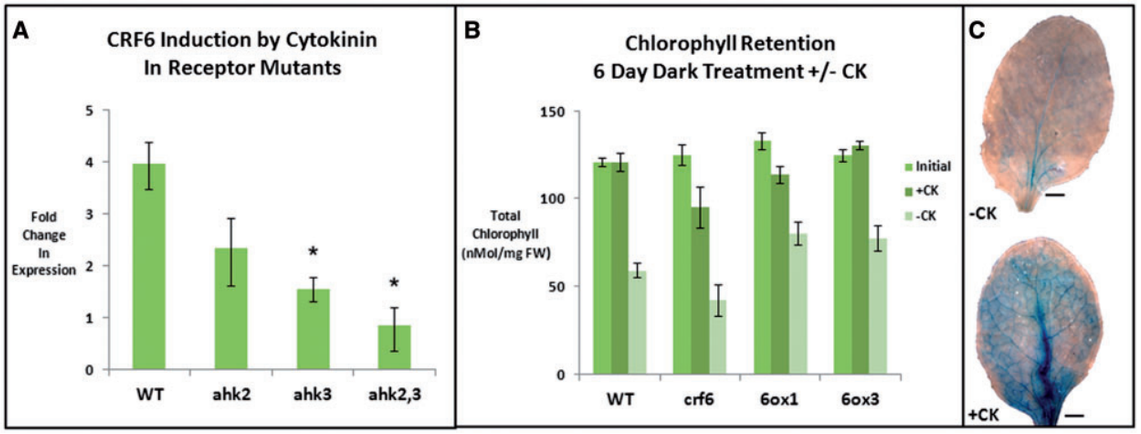

Fig. 1 (A) Fold change in CRF6 transcript levels in WT and cytokinin receptor mutant backgrounds following 6 h treatment with 1 μM BA. *Significant difference from the WT, $\alpha = 0.05$. (B) Chl retained during 6 d dark treatment of detached leaves in the presence and absence of 1 μM BA. (C) proCRF6::GUS expression is induced during dark incubation in the presence of cytokinin (2 d incubation, 1 μM BA).

regulation of leaf senescence, we decided to analyze leaves overexpressing CRF6 to determine whether increased expression of CRF6 without application of exogenous cytokinin was sufficient to delay senescence. After dark incubation in the absence of exogenous cytokinin, the leaves of plants constitutively expressing CRF6 ($6ox^1$ and $6ox^3$) (**Supplementary Fig. S1**) had retained a greater amount of Chl [$6ox^1$ $final_{-CK} = 80 \pm 7$ (60%), $6ox^3$ $final_{-CK} = 77 \pm 7$ (62%) nmol mg^{-1} FW] than the WT (**Fig. 1B**). Although greater, this increased level of Chl retention was not to the extent observed in the WT treated with cytokinin (100%). This partial compensation for the lack of exogenous cytokinin by increased CRF6 levels was taken as further support for CRF6 as a key downstream component of the AHK3-mediated senescence-delaying pathway.

To determine whether the CRF6 promoter is differentially regulated during this assay in response to cytokinin, promoter::reporter transgenic lines were examined. Leaves from three independent proCRF6::GUS (β-glucuronidase) lines were subjected to the conditions from the assay above and expression of the transgene was monitored. After 2 d, leaves which had been incubated with cytokinin clearly showed greater GUS activity than those incubated without cytokinin (**Fig. 1C**). The activation of the CRF6 promoter by cytokinin specifically under the conditions of this assay is in agreement with the altered responses seen in the mutant and overexpression lines.

Altered expression of CRF6 causes accelerated vegetative growth and development

To gain further insight into the role of CRF6 as a negative regulator of senescence, *crf6* and CRF6-overexpressing ($CRF6ox^1$) plants were examined for phenotypic differences associated with senescence during normal development (**Fig. 2A–E**). On average, *crf6* plants produced fewer leaves (10.5 ± 0.4 vs. 14.0 ± 0.4 for the WT) from the primary meristem prior to bolting (**Fig. 2C, E**), suggesting an early transition to reproductive development (although no significant change in days to bolting was observed). CRF6ox plants also displayed a more rapid transition from the vegetative to reproductive stage of growth and development as compared with the WT, but to a greater extent and with additional significant differences. The most visually obvious change in growth was that rosettes of CRF6ox plants reached a maximum diameter much sooner than WT or *crf6* plants (**Fig. 2A**). This was quantified by measuring the rosette diameter at a time when the difference was most obvious, at 18 days after germination (DAG) (**Fig. 2D**). The final size achieved by rosettes of all genotypes was similar (data not shown). In addition to producing fewer leaves (9.0 ± 0.1) (**Fig. 2C, E**), CRF6ox plants bolted in fewer days after germination than the other genotypes (20.2 ± 0.2 d vs. 24.6 ± 0.5 d for *crf6* and 25.0 ± 0.4 d for the WT) (**Fig. 2B**). Germination times were unchanged (data not shown), indicating that the changes in timing were associated with vegetative growth and development.

As early bolting could correspond to an early onset of monocarpic senescence, the Chl content of leaves was measured at regular intervals to determine if the timing of senescence was altered in these lines (**Fig. 2F**). All genotypes had similar levels of Chl at 14 DAG. At 18 DAG, prior to bolting in any lines, leaves of WT plants had lost on average about 14% of their Chl content, which across the sample population did not constitute a significant change. At this time, *crf6* and CRF6ox leaves had lost a much greater proportion of total Chl (58% for *crf6* and 71% for CRF6ox, $P < 0.01$; **Fig. 2F**), indicating that senescence had begun in these leaves. By 28 DAG (3–8 d after all genotypes had bolted) a difference in Chl content compared with the WT was no longer observed in either of the altered expression lines

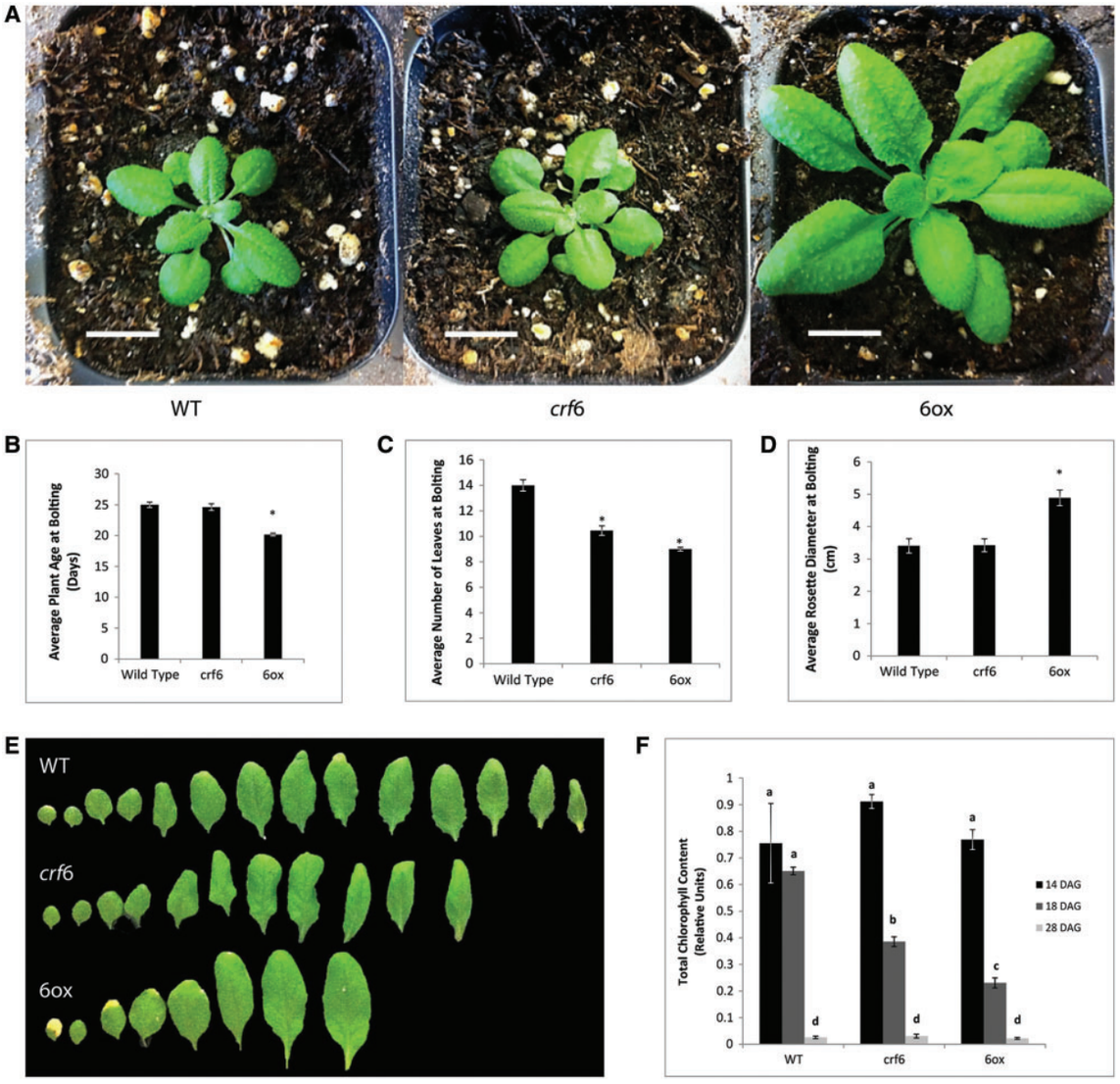

Fig. 2 Constitutive expression of CRF6 leads to accelerated growth and development. (A) Rosettes at 18 DAG. (B) Average age of plants (DAG) when inflorescence reaches 1 cm in each genotype. (C) Average number of primary rosette leaves which were present at bolting in each genotype. (D) Average diameter of rosettes at 18 DAG. (E) Leaves which were present on the primary rosette at the time of bolting pictured at 28 DAG. Note that 6ox[1] leaves are about 5 d older relative to bolting than the other genotypes (see B), but total Chl of leaves 5 and 6 is similar (see F, 28 DAG). (F) Average Chl content of fifth and sixth leaves at 14, 18 and 28 DAG. Lower case letters identify values as significantly different ($P < 0.05$ one-tailed Student's *t*-test) from those labeled with a different letter. Error bars in all graphs represent the standard error of the population, * indicates that the difference compared with the WT is significant, $P < 0.05$ (two-tailed Student's *t*-test).

(**Fig. 2F**), suggesting a change specifically in the onset as opposed to the latter progression of senescence. These two senescence processes have been suggested to be under regulation of distinct mechanisms (Balazadeh et al. 2008). Based on these findings, it appears that altered levels or spatial expression of CRF6 may cause accelerated development of the vegetative rosette, resulting in an earlier transition to reproductive growth and onset of monocarpic senescence.

CRF6 expression in source leaves decreases with age

We have previously reported that CRF6 promoter-driven GUS expression is absent in newly emerged and non-expanded leaves, but is expressed in vascular tissues, particularly the phloem in older leaves (Zwack et al. 2012). To better understand these changes, proCRF6::GUS expression in rosette leaves

was monitored over the later stages of leaf development. Expression of CRF6 remained limited to the vasculature of leaves throughout development (**Fig. 3A**). As each leaf matured, proCRF6::GUS expression was first apparent in the vasculature at the distal end and progressively expanded proximally to the vasculature throughout the entire organ. This expansion of expression took place over the course of two to several days, occurring more rapidly in the smaller early leaves (i.e. leaves 1–4) and more slowly in larger later emerging leaves (i.e. leaf 8+). Expression was observed throughout the leaf vascular network until visible yellowing of senescence could be observed at the distal tip, at which time expression was no longer observed in this region. In the earliest emerging leaves (1–4) which reached maximum expansion well before bolting, progressive loss of proCRF6::GUS expression continued, again proceeding in a distal to proximal manner generally corresponding to visible senescence or yellowing of the leaf (data not shown). Additionally, the GUS staining which was present became weaker in all leaves as the plant aged, suggesting a decrease in expression correlated with both the age of the individual leaf and the developmental age of the whole plant. The strongest intensity of GUS staining was observed just prior to the emergence of the primary inflorescence (18–21 DAG), with a large drop in expression by 28 DAG, after which staining continued to decrease until full senescence. Importantly, later emerging leaves which were not fully expressing the GUS reporter at the time of bolting continued to gain expression in the whole vasculature, but only to the reduced level found in other leaves at the particular age of the plant.

qPCR was used to determine whether native CRF6 transcript levels in WT plants followed a similar pattern to that seen for proCRF6-driven GUS expression. The relative abundance of CRF6 transcripts in the third and fourth leaves at 21, 28 and 35 DAG was compared. These leaves were chosen because they had consistently achieved proCRF6::GUS expression throughout the vasculature by the first time point to be examined, thus ensuring that data were not skewed due to concurrent increases in area of and decreases in degree of expression. In agreement with findings from the GUS reporter analysis, CRF6 transcript levels in these leaves decreased greatly (approximately 5-fold) from 21 to 28 DAG and were further reduced by 35 DAG (**Fig. 3B**).

Modulation of CRF6 expression levels in response to stress

The differential expression of CRF6 correlated to developmental age supports its role in the negative regulation of developmental senescence. A similar antagonistic role could exist in the context of premature, stress-induced senescence. Such a role is suggested by transcriptional data from numerous microarray experiments showing that CRF6 is transcriptionally induced by multiple abiotic stresses (**Table 1**) (Kleine et al. 2007, Winter et al. 2007, Usadel et al. 2008, Mizoguchi et al. 2010, Pecinka et al. 2010, Inze et al. 2012). To confirm this, we examined proCRF6::GUS under heat shock, salt and oxidative (H_2O_2) stress conditions. In response to each of these stresses, GUS levels were clearly elevated over a range of tissues (**Fig. 4A–C**). A transcriptional analysis of CRF6 levels by qPCR showed a 5.22 ± 1.45-fold increase in response to treatment with 200 mM NaCl. Although the induction level of CRF6 found in this qPCR analysis was not as great as those found in the microarray experiments in **Table 1**, the responsiveness of CRF6 to these stimuli is supported. Moreover, the findings from

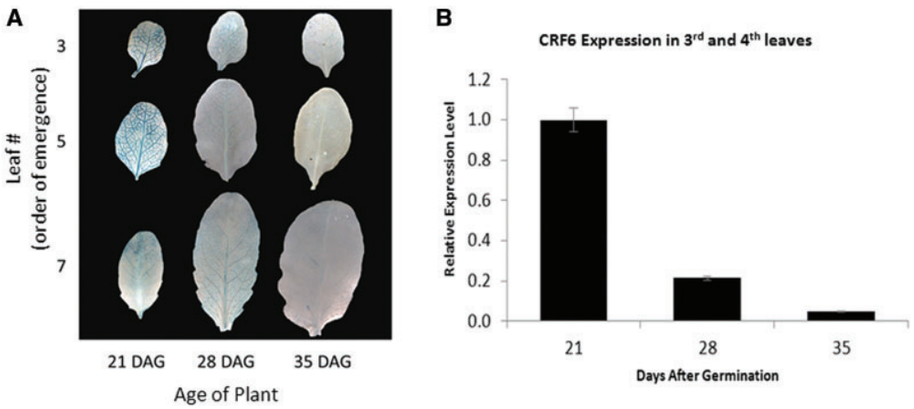

Fig. 3 CRF6 expression in the vasculature of mature leaves decreases after bolting. (A) proCRF6::GUS leaves from plants of the indicated ages. Leaf number reflects the order of emergence (i.e. leaf 3 is the oldest, leaf 7 is the newest). A dramatic decrease in GUS activity can be seen from 21 DAG (pre-bolting) to 28 DAG (post-bolting); this trend continues at 35 DAG. Note that leaf 7 at 21 DAG is not fully expanded and does not show expression throughout the vasculature, yet the level of GUS appears to be greater where it is present vs. 28 DAG. (B) qPCR expression analysis of native CRF6 transcript levels in the third and fourth leaves (pooled) of WT plants were taken at the time points indicated on the *x*-axis; again a large decline is observed from 21 to 28 DAG. Error bars represent the standard deviation between two biological replicates using pooled tissue from at least three plants each. Reactions were performed in triplicate. Relative transcript abundance at 21 DAG is arbitrarily set to 1.

Table 1 CRF6 expression is induced by abiotic stress

Stress	Treatment	Fold change	Reference
Osmotic	300 mM mannitol 12 h	36.8	Winter et al. (2007)
UV-B	6 h after 15 min exposure to UV-B light	26.0	Winter et al. (2007)
Salt	150 mM NaCl 12 h	21.1	Winter et al. (2007)
Oxidative	10 μM methyl viologen 24 h	19.7	Winter et al. (2007)
Oxidative	H_2O_2-accumulating mutant, high light 8 h	2.2	Inze et al. (2012)
Cold	12 h at 4°C	7.5	Winter et al. (2007)
Drought	Dehydration 1 h	2.1	Mizoguchi et al. (2010)
Heat	30 h at 37°C	6.8	Pecinka et al. (2010)
Dark	48 h extended dark	2.8	Usadel et al. (2008)
High light	3 h 1,000 μE	3.0	Kleine (2007)

Sample of microarray experiments in which CRF6 expression increased in response to stress. Treatments are briefly described and classified by general type of stress. Values shown are fold change from control with multiple samples and P-values < 0.05.

both the qPCR and GUS expression analyses support the overall trend of CRF6 induction by multiple abiotic stresses, which may imply a role in delaying induced as well as developmental senescence.

Discussion

CRF6 regulation of leaf longevity is downstream of cytokinin signaling

Since the regulation of senescence by cytokinin was first demonstrated >60 years ago (Richmond and Lang 1957), cytokinin-delayed senescence has been observed in numerous species and under a wide variety of experimental conditions (Gan and Amasino 1996). More recently, the model system Arabidopsis has facilitated the elucidation of some aspects of the molecular mechanisms underlying this phenomenon. Kim et al. (2006) showed that perception of cytokinin specifically by the receptor AHK3 initiates a signaling cascade involving the type-B response regulator ARR2 and that this signal is necessary for increased leaf longevity in response to cytokinin.

We have previously shown that CRF6 is among a subset of CRFs which are transcriptionally induced by cytokinin (Rashotte et al. 2006). Given the regulation of CRF6 by cytokinin as well as its expression in the phloem of mature leaves, it was hypothesized that CRF6 may also be involved in negatively regulating senescence. In this study, we have shown that induction of CRF6 by cytokinin in leaves is greatly reduced in *ahk3* mutants (**Fig. 1A**), indicating that CRF6 is transcriptionally downstream of this receptor in the cytokinin signaling pathway, linked to senescence. To connect CRF6 to leaf senescence, altered CRF6 expression lines were examined in a dark-induced senescence cytokinin bioassay. We found that leaf senescence in *crf6* mutants is not delayed by cytokinin to the same extent as in WT leaves, similar to the phenotype previously found for *ahk3* mutants. We further showed that leaves with constitutively higher levels of CRF6 expression retain greater levels of Chl in the absence of exogenous cytokinin (**Fig. 1B**). These findings suggest that elevated expression of CRF6, either from induction by cytokinin or under regulation of a constitutive promoter (**Fig. 1C**; **Supplementary Fig. S1**), is sufficient partially to delay senescence in detached dark-incubated leaves and link CRF6 to senescence.

Although there are 12 CRF proteins in Arabidopsis that could have partially redundant roles, we have recently shown that CRF proteins in all flowering plants belong to five distinct phylogenetic clades, indicating that those from different clades have not shared a common ancestor since at least the evolution of the angiosperms (Zwack et al. 2012). The maintenance of these five groups in all lineages of flowering plants suggests that each may be involved in distinct processes without a great deal of overlap in function. In Arabidopsis, CRF6 is a member of the clade III CRFs, along with one another CRF: CRF5. Despite the sequence similarity of these two proteins and their shared expression in vascular tissue, CRF5 transcript levels do not show the same correlation with senescence as CRF6 does (data not shown). Additionally, we have found using qPCR that CRF5 transcript levels were somewhat reduced (fold change = 0.5 ± 0.08) in *crf6* mutants, indicating that CRF5 is not being transcriptionally up-regulated in order to compensate for the loss of CRF6. Further testing of the relationship between these two genes is difficult as the *crf5*,*crf6* double mutant is embryo lethal (Rashotte et al. 2006).

CRF6 expression levels influence the transition to reproductive development

crf6 mutant and CRF6ox plants were examined throughout development to determine whether leaf senescence was also affected by altered CRF6 levels in a whole-plant context (**Fig. 2**). Mutant plants produced fewer leaves prior to bolting and began to lose Chl sooner than WT plants suggesting precocious monocarpic senescence (Noodâen and Leopold 1988) (**Fig. 2C, E, F**), in agreement with the *crf6* phenotype observed specifically in detached leaves used in the dark-induced senescence cytokinin bioassay. Interestingly, CRF6ox plants exhibited greatly accelerated vegetative growth, with both flowering and visible senescence also occurring in fewer days after

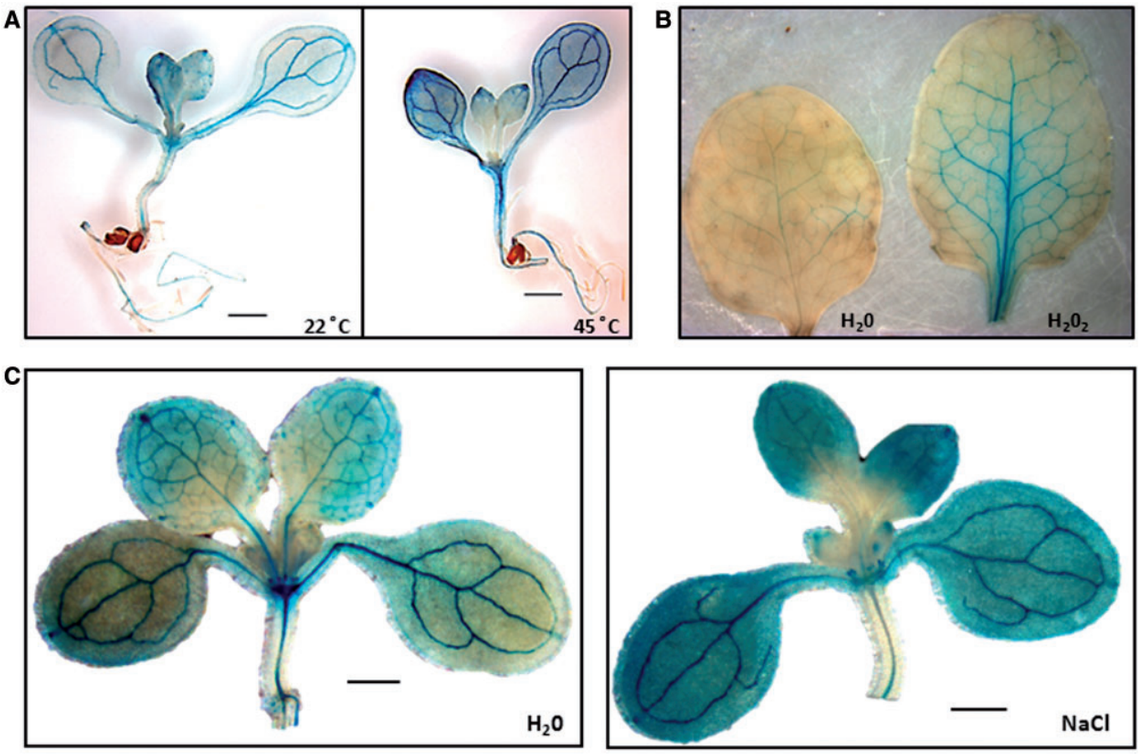

Fig. 4 CRF6 expression is induced in response to stress. (A–C) Increased proCRF6::GUS expression in response to (A) 45°C heat shock, 1 h (7 DAG seedling), (B) exposure to 20 mM H_2O_2, 6 h (18 DAG rosettes, leaf 4), (C) exposure to 400 mM mannitol or 200 mM NaCl, 6 h (18 DAG rosettes, close up of vein from leaf 4). (D) qPCR analysis of CRF6 expression in 18 DAG rosettes. The relative transcript level in the osmotic control is arbitrarily set to 1.

germination than in the WT (**Fig. 2A–E**). The inconsistency of the effects of overexpression of CRF6 on senescence in detached leaves during dark-induced senescence (delaying) and whole plants during developmental senescence (accelerating) may result from the ectopic expression of CRF6 by the 35S viral promoter. Expression in non-vascular cells and in very young leaves or other sink tissues where CRF6 is not normally expressed could result in altered levels of downstream target genes involved in processes atypical for these cells. Given the correlation between normal CRF6 expression (**Fig. 3**) and source identity, these changes could disrupt sink–source relationships, indirectly triggering premature bolting and senescence. Additionally it is known that detached leaves and those of intact plants respond differently to cytokinin with respect to senescence (Gan and Amasino 1996). Regardless, the whole-plant phenotypes of both mutant and overexpression plants suggest that CRF6 is important in regulating processes associated with the change from vegetative to reproductive development, shown to be correlated with monocarpic senescence (Balazadeh et al. 2008). The importance of CRF6 at this developmental stage is supported by expression patterns found corresponding in plants around this period.

CRF6 expression in leaves decreases with age

In Arabidopsis, developmentally induced monocarpic senescence of rosette leaves is correlated with the transition to reproductive growth (Levey and Wingler 2005, Balazadeh et al. 2008). Using a combination of reporter gene and qPCR expression analyses, we have demonstrated a decrease in CRF6 expression in leaves which occurs across the whole rosette. The onset of this decrease is characterized by a strong decline around the time of bolting. Expression appears to continue decreasing in a relatively uniform manner, with complete loss at full senescence (**Fig. 3A, B**). Additionally, a loss of CRF6 promoter activity was found to occur in older (earlier emerging) leaves which began to undergo sequential senescence and progressed in a distal to proximal manner, mirroring that of the progression of yellowing or visible senescence. Together these patterns of expression indicate a negative correlation between CRF6 expression and senescence, consistent with an antagonistic role in senescence.

Similar developmental expression patterns have been previously shown for other genes which were implicated in the negative regulation of senescence (Besseau et al. 2012). Decreased expression of such genes during senescence is probably a

reflection of the crucial role of leaf senescence in plant development, allowing valuable nutrients and carbon to be reallocated to actively growing organs. While high levels of expression are maintained during vegetative development to inhibit premature senescence, such inhibition during later stages of development would adversely affect the reproductive phase of growth and the overall life cycle of a plant.

CRF6 expression increases in response to environmental stress

Just as the antagonism of senescence by cytokinin may play a large role in the regulation of senescence in young leaves as part of normal development, similar negative regulation is likely to be of particular importance in the context of stress-induced senescence. For plants growing in the wild, conditions which induce senescence as an adaptive response are inherently unpredictable, and the true benefit of this response will depend on several factors, such as the developmental stage of the plant, nutrient levels or the photosynthetic capacity of individual leaves (Noodâen and Leopold 1988, Buchanan-Wollaston et al. 2003, Wingler and Roitsch 2008). In some cases, these stresses are transient and the losses associated with premature senescence would not be outweighed by the benefits. A mechanism of negative regulation allows the senescence response to be fine-tuned based on the particular circumstances. The delay of senescence mediated by CRF6 may be one such mechanism, as its level of expression in leaves appears to be subject to modification in response to multiple cues.

Using a proCRF6::GUS line and qPCR, we were able to confirm the induction of CRF6 by several abiotic stresses (**Fig. 4**), suggesting that CRF6 can be transcriptionally regulated in response to signals other than cytokinin. Previous studies have indicated that cytokinin levels decrease as a result of various environmental stresses (Ambler et al. 1992, Bano et al. 1994, Shashidhar et al. 1996, Ghanem et al. 2008, Nishiyama et al. 2011), in contrast to what we find for CRF6, implying that it is regulated not only by cytokinin but also in response to numerous stresses (Rashotte et al. 2006; **Table 1**). In fact, hierarchical clustering analyses of microarray data indicate that among the Arabidopsis transcription factors most associated with cytokinin signaling (type-B ARRs, Clade I and Clade II CRFs), the regulation of CRF6 under stress conditions is quite distinct (**Supplementary Fig. S2**). Again, this seems to suggest that cytokinin levels are not the only factor influencing CRF6 expression and its effects on leaf senescence.

Based on this unique regulation and the presented evidence indicating a negative regulatory function for CRF6, we propose a model for the regulation of senescence by cytokinin which includes interactions with stress signals (**Fig. 5**). In this model, CRF6 acts downstream of cytokinin perception by AHK3 in a pathway mediating the delay of senescence. We have included a parallel AHK3–cytokinin-regulated leaf senescence pathway as loss of CRF6 does not fully phenocopy an AHK3 loss. Such a parallel pathway may partially overlap or interact with the one involving CRF6 and could contain ARR2 which has also been shown to function downstream of AHK3 in this response, although additional work is needed to resolve this relationship (Kim et al. 2006). The influence of stress upon cytokinin levels, CRF6 expression and in general upon leaf senescence is also indicated. The unique relationship which exists between CRF6 and both cytokinin and abiotic stress may be indicative of a specific functional role for this transcription factor in integrating stress response into the negative regulation of senescence.

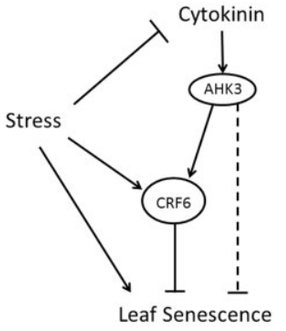

Fig. 5 Model representing relationships among CRF6, cytokinin and stress in the regulation of leaf senescence. Expression of CRF6 is induced by cytokinin via the TCS and in response to stress. Stress also leads to reduced cytokinin levels and can cause premature senescence. Loss of CRF6 does not completely phenocopy loss of cytokinin perception by AHK3, suggesting an additional parallel pathway or step in the negative regulation of senescence by cytokinin.

Further elucidation of this pathway will require identification of the downstream targets of CRF6 specific to this regulation; however, the findings presented in this study indicate an important novel functional role for CRF6. In fact, the negative regulation of senescence by CRF6 is the first function directly linked to cytokinin which has been demonstrated for a CRF protein in any species. Moreover, CRF6 is a novel downstream component in the pathway by which AHK3 and the TCS mediate enhanced leaf longevity in response to cytokinin.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (Col-0) sterilized seeds were germinated on Petri dishes containing 0.8% agar gel with MS salts (4.8 g l^{-1}) plus 1% sucrose medium of pH 5.7. Plants were grown under 100 μmol m^{-2} s^{-1} of light with a light–dark cycle of 16 h, 22°C/8 h, 18°C in controlled environment chambers. For extended growth, seedlings were transferred to soil (sunshine mix #8) and grown under conditions as above, but under approximately150 μmol m^{-2} s^{-1} light.

Developmental measurements

Germination time was measured on Petri dishes as above; seeds were stratified for 48 h in dim light at 4°C. Germination was considered to have occurred when radicle emergence was

visible. Plants were observed twice daily. Bolting time was measured on soil-grown plants and was considered to have occurred when inflorescences had reached 1 cm in height. For the Chl retention assay, leaves were considered to have emerged when they were visually distinguishable from the shoot apex.

Generation of transgenic plants

PromoterCRF6::GUS (proCRF6::GUS) and *crf6*$^{-2}$ insertional lines used were described in (Zwack et al. 2012). To generate overexpression lines, the coding sequence of CRF6 (At3g61630) was PCR amplified using sequence-specific primers with GATEWAY™ att-B sites. PCR products were cloned into pDONR221 entry vector (Invitrogen) and plasmids were generated using the Invitrogen GATEWAY™ system according to the manufacturer's instructions. The destination vector pEARLYGATE103, ordered from the Arabidopsis stock center ABRC, was used to create expression clones. The plasmid was transformed into *Agrobacterium tumefaciens* C58 via electroporation, and plants were transformed by agrobacteria using the floral dip method (Clough and Bent 1998). Transgenic T_1 plants were selected based on BASTA resistance. Multiple lines (six) showed similar phenotypes in the T_3 generation from which the homozygous lines $6ox^1$ and $6ox^3$ were arbitrarily chosen for further analysis.

RNA isolation, cDNA synthesis and quantitative PCR

RNA was isolated from pooled tissue with a Qiagen RNeasy Kit according to the manufacturer's instructions. RNA concentrations were equalized and reverse transcription was carried out using Quanta qScript cDNA supermix. The resulting cDNA samples were again normalized if necessary and were diluted prior to qPCR performed with the SYBR-Green chemistry in an Eppendorf Mastercycler ep realplex thermocycler. Reactions contained 9 µl of SYBR-Green supermix, 10.76 µl of cDNA template and 0.12 µl of 100 µM gene-specific primers: CRF6-F, GAA GTTCACAGAGAATCG; CRF6-R, CCTTGGCAGAATCTAATC; CRF5-F, ACACCGTTCGACGGTTTCCCG; CRF5-R, GCAGCCG CCGGGGACTTTTT; TUB4-F, ACCAATGAAAGTAGACGCCA; and TUB4-R, AGAGGTTGACGAGCAAGATGA. The qPCR program consisted of one cycle at 95°C, followed by 40 cycles of 15 s 95°C, 45 s 57°C and 30 s 68°C. Relative expression data are the mean of two or three biological replicates each consisting of a pool from at least three individual plants. Each reaction was performed in triplicate. Expression levels of the reference gene (β-tubulin 4) for normalization were confirmed with RT–PCR as in Zwack et al. (2012).

Histochemical analysis

Analysis of GUS activity from tissues of various ages as well as stress treatments as noted in the text were vacuum infiltrated for 10 min with X-gluc buffer (Weigel and Glazebrook 2002) then further incubated at 37°C for 2 h (developmental assay) or a time at which differences could be observed (stress assays). Tissue was then cleared in 70% and 95% ethanol at room temperature and examined.

Dark-induced senescence

Third and fourth leaves from 18–24 plants of each genotype were detached 10 d after emergence and weighed prior to treatment. Chl from 6–8 leaves was measured for initial content. For treatment, 6–8 leaves per genotype per treatment were individually floated adaxial side up in a single well of a 24-well microtiter plate containing either 1 µM benzyl adenine (BA) or 0.1% dimethylsulfoxide (DMSO; vehicle control) which was sealed in a dark box at room temperature for 6 d. After treatment, Chl was extracted in methanol at 4°C overnight. Chl content was measured spectrophotometrically as described by Ritchie (2006). Chl content was normalized to fresh weight of each leaf prior to treatment.

Stress treatment

Intact rosettes of 10 or 18 DAG plants (as indicated in the figure legends) with most of the root system removed were floated and gently shaken for 6 h on water, a solution of 200 mM NaCl (for salt stress) or a solution of 20 mM H_2O_2 (for oxidative stress). Following treatment, tissues were briefly dried to remove excess water and the remaining root tissue was removed just prior to freezing in liquid nitrogen for RNA extraction or incubation in X-gluc buffer for GUS analysis. Heat treatments were carried out on 7 DAG seedlings which remained on agar plates during treatment. Plates were moved from 22°C and floated on a 45°C water bath with dim light for 1 h, after which plants were returned to 22°C for an additional 4 h prior to incubation in X-gluc buffer.

Hierarchical clustering analysis

Clustering analyses were performed for genes, as designated, on ATH1 microarrays of all microarray experiments within the abiotic subset of the perturbations group available at Genevestigator using default settings and displayed as a Pearson correlation.

Supplementary data

Supplementary data are available at PCP online.

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Interactions between Cytokinin Signaling and Abiotic Stress Responses

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Abstract

Plants have evolved elaborate mechanisms for sensing and responding to sub-optimal environmental conditions. Abiotic stresses caused by these conditions trigger a wide range of local and long distance signals which must be coordinated and integrated into whole-plant processes such as development for the plant to properly respond and survive. Several hormones function as key regulators of stress tolerance, connecting local stimuli to systemic responses. Cytokinin is a hormone well known for its role in numerous aspects of growth and developmental, although abundant evidence also indicates that cytokinin functions in stress responses as well. At present a full understanding of the effects of cytokinin on plant resistance to stress is lacking, possibly as a result of complex interactions between cytokinin and stress signaling. Here we examine the current knowledge of the physiological relationship between cytokinin and abiotic stress based on measurements of cytokinin levels under stress conditions and the effects of cytokinin treatment on stress tolerance. We also identify a pattern of transcriptional regulation of stress related genes by cytokinin in different plant species. Additionally, research regarding the role of specific cytokinin signaling components in a variety of stress responses is presented. We discuss what this body of research collectively implies with regard to crosstalk between cytokinin and abiotic stress tolerance.

Keywords: Cytokinin, Abiotic Stress, Signal Transduction, Transcription Factor, Hormones, Stress Tolerance

Introduction

Cytokinin is a plant hormone that regulates a wide array of processes crucial to plant development (Werner and Schmulling, 2009; Kieber and Schaller 2014). Cytokinin was first identified as a potent inducer of cell division in tissue culture, and is now known to be a key regulator of the cell cycle (Miller, et al., 1955; 1956; Schaller et al., 2014). Along with auxin, cytokinin plays an important role in regulating cell divisions and the maintenance of stem cells in apical meristems of both shoots (Gordon, et al, 2009; Zhao et al, 2010; Su et al, 2011) and roots (Dello Ioio et al, 2008; Zhang et al, 2013). Functional roles of cytokinin in growth and development at the tissue and organ level include: inhibition of lateral root initiation (Laplaze et al, 2007; Bielach et al, 2012), differentiation of phloem and metaxylem in roots (Mähönen et al, 2000; Bishopp et al, 2011), regulation of cell division and photomorphogenic differentiation in expanding leaves (Chory et al., 1994; Chiang et al., 2012; Efroni et al, 2013) and inhibition of leaf senescence (Gan and Amasino, 1996; Zwack and Rashotte, 2013). In contrast, interactions between cytokinin signaling, environmental stimuli and stress have only more recently begun to be well characterized (Argueso et al., 2009; Ha et al., 2012; O'Brien and Benkova, 2013).

Several other plant hormones are known to function in abiotic stress responses. Abscisic acid (ABA) has long been recognized as the primary plant "stress hormone", regulating a wide range of mechanisms leading to enhanced stress tolerance (Cutler et al., 2010; Danquah et al., 2014). Ethylene is also involved in abiotic stress response, in part by limiting growth in favor of increased stress tolerance (Morgan and Drew, 1997; Wang et al., 2013). Salicylic acid and jasmonic acid are important in stress responses as well, however these hormones are primarily linked to biotic stress (Wasternack, 2007; An and Mou, 2011). Additionally, evidence exists in support of the involvement of auxin and gibberellins in stress adaptation, which may occur indirectly through alterations in growth (Achard et al., 2008; Tognetti et al., 2012).

Cytokinin Plays an Important and Complex Role in Abiotic Stress Responses

Cytokinin is generally considered to play a negative role in plant adaptation to stress, however this is not always clearly supported. In fact, evidence exists for cytokinin having both positive and negative effects on stress tolerance. Much of the conflicting evidence has come from physiological investigations examining endogenous cytokinin levels during and after stress treatments and/or the effects that exogenous cytokinin application has on stress tolerance. Numerous studies, conducted in a wide range of plant taxa, have found that cytokinin concentrations decrease in response to extended stress (Itai and Vaadia, 1965; Itai et al., 1973; Walker and Dumbroff, 1981; Caers et al., 1985; Hubick et al., 1986; Hansen and Dörffling, 2003; Kudoyarova et al., 2007; Albacete et al., 2008; Ghanem et al., 2008). In contrast, other investigations have reported both short-term and

sustained increases in cytokinin levels, particularly in response to severe stress (Walker and Dumbroff, 1981; Hansen and Dörffling, 2003; Pospisilova et al., 2005; Alverez et al., 2008). The measurement of cytokinin in xylem sap of stress treated plants has indicated that cytokinin long distance transport is reduced in some species under certain conditions, but the physiological relevance of these findings has been questioned (Burrows, et al., 1969; Bano et al., 1993; Hare et al., 1997). Expression analyses of Arabidopsis cytokinin biosynthesis (*IPT*) and catabolism (*CKX*) genes following NaCl treatment showed that *IPT5* and *IPT7* are up-regulated for the first hour or two before returning to pretreatment levels. Conversely, *CKX1*, *CKX3* and *CKX6* are repressed after one hour followed by a gradual increase with elevated levels sustained for 5 to 10+ hours (Nishiyama et al., 2011). Taken together, analyses of cytokinin levels during stress response could be interpreted as indicating that cytokinin concentrations first undergo a transient increase upon exposure to stress followed by either an overall decrease under sustained moderate stress or the maintenance of initially increased higher levels when challenged with more severe conditions (**Fig. 1**).

The effects of an exogenous cytokinin application towards regulating stress tolerance have also been examined using a variety of hormone-treatment strategies. Bean plants treated with cytokinin by foliar spray were more susceptible to salt treatment than control plants (Kirkham, et al., 1974). However wheat seedlings grown on cytokinin supplemented media and sprouted potato tubers pretreated with cytokinin demonstrated improved salt tolerance (Naqiv et al., 1982; Abdullah and Ahmad, 1990). Pospislova and Batkova (2004) found irrigation with cytokinin prior to drought increased the tolerance of bean plants, but had no effect and a negative effect on tolerance in maize and sugar beet, respectively. Cytokinin aided in recovery of leaf photosynthetic performance when applied following heat shock in maize and tobacco; however cytokinin pretreatment exacerbated the negative effects of the stress (Itai et al., 1978; Caers et al., 1985). Arabidopsis plants grown on media supplemented with cytokinin had a higher survival rate than non-supplemented plants when exposed to freezing or dehydrating conditions (Jeon et al., 2010; Kang et al., 2012). While in these studies the plants did survive, the exogenous cytokinin resulted in overall inhibited growth. Together, these studies indicate that multiple factors influence how cytokinin treatment affects stress signaling and that the spatial, temporal and developmental context may be an important factor in the downstream stress response.

More recently a novel approach has been taken to examine the effects of increasing cytokinin levels in relation to stress tolerance. The isopentenyl transferase (*IPT*) gene from *Agrobacterium tumfaciens*, which catalyzes the rate limiting step of cytokinin biosynthesis, was inserted into the genome of tobacco plants under the regulation of a stress inducible promoter. The resulting transgenic plants increase cytokinin levels specifically in response to stress and have enhanced drought tolerance (Rivero et al.,

2007). Subsequent work using this same stress induced cytokinin circuit has replicated these findings in transgenic rice and peanut plants (Peleg et al., 2011; Qin et al., 2011). In apparent contrast to these findings, Arabidopsis *ipt* mutants, which have reduced cytokinin levels are also more drought resistant as compared to WT (Nishiyama et al., 2011). Decreased cytokinin levels achieved by over-production of the cytokinin degrading enzyme cytokinin oxidase (CKX) in either a constitutive or root specific manner, also has a positive effect on drought stress tolerance (Werner et al., 2010; Nishiyama et al., 2011; Mackova et al., 2013). Importantly, the root specific (*WRKY6*) promoter used in this study is repressed in response to stress so the over-production of CKX does not persist under drought. Therefore the increased tolerance was attributed to the plants having enhanced root systems as a result of diminished cytokinin (which inhibits root growth) during development (Mackova et al., 2013). Collectively these experiments clearly demonstrate the potential impact of altered cytokinin metabolism on stress responses, and further emphasize the complex role it plays in such responses.

Transcriptional Regulation of Stress Related Genes by Cytokinin

Transcriptome wide responses to cytokinin treatment have been examined many times. These have in turn been recently used in comprehensive meta-analyses of transcriptional responses to cytokinin (Brenner et al., 2012; Bhargava et al., 2013; Brenner and Schmulling, 2015). Among the processes from GO term/MapMan analyses, implicated as being regulated by cytokinin were abiotic stress responses, particularly response to oxidative stress – with an abundance of redox associated enzyme activities (such as peroxidase and glutathione transferase) (Brenner et al., 2012; Bhargava et al., 2013). It was also shown that genes that are most highly responsive to cytokinin tend to be repressed in response to external stimuli including stress conditions (Brenner et al., 2012).

To better understand the regulation of stress responsive genes by cytokinin, we separately examined induced and repressed genes from two different cytokinin transcriptome profiling experiments in Arabidopsis for additional connections to stress (Rashotte et al., 2003; Brenner et al., 2005). Functional clusters of enriched GO biological process terms were generated using tools available at the DAVID bioinformatics resources website (**Table 1)** (Huang et al., 2009a,b). For both experiments, the most highly enriched cluster of induced genes included the term “response to hormone stimulus” along with other similar terms. From both sets of down-regulated genes the cluster with the greatest degree of enrichment was characterized by oxidative stress related terms. A similar analysis of individually enriched terms was also conducted with publicly available transcriptome datasets from cytokinin treated Rice (*Oryza sativa*) and Tomato (*Solanum lycopersicum*) plants to determine if comparable responses occurred in these species (**Table 2)** (Gupta et al., 2013; Sato et al., 2013; Shi et al., 2013). In both species datasets, genes with molecular functions related to detoxification of reactive oxygen were over-represented

among induced genes. Interestingly the same terms were enriched for the repressed genes, generally at a higher threshold of significance. Tomato and rice genes repressed by cytokinin were also enriched with the GO terms “response to stress” and “response to oxidative stress”, respectively. In agreement with this trend, examination of differentially expressed Arabidopsis genes in cytokinin deficient (and salt tolerant) *ipt1,3,5,7* mutant plants revealed that genes involved in response to and breakdown of ROS were greatly over-represented among those with increased expression relative to WT under normal conditions (Nishiyama et al., 2012). This pattern of regulation suggests that cytokinin may act to transcriptionally alter responses to ROS, which are known to be produced as a result of a wide range of abiotic stress conditions.

The Cytokinin Signaling Pathway Interacts with Multiple Abiotic Stress Responses

Identification of cytokinin receptors and subsequent characterization of the cytokinin signaling pathway began in the late 1990s; it is now known to be a modified version of the bacterial two-component signaling system (Kieber and Schaller, 2014). The two-component cytokinin signaling pathway (TCS) includes membrane-bound (primarily ER) hybrid histidine kinases (HKs) with a cytokinin binding input domain (CHKs) (Wulfetange et al., 2011). Upon binding of cytokinin, CHK dimers become auto-phosphorylated (Shi and Rashotte, 2012). A subsequent phosphorelay involves histidine phosphotransfer proteins (HPts) that carry the signal into the nucleus where they phosphorylate the receiver domain of two types of response regulator proteins (RRs). Phosphorylation of B-type RRs (RRBs) activates these GARP domain containing MYB-like transcription factors, which regulate the expression of cytokinin responsive genes. Among the most highly induced genes are the A-type RRs (RRAs) which are similar to RRBs but lack a DNA binding GARP domain. As such, these proteins act as negative regulators of the cytokinin signal primarily by competing with RRBs for phosphorylation by HPts (Gupta and Rashotte, 2012). Like the RRAs, C-type RRs (RRCs) lack a DNA binding domain and are also believed to act as negative regulators of cytokinin signaling. The elucidation of this pathway and identification of its components, particularly in Arabidopsis has enabled researchers to use reverse genetic approaches to examine in greater detail specific roles of and interactions involving cytokinin signaling components in response to abiotic stress (summarized in **Fig. 2**).

Osmotic Stress: Drought and Salt

Loss of water potential as a result of drought or dehydration reduces transpiration and negatively impacts numerous processes including solute transport and photosynthesis (Farooq et al., 2009). Arabidopsis possesses three CHKs (*AHK2*, *AHK3* and *AHK4*). The expression of two of these receptors, *AHK2* and *AHK3*, is induced by drought and osmotic stress suggesting a state of heightened cytokinin sensitivity under these conditions. Plants lacking either or both of these receptors have enhanced tolerance to

drought treatment as well as increased sensitivity to ABA (Tran, et al., 2007). This indicates that these receptors (and presumably the downstream output of the cytokinin signal) negatively impact drought tolerance. In contrast to the AHKs expression of Arabidopsis HPts (AHPs) is repressed by drought stress. *ahp2,ahp3,ahp5* triple mutants do however demonstrate a drought tolerance phenotype similar to receptor mutants (Nishiyama et al., 2013). Interestingly, drought induction of Arabidopsis A-type RRs (ARRs) *ARR5* and *ARR15* and the C-type RR *ARR22* is independent of the cytokinin receptors, implying that these downstream components of the TCS may be regulated by other additional signaling pathways (Kang et al., 2012). Moreover, this same work demonstrated a positive effect of cytokinin pretreatment on drought tolerance which was enhanced in *ahk2* and *ahk3* mutants, indicating that enhancement of survival by cytokinin was not mediated by these receptors. Because loss of *AHK4* did not suppress the enhanced survival, this effect of cytokinin may have been independent of TCS signaling.

Salt and drought stress have similar effects on water potential, but salinity has additional cytotoxic effects within the cell; accordingly, regulatory responses to these conditions may also be distinct (Zhu, 2002). The role of cytokinin signaling in salt stress has also been examined. As is the case for drought, *AHK2* and *AHK3* are induced by saline conditions, and single *ahk2*, *ahk3* and double *ahk2,ahk3* mutants are more tolerant of these conditions (Tran et al., 2007). Consistent with this finding implying a negative regulation of salt responses by cytokinin signaling, the B-type RR double mutant *arr1,arr12* demonstrates increased salt tolerance (Mason et al., 2010). However, despite greater cytokinin-sensitivity, *arr3,arr4,arr5,arr6* quadruple RRA mutants are also more salt tolerant (Mason et al., 2010). This further supports the notion of RRAs interacting with other signaling systems independent of cytokinin.

In addition to the three cytokinin receptor HKs, the Arabidopsis genome encodes eight other HK proteins. Five of these are ethylene receptors that function independent of histidine kinase activity. The remaining three (AHK1, AHK5 and CKI1) are functional HKs, but lack cytokinin binding capability (Schaller et al., 2008). *AHK1* is induced by drought and is a positive regulator of drought tolerance, possibly acting as an osmosensor (Tran et al., 2007; Wohlbach et al., 2008; Kumar et al., 2013). It is unknown whether this regulation is mediated through downstream TCS components. Alternatively AHK5, a cytoplasmic HK, is a negative regulator of both drought and salt stress, directly interacting with AHP1, AHP2 and AHP5 (Mira-Rodado et al., 2012; Pham et al., 2012). Although these HK proteins are not activated by cytokinin, they may act through shared downstream components, integrating stress signaling with cytokinin responses (**Fig. 2**).

Low Temperature Stress: Cold and Freezing

Cold conditions negatively impact membrane fluidity and transpiration rates leading to dysfunctions in primary metabolism. Chilling also initiates an acclimation response that

prepares the plant for the more severe stress associated with freezing temperatures (Beck et al., 2007). Exposure to cold, non-freezing temperatures results in transiently induced expression of the type A RRs *ARR5*, *6*, *7* and *15*, which is dependent upon the function of the receptors AHK2 and AHK3, along with AHP2, 3 or 5, and the RRB ARR1 (Jeon et al., 2010; Jeon and Kim, 2013). Upon extended cold treatment (6 hours) EIN3, a positive regulator of ethylene signaling, represses the expression of *ARR5*, *7* and *15* (Shi et al., 2012b). This repression can be overcome by cytokinin treatment which, in addition to constitutive overexpression of *ARR5*, *7* or *15*, enhances tolerance to freezing conditions (Shi et al., 2012b). Plants overexpressing *ARR1* also maintain elevated levels of these RRAs during extended cold and when they are pretreated with cytokinin become more freeze-tolerant than WT controls (Jeon and Kim, 2013). These finding indicate that a subset of RRAs positively regulate resistance to freezing temperatures (**Fig. 2**).

In contrast, *arr5, arr6* and *arr7* mutants are reported to have enhanced freeze tolerance and the overexpression of *ARR7* results in greater susceptibility to freezing (Jeon et al., 2010). Additionally, inducible overexpression of the type-C RR *ARR22*, represses the expression of several RR-As, including *ARR5*, *6* and *7*; and results in increased resistance to cold (and drought) stress (Kang et al., 2013). These conflicting results make it difficult to determine the precise role of cytokinin signaling in cold-acclimation, yet it seems clear that proper expression particularly of a subset of RRAs is crucial for optimal tolerance of freezing conditions.

Photooxidative Stress

Emerging evidence has implicated cytokinin signaling in additional abiotic stresses related to photosynthetic dysfunction leading to the production of reactive oxygen species (ROS) (**Fig. 2**). Loss of *AHK2* and *AHK3* resulted in greater photooxidative stress tolerance as indicated by decreased proline accumulation, lipid peroxidation and chlorophyll loss. This was interpreted as an indication that these receptors negatively regulate tolerance under such conditions (Danilova et al., 2014). A recent report that *ahk2,3* and *arr1,12* double mutants were hypersensitive to high-light stress, indicates that cytokinin signaling may play a positive role in this response again illustrating the complex nature of interaction between cytokinin and abiotic stress responses (Cortleven et al., 2014).

It should be noted that clear connections also exist between cytokinin signaling and biotic stress. These important interactions have been reviewed elsewhere and are outside the scope of this review (Argueso et al., 2009; O'Brien and Benkova, 2013).

Crosstalk between ABA and Cytokinin Signaling

Some of the changes in stress tolerance associated with cytokinin signaling and metabolism mutants described above are also accompanied by alterations in ABA

sensitivity (Tran et al., 2007; Jeon et al., 2010; Nishiyama et al., 2011). Additionally, cytokinin deficient plants have decreased levels of ABA, due to a repression of ABA biosynthesis (Nishiyama et al., 2011; Mackova et al., 2013). Treatment of Arabidopsis plants with ABA has been shown to repress the expression of the type-A RRs *ARR6* and *ARR9*, an effect greatly diminished in *ahk2,ahk4* and *ahk3,ahk4* double mutants. This implies that this RRA repression occurs at least in part by direct interaction with the TCS pathway (Yang et al., 2014).

Direct interactions between the cytokinin and ABA signaling pathways have been described in the regulation of other (non-stress) processes. For example, during seed germination ARR4, 5 and 6 proteins interact with and inhibit the function of the transcription factor ABI5, a positive regulator of ABA signaling (Wang et al., 2011). Another component of the ABA signaling pathway, ABI4, which functions in a manner similar to ABI5, induces the expression of *ARR5* in lateral root formation (Shkolnik-Inbar and Bar-Zvi, 2010). Additionally, cytokinin signaling enhances cotyledon greening by promoting degradation of ABI5 in a manner dependent upon several different parts of the TCS: AHK4, AHP2, AHP3, AHP5 and ARR12 (Guan et al., 2014). Although such interactions have not been demonstrated in the regulation of abiotic stress response, these findings support the feasibility of similar mechanisms of crosstalk.

Recently, a very different cytokinin-ABA interaction, not involving the TCS, was reported. ABA-mediated stomatal closure in response to drought involves the production of nitric oxide (NO). Cytokinin inhibits this process through direct interaction with and reduction of the NO molecule, acting as a scavenger of this signaling molecule (Liu et al., 2013a). Thus, in at least this case cytokinin itself is directly involved in modulating the ABA response.

Cytokinin Response Factors: Integrators of Cytokinin and Abiotic Stress Responses

Cytokinin Response Factors or CRFs comprise a small monophyletic group of AP2/ERF transcription factors that function as a side branch of the TCS pathway (Rashotte et al., 2006). CRF proteins interact with TCS components, primarily HPts and to a lesser extent type-A and type-B RRs, however the function of these interactions is unclear (Cutcliffe et al., 2010). An evolutionarily conserved subset of CRFs are transcriptionally induced by cytokinin placing them downstream of the TCS pathway, yet CRFs themselves regulate transcriptional responses to cytokinin in concert with the RRBs (Rashotte et al., 2006; Shi et al., 2012a; Zwack et al., 2012). CRFs have been functionally linked to classical cytokinin regulated processes (Rashotte et al., 2006; Okazaki et al., 2009; Zwack and Rashotte, 2013), and a large amount of correlative (expression-based) evidence suggest roles in stress response, but to date few such roles been demonstrated experimentally.

Analysis of microarray data indicates that Arabidopsis *CRF*s are responsive to various abiotic stresses (Argueso et al., 2009). Similar expression patterns have been shown for *CRF*s from tomato (*Solanum lycopersicum*) and Chinese cabbage (*Brassica rapa*) (Shi et al., 2012a; Liu et al., 2013b; Shi et al., 2014; Gupta and Rashotte, 2014). Additional studies have provided more detailed information about the regulation of *CRF*s in response to specific abiotic stresses.

Lee et al. (2005) demonstrated that *CRF3* is induced at low temperatures and that this regulation is mediated by the cold responsive transcription factor ICE1. In Arabidopsis *CRF4* has the greatest sequence similarity to *CRF3*. A separate study using reporter genes and quantitative PCR (qPCR) showed that this gene is highly induced by cold as well. A role in cold-acclimation was demonstrated in this instance as constitutive overexpression of *CRF4* enhanced freeze tolerance in non-acclimated plants (Compton, 2012).

The tobacco CRF referred to as Tobacco stress-induced gene 1 (*Tsi1*) was originally identified in a screen for salt inducible transcripts. Overexpression of the Tsi1 protein was found to confer greater tolerance to not only salt but also pathogen attack which was taken to suggest that it was a positive regulator of two distinct responses (Park et al., 2001). Expression of the Tomato ortholog of *Tsi1* (SlCRF1) is also induced by salt but a similar functional role in salt stress has not been demonstrated (Shi et al., 2012a).

CRF6 is the most widely stress regulated Arabidopsis *CRF*s and is particularly responsive to oxidative stress (Zimmermann et al., 2004; Inze et al., 2011; Zwack et al., 2013; Ramireddy et al., 2014). In agreement with this trend qPCR and promoter:GUS experiments have demonstrated *CRF6* induction by salt, heat shock and H_2O_2 (Zwack et al., 2013). This expression pattern may be evolutionary conserved as qPCR analyses showed that the tomato *CRF6* ortholog (*SlCRF5*) is also induced by heat and H_2O_2 as well as drought (Gupta and Rashotte, 2014). A recent study identified Arabidopsis *CRF6* as a member of the mitochondrial dysfunction (MD) stimulon, a set of 24 genes which are highly responsive to MD leading to the production of ROS (De Clercq et al., 2013). This retrograde signal is mediated largely by the transcription factors ANAC013 and ANAC017 which directly target *CRF6* and the closely related *CRF5* (Ng et al., 2013). This finding suggests that CRF5 and CRF6 along with their downstream targets may play a role in the response to stress induced mitochondrial dysfunction, yet this remains to be experimentally tested.

Conclusions

Although the majority of cytokinin research has focused primarily on the regulation of developmental processes, the involvement of this hormone in stress response has long been the subject of investigation. However much about the relationship between cytokinin and stress tolerance and/or susceptibility remains unclear. Examinations of

endogenous cytokinin levels under various conditions have suggested that cytokinin metabolism is highly regulated during response to abiotic stress. Collectively the findings of these hormone quantification studies seem to indicate a transient spike of cytokinin occurs in the initial response to stress followed by a subsequent reduction in overall levels. This reduction however may not occur in the case of more severe stress conditions. Experiments involving treatment of plants with exogenous cytokinin or biotechnological manipulation of endogenous levels have demonstrated both positive and negative effects on tolerance of different stresses; therefore generalizations about the effect of manipulating cytokinin levels with respect to overall stress tolerance are more difficult to make at this time.

Cytokinin signal transduction occurs via a multi-step His to Asp phosphorelay pathway and the signaling components have been identified in several species, but are most extensively characterized in Arabidopsis. The CHK receptor proteins from Arabidopsis (primarily AHK2 and 3) are antagonistic of drought, salt and cold stress tolerance; yet have a positive role in adaptive response to high-light stress. Similarly conflicting results have been found for type-A RRs as well. Overall the current body of research suggests that the interactions between components of the cytokinin signaling pathway and stress responses are variable and context specific. It is likely that many of these interaction occur as a result of crosstalk between cytokinin and ABA signaling and metabolism.

CRF proteins are emerging as potential integrators of cytokinin and stress responses. These transcription factors are (along with RRBs) co-regulators of cytokinin responses, yet little is known about the mechanisms of this co-regulation. It seems likely that CRFs are involved in the regulation of other transcriptional responses independent of RRBs. As such CRFs could serve to coordinate such responses along with the transcriptional output from cytokinin signaling.

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Tables

Table 1. Most enriched clusters of biological process GO terms from cytokinin transcriptome datasets.

Clustering and enrichment analyses performed using DAVID Bioinformatic Resources Functional Annotation Clustering at high stringency.

Dataset	Representative Term from Top Cluster	% of genes in cluster	Enrichment Score
Rashotte et al., 2003 Induced	Response to Hormone Stimulus	24	9.71
Rashotte et al., 2003 Repressed	Response to Oxidative Stress	4.5	3.13
Brenner et al., 2005 Induced	Response to Hormone Stimulus	13	16.57
Brenner et al., 2005 Repressed	Response to Oxidative Stress	2.3	7.84

*All genes induced or repressed at any time-point ≥ 1 hour were combined

Table 2. Enrichment of molecular function GO terms related to alleviation of oxidative stress in rice and tomato

p-values of enrichment generated using DAVID Bioinformatic Resources for rice and Blast-2-Go software for tomato. Values not present were not below the threshold of $p < 0.05$.

Data set	Antioxidant Activity	Glutathione Transferase Activity	Peroxidase Activity
Rice Induced	-	2.00E-03	-
Rice Repressed	3.20E-06	-	4.20E-07
Tomato Induced	4.80E-02	-	2.40E-02
Tomato Repressed	4.20E-02	2.30E-02	-

*All genes induced or repressed at any time-point ≥ 1 hour in roots or shoots/leaves were combined (Gupta et al., 2013; Sato et al., 2013; Shi et al., 2013).

Figure Legends

Fig. 1. Graphical representation of possible effects of stress on cytokinin content. Studies measuring cytokinin concentrations and expression of cytokinin metabolism genes during and after various stress stimuli suggest that as a general stress response cytokinin production/accumulation is rapidly elevated. This initial induction is usually transient (< 3 hours), but may be sustained under certain conditions including severe stress.

Fig. 2. Diagram illustrating the two-component cytokinin signaling (TCS) pathway and the relationship of its components with various abiotic stress conditions. Large solid black arrows represent known TCS interactions; dashed black blocked arrow represents predicted negative regulation; solid grey arrow represents the interaction between AHK5 and AHPs that is predicted to involve downstream components. Within the boxes associated with each class of TCS components are the abiotic stress conditions regulated by that class, the + and – indicate a positive and negative role in tolerance respectively. CHK: Cytokinin Receptors, HK: Non-cytokinin binding histidine kinases, HPt: Histidine containing phosphotransfer proteins, RRB: Type-B response regulators, RRA: Type-A response regulators, RRC: Type-C response regulator.

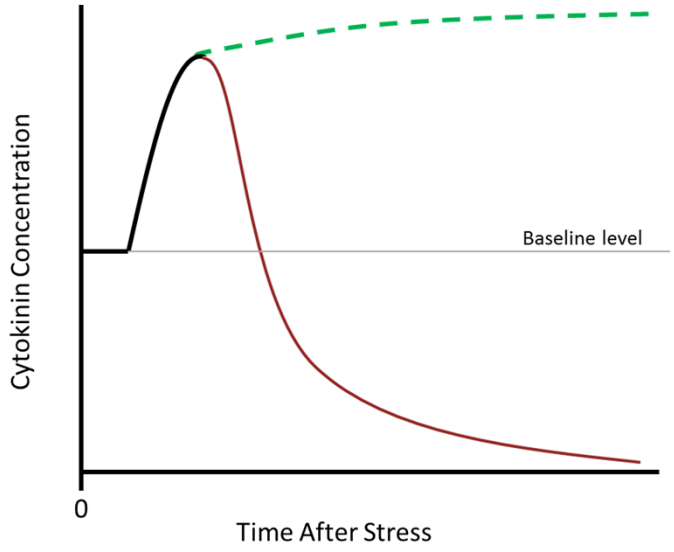

Figure 1

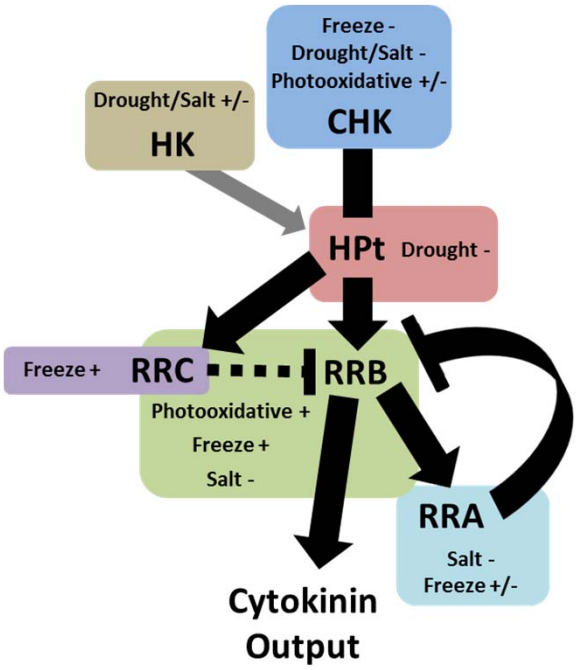

Figure 2

Chapter 6. Cytokinin Response Factor 6 Represses a Subset of Cytokinin Metabolism, Transport and Signaling Genes in Response to Oxidative Stress

6.1 Introduction

The environment in which a plant exists is often subject to frequent change. These changes can lead to physiological alterations and disruption of normal metabolism. In particular the energetic reactions which take place in chloroplasts, peroxisomes and mitochondria are susceptible to dysfunction resulting in production of excessive amounts of reactive oxygen species (ROS). In fact many common abiotic stress conditions encountered in agriculture including cold, drought, soil salinity and pollution are known to have an oxidative stress component. Cellular levels of ROS are carefully maintained at relatively low levels through a wide range of mechanisms of avoidance and detoxification. If the balance between ROS production and removal is shifted to far towards production (for example under stress conditions), cellular damage as a result of oxidation of macromolecules such as lipids, proteins and nucleic acids can occur(Mittler, 2002; Gill and Tuteja, 2010). Accumulation of ROS beyond some threshold triggers programmed cell death as a response. Therefore ROS are thought to serve as indicators of oxidative stress within a cell, but may also play a role in systemic stress signaling (Dat et al., 2000; Apel and Hirt, 2004; Suzuki et al, 2012).

Phytohormones also play important roles in stress responses signaling (O'Brien and Benkova, 2013). One such hormone, cytokinin is generally considered to be an antagonist of stress tolerance (Argueso et al., 2009; Ha et al., 2012). In Arabidopsis reduced cytokinin levels as a result of decreased synthesis or increased degradation has been found to increase tolerance to drought stress (Nishiyama, et al., 2011; Mackova et al., 2013). Compromised cytokinin signaling in mutants of various components of the cytokinin signaling pathway enhanced drought, salt and freeze tolerance (Tran et al., 2007; Jeon et al., 2010; Mason et al., 2010;). Each of these conditions are known to stimulate ROS production, however oxidative stress has not been directly examined in these mutants.

Cytokinin response factors (CRFs) are a subset of AP2/ERF transcription factors that function both downstream and as a side branch of the primary cytokinin signaling pathway (Rashotte et al., 2006; Cutcliffe et al, 2011). CRF6 from Arabidopsis is

transcriptionally induced by cytokinin and plays a role in delaying leaf senescence (Zwack et al., 2013). It is also induced in response to a wide range of stress stimuli, including oxidative stress in the form of H_2O_2 (Inze et al., 2011; Zwack et al., 2013). Additionally, CRF6 is a direct target of retrograde signaling in response to mitochondrial dysfunction (De Clercq et al., 2013; Ng et al., 2013). As such this gene has been proposed to integrate cytokinin and stress responses as part of a finely tuned response network (Zwack et al. 2013).

Here we demonstrate that CRF6 enhances oxidative stress tolerance. This is in part a result of its repression of a set of genes involved in cytokinin metabolism, transport and signaling. Thus we propose that CRF6 acts to attenuate cytokinin responses as part of an adaptive response to stress.

6.2 CRF6 partially mediates transcriptional response to H_2O_2

We hypothesized that as a transcription factor, CRF6 may be regulating the expression of stress responsive genes. To test this, we examined global transcriptome changes in response to H_2O_2 in WT and *crf6* mutant plants. After a six hour treatment, there were 606 and 351 transcripts induced and repressed respectively in WT. As expected, many of these genes were associated with stress responses as indicated by enriched GO terms **Sup Fig. 1**. Examination of the expression of these genes under identical conditions in the *crf6* mutant background revealed that a subset were regulated in a CRF6-dependent manner (>50% decrease in magnitude of fold change as compared to WT, see Materials and Methods for further details) **Fig 1 A**. Among the induced genes, 49 (8%) were CRF6 dependent (putative positive targets) **Sup table 1**. Strikingly, 28% (98 of 351) of the genes repressed in WT showed CRF6 dependence (putative negative targets; **Sup table 2**), possibly suggesting that CRF6 plays a role in repressing the expression of genes in response to oxidative stress.

Although the DNA binding specificity of CRF6 has not been demonstrated other CRF proteins have been shown to interact with both DRE ([A/G]CCGAC) and GCC (GCCGCC) motifs. The 2kb upstream promoters of these genes were examined for the presence of these motifs. 27 of the 49 putative positive targets and 63 of the 98 putative

negative targets contained at least one DRE or GCC element. Therefore, regulation of many of these genes by CRF6 could be mediated by direct DNA interactions.

Because CRF6 is induced by H_2O_2 we investigated whether the constitutively elevated levels of CRF6 in CRF6oe plants was sufficient to regulate the putative target genes we had identified. To this end transcriptomes of untreated WT and CRF6oe plants were compared. Examination of putative positive targets indicated that 16 were expressed significantly more in the CRF6oe background. Similarly, 41 putative negative targets were significantly lower. Thus for these genes CRF6 was necessary for normal H_2O_2 response and elevated levels of CRF6 independent of H_2O_2 stimulus was sufficient for a similar response. Those putative targets for which CRF6 overexpression was not sufficient may require other context specific factors for proper regulation.

CRF6 expression is induced by cytokinin in addition to H_2O_2. A similar approach was taken to identify putative targets of CRF6 in response to cytokinin. Transcriptome changes were examined in plants treated with cytokinin (2 µM Benzyl Adenine, BA) under otherwise identical conditions to H_2O_2 treatments. There is a relatively large set of transcriptome analyses of cytokinin response and our data were largely in keeping with established trends; however our six hour time-point is highly under-represented and may offer greater resolution of the dynamics of cytokinin response.

We investigated whether there was any overlap of the transcriptional changes resulting from treatment with cytokinin and H_2O_2. Comparison of differentially expressed genes from the two treatments in the WT background revealed little overlap with the exception of those transcripts repressed under both conditions **Fig 1 B**. In this case, of the 351 and 147 genes down-regulated by cytokinin and H_2O_2 respectively, 51 were found in both sets representing 15% of the H_2O_2 and 35% of the cytokinin repressed genes **Sup tables 3**. Moreover, the cytokinin repressed (but not induced) genes showed significant enrichment of the GO terms “response to stress” and “response to abiotic or biotic stimulus”. Although circumstantial, these findings may suggest common targets of repression in these two responses.

Identification of putative CRF6 targets in response to cytokinin was carried out as for H_2O_2 **Fig 1 A**. Only seven of the 125 (6%) transcripts induced by cytokinin were found to be CRF6 dependent and none of these were elevated in the overexpression

background. In contrast, 41 of the 147 (28%) cytokinin repressed showed CRF6 dependence; 16 also had decreased expression in CRF6oe plants and 21 have DRE/GCC promoter elements.

6.3 CRF6 Represses Cytokinin Related Genes in Response to Oxidative Stress

We sought to determine if H_2O_2 exposure affected the expression of genes involved in various aspects of cytokinin physiology. Of 74 genes encoding proteins involved in cytokinin metabolism transport and signaling, seven were found to be H_2O_2 regulated in our experiment. One, LOG5 (involved in cytokinin activation) was induced (2.7 fold); the other six were repressed by H_2O_2. Significantly, all six repressed genes were among the putative negative targets of CRF6 and all but one (AHP1) had lower expression in CRF6 overexpressing plants **Table 1**.

6.4 Materials and Methods

Plant Material and Growth Conditions

Plants of each genotype were sterilized and sown on plates containing full strength MS salts and 1% sucrose buffered by MES at pH 5.7. After 2 days at 4°C, plates were moved to a controlled environmental chamber and grown under diurnal conditions of 16 h light (100 μE) at 22°C, and 8 h dark at 18°C.

Treatments and Expression Analyses

Ten day old plants were removed from plates and floated on 3mM MES buffer pH 5.7. After 1h, 6-benzyl adenine or hydrogen peroxide were added to a final concentration of 2μM and 20mM, respectively. Each solution also contained 0.2% DMSO. Plants were treated for 6h, then immediately frozen in liquid nitrogen. For each treatment approximately 10 individual plants were pooled for analysis.

RNA from 2 independent experiments was isolated using the Qiagen RNeasy Plant Mini-kit according to the manufacturer's instructions. Hybridization to Affymetrix Arabidopsis Gene 1.0 ST arrays, scanning and raw data pre-processing were performed

as a service by the Heflin Center for Genomic Science at the University of Alabama-Birmingham.

Normalization and differential expression analyses were performed using the FlexArray 1.6 software package from McGill University and Genome Quebec. Normalization was performed using algorithms provided by Affymetrix. Fold change in expression was calculated by Cyber-T analysis and adjusted p-values were determined using the Benjamini Hochberg method of False Discovery Rate.

qRT-PCR verification was carried out using Sybr-Green and sequence specific primers. Reactions were carried out as previously described (Zwack et al., 2013).

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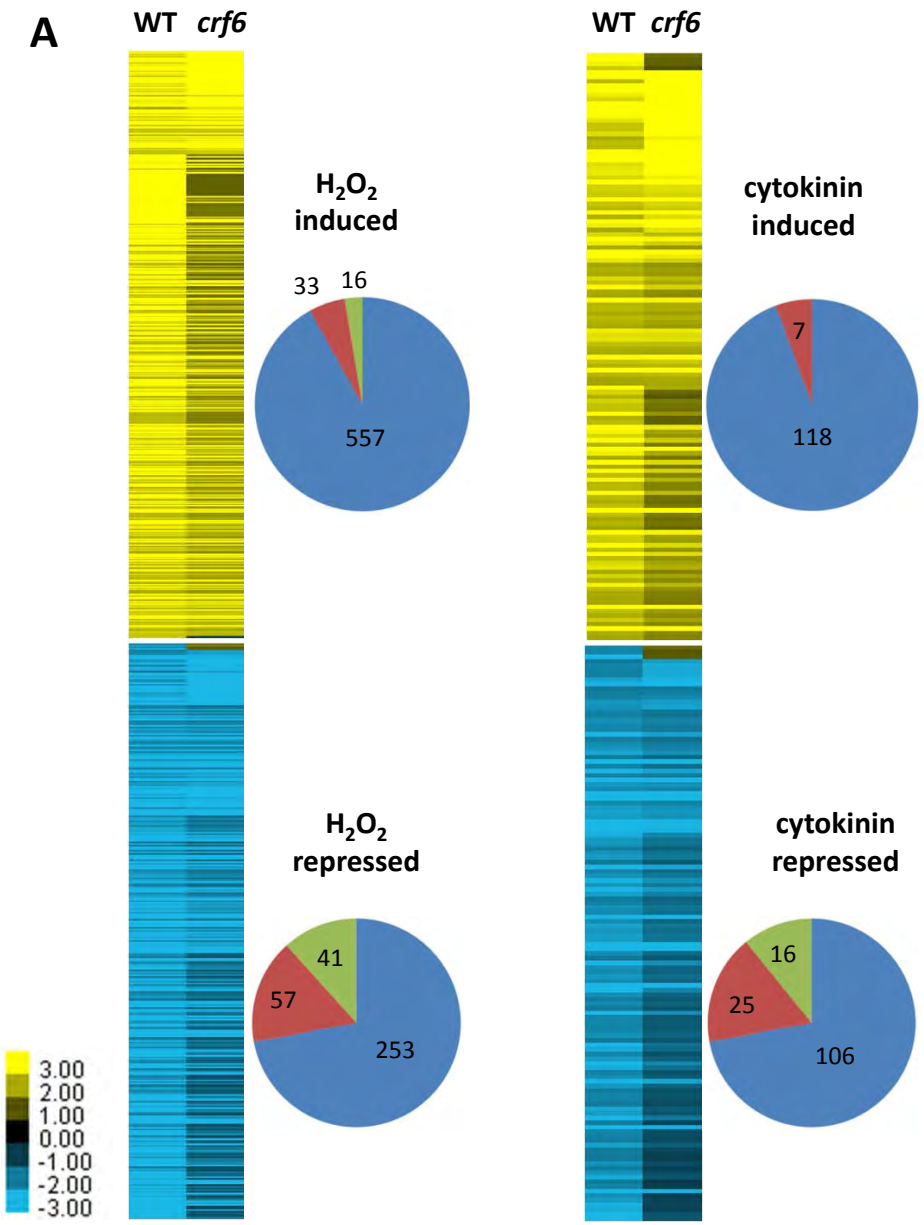
	Fold Change H_2O_2		CRF6oe/WT
	WT	crf6	
AHP1	**-2.61**	-1.52	**1.85**
ABCG14	**-3.76**	**-2.28**	**-2.54**
ARR11	**-2.78**	-1.51	**-1.91**
ARR9	**-2.24**	-1.29	**-3.57**
LOG7	**-2.06**	-1.35	**-2.72**
ARR6	**-2.59**	-1.37	**-2.77**

Table 1. Fold change of cytokinin related genes in response to H_2O_2 in WT and mutant backgrounds and fold change in CRF6oe compared to WT. Values in bold font have a corresponding adjusted p-value < 0.05.



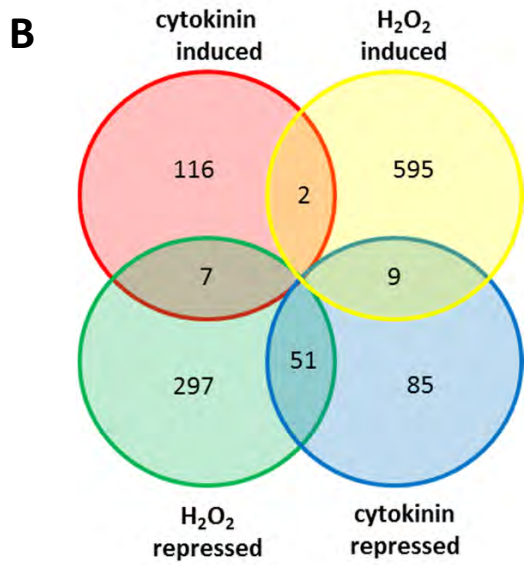


Figure1. A. Heat-maps comparing WT and crf6 fold change of genes differentially expressed in WT. Pie charts show numbers of genes in each comparison that are CRF6 independent (blue), dependent (red) and CRF6 dependent as well as similarly changed in CRF6oe/WT comparison. **B.** Venn diagram of differentially expressed genes in WT from the two treatments.

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Appendix 2

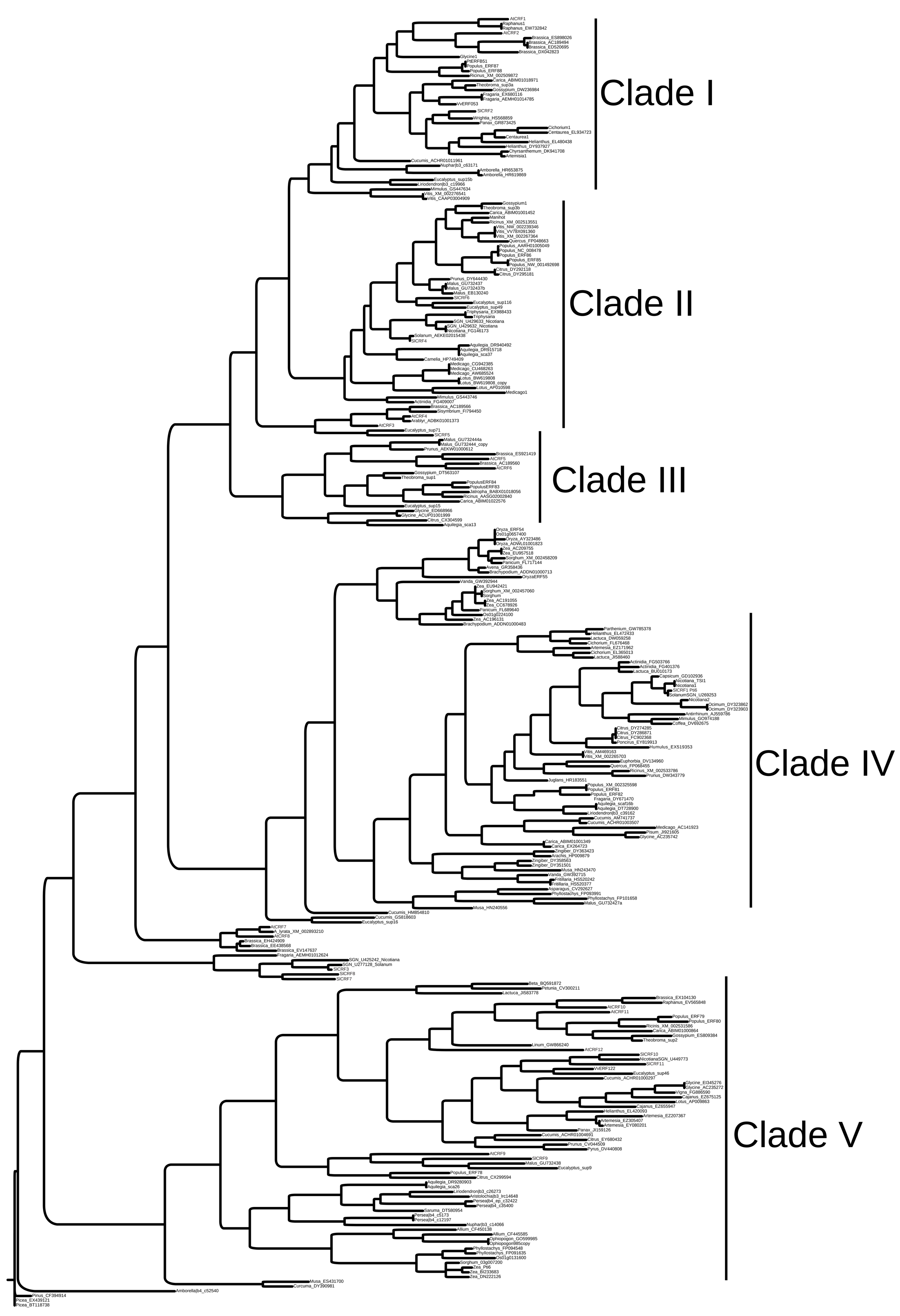
Supplemental Materials

Supplemental Materials:

Chapter 2

	Areole Number	Density of Free Ending Veins (mm^{-2})
WT Col	91.29 (+/-) 12.21	0.507 (+/-) 0.054
crf1 (I)	*73.47 (+/-) 2.734	0.364 (+/-) 0.014
crf2 (I)	*62.07 (+/-) 3.884	0.575 (+/-) 0.050
crf3 (II)	88.75 (+/-) 9.597	0.563 (+/-) 0.041
crf5 (III)	125.1 (+/-) 11.15	*0.889 (+/-) 0.040
crf6 (III)	100.9 (+/-) 4.348	*0.643 (+/-) 0.053
crf9 (V)	107.2 (+/-) 8.420	*0.755 (+/-) 0.062
crf2,crf5 (I,III)	92.25 (+/-) 6.452	0.618 (+/-) 0.029

Table S1 Preliminary vein patterning data. Mean values with standard errors. Leaves were treated as with those used in extensive analysis; however the entire leaf was measured. Sample sizes range from 15 to 30 per genotype.


Clade I
Clade II
Clade III
Clade IV
Clade V
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Raphanus1
Raphanus_EW732842
AtCRF2
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Brassica_AC189494
Brassica_ED520695
Brassica_DX042823
Glycine1
PtERFB51
Populus_ERF87
Populus_ERF88
Ricinus_XM_002509872
Carica_ABIM01018971
Theobroma_sup3a
Gossypium_DW236984
Fragaria_EX680116
Fragaria_AEMH01014785
VvERF053
SlCRF2
Wrightia_HS568859
Panax_GR873425
Cichorium1
Centaurea_EL934723
Centaurea1
Helianthus_EL480438
Helianthus_DY937927
Chrysanthemum_DK941708
Artemisia1
Cucumis_ACHR01011961
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Amborella_HR619869
Eucalyptus_sup15b
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Vitis_XM_002276541
Vitis_CAAP03004909
Gossypium1
Theobroma_sup3b
Carica_ABIM01001452
Manihot
Ricinus_XM_002513551
Vitis_NW_002239346
Vitis_VV78X091360
Vitis_XM_002267364
Quercus_FP048663
Populus_AARH01005049
Populus_NC_008478
Populus_ERF86
Populus_ERF85
Populus_NW_001492698
Citrus_DY292118
Citrus_DY295181
Prunus_DY644430
Malus_GU732437
Malus_GU732437b
Malus_EB130240
SlCRF6
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Eucalyptus_sup49
Triphysaria_EX988433
Triphysaria
SGN_U429633_Nicotiana
SGN_U429632_Nicotiana
Nicotiana_FG146173
Solanum_AEKE02015438
SlCRF4
Aquilegia_DR940492
Aquilegia_DR915718
Aquilegia_sca37
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Medicago_CG942385
Medicago_CU468263
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Medicago1
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Brassica_AC189566
Sisymbrium_FI794450
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AtCRF3
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Helianthus_EL472433
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Cichorium_FL676468
Artemesia_EZ171962
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Lactuca_JI588460
Actinidia_FG503766
Actinidia_FG401376
Lactuca_BU010173
Capsicum_GD102936
Nicotiana_TSI1
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SlCRF1 Pti6
SolanumSGN_U269253
Nicotiana2
Ocimum_DY323862
Ocimum_DY323903
Antirrhinum_AJ559786
Mimulus_GO974188
Coffea_DV692675
Citrus_DY274285
Citrus_DY286871
Citrus_FC902368
Poncirus_EY819913
Humulus_EX519353
Vitis_AM469163
Vitis_XM_002265703
Euphorbia_DV134960
Quercus_FP068455
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Fragaria_DY671470
Aquilegia_scaf16b
Aquilegia_DT728900
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Glycine_AC235742
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Musa_HN243470
Vanda_GW392715
Fritillaria_HS520242
Fritillaria_HS520377
Asparagus_CV292627
Phyllostachys_FP093991
Phyllostachys_FP101658
Malus_GU732427a
Musa_HN240556
Cucumis_HM854810
Cucumis_GS818603
Eucalyptus_sup16
AtCRF7
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Fragaria_AEMH01012624
SGN_U425242_Nicotiana
SGN_U277128_Solanum
SlCRF3
SlCRF8
SlCRF7
Beta_BQ591872
Petunia_CV300211
Lactuca_JI583778
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Linum_GW866240
AtCRF12
SlCRF10
NicotianaSGN_U449773
SlCRF11
VvERF122
Eucalyptus_sup46
Cucumis_ACHR01000297
Glycine_EI345276
Glycine_AC235272
Vigna_FG886590
Cajanus_EZ675125
Lotus_AP009863
Cajanus_EZ655947
Helianthus_EL420093
Artemesia_EZ207367
Artemesia_EZ305407
Artemesia_EY080201
Panax_JI159126
Cucumis_ACHR01004691
Citrus_EY680432
Prunus_CV044509
Pyrus_DV440808
AtCRF9
SlCRF9
Malus_GU732438
Eucalyptus_sup9
Populus_ERF78
Citrus_CX299594
Aquilegia_DR9280903
Aquilegia_sca26
Liriodendron|b3_c26273
Aristolochia|b3_lrc14648
Persea|b4_ep_c32422
Persea|b4_c35400
Saruma_DT580954
Persea|b4_c5173
Persea|b4_c12197
Nuphar|b3_c14066
Allium_CF450138
Allium_CF445585
Ophiopogon_GO599985
Ophiopogon985copy
Phyllostachys_FP094548
Phyllostachys_FP091635
Os01g0131600
Sorghum_03g007200
Zea_Pti6
Zea_BI233683
Zea_DN222126
Musa_ES431700
Curcuma_DY390981
Amborella|b4_c52540
Pinus_CF394914
Picea_EX439121
Picea_BT118738

Fig S1 Neighbor joining tree of CRF proteins based on conserved CRF and AP2 domains from 241 sequences identified by genus name and numerical identifiers from respective databases. The clustered sequences corresponding to the different clades illustrate the similarity of proteins within each clade even in these evolutionarily maintained domains. The position of most clades relative to one another is not strongly supported by bootstrap analysis (see Fig S2), but can be inferred by shared characters within C-terminal alignments shown in (Figure 1).

```
Bootstrap method with neighbor-joining search:
  Number of bootstrap replicates = 1000
  Starting seed = 2142912777
  Ties (if encountered) will be broken randomly
  Distance measure = mean character difference
  (Tree is unrooted)

   Note: Ties were encountered in one or more replicates

   Time used for neighbor-joining bootstrap = 00:03:16 (CPU time = 00:03:16.1)

Bootstrap 50% majority-rule consensus tree

/------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------ Picea BT118738(273)
|                                                                                                                                                                            /------------------------------------------------- Brassica ES898026(1)
|                                                                                                                                                   /-----------89-----------+                        /------------------------ Brassica AC189494(6)
|                                                                                                                          /----------100-----------+                        \----------63------------+------------------------ Brassica ED520695(8)
|                                                                                                 /----------89------------+                        \-------------------------------------------------------------------------- Brassica DX042823(7)
|                                                                                                 |                        \--------------------------------------------------------------------------------------------------- AtCRF2(5)
|                                                                         /-----------86----------+                                                                          /------------------------------------------------- AtCRF1(2)
|                                                                         |                       \------------------------------------83------------------------------------+                        /------------------------ Raphanus1(3)
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|                                                                         |                                                                                                  /------------------------------------------------- Carica ABIM01018971(9)
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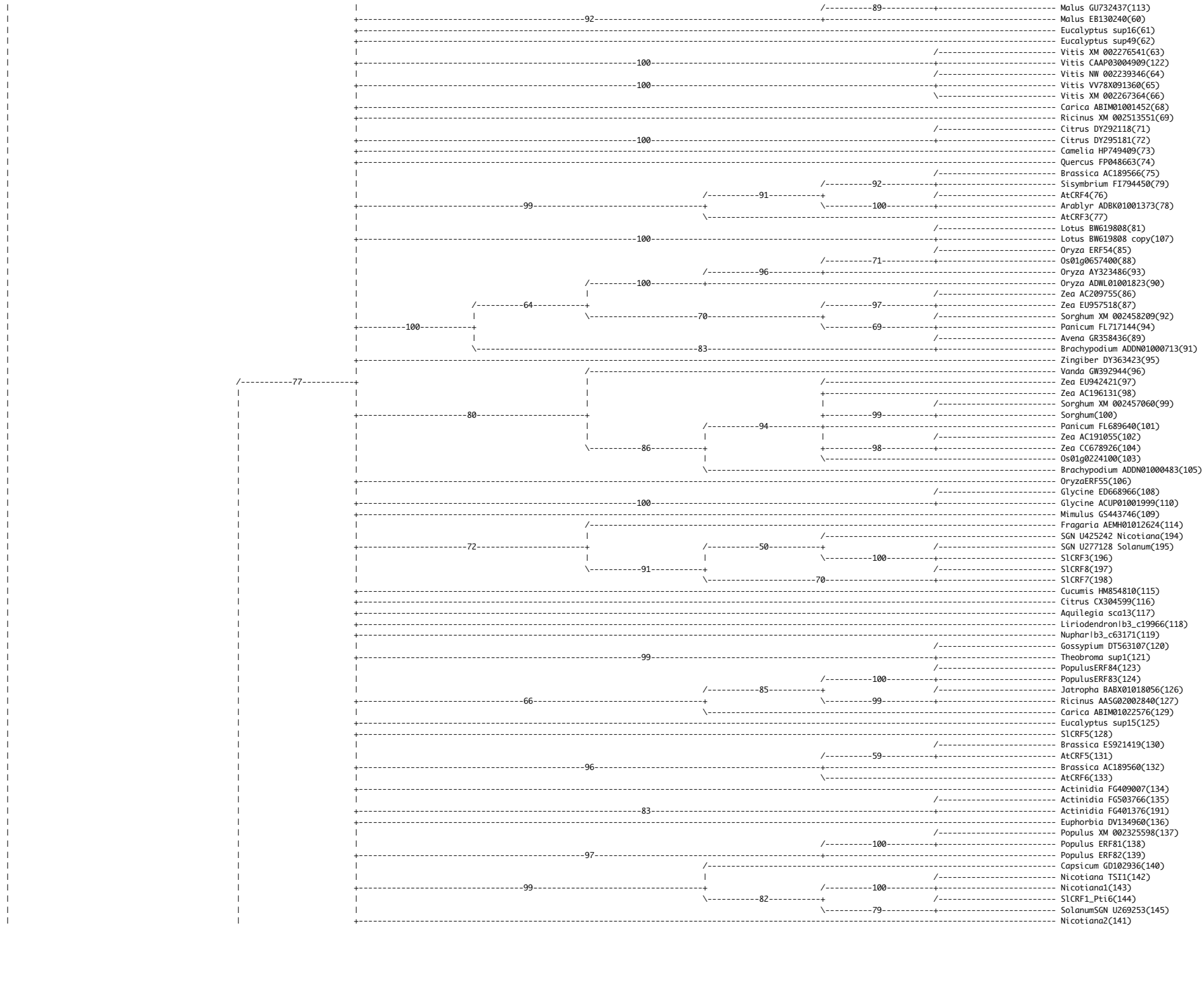
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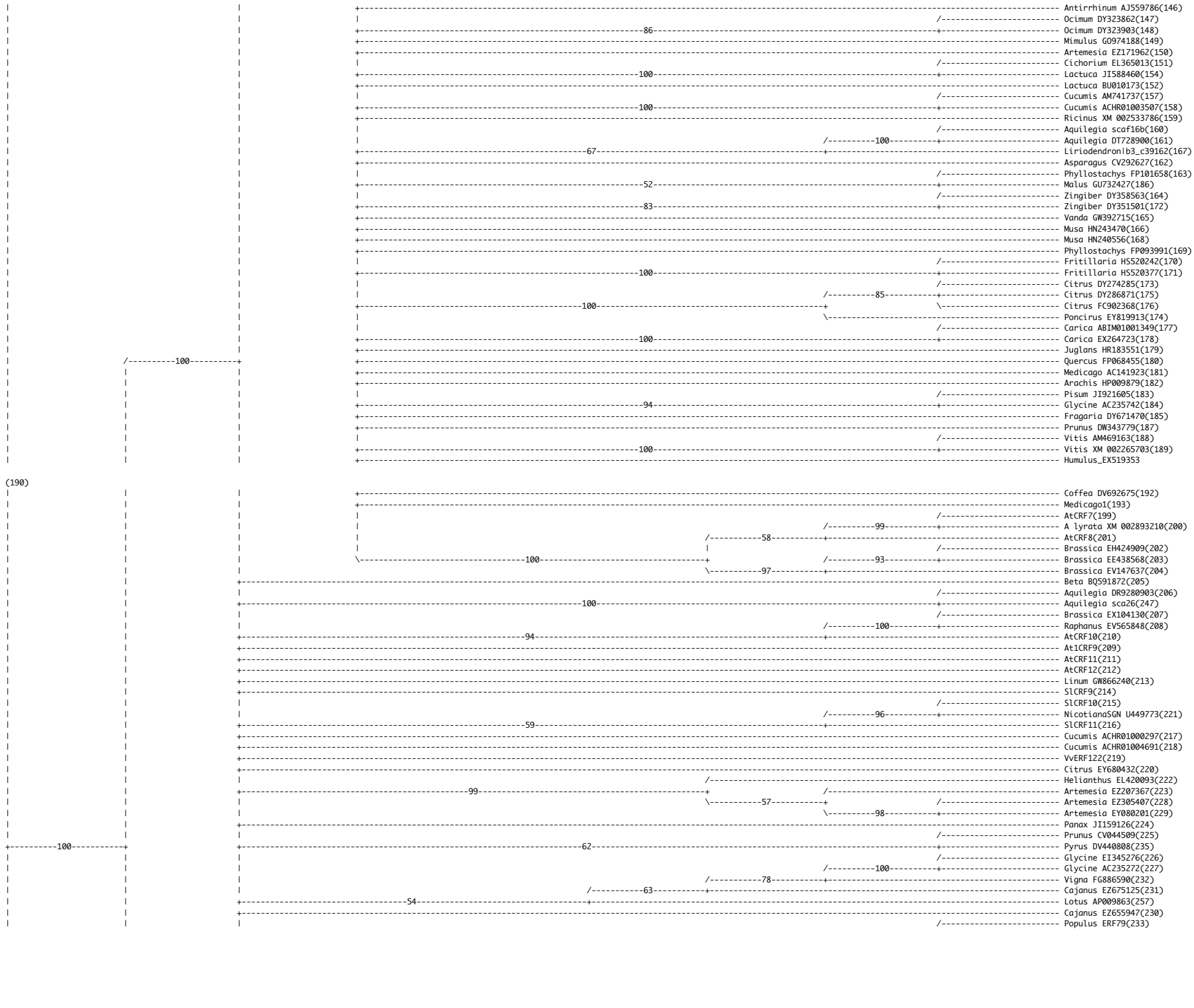
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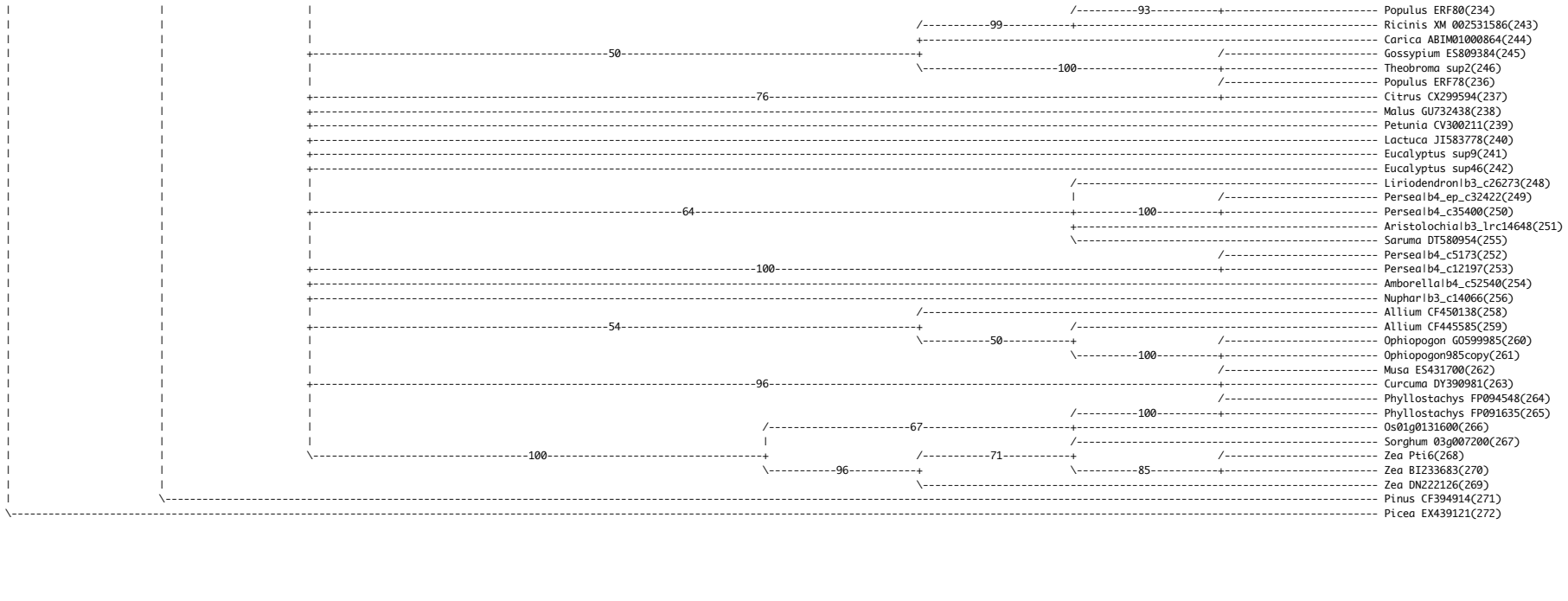
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Fig S2 Bootstrap analysis of CRF protein neighbor joining tree in Fig S1. Consensus tree of sequences used in Figure S1 with bootstrap values greater that 50% shown. Nodes with less than 50% support have been collapsed. Bootstrap analyses were performed using 1000 pseuodreplicates and 10 random addition replicates with TBR branch swapping within each bootstrap replicate.

Fig S3 Maximum likelihood tree of sequences used in Figure 1. Bootstrap values of nodes based on analyses using 1000 pseuodreplicates and 10 random addition replicates with TBR branch swapping within each bootstrap replicate. Clustered sequences labeled as to which clade they belong.

Fig S4 Hierarchical Cluster Analysis of Arabidopsis and Populus CRFs. Arabidopsis and Populus clade specific CRF genes were examined by hierarchical clustering analysis on publically available microarrays with clade designation indicated. (a) For Arabidopsis all ATH1 microarrays experiments available at Genevestigator were examined using default settings, and a Pearson Correlation, here shown are Heatmaps from those analyses from perturbations (1473) and anatomical/development (74) array data sets. (b) For Populus all 384 microarray experiments available at the Populus Genome Integrative Explorer: PopGenIE v2.0 were displayed by ExCluster-Heatmap with the default settings of a Euclidean Distance function and Ward Hierarchical function.

Supplemental Materials:

Chapter 4

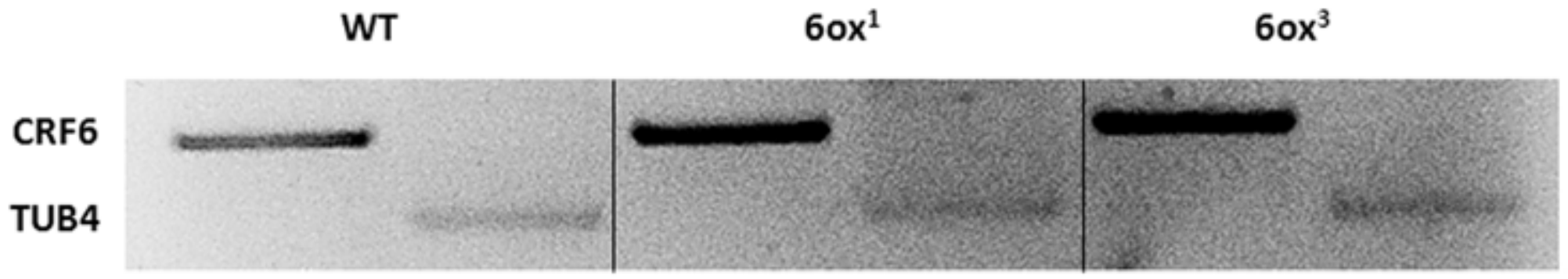

Fig. S1 Electrophoresis gel showing the products of RT PCR amplification of CRF6 in WT and overexpression backgrounds. RNA was extracted and pooled from rosettes of 3 plants per genotype at 14 DAG. The housekeeping gene β-tubulin 4 was also amplified as an input control. Products shown are from 28 PCR cycles. Image has been black and white inverted for clarity.

Supplementary Figure S2. Hierarchical Cluster Analysis of CRF and ARR genes. Arabidopsis CRF and ARR genes were examined by hierarchical clustering analysis of publically available microarray data. For both clusters all ATH1 perturbation microarray experiments in leaf tissues available at Genevestigator were examined using default settings. The first clustering is of "cytokinin regulated transcription factors" which includes type-B ARRs 1, 2, 10, 11, and 12 along with the CRFs 1, 2, 5, and 6 which are induced by cytokinin. The second clustering includes all type-A and B ARRs and All CRFs available on the ATH1 chip. In both analyses CRF6 expression patterns are distinct from all other genes.

Supplemental Materials:

Chapter 6

AGI	Gene Symbol	Gene Name	Fold change Col	Adjusted p-value Col	Fold change crf6	Adjusted p-value crf6	Fold change 6oe	Adjusted p-value	Fold change crf6	Adjusted p-value
At1g03070	---	Bax inhibitor-1 family protein	4.087	0.000614	2.021	0.125643	0.745159	0.283161	0.969105	0.994996
At1g05575	---	---	4.502	0.001429	1.749	0.269116	2.98596	5.12E-05	0.914665	0.97213
At1g05675	---	UDP-Glycosyltransferase superfamily protein	13.079	1.48E-05	6.071	1.17E-05	1.569696	0.232403	0.628707	0.531569
At1g05680	UGT74E2	Uridine diphosphate glycosyltransferase 74E2	8.847	2.00E-06	3.200	0.002694	1.188202	0.45074	1.124361	0.934461
At1g07160	---	Protein phosphatase 2C family protein	4.213	0.002312	1.529	0.447808	1.394028	0.156764	1.226553	0.747377
At1g09500	---	NAD(P)-binding Rossmann-fold superfamily protein	6.669	0.000918	2.400	0.096716	2.79792	0.010122	0.858601	0.955252
At1g17170	GSTU24	glutathione S-transferase TAU 24	27.295	1.67E-09	13.400	4.67E-08	2.233653	0.008677	1.112427	0.963099
At1g28480	GRX480	Glutaredoxin family	3.828	0.005106	1.810	0.109141	2.049639	0.01009	1.161742	0.918619
At1g54050	---	HSP20-like chaperones superfamily protein	8.376	2.40E-07	3.164	0.000245	0.641511	0.11875	1.199198	0.88313
At1g56240	PP2-B13	phloem protein 2-B13	15.291	8.49E-07	6.383	2.84E-06	1.433906	0.126194	0.908762	0.943674
At1g59860	---	HSP20-like chaperones superfamily protein	5.623	7.64E-05	2.708	0.001656	2.297134	0.002947	0.95089	0.986676
At1g64950	CYP89A5	cytochrome P450, family 89, subfamily A, polypeptide 5	5.910	9.19E-05	1.694	0.315712	1.753582	0.064667	1.229754	0.895368
At1g65490	---	---	4.771	0.00053	2.179	0.083004	2.790986	0.034586	0.754342	0.813278
At1g65790	RK1	receptor kinase 1	4.058	0.000711	1.174	0.867683	0.572061	0.144183	1.105256	0.964688
At1g66570	SUC7	sucrose-proton symporter 7	9.844	2.23E-05	3.323	0.036745	0.745213	0.353946	0.962551	0.988659
At1g71520	---	Integrase-type DNA-binding superfamily protein	11.644	2.68E-07	4.371	1.10E-05	3.499309	0.000474	0.909806	0.945899
At1g73120	---	---	2.464	0.006099	1.132	0.921862	2.115781	0.001037	1.079123	0.971878
At1g76600	---	---	8.184	2.77E-06	4.021	5.62E-05	1.347813	0.337194	0.768912	0.690988
At2g02930	GSTF3	glutathione S-transferase F3	3.351	0.01339	1.432	0.632801	4.420976	0.00018	1.132154	0.962977
At2g18660	PNP-A	plant natriuretic peptide A	7.031	6.59E-05	2.475	0.117142	0.902798	0.787152	1.863649	0.523989
At2g19310	---	HSP20-like chaperones superfamily protein	2.326	0.018839	1.137	0.80123	3.619311	1.83E-05	1.997748	0.039703
At2g20560	---	DNAJ heat shock family protein	3.599	0.006777	1.684	0.190636	1.401625	0.268885	1.047235	0.986351
At2g25510	---	---	4.001	2.87E-05	1.794	0.250035	2.490119	6.63E-05	2.389684	0.043263
At2g31945	---	---	4.308	0.016834	2.073	0.111781	3.287019	5.53E-05	0.775538	0.786758
At2g42530	COR15B	cold regulated 15b	2.872	0.033637	1.395	0.068141	0.222024	0.000198	3.553313	0.006258
At2g42540	COR15A	cold-regulated 15a	5.023	4.74E-06	1.738	0.000741	0.529624	0.002381	4.22964	2.48E-07
At3g16030	CES101	lectin protein kinase family protein	2.376	0.011976	0.889	0.930823	0.461466	0.002221	1.201424	0.862305
At3g16050	PDX1.2	pyridoxine biosynthesis 1.2	2.805	0.001067	1.319	0.349888	1.279101	0.267407	1.162636	0.82232
At3g25010	RLP41	receptor like protein 41	2.583	0.002212	1.285	0.874394	0.508736	0.08861	1.31911	0.900542
At3g46080	---	C2H2-type zinc finger family protein	5.347	0.000511	2.666	0.000689	3.178597	0.000347	0.924458	0.973011
At3g47090	---	Leucine-rich repeat protein kinase family protein	3.175	0.006022	1.556	0.529003	1.117574	0.739698	1.142429	0.908146
At3g56710	SIB1	sigma factor binding protein 1	6.166	0.003203	2.651	0.002748	1.282537	0.558818	1.146559	0.953794
At4g08555	---	---	8.309	0.000107	2.763	0.001425	1.201732	0.547516	0.817112	0.676432
At4g12400	HOP3	stress-inducible protein, putative	10.056	1.17E-05	4.944	2.96E-05	1.659899	0.163413	1.110558	0.97199
At4g14400	ACD6	ankyrin repeat family protein	4.841	2.60E-06	2.131	0.046115	0.38226	0.026489	2.700815	0.015467
At4g34131	UGT73B3	UDP-glucosyl transferase 73B3	6.369	9.35E-05	2.707	0.002293	1.740691	0.142685	1.298958	0.789332
At5g12020	HSP17.6II	17.6 kDa class II heat shock protein	43.879	1.22E-08	14.813	5.01E-09	1.409804	0.37696	1.192736	0.911474
At5g22140	---	FAD	14.377	1.53E-07	5.077	1.75E-06	0.746503	0.456183	1.156812	0.919569
At5g24110	WRKY30	WRKY DNA-binding protein 30	4.709	0.001709	2.265	0.029944	1.422743	0.359308	0.954916	0.98892
At5g24150	SQP1	Squalene monooxygenase 1	3.327	0.001278	1.487	0.539497	2.144037	0.012205	1.002787	0.999186
At5g26170	WRKY50	WRKY DNA-binding protein 50	2.445	0.044762	0.981	0.995677	1.276838	0.540037	1.791024	0.374908
At5g37260	RVE2	Homeodomain-like superfamily protein	2.459	0.028522	0.853	0.869761	6.875764	2.77E-07	3.759967	0.004782
At5g37670	---	HSP20-like chaperones superfamily protein	3.936	0.0024	1.291	0.783856	0.840442	0.61076	1.379093	0.722411
At5g39090	---	HXXXD-type acyl-transferase family protein	3.217	0.00212	1.306	0.675673	1.114043	0.798373	0.978657	0.994996
At5g48850	ATSDI1	Tetratricopeptide repeat (TPR)-like superfamily protein	2.844	0.002406	1.054	0.989779	0.496447	0.016463	5.181519	0.000129
At5g54165	---	---	25.096	3.84E-08	7.748	4.31E-07	19.86539	9.75E-10	1.36924	0.731665
At5g54610	ANK	ankyrin	2.786	0.020643	1.149	0.957144	0.8377	0.604483	1.602012	0.693988
At5g59310	LTP4	lipid transfer protein 4	5.808	0.002223	1.041	0.992717	2.102455	0.064062	1.825871	0.499735
At5g59320	LTP3	lipid transfer protein 3	9.812	1.49E-07	1.644	0.409198	1.28915	0.504588	1.717964	0.411951

Supplemental Table 1. H_2O_2 Induced, CRF6 dependent genes

AGI	Gene Symbol	Gene Name	Fold change Col	Adjusted p-value Col	Fold change crf6	Adjusted p-value crf6	Fold change 6oe	Adjusted p-value	Fold change crf6	Adjusted p-value
At1g02810	---	Plant invertase	0.441578	0.046409	0.670486	0.177772	0.8349085	0.5466505	0.8994344	0.9257
At1g12040	LRX1	leucine-rich repeat	0.211628	6.59E-05	0.480203	0.037065	2.69541	0.000113084	0.7306463	0.5873
At1g13480	DUF1262	Protein of unknown function (DUF1262)	0.226882	0.00028	0.601931	0.254036	1.828309	0.004471545	0.620589	0.2622
At1g18860	WRKY61	WRKY DNA-binding protein 61	0.184839	2.42E-05	0.299275	6.96E-05	1.015475	0.9580303	0.797408	0.5384
At1g20900	ESC	Predicted AT-hook DNA-binding family protein	0.484922	0.033229	0.775213	0.566536	1.159168	0.6133178	0.6467836	0.113
At1g26250	---	Proline-rich extensin-like family protein	0.102532	1.36E-07	0.219961	3.24E-06	1.544291	0.04538846	1.804576	0.0488
At1g33750	---	Terpenoid cyclases	0.342118	0.018269	0.577173	0.394614	0.6273533	1.63E-01	0.8114871	0.9035
At1g44160	---	HSP40	0.431574	0.040388	0.648294	0.440378	0.6109093	0.0957137	0.8364986	0.902
At1g51850	---	Leucine-rich repeat protein kinase family protein	0.080043	2.81E-05	0.129188	1.79E-06	0.6639999	0.142795	0.6348026	0.4862
At1g60960	IRT3	iron regulated transporter 3	0.388766	0.010548	0.612561	0.159005	0.7506774	0.1691606	0.948211	0.972
At1g73300	scpl2	serine carboxypeptidase-like 2	0.255773	0.001286	0.526775	0.417253	1.341033	0.3657201	0.3673711	0.08
At1g80240	DGR1	Protein of unknown function, DUF642	0.276552	0.001278	0.474957	0.001257	0.9959257	0.9919067	0.8391089	0.7846
At2g05510	---	Glycine-rich protein family	0.422936	0.01006	0.718545	0.09317	1.152678	5.19E-01	0.9922393	0.9992
At2g18370	---	Bifunctional inhibitor	0.445785	0.007074	0.753182	0.268221	0.9702578	0.9150813	0.9965783	0.9992
At2g19190	FRK1	FLG22-induced receptor-like kinase 1	0.321869	0.031979	0.601351	0.383704	0.5142404	0.03283318	0.3926117	0.081
At2g30840	---	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily	0.265834	0.000833	0.565116	0.104695	0.6518198	0.1188472	0.726032	0.4579
At2g38940	PHT1;4	phosphate transporter 1;4	0.287895	0.012516	0.472215	0.077865	0.9235199	0.8559113	0.7071705	0.7669
At2g42060	---	Cysteine	0.308532	0.005001	0.487955	0.098507	0.5326446	0.06000841	0.6172963	0.4922
At2g46740	GOLLU5	D-arabinono-1,4-lactone oxidase family protein	0.23428	0.000512	0.415196	0.006517	2.267252	0.006553709	0.8266938	0.7708
At2g46750	GULLO2	D-arabinono-1,4-lactone oxidase family protein	0.176999	1.44E-05	0.37878	0.000918	2.144647	0.001060559	0.7019937	0.2465
At2g47540	---	Pollen Ole e 1 allergen and extensin family protein	0.326007	0.034135	0.50242	0.073038	0.5931833	0.1317369	0.8176328	0.902
At2g47550	---	Plant invertase	0.499861	0.022094	0.922736	0.936734	1.133324	0.6098896	0.6851539	0.2478
At3g01420	DOX1	Peroxidase superfamily protein	0.097591	3.61E-07	0.168722	1.79E-09	1.187021	0.4632241	0.9929239	0.9992
At3g12977	---	NAC (No Apical Meristem) domain transcriptional regulator superfamily	0.321663	0.003375	0.544551	0.106145	1.147994	0.7177352	0.8752093	0.9408
At3g13403	---	Defensin-like (DEFL) family protein	0.31387	0.003343	0.774343	0.807947	0.7791781	0.3972265	0.5912458	0.3043
At3g13760	---	Cysteine	0.299778	0.009689	0.508354	0.007834	0.6840957	0.1941138	0.7823223	0.6855
At3g16770	EBP	ethylene-responsive element binding protein	0.41285	0.004758	0.74856	0.42847	2.565331	5.38E-05	0.8240507	0.7368
At3g21351	---	---	0.356267	0.004758	0.616272	0.413347	2.781783	2.75E-05	0.5634775	0.2067
At3g21510	AHP1	histidine-containing phosphotransmitter 1	0.383179	0.011158	0.657528	0.207698	1.846201	0.005505223	7.69E-01	0.5538
At3g22800	---	Leucine-rich repeat (LRR) family protein	0.34746	0.039238	0.730393	0.56925	1.452552	0.1505651	0.7221323	0.584
At3g23125	---	MIR173; miRNA	0.478934	0.049518	1.14254	0.938685	1.03967	0.9374664	0.8130241	0.9074
At3g28550	---	Proline-rich extensin-like family protein	0.10792	0.000109	0.162075	1.41E-07	5.084056	8.57E-06	1.074474	0.9771
At3g55090	ABCG16	ABC-2 type transporter family protein	0.352622	0.00668	0.676527	0.677186	0.6028016	0.07806669	0.6750705	0.5947
At4g11650	OSM34	osmotin 34	0.15065	2.04E-06	0.234222	3.57E-08	3.61906	5.74E-07	1.454154	0.1304
At4g12090	---	Cornichon family protein	0.455408	0.013273	0.683671	0.274267	1.760378	0.005001348	0.7283531	0.3895
At4g12470	AZI1	azelaic acid induced 1	0.196618	0.001015	0.652792	0.486316	1.313713	4.81E-01	0.576483	0.565
At4g12480	EARLI1	Bifunctional inhibitor	0.287914	0.000883	0.881499	0.928204	4.537649	1.05E-06	0.5236573	0.2426
At4g13280	TPS12	terpenoid synthase 12	0.451656	0.02037	0.705054	0.553149	0.6414261	1.13E-01	0.8132242	0.8404
At4g13300	TPS13	terpenoid synthase 13	0.236941	0.000719	0.783812	0.73812	3.687952	0.000323709	0.4623236	0.0641
At4g14630	GLP9	germin-like protein 9	0.157695	1.61E-05	0.25815	2.24E-06	0.7367311	0.1254133	0.8693476	0.7893
At4g20362	---	other RNA	0.364083	0.016707	0.728166	0.697656	3.165449	0.000346452	0.6155871	0.5094
At4g22610	---	Bifunctional inhibitor	0.361105	0.024606	0.627316	0.124936	2.893529	0.001216563	0.5908976	0.3191
At4g22810	---	Predicted AT-hook DNA-binding family protein	0.437147	0.025555	1.034573	0.991285	0.5863707	5.21E-02	0.7569264	0.7569
At4g24310	DUF679	Protein of unknown function (DUF679)	0.263581	0.000359	0.40745	0.012035	1.262803	0.3425053	0.9374671	0.972
At4g28720	YUC8	Flavin-binding monooxygenase family protein	0.385445	0.028314	0.602321	0.195387	0.9212854	7.83E-01	7.98E-01	0.7726
At4g36570	RL3	RAD-like 3	0.430624	0.035423	0.71707	0.424734	0.5640891	0.115356	0.9742609	0.99207
At4g38080	---	hydroxyproline-rich glycoprotein family protein	0.36724	0.005424	0.562202	0.008362	1.250946	0.2221812	0.6574007	0.0809
At4g38780	---	Pre-mRNA-processing-splicing factor	0.322422	0.029371	0.989482	0.998744	1.038622	9.20E-01	0.8084273	0.7877
At4g40010	SNRK2.7	SNF1-related protein kinase 2.7	0.379396	0.039666	0.654434	0.448085	1.336181	2.54E-01	1.18E+00	0.82526
At5g05500	MOP10	Pollen Ole e 1 allergen and extensin family protein	0.456379	0.015003	0.716921	0.51875	1.495751	0.1000946	0.6912694	0.557
At5g19520	MSL9	mechanosensitive channel of small conductance-like 9	0.366861	0.003758	0.657127	0.179768	0.7450789	0.2744474	0.8851078	0.908
At5g19890	---	Peroxidase superfamily protein	0.129261	7.23E-06	0.390443	0.001041	2.601475	0.000420032	0.761906	0.4684
At5g35190	EXT13	proline-rich extensin-like family protein	0.155533	0.000268	0.299434	0.000536	1.898184	0.01970653	0.5602198	0.102
At5g43580	UPI	Serine protease inhibitor, potato inhibitor I-type family protein	0.19404	0.00028	0.300082	0.000671	3.996356	5.61E-07	0.6963744	0.4013
At5g44610	MAP18	microtubule-associated protein 18	0.316168	0.04136	0.519905	0.10939	0.6194952	0.1150847	0.6209031	0.5103
At5g66870	ASL1	ASYMMETRIC LEAVES 2-like 1	0.301691	0.002264	0.517376	0.077865	0.8768671	0.6696597	0.9045768	0.9322
At4g22470	---	protease inhibitor	0.176948	0.000681	0.458531	0.101147	6.70915	3.65E-08	0.3118275	0.0121
At5g55050	---	GDSL-like Lipase	0.427549	0.001182	0.67219	0.209208	1.7117	0.003504634	0.5643766	0.0409
At1g07560	---	Leucine-rich repeat protein kinase family protein	0.172526	1.23E-05	0.323289	0.000722	0.3801406	0.001900521	0.7726115	0.734
At1g08090	NRT2:1	nitrate transporter 2:1	0.087416	3.07E-06	0.184781	5.51E-07	0.1362176	3.10E-08	0.9291929	0.9487
At1g12740	CYP87A2	cytochrome P450, family 87, subfamily A, polypeptide 2	0.340241	0.026451	0.607615	0.174888	0.4246644	0.002604611	0.8896779	0.9181
At1g30280	---	Chaperone DnaJ-domain superfamily protein	0.444677	0.006106	0.712791	0.175845	0.1412209	9.00E-07	0.7926962	0.4071
At1g31770	ABCG14	ATP-binding cassette 14	0.266095	0.001735	0.438516	0.000772	0.3932722	0.000204069	0.8687662	0.8294
At1g49860	GSTF14	glutathione S-transferase (class phi) 14	0.092764	3.34E-07	0.204124	0.000778	0.1531455	1.49E-07	0.958347	0.9889
At1g49960	---	Xanthine	0.283149	0.00584	0.513012	0.006943	0.4991672	0.005505079	0.6884577	0.374
At1g52450	---	Ubiquitin carboxyl-terminal hydrolase-related protein	0.3601	0.023804	1.106739	0.941671	0.4219898	0.00413277	0.5982975	0.2424
At1g53610	---	---	0.302291	0.000745	0.565669	0.115286	0.5569057	4.76E-02	0.6018559	0.3478
At1g64920	---	UDP-Glycosyltransferase superfamily protein	0.381061	0.020067	0.789108	0.834358	0.3441156	0.000210482	0.7263793	0.7339
At1g67710	ARR11	response regulator 11	0.359651	0.003384	0.662368	0.386545	0.5239133	0.003647742	0.650459	0.1632
At1g71380	CEL3	cellulase 3	0.251193	0.000314	0.401594	0.008189	0.3656246	0.00067118	0.5059494	0.1044
At1g78000	SULTR1;2	sulfate transporter 1;2	0.391363	0.00084	0.590396	0.021154	0.5630735	0.00525388	0.7609771	0.4557
At2g21045	---	Rhodanese	0.157122	0.000296	0.273946	2.27E-05	0.5545713	0.004894671	1.216743	0.6471
At2g23540	---	GDSL-like Lipase	0.275745	0.000136	0.448964	0.000171	0.2938639	4.79E-06	0.8165058	0.6429
At2g35770	scpl28	serine carboxypeptidase-like 28	0.233414	0.000652	0.395805	9.46E-05	0.5589922	2.27E-02	0.9593472	0.9854
At2g37280	PDR5	pleiotropic drug resistance 5	0.484789	0.012315	0.744465	0.513751	0.3638738	0.000162113	0.8654666	0.8326
At3g02850	SKOR	STELAR K+ outward rectifier	0.357237	0.04412	0.538557	0.259018	0.4395076	0.0237796	0.8411646	0.9074
At3g13610	---	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily	0.401894	0.013065	0.638266	0.07611	0.4772113	1.26E-02	0.9828206	0.9957
At3g20380	---	TRAF-like family protein	0.086001	1.02E-07	0.16778	1.20E-05	0.3935626	0.005891342	0.7969052	0.7339
At3g22770	---	F-box associated ubiquitination effector family protein	0.391359	0.022723	1.566565	0.454912	0.4547892	0.005667679	0.5810221	0.2799
At3g46330	MEE39	Leucine-rich repeat protein kinase family protein	0.274782	0.001562	0.441788	0.077281	0.2714334	3.45E-05	0.6278949	0.5103
At3g54040	---	PAR1 protein	0.110521	2.20E-07	0.214725	6.82E-07	0.5165035	0.000733071	0.9148836	0.9273
At3g57040	ARR9	response regulator 9	0.445712	0.011147	0.775068	0.593974	0.2799896	0.000547737	0.4996051	0.0182
At4g28410	---	Tyrosine transaminase family protein	0.313058	0.001278	0.536586	0.040873	0.3578533	0.005026096	0.7088587	0.4073
At4g30170	---	Peroxidase family protein	0.124939	2.77E-06	0.201803	4.37E-08	0.5638116	0.003670487	1.0532	0.968
At4g37070	PLP1	Acyl transferase	0.260217	0.000562	0.524838	0.177817	0.4918925	0.02615641	0.4440318	0.1318
At5g02360	---	DC1 domain-containing protein	0.233377	0.001138	0.399898	0.124384	0.3783216	0.002736377	0.6640643	0.3977
At5g05880	---	UDP-Glycosyltransferase superfamily protein	0.314507	0.004758	0.473242	0.121163	0.4672317	0.008912377	1.05862	0.9856
At5g06090	GPAT7	glycerol-3-phosphate acyltransferase 7	0.239559	0.006542	0.37665	0.003164	0.5244565	0.01252894	0.8935652	0.92734
At5g06300	LOG7	Putative lysine decarboxylase family protein	0.484885	0.02315	0.740476	0.125643	0.3677265	0.000117287	0.6562375	0.1354
At5g13580	---	ABC-2 type transporter family protein	0.375926	0.022623	0.615602	0.195445	0.5232419	1.20E-02	0.7698264	0.5942
At5g22550	DUF247	Plant protein of unknown function (DUF247)	0.424329	0.043649	0.713198	0.511257	0.3306393	8.22E-05	0.6461787	0.2779
At5g25110	CIPK25	CBL-interacting protein kinase 25	0.268925	0.004461	0.509146	0.152105	0.4607002	0.009032487	7.60E-01	0.7685
At5g40510	---	Sucrase	0.265064	0.000455	0.480131	0.074172	0.519905	0.01156377	0.8697605	0.8831
At5g43350	PHT1;1	phosphate transporter 1;1	0.13528	1.06E-05	0.2387	2.50E-06	0.5173025	2.28E-02	0.9990578	0.9995
At5g43520	---	Cysteine	0.202365	6.55E-05	0.430027	0.02319	0.2407537	1.48E-06	0.5705202	0.0574
At5g62920	ARR6	response regulator 6	0.386025	0.017458	0.731247	0.732309	0.3612864	0.001304932	0.4172286	0.0856
At5g64100	---	Peroxidase superfamily protein	0.249662	0.000203	0.449512	6.69E-05	0.6121491	0.01815922	0.7483681	0.2604
At5g65790	MYB68	myb domain protein 68	0.36694	0.003809	0.809484	0.803889	0.4084963	0.000346421	0.6248232	0.1965

Supplemental Table 2. H_2O_2 Repressed, CRF6 dependent genes

A

AGI	Gene symbol	Gene Name	Fold change Col	Adjusted p-value Col	Fold change crf6	Adjusted p-value crf6	Fold change 6oe	Adjusted p-value	Fold change crf6	Adjusted p-value
At2g21770	CESA9	cellulose synthase A9	2.113289	0.04123227	1.052106	0.9529763	1.082393	0.811507	1.491537	0.35296
At5g19257	---	---	2.522152	0.04233433	1.245802	0.766144	1.168429	0.646782	1.280414	0.830141
At4g19030	NLM1	NOD26-like major intrinsic protein 1	4.129609	0.000687869	2.021512	0.02406952	1.058789	0.872205	1.705158	0.263625
At5g18020	SAUR20	SAUR-like auxin-responsive protein family	2.870068	0.0121343	1.17349	0.889982	0.503785	0.037999	0.696437	0.773173
At3g06020	FAF4	Protein of unknown function (DUF3049)	2.442333	0.03652287	0.9543266	0.944927	1.000999	0.998181	1.810132	0.144044
At5g50915	---	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	3.549215	8.62E-05	1.301919	0.434847	1.484272	0.211785	1.415356	0.419035
At1g59940	ARR3	response regulator 3	3.419286	0.005938978	1.185598	0.8435807	1.09179	0.788754	1.418361	0.606283

B

AGI	Gene symbol	Gene Name	Fold change Col	Adjusted p-value Col	Fold change crf6	Adjusted p-value crf6	Fold change 6oe	Adjusted p-value	Fold change crf6	Adjusted p-value
At1g02850	BGLU11	beta glucosidase 11	0.445186	0.03701988	0.7228033	0.3346331	3.126601	8.01E-06	0.744645	0.617087
At1g06160	ORA59	octadecanoid-responsive Arabidopsis AP2	0.4086025	0.0460856	0.7701279	0.79436	0.837717	0.659783	0.479448	0.234852
At1g07560	---	Leucine-rich repeat protein kinase family protein	0.374009	0.01931315	0.6433162	0.2097779	0.380141	0.001901	0.772612	0.733956
At1g15125	---	S-adenosyl-L-methionine-dependent methyltransferases superfamily	0.4576241	0.005938978	0.7043576	0.002416491	1.216242	0.458046	0.850935	0.662219
At1g32450	NRT1.5	nitrate transporter 1.5	0.4821955	0.004754344	0.7576876	0.08565673	0.369134	0.000554	1.521016	0.085965
At1g51470	BGLU35	beta glucosidase 35	0.4569031	0.01370738	1.045367	0.9576731	0.524687	0.012104	0.760144	0.564252
At1g54890	---	Late embryogenesis abundant (LEA) protein-related	0.2809655	0.001380952	0.497385	0.00801003	0.309421	0.000237	0.774324	0.730391
At1g62620	---	Flavin-binding monooxygenase family protein	0.4593752	0.02665674	1.257981	0.7454764	0.423097	0.003219	0.577369	0.245357
At2g02820	MYB88	myb domain protein 88	0.4751797	0.02058503	0.7403119	0.1482131	0.483079	0.004588	1.114808	0.895485
At2g20030	---	RING	0.3582342	0.001584255	0.6480176	0.06112535	0.409084	0.001032	1.131361	0.866122
At2g32620	CSLB02	cellulose synthase-like B	0.4716358	0.03588106	0.7917984	0.7615579	0.630877	0.068743	1.272733	0.836309
At2g43120	---	RmlC-like cupins superfamily protein	0.460499	0.01835485	0.7752508	0.247936	0.579859	0.028559	0.657763	0.193763
At3g02850	SKOR	STELAR K+ outward rectifier	0.3872199	0.03251222	0.6206815	0.1321276	0.439508	0.02378	0.841165	0.907391
At3g10320	---	Glycosyltransferase family 61 protein	0.4248616	0.006995054	0.6487502	0.1959062	1.717	0.044269	0.708436	0.439767
At3g12580	HSP70	heat shock protein 70	0.4657653	0.006634837	0.7290906	0.2175617	2.326321	0.000143	0.631778	0.192633
At3g13100	MRP7	multidrug resistance-associated protein 7	0.4920988	0.03434855	0.9315628	0.9252105	0.773429	0.445046	0.739035	0.399532
At3g13760	---	DC1 domain containing protein	0.4454036	0.02861287	0.7036598	0.2175617	0.684096	0.194114	0.782322	0.685546
At3g20380	---	TRAF-like family protein	0.2069236	5.17E-06	0.3426316	0.000992968	0.393563	0.005891	0.796905	0.733931
At3g23800	SBP3	selenium-binding protein 3	0.4826302	0.02682966	0.8336392	0.853295	0.454873	0.002143	0.814669	0.802859
At3g46330	MEE39	Leucine-rich repeat protein kinase family protein	0.3573253	0.03588106	0.7001058	0.5786537	0.271433	3.45E-05	0.627895	0.510349
At3g49960	---	Peroxidase superfamily protein	0.2966472	0.007975142	0.7075676	0.4296018	1.238312	0.466294	1.046509	0.985583
At3g54590	HRGP1	hydroxyproline-rich glycoprotein	0.3875099	0.005280052	0.8573077	0.9073865	0.926152	0.837577	1.043724	0.98892
At3g60330	HA7	H(+)-ATPase 7	0.4079106	0.007975142	0.7820106	0.2857871	0.748023	0.360881	0.672113	0.127216
At4g02270	RHS13	root hair specific 13	0.3980372	0.01020888	0.6185812	0.1325597	0.63364	0.104276	0.843117	0.881365
At4g08770	PRX37	Peroxidase 37	0.2311234	6.53E-05	0.3594069	3.95E-07	1.396896	0.14392	1.303115	0.52096
At4g12480	PEARLI11	Bifunctional inhibitor	0.4871372	0.03512343	0.9126034	0.9279965	4.537649	1.05E-06	0.523657	0.2426
At4g13420	HAK5	high affinity K+ transporter 5	0.166967	1.67E-05	0.3398311	0.01741581	3.11655	0.000608	0.369312	0.017772
At4g14020	---	Rapid alkalinization factor (RALF) family protein	0.2932098	0.004398344	0.4443536	0.001762013	1.95671	0.001376	1.748121	0.067891
At4g21680	NRT1.8	NITRATE TRANSPORTER 1.8	0.3885656	0.02145855	0.694006	0.2686936	1.248512	0.558818	0.575752	0.199537
At4g22940	---	Protein kinase superfamily protein	0.4123768	0.02652598	0.9223716	0.9364609	0.57415	0.122391	0.807445	0.873836
At4g27830	BGLU10	beta glucosidase 10	0.4789825	0.01460666	0.9854879	0.9845004	1.071547	0.799397	0.607365	0.09555
At4g36430	---	Peroxidase superfamily protein	0.4427088	0.002461844	0.7527277	0.1149937	0.316039	1.95E-05	1.106605	0.911474
At4g37990	ELI3-2	elicitor-activated gene 3-2	0.3332138	0.01033603	1.08942	0.9504738	3.993822	1.16E-05	0.490182	0.159849
At5g05500	---	Pollen Ole e 1 allergen and extensin family protein	0.4757778	0.040305	0.9069783	0.9102198	1.495751	0.100095	0.691269	0.557002
At5g07690	MYB29	myb domain protein 29	0.4844535	0.04397743	0.8528625	0.800916	0.17291	1.29E-06	0.789965	0.789221
At5g09690	MGT7	magnesium transporter 7	0.4486617	0.006995054	0.9287325	0.9028203	0.62976	0.02352	0.683783	0.226616
At5g27060	RLP53	receptor like protein 53	0.2857459	0.01010253	0.7147249	0.744624	3.775742	0.001334	0.944685	0.991472
At5g35190	---	proline-rich extensin-like family protein	0.3249343	0.006099199	0.5240204	0.1391553	1.898184	0.019707	0.56022	0.102003
At5g54020	---	Cysteine	0.3500918	0.002423697	0.5367901	0.04873308	0.516006	0.007821	1.192517	0.813149
At5g61160	AACT1	anthocyanin 5-aromatic acyltransferase 1	0.3569812	0.009986519	0.889234	0.9291304	1.040722	0.9388	0.407268	0.094644
At5g63850	AAP4	amino acid permease 4	0.3571792	9.08E-05	0.5767908	0.00340045	0.87524	0.555125	0.862541	0.711622

Supplemental Table 3. Cytokinin Induced (A) and Repressed (B), CRF6 dependent genes