Defining the role of the Cytokinin Response Factors (CRFs) in *Arabidopsis thaliana*

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ABSTRACT

Tracy Michelle Raines: Defining the role of the Cytokinin Response Factors (CRFs) in *Arabidopsis thaliana*

(Under the direction of Joseph Kieber)

Cytokinin is a phytohormone that plays an integral role in regulating the growth, development and physiological responses of a plant. Among the processes linked to cytokinin signaling are meristem maintenance, root growth, rosette size, seed count, pathogen defense and leaf senescence. The signaling pathway consists of a hybrid two-component system consisting of the histidine kinase receptors, phosphotransfer proteins and response regulators. Numerous genes are activated or repressed downstream of the primary signaling pathway. A large number of these genes are characterized as transcription factors and therefore drive appropriate downstream gene expression in response to cytokinin. This transcriptional cascade is complex and consists of many components and feedback loops, many that have yet to be determined

The *Cytokinin Response Factors* (*CRF*s) are a family of genes activated downstream of the cytokinin signaling pathway and were first identified by their induction in response to cytokinin. The *CRF*s are members of the AP2/ERF transcription factor family, one of the largest found in plants. Through mutant analysis, we show that the CRFs negatively regulate several cytokinin related processes, dependent on their interaction with the Arabidopsis Histidine

Phosphotransfer proteins (AHPs). The CRFs positively regulate meristem size, hypocotyl elongation in the dark and the rate of leaf senescence.

Additionally, through expression analyses and protein binding microarray, we were able to uncover some of the downstream processes and targets with which the CRFs are involved. We show that CRFs bind the GCC box with high affinity, as is common of other AP2/ERF members. Microarray results show the CRFs regulate genes involved in many downstream processes and also regulate some of the cytokinin signaling components. Combining both phenotypic and expression analyses, we are able to elucidate many developmental processes in which the CRFs participate, as well as to define them as negative regulators of many cytokinin regulated processes.

To my beautiful sons, Wyatt and Brodie, and my best friend and soul mate, Ryan.

I love you all dearly.

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LIST OF ABBREVIATIONS

ABPH1 - aberrant phyllotaxy1

ABA – abscisic acid

AHK – ARABIDOPSIS HISTIDINE KINASE

AHP – ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN

AP2/ERF - APETALA2/Ethylene Responsive Factor

ARF - AUXIN RESPONSE FACTOR

ARR - ARABIDOPSIS RESPONSE REGULATOR

AS2 - ASYMMETRIC LEAVES 2

Asp – aspartic acid

AUX1/LAX - AUXIN RESISTANT 1/LIKE AUX1

Aux/IAA - AUXIN RESPONSE FACTOR/INDOLE-3-ACETIC ACID INDUCIBLE 22

AuxRE - auxin responsive element

BA – benzyladenine

BiFC – bimolecular fluorescence complementation

bp – base pair

bZIP - basic leucine zipper

CaMV – cauliflower mosaic virus

CDK – CYCLIN DEPENDENT KINASE

CGA 1 - CYTOKININ-RESPONSIVE GATA FACTOR 1

ChIP - Chromatin immunoprecipitation

ChIP-chip - Chromatin immunoprecipitation coupled with microarray

ChIP-seq - Chromatin immunoprecipitation coupled with sequencing

CLV – CLAVATA

CRF – CYTOKININ RESPONSE FACTOR

CRY1 - CRYPTOCHROME 1

CUC - CUP SHAPED COTYLEDON

CYCD- CYCLIN D1

dpg – days past germination

DSC – distal stem cell

DR5 - auxin responsive promoter reporter system

GA –gibberellic acid

GA2OX - GIBBERELLIN 2-OXIDASE

GeBP/GPL – GLABROUS 1 enhancer-binding protein/GPL (GLABROUS-LIKE)

GFP – green fluorescent protein

GOF – gain-of-function

His – histidine

HY5 - ELONGATED HYPOCOTYL 5

IPT - ISOPENTENYLTRANSFERASE

KNOX - KNOTTED1

LOB - LATERAL ORGAN BOUNDARIES

LOG – LONELY GUY

LOF – loss-of-function

MP – MONOPTEROS

PBM11 – protein binding microarray 11

PDV2 - PLASTID DIVISION 2

phyA - PHYTOCHROME A

phyB - PHYTOCHROME B

PID – PINOID

PIL5 - PHYTOCHROME INTERACTING FACTOR 3-LIKE 5

PIN – PIN-FORMED

PLT – PLETHORA

QC – quiescent center

qRT-PCR - quantitative real-time polymerase chain reaction

RAM – root apical meristem

RGA - REPRESSOR OF GA

RNA:seq - next-generation sequencing to reveal a snapshot of RNA presence and quantity from a genome at a given moment in time

SAM – shoot apical meristem

SCR – SCARECROW

SHY2 - SHORT HYPOCOTYL 2

STIP – STIMPY

STM - SHOOT MERISTEMLESS

TCS – two-component system

WOX – WUSCHEL RELATED HOMEOBOX

WUS - WUSCHEL

PREFACE

Chapter 1 is published as Cristiana T Argueso, Tracy Raines and Joseph J Kieber, “Cytokinin signaling and transcriptional networks” in Current Opinions in Plant Biology, Volume 13, issue 5, pages 533–539 in October of 2010; 10.1016/j.pbi.2010.08.006.

In Chapter 2, the NanoString® time course treatment (Figure 2.1B) was carried out by Cristiana Argueso, the NanoString® experiment with the type-B arr mutants (Supplementary Figure 2.1A) was carried out by Apurva Bhargava, and the hybridization and analysis of the PBM11 experiment (Figure 2.4) was done by Jose Manuel Franco.

CHAPTER 1: BACKGROUND AND SIGNIFICANCE

While mostly known for their role in the control of cell division in plants, cytokinins, N6-substitutedadenine derivatives, regulate diverse aspects of plant growth and development, including the function of meristems, chloroplast development, vascular differentiation, leaf senescence, the modulation of sink–source relationships, nutrient acquisition, nodulation, and the response to biotic and abiotic stresses [1,2]. Deciphering the mechanisms by which cytokinins and other phytohormones regulate such diverse responses remains a central challenge in plant biology. Emerging evidence indicates that complex transcriptional cascades play an important role in mediating cytokinin responses, including changes in the expression of components of the cytokinin signaling, biosynthetic, and metabolic pathways, as well as the induction of various transcription factors. Here we review the cytokinin response pathway, with a focus on the transcription networks regulated by this phytohormone.

Cytokinin signal transduction

Cytokinin signaling involves a phosphotransfer cascade comprised of a modular system similar to the two- component signal transduction pathways present in most bacteria and in fungi [3] (Figure 1.1). Hybrid histidine kinase receptors,

known as CRE1/WOL/AHK4, AHK3 and AHK2, bind to cytokinin, which induces them to autophosphorylate on a conserved His residue within their kinase domain. This phosphate group is subsequently transferred to a conserved Asp residue within the receiver domain of these AHK proteins, and then transferred to the histidine phosphotransferproteins (AHPs). The AHPs actively move in and out of the nucleus in a manner that is independent of their phosphorylation status and is not altered by exogenous cytokinin [4]. In the nucleus, phosphorylated AHPs transfer the phosphate to a set of response regulators (ARRs), which are classified according to their C-terminal domains. Type-A and type-C ARRs have short C-termini, while type-B ARRs have longer C-termini that function in DNA binding and activation of transcription. Aside from the differences in their C-terminal regions, all ARRs share a similar receiver domain with conserved residues targeted for phosphorylation.

The type-B ARRs are encoded by a family of eleven transcription factors comprising three subfamilies; seven belonging to subfamily I and two comprising each of subfamily II and III. Genes belonging to subfamily I act directly downstream of cytokinin perception to initiate the appropriate transcriptional cascades [5–7]. Their C-termini contain conserved GARP DNA binding and activation domains that function in plant-specific processes. Disruption of multiple type-B *ARR* genes or expression of a dominant negative form of *ARR1* results in a phenotype strikingly similar to that observed in loss-of-function cytokinin receptor mutants [5,6,8,9], implicating type-B ARRs as positive elements in cytokinin signaling. The type-A ARRs, which are direct targets of the type-B *ARR* transcription factors [10,11], are negative regulators of cytokinin signaling [12]. In addition to their transcriptional up-regulation by type-B ARRs,

phosphorylation of type-A ARRs on a conserved Asp residue in the receiver domain is required for their activation and function, and in most cases decreases their turnover [13]. While lacking a DNA binding domain, most type-A ARRs are predominantly localized to the nucleus, and may act as transcriptional regulators in conjunction with other transcription factors, such as type-B ARRs.

Other transcription factors involved in cytokinin signaling

Microarray analyses of cytokinin-treated seedlings have revealed multiple transcription factors regulated by cytokinin [14–16]. Among these, a subset of AP2 transcription factors, known as the Cytokinin Response Factors (CRFs), are implicated in cytokinin signaling. The CRFs consist of six core family members, three of which are transcriptionally up-regulated by cytokinin [17]. Microarray analysis of cytokinin-regulated genes in a multiple *crf* mutant background revealed that many genes regulated by type-B ARRs are also regulated by CRFs [17]. Cytokinin-inducible expression of the *CRF* genes is compromised in an *arr1,12* mutant, placing them downstream of the type-B ARRs.

Transcription factors of the non-canonical leucine-zipper GeBP (GLABROUS1enhancer-binding protein)/GPL (GLABROUS-LIKE) family also may influence cytokinin signaling. Disruption of combinations of four of the *GeBP1/GPL* genes results in reduced sensitivity to exogenous cytokinin, coupled with increased expression of type-A *ARR*s [18]. This, along with overlapping expression patterns with type-A ARRs, indicates that a subset of *GeBP/GPL* genes inhibits the induction of the

type-A *ARR*s and thus may antagonize the negative feedback regulation of cytokinin signaling [18].

Cytokinin-regulated transcriptional networks in plant development

Cytokinin-regulated transcriptional cascades involved in shoot development

A requirement for cytokinin in shoot development was first suggested decades ago from their role in stimulating shoot formation in cultured callus tissue. Further evidence for a role of cytokinin in shoot development came from studies that demonstrated that reducing cytokinin levels in transgenic plants led to decreased activity of the shoot apical meristem (SAM) [19]. Subsequently, a number of transcription factors were found to control SAM function by regulating levels of cytokinin and cytokinin signaling in different SAM domains.

The class I KNOTTED1-like homeobox (KNOX) transcription factor, SHOOT MERISTEMLESS (STM), is necessary for SAM formation, maintaining cell division and preventing cell differentiation within the SAM [20]. Expression of STM is up-regulated by cytokinin [21], which in turn, up-regulates the expression of the cytokinin biosynthetic gene, *IPT7*, creating a positive feedback loop that increases cytokinin levels in the SAM [22,23]. Exogenous application of cytokinin, as well as expression of *IPT7* from the *STM* promoter, can rescue the phenotype of *stm* mutants, suggesting that the induction of cytokinin biosynthesis is a primary function of STM [23].

Increased levels of cytokinin in the SAM, due to inducible overexpression of *STM*, resulted in localized increases in the expression of the gibberellin (GA) catabolism gene *GA2OX*, which acts to reduce GA levels [22]. Thus, STM and related KNOX

proteins may act by maintaining a hormonal regimen of high cytokinin/low GA required for proper SAM formation.

STM expression in the SAM is positively regulated by transcription factors from the CUP-SHAPED COTYLEDONS (CUC) family and negatively regulated by the ASYMMETRIC LEAVES 2/LATERAL ORGAN BOUNDERIES (AS2/LOB) family of transcription factors [24]. *CUC* gene products and *STM* are required for SAM formation and cotyledon separation [25]. The *CUC2* and *CUC3* genes are up-regulated by cytokinin in the inflorescence meristem of transgenic plants overexpressing *IPT4* under the control of the *APETALA1* promoter, in a manner dependent on AHK3 and AHK4 [26]. *ASL9*, a member of the AS2-LIKE family of transcription factors, is also rapidly and specifically regulated by cytokinin, with expression kinetics similar to that of type-A *ARR*s [26,27]. The regulation of *CUC* and *AS2-like* genes is likely to be a key regulatory output of cytokinin in the regulation of SAM function.

The control of stem cell fate in the shoot apical meristem (SAM) is regulated by the homeodomain transcription factors WUSCHEL (WUS) and CLAVATA3 (CLV3) [20]. WUS expression in the organizing center of the meristem induces stem cell fate to the overlaying cells. CLV3, a target of WUS, encodes a small, secreted peptide that is recognized by the CLV1–CLV2 heteromeric receptors, ultimately leading to suppression of WUS expression in a negative feedback loop that regulates the size of the stem cell pool. Cytokinin up-regulates *WUS* through CLV-dependent and CLV-independent pathways [28,29]. WUS, in turn, binds to the promoter of a subset of type-A *ARR* genes, repressing their expression and increasing cytokinin signaling in certain SAM domains [30]. Computational modeling of the *in vivo* expression patterns of WUS, CLV3 and

AHK4 in the SAM, as revealed by confocal analyses of transgenic plants harboring promoter::florescent protein reporter constructs, led to the development of a model in which a gradient of cytokinin signaling is created in specific domains of the SAM through the regulation of the expression of cytokinin signaling components, thus controlling WUS expression [28]. Validation of the model showed that expression of WUS is positively correlated with AHK4 expression and cytokinin accumulation, and negatively correlated with the expression of type-A ARRs [28]. Furthermore, studies in rice indicate that the spatial distribution of gradients of active cytokinin is generated via the action of the phosphoribohydrolase LONELY GUY (LOG) [31]. Together, these observations indicate that gradients of cytokinin accumulation and signaling are major regulators of SAM function and stem cell fate.

The function of the type-A response regulators seems to be essential for appropriate definition of cytokinin signaling domains in the SAM and consequently proper stem cell fate. As noted above, WUS represses type-A ARR expression, and elevated type-A ARR7 function also leads to reduced WUS expression, suggesting a negative feedback loop between type-A ARR expression and WUS [30]. Transgenic plants overexpressing a constitutively active form of ARR7 show developmental phenotypes, including SAM arrest, and disruption of multiple type-A ARRs leads to defects in SAM function, including altered leaf phyllotaxy [30], similar to the effect of loss-of-function mutations in the maize type-A RR gene *ABPH1* [32]. In addition to cytokinin and WUS, auxin also regulates the expression of *ARR7* and *ARR15* in the SAM [33] via the auxin response factor ARF5/MONOPTEROS (MP) [33]. Together, these findings suggest a model in which these two type-A ARRs integrate cytokinin and

auxin signaling in the SAM, and in turn connect these hormonal inputs into the WUS/CLV3 regulatory circuit.

Additional evidence for cytokinin regulation of SAM function comes from the WUS-related transcription factor STIMPY (STIP/WOX9), which is required for proper establishment of meristematic tissues of the shoot and root [34]. *stip* loss-of-function mutants show reduced sensitivity to cytokinin in the SAM, but wild-type cytokinin responses in other parts of the plant, indicating a compartmentalized role in cytokinin responsiveness [35]. The expression of *STIP* is not rapidly regulated by exogenous cytokinin treatment, yet there is reduced *STIP* expression in the SAM of *ahk* and type-B *arr* mutants. Thus, *STIP* expression is dependent on cytokinin two-component signaling, but it is most likely not a direct transcriptional target of type-B ARRs. STIP is likely involved in the transcriptional activation of cytokinin-regulated genes in the SAM, as disruption of *STIP* results in reduced expression of type-A *ARR*s and *CRF*s in the SAM [35].

Cytokinin-regulated transcriptional cascades involved in root development

Cytokinin negatively regulates the size of the root apical meristem (RAM); lowering endogenous cytokinin levels or responsiveness leads to an increase in RAM size [19], while increased cytokinin levels or sensitivity causes a reduction in the size of the RAM [36]. Expression of cytokinin oxidases in different domains of the RAM revealed that cytokinin acts primarily within the root transition zone, where it promotes cell differentiation and thus decreases the number of cells in the meristematic zone [36].

Loss-of-function *arr1* mutants displayed an increase in RAM size, while elevated ARR1 function causes the RAM to become smaller, suggesting that this particular type-B transcription factor plays a role in regulating RAM function [36,37]. The regulation of RAM size by ARR1 likely occurs via a direct induction of the *SHY2* gene [37], a negative transcriptional regulator of auxin signaling. *SHY2* induction leads to decreased PIN expression, causing a redistribution of auxin and cell differentiation in the transition zone. The interplay between these cytokinin and auxin-regulated transcriptional regulators (ARR1 and SHY2) mediates the control of root meristem size and root growth [37]. During the meristem growth phase immediately following germination, a distinct type-B ARR, ARR12, mediates the regulation of SHY2 expression [38].

Cytokinin and auxin play an antagonistic role in root stem cell specification during embryo development [39]. In these cells, auxin up-regulates the expression of ARR7 and ARR15, suppressing cytokinin signaling in the basal daughter cell of the hypophysis, ultimately regulating the expression of transcription factors controlling stem cell specification, such as SCARECROW (SCR), WUS-RELATEDHOMEOBOX5 (WOX5) and PLETHORA (PLT1) [39]. This is in contrast to the SAM, in which auxin represses expression of ARR7 and ARR15 [33]. This difference in the response of these type-A ARRs to auxin in these meristems may explain why auxin and cytokinin act antagonistically in the RAM, but have a synergistic effect in the SAM.

Cytokinin and light

Growth of etiolated seedlings in the presence of cytokinin results in morphology similar to that of light-grown seedlings and induces the expression of many light-

regulated genes [2]. Furthermore, ARR4 directly interacts with the red light photoreceptor, phyB, and stabilizes it in the active, Pfr form [40]. This interaction depends on cytokinin-dependent phosphorylation of ARR4 on the canonical Asp residue within its receiver domain [41], providing a link between cytokinin signaling and light responses. A second potential convergence point of light and cytokinin signaling occurs at the basic leucine zipper (bZIP) transcription factor LONG HYPOCOTYL5 (HY5), which is involved in the response to blue light, acting downstream of the CRY1 blue light photoreceptor [42]. Cytokinin treatment increases HY5 protein levels by decreasing its rate of degradation [43]. Loss-of-function *hy5* mutants result in partial resistance to cytokinin as measured by root elongation and shoot regeneration assays [43,44]. *hy5* mutants also are unable to form callus tissue on regeneration media, a phenotype that is partially rescued by exogenous cytokinin [44]. Together, these data suggest that cytokinin signaling may affect light responsiveness via effects on HY5 protein stability, though the mechanism by which the cytokinin two-component pathway is linked to the regulation of HY5 protein turnover is unclear.

PIL5 is a basic helix–loop–helix transcription factor that inhibits seed germination in the dark by inducing GA catabolism and the expression of ABA anabolic genes [45,46]. In response to light, PIL5 directly interacts with phytochromes and is then rapidly degraded, which switches the environment to high GA, low ABA content, promoting seed germination [47]. Recent ChIP:chip analysis has revealed that PIL5 binds to the upstream regions of the genes encoding the cytokinin-responsive transcription factors CRF1, CRF2 and CRF3. PIL5 directly represses the expression of these *CRF* genes, and also indirectly elevates *AHP5* transcript levels [48], suggesting a

direct effect of PIL5 on cytokinin responsiveness. Like *pil5*, cytokinin receptor mutants (*ahk2, ahk3* and *ahk4*) also display an increased germination rate [49], suggesting that cytokinin may work through PIL5 to restrict seed germination in dark conditions [48]. The GATA transcription factor, CGA, provides another link between light and cytokinin signaling. *CGA1* expression is induced by white and red light in a phyA-dependent and phyB-dependent manner, but is also up-regulated in response to cytokinin [50]. The induction of *CGA1* by cytokinin is independent of phyA and phyB, suggesting that the two pathways act in parallel.

Cytokinin may also influence chloroplast number via the CRF transcription factors [51]. While single *crf* loss-of-function mutants do not display any obvious phenotypes, overexpression of CRF2 causes an increase in the number of chloroplast bodies and an increase in the PLASTID DIVISION2 (PVD2) protein level, indicating that CRF2 directly regulates the expression of genes involved in plastid division [51]. Addition of exogenous cytokinin also leads to increased chloroplast number and elevated *PVD2* expression, possibly via CRF2 [51].

Targets of the cytokinin transcriptional network

Some of the target genes of the cytokinin-regulated transcriptional network have been identified, primarily through microarray analyses [14, 15, 17]. From these studies, it is clear that cytokinin regulates waves of transcriptional effects. The first response (<30 min) includes a preponderance of transcription factors, which presumably contribute to the regulation of the subsequent waves of transcription in response to cytokinin. A second clear signature from the early response genes is a down-regulation

of cytokinin signaling (i.e. type-A ARRs) and levels (i.e. genes encoding cytokinin oxidases), likely acting as a negative feedback loop. The rapid regulation of multiple genes located in the chloroplast genome further suggests that the cytokinin signal is rapidly propagated into this organelle by an unknown mechanism to affect transcription of the plastid genome [14].

The functions of the cytokinin-regulated genes reflects processes known to be targets of cytokinin signaling, including genes involved in cell expansion, other phytohormone pathways (auxin, ethylene and GA), pathogen-responsive and light-regulated genes. Other, more directed approaches have identified individual genes regulated by cytokinin, including cyclinD3 [52] which provides a mechanistic link between cytokinin and the regulation of the cell cycle. Additionally, other clusters of genes suggest unsuspected targets of cytokinin, including trehalose-6-phosphate metabolism and potential effects on the redox state of the cell. Undoubtedly, there are many additional targets that remain to be identified and the transcription factors responsible for the regulation of these targets, and how they interact remains to be determined.

Conclusions

Cytokinin controls many aspects of development and responses to the environment. Recent research highlights the importance of cytokinin-regulated transcriptional networks in the regulation of these processes. While type-B ARRs play a predominant role as master regulators, it is clear that additional classes of transcription factors participate in the control of cytokinin-regulated gene expression (Table 1.1), and

that cross talk between cytokinin and other plant hormones at the transcriptional level is widespread.

Genetic and genomic analyses have been paramount in the identification of a suite of cytokinin-regulated transcription factors; however only a small set of transcriptional regulators and their target genes have been analyzed. Because transcription factors are generally expressed at low levels and may be poorly detected by hybridization to oligonucleotide arrays [53], the emergence of expression profiling technologies that overcome the limitations of microarrays, such as large-scale qReal-Time PCR and RNA:seq will most likely extend the suite of transcription factors regulated by cytokinin, shedding light into new transcriptional modules. Furthermore, ChIP:chip and ChIP:seq approaches of the master regulators of the cytokinin transcriptional network will undoubtedly reveal additional details regarding the transcriptional targets regulated by cytokinin and the crosstalk with other hormonal, developmental and environmental response pathways. The challenge facing plant biologists will be to integrate these large-scale datasets and use them to predict interactions among transcriptional networks, using computational modeling and systems biology approaches. Ultimately, these approaches should help elucidate how one simple signaling molecule, like cytokinin, can mediate such divergent responses.

Table 1.1 – Genes involved in the transcriptional cascade of the cytokinin signaling pathway

Transcription factor	Protein name	AGI code	TF family	Regulated by cytokinin?
Cytokinin responses				
Type-B ARRs	Class I Type-B ARRs	AT3G16857 AT4G16110 AT4G31920 AT1G67710 AT2G25180 AT2G01760 AT5G58080	GARP	Not regulated [11,12,13**]
CRFs	CYTOKININ RESPONSE FACTORS	AT4G11140 AT4G23750 AT5G53290 AT4G27950 AT2G46310 AT3G61630	ERF/Ap2	CRF2, CRF5 and CRF6 UP [21]
GeBP, GPL1, GPL2, GPL3	GLABROUS1 enhancer-binding protein	AT4G00270 AT2G25650 AT5G14280 AT2G36340	GeBP-like	Not regulated [25]
Shoot development				
STM	SHOOT MERISTEMLESS	AT1G62360	Class I knotted1-like homeodomain	UP [28**]
WUS	WUSCHEL	AT2G17950	WUS type homeodomain	UP [38,39]
STIP/WOX9	STIMPY	AT2G33880	WUS type homeodomain	Not regulated [47]
CUC2, CUC3	CUP-SHAPED COTYLEDON	AT5G53950 AT1G76420	NAC	UP [35,36]
ASL9	ASYMMETRIC LEAVES 2 LIKE 9	AT1G16530	LOB domain	UP [35,36]
GIS	GLABROUS INFLORESCENCE STEMS	AT3G58070	C2H2 domain zinc finger	Not regulated [37**]
ZFP8	ZINC FINGER PROTEIN 8	AT2G41940	Zinc finger	Not regulated [37**]
GIS2	GLABROUS INFLORESCENCE STEMS 2	AT5G06650	C2H2 domain zinc finger	UP [37**]
GL1	GLABROUS1	AT3G27920	myb domain	UP [37**]
Root development				
SHY2	SHORT HYPOCOTYL 2	AT1G04240	AUX/IAA	UP [22,49]
SCR	SCARECROW	AT3G54220	GRAS	DOWN [51]
WOX5	WUS-RELATED HOMEOBOX 5	AT3G11260	WUS type homeodomain	DOWN [51]
PLT1	PLETHORA	AT3G20840	Ap2/EREBP	DOWN [51]
Light responses				
HY5	LONG HYPOCOTYL 5	AT5G11260	Basic leucine zipper (bZIP)	Stabilizes protein [56]
PIL5	PHYTOCHROME INTERACTING FACTOR 3-LIKE 5	AT2G20180	myc-related bHLH	Unknown [61]
CGA1	CYTOKININ-RESPONSIVE GATA FACTOR 1	AT4G26150	GATA factor family of zinc finger	UP [63]

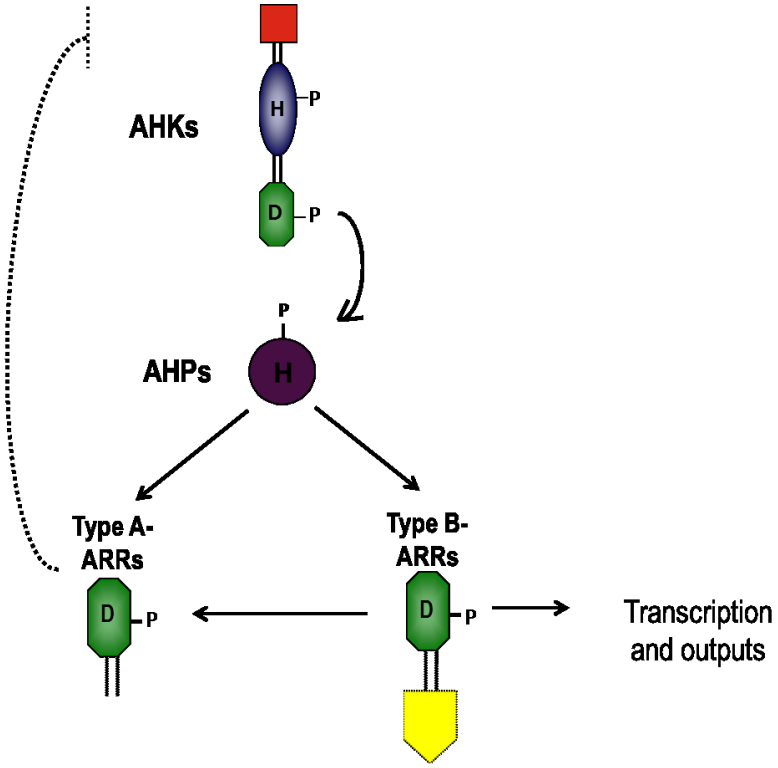

Figure 1.1 – Model for cytokinin signal transduction – The signaling pathway is a hybrid two component system with histidine kinase receptors which autophosphorylate upon cytokinin binding, subsequently transferring the phosphate to one of five phosphotransfer proteins (AHPs) which move in and out of the nucleus to shuttle phosphates to the response regulators (ARRs).

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CHAPTER 2: THE CYTOKININ RESPONSE FACTORS ACT DOWNSTREAM OF CYTOKININ SIGNALING TO REGULATE LEAF SENESCENCE AND OTHER DEVELOPMENTAL PROCESSES

ABSTRACT

A complex network of transcription factors is necessary to drive the downstream processes that cytokinin signaling regulates. Among these, the CRFs are a group of AP2/ERF transcription factors whose transcript levels are directly regulated by cytokinin. Genetic analysis indicates that the CRFs play an important role in promoting leaf senescence and other developmental processes. Specifically, disruption of multiple CRFs results in a substantial delay in leaf senescence, while overexpression of CRF3 or CRF5 results in premature leaf senescence. In addition to senescence, disruption of multiple CRFs results in stunted shoot growth, with the *crf1/+,2,5,6* mutant showing smaller leaves and inflorescences as compared to wild-type plants. Further, disruption of the CRFs leads to reduced primary root growth and lateral root growth, which are processes previously shown to be inhibited by cytokinin. Finally, we show that CRFs interact directly with the AHPs, which are phosphotransfer proteins integral to the primary cytokinin signaling pathway. CRF function appears to be dependent on the AHPs, as loss-of-function mutations in the AHPs suppress the phenotypes resulting from overexpression of CRF3. These findings suggest the CRFs are transcription factors directly downstream of the

cytokinin signaling pathway that negatively regulate cytokinin-related processes through their interaction with the AHPs.

INTRODUCTION

Cytokinins are N^6-substituted adenine derivatives that were first described by their ability to promote cell division in tobacco tissue culture [1]. Cytokinin plays pleiotropic roles in plant growth and development, including meristem maintenance, germination, the modulation of sink–source relationships, nutrient acquisition, nodulation, shoot and root development and the response to biotic and abiotic stresses [2, 3]. Cytokinin signaling involves a phosphotransfer cascade comprised of a modular system similar to two-component signal transduction pathways present in most bacteria and fungi [4, 5]. In Arabidopsis, there are three transmembrane histidine kinase cytokinin receptors localized on the membrane of the endoplasmic reticulum, known as CRE1/WOL/AHK4, AHK3 and AHK2 [6, 7]. These receptors contain a CHASE domain that binds cytokinin, which induces autophosphorylation on a conserved histidine residue within their kinase domain [8]. This phosphate group is subsequently transferred to a conserved aspartic acid residue within the receiver domain of the AHK proteins, and then to the histidine phosphotransfer proteins (AHPs), which move between the cytosol and nucleus independent of the phosphorylation state [9]. Finally, the AHPs transfer the phosphate to one of twenty-three response regulators (ARRs), all of which share a common receiver domain, but which have variable C-terminal domains. Type-A ARRs have short C-termini that do not contain a DNA binding domain while the type-B ARRs have longer C-termini that function in DNA binding and act as transcription factors mediating cytokinin

regulated gene expression [10, 11, 12, 13]. While the transcription of the type-A ARRs is rapidly induced in response to cytokinin, the levels of type-B ARR transcripts show little or no change [14]. The type-A ARRs provide a negative feedback loop for cytokinin responses, playing a key role in determining the responsiveness of a cell to this phytohormone [14].

While the type-B ARRs are at the top of a transcriptional cascade and are induced in response to cytokinin, there are numerous additional transcription factors regulated by cytokinin that likely mediate secondary waves of transcription. Multiple transcription factors regulated by cytokinin have been identified by transcriptome analyses [15, 16, 17, 18]. Among these, a subset of AP2/ERF transcription factors known as the Cytokinin Response Factors (CRFs) has been implicated in cytokinin signaling. The core *CRF* family consists of six genes, three of which have been previously shown to be transcriptionally induced by cytokinin [19, 20]. The cytokinin inducible expression of these *CRF* genes is compromised in an *arr1,12* mutant, placing their induction downstream of the type-B ARRs [19]. Recent studies have also shown that the CRFs are able to form homo- and heterodimers with each other and that they interact with proteins within the primary cytokinin signaling pathway [21]. Together, these data suggest the CRFs play a role in regulating the cytokinin-driven transcriptional response, potentially downstream of their induction by the type-B ARRs.

Here, we show that *crf* loss of function (LOF) results in reduced sensitivity to exogenous cytokinin in the root, while overexpression leads to hypersensitivity to cytokinin. We describe various developmental processes in which CRFs play a role,

including the positive regulation of leaf senescence. Many of the phenotypes observed by CRF overexpression can be suppressed by disruption of the Arabidopsis histidine phosphotransfer proteins (AHPs), suggesting the CRFs control these processes directly by their interaction with primary members of the cytokinin signaling pathway.

RESULTS

A subset of the *CRF*s are induced by treatment with cytokinin

It has been previously shown by northern blot and microarray analysis that some of the *CRF* family members are induced after the addition of cytokinin to Arabidopsis seedlings [19]. To further assess cytokinin responsiveness amongst the *CRFs*, we examined transcription levels of the *CRF*s in Arabidopsis seedlings after treatment with cytokinin using the NanoString© nCounter gene expression technology [22]. This analysis confirmed that *CRF2, CRF5* and *CRF6* were induced in response to cytokinin in whole seedlings (Figure 2.1A). *CRF2* and *CRF5* are induced in response to cytokinin within 15 minutes of treatment, and induction was sustained throughout the duration of treatment (Figure 2.1B). The induction kinetics of these *CRF*s is similar to those of the type-A ARRs, which are cytokinin primary response genes [23, 24].

When root and shoot tissues were examined separately, all *CRF*s were significantly up-regulated in the shoots in response to cytokinin, with the exception of *CRF1*, which was slightly down-regulated (Figure 2.2A). This suggests that regulation by cytokinin is a more general feature of the *CRF* gene family than

previously realized. In contrast, only *CRF6* was induced in roots (Figure 2.2A, 2.2B). Thus, the *CRF*s are generally only induced by cytokinin in shoot tissue, despite the somewhat comparable basal levels of expression of each gene in roots and shoots in the absence of cytokinin. These data are consistent with the opposing effects of cytokinin on root and shoot growth, [25, 26, 27, 28] and suggest unique transcriptional responses in root verse shoot tissues.

CRF localization does not change upon addition of cytokinin in intact plants

The CRFs belong to the B-5 AP2/ERF family of transcription factors and our previous analysis, primarily in Arabidopsis mesophyll protoplasts, suggested that these proteins move into the nucleus in response to cytokinin [19]. To determine to the localization of CRFs in whole plants we analyzed the intracellular localization of *CRF1, CRF3* and *CRF5* as fusions to GFP in stable, transgenic plants expressed from the CaMV 35S promoter. In the roots of five-day-old seedlings, the majority of the signal is present in the nucleus, consistent with the idea that the CRFs act as transcription factors (Figure 2.3). The pattern of localization for both 35S:CRF3-GFP and 35S:CRF5-GFP remains unchanged after treatment with cytokinin, both in the root and hypocotyl tissues (data not shown). These results demonstrate that *in planta*, the CRFs are present in the nucleus in the absence of treatment with exogenous cytokinin, and that their level of nuclear localization does not substantially increase in response to elevated cytokinin.

CRFs bind the GCC box with high affinity

In order to identify the preferred *cis* acting sequences recognized by the CRFs, we expressed CRF5 fused to a maltose binding protein domain in *E. coli* and used this to probe the PBM11 microarray [29]. Similar to other AP2/ERF transcription factors [30, 31], CRF5 preferentially bound the GCC box (Figure 2.4A). Amongst the different 7mer motifs, CRF5 preferentially bound to a [C/A]GCCGCC sequence, with the 6th position somewhat variable since CRF5 bound equally well to CGCCGTC and CGCCGAC (Figure 2.4B). To assess the frequency these motifs are found in genes potentially regulated by the CRFs, we screened for genes co-regulated with CRF5 using the "perturbations series" in Genevestigator. Both elements [C/A]GCCGCC were over-represented in the promoters of the top 200 of these genes (Figure 2.4C) as compared to genes not co-expressed with *CRF5*. The other variants identified (CGCCGTC and CGCCGAC) were also over-represented in the 5' upstream regions of genes co-regulated with *CRF5* (Figure 2.4D). These results suggest that CRF5 activates genes by binding several variants of the canonical GCC-box.

***CRF* induction by cytokinin is dependent on the type-B ARRs**

The type-B ARRs are involved in mediating the transcriptional response to cytokinin, including the induction of the type-A ARRs [23, 24]. We next examined the role of the type-B ARRs in the induction of the *CRF*s. After two hours of cytokinin treatment, the induction of *CRF2* and *CRF5* observed in wild-type seedlings was nearly absent in *arr1,2,10,12* mutant seedlings (Supplementary Figure 2.1A).

Further, the basal level of *CRF2* and *CRF5* was reduced in the *arr1,10,12* mutant seedlings, suggesting that these *CRF*s are regulated by endogenous cytokinin (Supplementary Figure 2.1A). The loss of *CRF* induction in the absence of type-B ARRs places the *CRF*s downstream of these genes in the cytokinin signaling pathway. Conversely, disruption of multiple type-A ARRs resulted in a hyperinduction of *CRF5* in response to cytokinin, as well as an elevated basal level (Supplementary Figure 2.1B).

Isolation of Arabidopsis lines with altered CRF function

To help uncover the specific roles of the CRFs within the plant, we obtained loss of function T-DNA lines containing single insertions within the cDNA regions for *CRF1* (AT4G11140), *CRF2* (AT4G23750), *CRF3* (AT5G53290), *CRF5* (AT2G46310) and *CRF6* (AT3G61630). Previous work with mutant crf lines also focused on T-DNA alleles containing insertions, some of which were not within the coding region of the gene [19], and therefore, we selected lines more likely to interrupt CRF function. There were no available insertions near the coding region of *CRF4* (AT4G27950), and as a result, we omitted this gene from the analyses below.

We next examined the expression of the type-A ARRs in *CRF* loss-of-function lines to determine if the CRFs are involved in the regulation of these cytokinin-primary response genes (Supplementary Figure 2.1C). In wild-type plants, the type-A *ARR*s are rapidly induced after the addition of cytokinin [6] in a manner dependent on the type-B ARRs [10]. We quantified type-A *ARR* transcript levels in both the *crf1,3,5,6* quadruple loss-of-function mutant and an overexpression line, CRF3OX.

The expression levels of the type-A *ARR*s did not change substantially in either case (Supplementary Figure 2.1C), suggesting that the CRFs do not play a role in regulating type-A *ARR* gene expression, consistent with previous results [19].

Effect of *crf* mutations on root growth

Primary and lateral root growth is regulated by cytokinin [25, 26, 27], therefore we examined root growth in the various *crf* mutant lines. We failed to detect significant differences in the elongation of the primary root in the overexpression lines (Supplementary Figure 2.2A). When the *crf1, crf2, crf3, crf5* and *crf6* single mutant combinations were assayed at 7- and 10- days after germination (Supplementary Figure 2.2B), we only saw significant differences in *crf1* which displayed longer root lengths than wild type. This is interesting in that CRF1 is the only *CRF* down-regulated by cytokinin and has the lowest levels of expression in the root. Root lengths in the triple mutants, however, were significantly reduced relative to wild type at day 10 (Figure 2.5A, Supplementary Figure 2.2B). Most strikingly, the *crf1,3,5,6* quadruple mutant had decreased root growth at every time point (Figure 2.5A) Together, these data suggest that the CRFs play redundant roles as positive regulators of root growth. We focused the remaining studies on the CRF3OX or CRF5OX overexpression lines and the *crf2,5,6* and *crf1,3,5,6* mutants as these lines generally displayed the strongest phenotypes.

We also examined lateral root formation in lines altered in CRF function. The total number of lateral roots in ten-day-old seedlings was increased in both the CRF3OX and CRF5OX lines as compared to the wild type (Figure 2.5B).

Conversely, the *crf2,5,6* and *crf1,3,5,6* multiple mutants had fewer lateral roots than wild-type roots. Thus, the CRFs play a positive role in the formation of lateral roots, in addition to a positive role in the elongation of the primary root.

To determine if the CRFs play a role in cytokinin responsiveness in roots, we examined root growth in the presence of cytokinin. Consistent with the involvement of CRFs in cytokinin responsiveness, overexpression of *CRF5* resulted in shorter roots in the presence of cytokinin, indicative of increased sensitivity to cytokinin (Figure 2.6A, 2.6B). Single and double *crf* mutants showed no significant difference in root elongation inhibition in comparison to wild-type plants (data not shown). However, the triple mutant *crf2,5,6* showed partial insensitivity to cytokinin treatment (Figure 2.6A, 2.6B). On increasing doses of BA, the triple mutants did not show reduced root elongation to the same extent as the wild-type control. By contrast, in the absence of cytokinin the *crf1,3,5,6* root displays a much shorter length than wild-type seedlings. In fact, the root length of the quadruple mutant showed almost no reduction in length in response to cytokinin, (Figure 2.6A, 2.6B). These data suggest that the CRFs may play a role in positive regulation of cytokinin signaling outputs with respect to root elongation.

CRFs positively regulate hypocotyl elongation in the dark

Cytokinin is known to inhibit hypocotyl elongation in the dark [32]. We examined the effect of loss of CRF function on cytokinin response of etiolated seedlings. The CRF5OX line had longer hypocotyls as compared to the wild type when grown in the absence of cytokinin (Figure 2.7A, 2.7B). Consistent with this, the

quadruple mutant, *crf1,3,5,6* had slightly shorter hypocotyls than wild-type seedlings (Figure 2.7A, 2.7B). Likewise, the *crf2,5,6* mutant also had slightly shorter hypocotyls, though the difference was not statistically significant.

We examined the response of etiolated seedlings to cytokinin to determine if perturbation of CRF function altered this response. As expected, the hypocotyls of wild-type plants were shorter when grown in the presence of cytokinin. The CRF3OX and CRF5OX lines as well as the multiple *crf1,3,5,6* and the *crf2,5,6* mutants showed a response to cytokinin (fold inhibition) comparable to the wild-type (Figure 2.7A, 2.7B).

Cytokinin causes an increased ethylene production in etiolated seedlings resulting in shortened hypocotyls [32]. To determine if the differences in hypocotyl lengths observed in the *CRF* mutants were due to changes in ethylene biosynthesis, we measured the amount of ethylene produced in four-day-old dark-grown seedlings, both in the presence and absence of exogenous cytokinin. The level of ethylene made by the *crf1,3,5,6* quadruple mutant seedlings was comparable to the wild type (Figure 2.7C). However, the level of ethylene produced by CRF5OX etiolated seedlings was greater than that of wild type both in the presence and absence of cytokinin, despite the fact that this mutant displayed elongated hypocotyls in these conditions. Thus the increased hypocotyl elongation is not the result of reduced ethylene synthesis, but rather reflects an effect on cell elongation independent of ethylene.

Effects of altered CRF function on shoot development

To determine if CRFs play a role in shoot development, we measured the rosette diameter of five-week-old CRF mutants and wild type plants. As shown in Figure 2.8, CRF3OX and CRF5OX plants were significantly smaller with smaller leaves as compared to wild-type plants. Conversely, various triple *crf* mutant lines had significantly larger rosettes as compared to the wild type (Figure 2.8A, 2.8B, data not shown). Surprisingly, the rosette of the quadruple *crf1,3,5,6* mutant was comparable to the wild type. These results highlight the functional redundancy of the CRFs and suggest that alteration of CRF function results in substantial effects on shoot growth.

Flowering time was also affected by alterations in CRF function. Flowering time was accelerated in the CRF3OX and CRF5OX lines, producing fewer rosette leaves before bolting of the primary shoot (Figure 2.8C). Conversely, the triple *crf2,5,6* mutant showed a delay in flowering time, with more rosette leaves than wild type (Figure 2.8C). Similar to the effects on rosette size, the quadruple *crf1,3,5,6* mutant was comparable to the wild type in its time of flowering.

Since we were unable to identify a *crf1,2,5,6* homozygous line, we set out to test the role of CRFs in embryo development. Self-fertilization of a *crf1/+,2,5,6* line resulted in only *crf1/+,2,5,6* and *crf2,5,6* seedlings, suggesting that the homozygous quadruple mutant was embryo lethal. Further, the rate of transmission of the *crf1/+,2,5,6* genotype among the selfed progeny of this line was substantially less than the expected 66% (~42%), suggesting that, in addition to the embryonic lethality of the quadruple mutant, a defect in transmission of this allelic combination.

We next examined the effect of disruption of CRF function on the development of the siliques. The siliques of the triple *crf2,5,6* and the quadruple *crf1,3,5,6* mutants were shorter and contained fewer seeds than wild type (Figure 2.9A through 2.9C). The *crf1/+,2,5,6* mutant had even shorter siliques than the triple or quadruple mutants and contained even fewer seeds. Both the *crf1,3,5,6* and the *crf1/+,2,5,6* line had a large number of missing seeds, which could result from the combination of gametophyte abortion and/or embryo lethality (Figure 2.9A, 2.9B, 2.9C). Indeed, examination of *crf1/+,2,5,6* flowers revealed 14% of defective female gametophytes (n=144), suggesting that the CRFs also play a role in their development.

CRFs positively regulate leaf senescence

We further characterized the phenotype of the *crf1/+,2,5,6* line. At five weeks, the rosette is much smaller than wild type and the leaves are curled and thin (Figure 2.10A), while the *crf2,5,6* siblings had the larger rosette size also observed in other *crf* mutants (Figure 2.8). The *crf1/+,2,5,6* inflorescence is bushy and, at 8 weeks of age, the overall height is extremely reduced relative to the wild type (Figure 2.10B). The rosette leaves of the mutant remain green at 8 weeks of age, while wild-type leaves are withered and yellow, suggesting that the mutant has a delay in senescence (Figure 2.10C).

We further explored the effect of altered CRF function on the timing of leaf senescence. Qualitatively, the various single and double *crf* mutants had no obvious effect on the timing of leaf senescence in either long- or short-day conditions (data not shown). In contrast, the *crf1,3,5,6* line displayed delayed leaf senescence

relative to the wild type (Figure 2.11A, 2.11B). Consistent with this, the CRF5OX line displayed substantially earlier leaf senescence. In order to confirm that the visual yellowing of the leaves we observed corresponded to senescence, we examined the expression of the senescence marker *SAG12 (SENESCENCE-ASSOCIATED GENE 12*), which is highly induced in senescing leaves, as well as the *CAB2* (*CHLOROPHYLL A/B-BINDING PROTEIN 2*) gene, whose expression decreases in senescing leaves as the level of functional chloroplasts decreases. We examined the sixth leaf to emerge from each plant from five-week old plants, which showed no visual yellowing at this time in either wild-type, CRF5OX or the *crf* loss-of-function lines, though the CRF3OX line did display slight yellowing at this time. There was a large induction of the *SAG12* gene and a corresponding reduction in *CAB2* expression in both the CRF3OX and CRF5OX lines, indicating that senescence was indeed occurring prematurely in these lines (Figure 2.11C, 2.11D). In contrast, *SAG12* and *CAB2* transcript levels in the various *crf* loss-of-function lines were comparable to wild-type leaves (Figure 2.11C, 2.11D), although *crf1,3,5,6* had slightly higher *CAB2* levels, consistent with delayed senescence.

The AHPs are required for CRF function

The AHPs are positive regulators of cytokinin signaling, involved in the relay of phosphate groups from the AHK receptors to the downstream response regulators [33]. It has recently been shown that the CRFs interact with the AHPs using a bimolecular fluorescence complementation (BiFC) assay in Arabidopsis mesophyll protoplasts [21]. We confirmed the interaction between AHP2 and CRF6 using both

yeast two-hybrid and a BiFC assay in Arabidopsis protoplasts (Supplementary Figure 2.3). CRF6 also interacted in the BiFC assay with a mutant version of AHP2 in which the His target of phosphorylation (H82) was altered to a glutamine or to a glutamic acid residue (Figure 2.12A, 2.12B), suggesting that the interaction of these proteins is not dependent on the phosphorylation state of the AHPs.

As the CRFs directly interact with the AHPs, we explored the role of the AHPs in CRF function. To this end, we introduced the *35S::CRF3:GFP* transgene into an *ahp1,3,4* line by crossing (CRF3OX/*ahp*). The mutant was grown in long day conditions together with wild-type and CRF3OX plants to compare the phenotypes observed. The transcript and protein levels of the *35S::CRF3:GFP* transgene were similarly overexpressed in the WT and *ahp* mutant background (Supplementary Figure 2.4). Upon comparison, the *ahp1,3,4* mutations suppressed both the rosette size and early leaf senescence phenotype of the CRF3OX (Figure 2.13 A, 2.13B, 2.13C and 2.13D), indicating that these AHPs are necessary for CRF function.

DISCUSSION

Cytokinin controls many important processes in the plant through a complex transcriptional network downstream of the signaling pathway. The *CRF*s have been implicated as early response genes downstream of cytokinin signaling [19], but the distinct processes they regulate have yet to be described. In this study, we extensively characterized the role of the *CRF* family of transcription factors in plant development as well as their role in cytokinin signaling. Consistent with the previous report of Rashotte *et al*. (2006), we observed that *CRF2*, *CRF5* and *CRF6* are

induced by cytokinin. Moreover, we demonstrated that all six of the core *CRF* genes are regulated by cytokinin in the shoot tissue, but that only *CRF6* is regulated in both shoots and the roots. All *CRF*s are up-regulated by cytokinin in the shoot, except for *CRF1*, which is down-regulated by the treatment. This suggests that cytokinin input is a general feature of CRF function, but primarily in shoot tissues. Our findings also support a mechanism whereby the transcriptional regulation of the *CRF*s by cytokinin is dependent on the type-B ARRs, consistent with the type-B ARRs acting at the top of a transcriptional cascade [10]. However, while the CRFs are downstream of the type-B ARRs, they do not regulate the induction of the type-A ARRs by cytokinin, and thus likely act downstream of the primary signaling pathway.

Based primarily on results in Arabidopsis mesophyll protoplasts, CRFs were previously reported to shift from being localized primarily in the cytosol to being localized primarily in the nucleus upon cytokinin treatment [19]. However, here we find that in stably transformed plants the CRF protein is localized primarily in the nucleus even in the absence of exogenous cytokinin treatment, and there is no substantial change in response to added cytokinin. This discrepancy could be the result of differences in protoplasts vs. intact plants, or other variables associated with the protoplasts used in that study. In any case, it seems clear from the analysis of the stable transgenic lines that the CRFs do not require cytokinin for their nuclear localization. The interaction with the AHPs occurs in both the cytosol and nucleus, confirming the presence of the CRFs with in both of these subcellular locations.

Many of the observed phenotypes of *crf* mutants indicate they negatively regulate cytokinin related functions within the plant. In the root, cytokinin restricts the

rate of cell division at the meristematic zone and promotes cell differentiation in the transition zone [26]. Cytokinin also inhibits cell division in the lateral root primordia, acting to inhibit the formation of lateral roots [25, 27]. Consistent with the idea that CRFs are acting as negative regulators of cytokinin function within roots, *crf* mutants displayed inhibited root growth and decreased number of lateral roots. Furthermore, CRF overexpression resulted in increased lateral root formation. However, our results showing the CRFs can be positive regulators of the response to cytokinin by root elongation are at odds with these findings, suggesting the roles of CRFs in the root are complex.

Cytokinin is also known to promote cell division in the shoot apical meristem, young leaves, and throughout embryogenesis [25, 28, 34]. Here we show that in the shoot, the rosette sizes of the *CRF* overexpression lines are much smaller than those of wild-type plants and their leaves are not fully expanded; while the loss of function lines showed larger rosettes and leaves. Thus, in addition to their negative regulation of cytokinin responses in roots, CRFs may also negatively regulate cytokinin signaling in leaves.

Our findings support a role for CRFs in embryonic development. Cytokinin regulated cell division is important in the developing embryo and aberrant cell division can result in embryonic defects and lethality. In the embryo, auxin up-regulates the expression of *ARR7* and *ARR15*, suppressing cytokinin signaling in the basal daughter cell of the hypophysis, ultimately regulating the expression of transcription factors controlling stem cell specification [28]. *MONOPTEROS* (*MP*), which directly regulates *CRF2* expression, plays an important role in embryo

development by regulating the transport of auxin from the apical embryo to the hypophysis precursor zone to control specification of these cells [35]. Removal of MP from the embryo results ectopic expression of *CRF2*, but lines overexpressing *MP* showed no change in transcript levels [35]. Higher order *crf* mutants show a reduced seed count, likely as a result of increasing penetrance of female gametophyte lethality, potentially combined with embryo lethality. The *crf1,2,5,6* quadruple mutant is clearly inviable as we failed to obtain the homozygous quadruple mutant line. It has previously been shown that a *crf5,6* double mutant was inviable [19]. However, we have utilized distinct insertional alleles for all the *CRF*s in this current study, and we are able to obtain viable *crf5,6* double mutants. It is, however, likely the *CRF5* and *CRF6* genes do play a role in embryonic development as they are lethal in combination with *crf1* and *crf2*. Further studies are needed to elucidate the role of the CRFs in embryo development.

The CRFs have clear roles in senescence. The addition of cytokinin exogenously to a plant or the ectopic expression of cytokinin biosynthetic genes has been demonstrated to substantially slow the senescence of leaves [36, 37]. Cytokinin signaling elements have been shown to directly control the rate of leaf senescence. An *AHK3* gain-of-function (GOF) mutation (*ore12*) resulted in delayed leaf senescence as well as reduced expression of senescence markers as measured by quantitative qRT-PCR [38]. While *AHK2* and *AHK3* have been shown to play an important role in the delay of senescence [39], the *ahk3* mutant is the only single cytokinin receptor mutant that displayed a senescence-associated phenotype, indicating its importance and specificity in controlling senescence through cytokinin

signaling [39]. In addition, *ARR2* overexpression resulted in the same delayed senescence phenotype, dependent on phosphorylation by AHK3, defining a cytokinin signaling pathway by which cytokinin inhibits senescence [39]. The CRFs could act downstream of cytokinin signaling to control the rate of senescence. Evidence that CRFs are positive regulators of leaf senescence comes from our observations that the rate of senescence is increased in the *CRF1*, *CRF3* and *CRF5* overexpression lines, manifesting as a premature yellowing of rosette leaves and higher levels of senescence related molecular markers than wild type. Conversely, multiple *crf* mutant lines show a delay in leaf senescence. It has recently been suggested that CRFs play a negative role in leaf senescence [40]. However, the data presented in that study examines effects of *crf* mutations on senescence only in detached leaf assays using dark-induced senescence, which may not reflect the effects in intact plants. The results presented here strongly suggest that the CRFs act as positive regulators of leaf senescence, possibly via negatively regulating cytokinin signaling and creating a negative feedback loop to tightly control the senescence process.

This study is the first to establish a functional relationship between the CRFs and AHPs. Specifically, we showed that AHPs are required for the robust rosette and senescence phenotypes resulting from *CRF* overexpression. Mutation of the phospho-accepting site of the AHPs does not disrupt or promote this interaction, suggesting the interaction may not be phospho-dependent. More studies are required to elucidate the mechanism by which the AHPs regulate CRF function. However, these results firmly place the CRFs within the cytokinin response pathway.

The exhaustive genetic analysis of CRFs presented here reveals they function downstream of the cytokinin signaling pathway to negatively regulate many cytokinin responses. These processes include primary and lateral root growth, rosette size, embryo development and leaf senescence. In addition, we show for the first time that processes regulated by the CRFs are dependent on their interaction with the AHPs, thereby suggesting a new role for the AHPs in driving cytokinin responses.

MATERIALS AND METHODS

Plant materials and treatment conditions

All Arabidopsis lines used in this study are in the Colombia (Col-0) ecotype. Insertions in *CRF1* (AT4G11140), *CRF2* (AT4G23750), *CRF3* (AT5G53290), *CRF5* (AT2G46310) and *CRF6* (AT3G61630) were obtained from the Salk collection of T-DNA insertion lines and are named, GABI_068G09, SAIL_371_D04, CS87573, SALK_024228 and GABI_541G11, respectively. Primers used for genotyping are found in Table 2.1. Multiple mutants were created by crossing homozygous lines to create higher order mutants. To generate transgenic plants over-expressing CRFs, the cDNA region of the gene was amplified and cloned into the pK7FWG2 vector [41]. This places the CRFs under the control of the CaMV 35S promoter and tagged C-terminally by the GFP reporter tag. Transgenic plants were generated by the previously described floral-dip method [42]. T1 lines were selected by plating surface sterilized seeds on 1x MS agar with 1% sucrose containing 50 µg/ml kanamycin. Single insertion lines were obtained by observing the segregation ratios of the T2

lines and selecting lines exhibiting the Mendelian 3:1 ratio. All PCR products and mutations were confirmed by DNA sequencing. The *arr1 arr2 arr10 arr12* and *ahp1 ahp2 ahp3 ahp4* mutants were previously described [19, 33]. Seedlings were grown as previously described [6] unless otherwise noted. Cytokinin treatment was carried out in 1X liquid MS containing 1% sucrose, constant light with mild shaking. Plants grown for senescence, rosette size, and flowering time assays were grown at 22ºC in 75 µE light under long-day conditions (16-h-light/8-h-dark).

RNA extraction and quantitative RT-PCR

Plant tissue was collected (5^{th} or 6^{th} leaf for senescence and CRFOX expression assays) and total RNA extracted using the RNeasy Plus kit (Qiagen). cDNA was prepared from the total RNA with the iScript cDNA Synthesis Kit as described by the manufacturer (BioRad). Quantitative RT-PCR was performed using 2X SYBR Premix ExTaq (TaKaRa) in an Applied Biosystems ViiA-7 real time machine. Primers were designed are described in Table 2.1. At least two biological samples were each analyzed with three technical replicates and the relative expression and standard errors were determined using REST 2009 software (Qiagen).

NanoString© nCounter gene expression analysis

Expression analysis of CRF transcripts was performed using the NanoString© nCounter gene expression assay essentially as described [22] by the UNC Genomics and Bioinformatics Core Facilities using 20 ng of total RNA extracted

using the method above. The probes targeting *CRF1*, *CRF2*, *CRF3*, *CRF4*, *CRF5*, *CRF6*, and the control probes for normalization of signal, *TUB4* (AT5G44340), *UBQ10* (AT4G05320) and *APT1* (AT1G27450) were designed and synthesized by NanoString© Technologies (www.nanostring.com) (Table 2.1). The expression level of each gene was normalized to the controls using protocols fund in the NanoString© Expression Assay Manual. (http://www.nanostring.com/uploads/Manual_Gene_Expression_Assay.pdf/).

Protein binding microarray

The cDNA for CRF5 was amplified and cloned into pDest-HisMBP obtained from Addgene (http://www.addgene.org/11085/) [43] to create CRF5 tagged with an N-terminal 6XHisMBP tag. This construct was sequence verified and transformed to BL21 E. coli for expression. Single colonies were grown overnight at 37ºC with shaking in 5 ml of expression broth (EB) from Zymo Research (catalog # M3011). This starter culture was then diluted into 15 ml of overexpression broth (OB) and grown at 30ºC overnight, adding 0.25 µM IPTG to induce expression.

For the identification of DNA sequence recognized by CRF5, a protein binding microarray strategy was followed, as in [29]. The design of the microarray covering all possible 11-bp sequences, DNA-binding reactions and immunological detection were as in [29]. Slides were scanned in a DNA Microarray Scanner (Agilent Technologies) at 5 um resolution and quantified with Feature Extraction 9.0 software (Agilent Technologies). Normalization of probe intensities and calculation of E-scores of all the possible 8-mers were carried out with the PBM Analysis Suite [44].

Perl scripts were modified to adapt them to different microarray dimensions and different input files generated by Feature Extraction software.

Lists of co-regulated genes with CRF5 were obtained from Genevestigator, and included top 200 genes with positive Pearson coefficient (positively co-regulated) and top 200 genes with negative Pearson coefficient (negatively co-regulated). Different lists were obtained from the “Anatomy” and “Perturbations” datasets in Genevestigator.

Identification of DNA motifs in the promoters of co-regulated genes was performed with Patmatch (http://www.Arabidopsis.org/cgi-bin/patmatch/nph-patmatch.pl) in the database TAIR10 Loci Upstream Sequences-1000 bp. We searched the same motifs in the promoters of all the genes unambiguously represented in the ATH1 microarray. Statistical over-representations of DNA motifs were evaluated by comparing the proportion of co-regulated genes containing the motif at their promoters relative to the corresponding proportion in the complete microarray, following a hypergeometric distribution.

Physiological assays

Root length was determined by growing seedlings as previously described (To *et al*., 2007) and scanning images at the indicated time points. The lengths of at least 30 roots per time point were measured using the software ImageJ software [45]. Root elongation assays were carried out as previously described [6] using increasing amounts of BA. Hypocotyl elongation assays were performed by plating seedlings as described [6] and placing them in the dark at 22ºC for 4 days. Plates were scanned

and hypocotyls were measured using ImageJ. Rosette size was determined by measuring the width of the rosettes at the widest point at 5 weeks of age. Flowering time was estimated by counting the number of rosette leaves present at the time of shoot emergence.

Transient expression in Arabidopsis protoplasts and immunoblot assay

For bimolecular fluorescence complementation (BiFC) assays, full-length *AHP2* and *CRF6* cDNAs were fused to plant expression vectors containing either amino- or carboxy-terminal fragments of YFP (YFP^N and YFP^C) [46]. Arabidopsis mesophyll protoplasts were transformed as described [47] and incubated overnight at 22°C under dim light (5 $\mu E \cdot m^{-2} \cdot s^{-1}$) and examined with an Axioplan2 fluorescent microscope (Carl Zeiss). To examine the protein levels, transfected cells were harvested and lysed with lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.1% Nonidet P-40, and protease inhibitor cocktail). The protein extracts were heated at 95°C for 5 min in SDS-PAGE sample loading buffer and separated on 10 % SDS-PAGE gels, and transferred to PVDF membranes [48]. The blot was probed with polyclonal anti-GFP antibody and horseradish peroxidase-conjugated anti-rabbit secondary antibody.

Yeast two-hybrid assays

The DupLEX-A™ system (OriGene Technologies) was used for yeast two-hybrid analysis of protein interactions. *AHP2* cDNA was cloned into the pGilda bait vector, which produces an in-frame fusion with the LexA DNA-binding domain. *CRF2*

and *CRF6* coding sequences were cloned into the pJG4-5 prey vector, which produces a B42 activation domain. The yeast strain EGY48 (*MATa*, *trp1*, *his3*, *ura3*, *leu2*::6 LexAop-*LEU2*) that contains the *lacZ* reporter plasmid pSH18-34 was transformed with the appropriate “bait” and “prey” plasmids. Interactions were tested on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-*gal*) medium [49].

Table 2.1 – List of primer and probe sequences

Primer/Probe Name	Sequence	Use
GAB_068G09 LP2	TCCTTACGCAACAGATTCGTC	genotyping crf1
GAB_068G09 RP2	CGTGAGACAACACGTGATACG	genotyping crf1
SAIL_371_D04 (crf2) LP	ATCACACGTGCCTCTCACCAAC	genotyping crf2
SAIL_371_D04 (crf2) RP	AGTTGTGGTCAAATGGAGTCG	genotyping crf2
CS875735 LP	TTCTGACCGGTGATTGAGAAC	genotyping crf3
CS875735 RP	TACCCGAAACTACCCTTGGAG	genotyping crf3
SALK_024228 LP	CCAGTCTCACGTGAGAGAAGG	genotyping crf5
SALK_024228 RP	GTGAGAAAATTCGTCTGACGG	genotyping crf5
GAB_541G11 LP	AAACAACGAAAGGTCCAAACC	genotyping crf6
GAB_541G11 RP	CGAACGAGACGAGTGAAGTTC	genotyping crf6
LB1.3	ATTTTGCCGATTTCGGAAC	genotyping SALK lines
LB3	TAGCATCTGAATTTCATAACCAATCTCGATACAC	genotyping SAIL lines
GAB KAT LB	ATATTGACCATCATACTCATTGC	genotyping GAB KAT lines
rtTUBr	ACCAATGAAAGTAGACGCCA	real time qPCR
rtTUBf	AGAGGTTGACGAGGAGATGA	real time qPCR
CRF1 RT F	CTCGACGTCGATGAGTG	real time qPCR
CRF1 RT R	GTTAAGACAGGATCCGACCCG	real time qPCR
CRF2 RT F	CCAGGATTCTTCGTATCTCAGTCAC	real time qPCR
CRF2 RT R	CCGGAGAAACAGTAGAACTCCG	real time qPCR
CRF3 RT F	CATCTCTCATCTCCTACTTCGG	real time qPCR
CRF3 RT R	GGAAATGAATCGTCTGATTCACC	real time qPCR
CRF5 RT F	GATGACGAACCTAAACCGGCG	real time qPCR
CRF5 RT R	CCAGAGTCTAGTACGACTCG	real time qPCR
CRF6 RT F	GTACAGAGGCGTGAGACAGAG	real time qPCR
CRF6 RT R	GCGGAGTTAGGAAATTCGTG	real time qPCR
SAG12 F	GATGAAGGCAGTGGCACACCAA	real time qPCR
SAG12 R	TCCCACACAAACACACAATTAAAAAGC	real time qPCR
CAB2 F	GGAACGGAHTCAAGTTTGGA	real time qPCR
CAB2 R	CAAAATGCTCTGAGCGTGAA	real time qPCR
CRF3twd GFP	GTGGTGAAACTATGAACATTG	real time qPCR of transgene
GFP twd CRF	CAGCTCGTCGCCCTTGC	real time qPCR of transgene
P P2AA3	ATCGCTCGGAACTTGGAAAGCAGCGTAATCGGTAGGGAGTGATTTGAGTTTGGTGAAGATGTCTATGGTTGAAGGCTTTATACCCGATTGCTGTGCT	NanoString probe (100 nmol)
UBQ10	AAGGCCAAGATCCAGGATAAGGAAGGAATCCCTCCGGAACGACAGAGGTTGATCTTTGCCGGAAAACAATTGGAGGATGGTCGTACTTTGGCGGATTACA	NanoString probe (100 nmol)
BETAT UBULIN 4	GAGAGGAAGAGTACGAGGAGGAAGAAGGAGTACGAGACTTAAGATGTTGTCAATGGCTCCCTCGGATTCGTAAGCTGTGTAAGCAAGCAGCATTCACT	NanoString probe (100 nmol)
ARR4	GCTCGTCTATGGCCAGAGACGGTGGTGTTTCTTGTTTACGAAGGTCGGTGGTATCGGAGGAATTGAATCTGCGCCGTTGGATTT	NanoString probe
ARR5	CTTTGCTGATAGAACCAAGACTGATCAAGAGTGACAAAGATGGGTTTTTATAAGATAGAAATGTAAATTGTAGACTTGGATTCATTAGAGAGGAGAAGA	NanoString probe
ARR6	AAACAGAGAAATGTCTACCCTGTTCACTGGCAGCTCAAACGCGCAAAGATCTGAGCTCTCCGATGCAAATTCCGTGACTGGATCTTAGAAAAAGCCTTTTC	NanoString probe
ARR7	ACTTATAATGAGGAATGAAGCTGAGGAATGCAAAATCTTAAGCCATTCTAACAAGAGAAAGCTTCAAGAAGACAGTGATACATCATCATCAAGTCATGAT	NanoString probe
ARR8	AGTCTTCATGTCAAGTAACTACAGTTGATTCAGGCTCTAAAGCTCTGGAATTTCTGGGTTTAAGATGTAGATGACAACGACCCAAATGCACTCTCTACATC	NanoString probe
ARR9	TATCAACACAAGAGGAAGTCAGTGGAAGAAGGGATTTCAAGTGATAGAGCACGTCCTAGATTCGACGGTATCGGCAACCGCTGTCTGATCTACCAGCTAT	NanoString probe
ARR15	TTAGCTGATGTGAAGCGTTTAAAAGAACTTATAATGAGAGGTGGTGAAGCTGAAGAAGGAAAAACCAAAAAACTTAGTCCTAAGAGAATCCTACAAAACG	NanoString probe
ARR16	AAAGTGACACAAGCAGAGAATGCGCTTAGAGCATTGGAGTATTTGGGTTTGGGAGATCAAAATCAGCATATTGATGCATTGACCTGTAACGTTATGAAGG	NanoString probe
CRF1	GGAAGTGGTGCTTGATTCGGTGGTTTCTGATAAAGAAGAAAGCCGATGAAGAAAGAAGGCGCTGTTGTTACTGTTCCAGTGGTTGTTACGACGGCG	NanoString probe
CRF2	AGAGAAACAAAGTCACAGTCTCGAGTCAAAAACACAGAAATACGAAAGAAAAATGGAAGCGGAGAAGAAAAATGGTTCTACCGAGAAATCAAATTCACAGAGC	NanoString probe
CRF3	CCTACTTCGGTTCTCAATCACCGGTCAGAAGAGATTCAACAAGTACAACAACCGTTTAAATCAGCTAAACCCGAACCGGGGGTTTCAAATGCACCATGGT	NanoString probe
CRF4	GGTTGGTTCGGGTCTCAATGACGGATCGTGACGCCACTGATTCATCAAGCGACGAGGAAGAGTTTCTGTTCCCTCGAAGACGTGTCAAGAGATTGATTAA	NanoString probe
CRF5	GAATTTTCTCAGTCCTCCTCTAGTCCGACGACTGAGGTGTTACCGGAAAACTCCGGTGATTGACCTTGAAACTGTCTCTGGTTGTGATTCGGCGAGGGAA	NanoString probe
CRF6	TCACAAACGTAGCAGGTACACCATCTAACGGGTCTCCGAGACTGGTCCGTATCACTGTTACTGATCCTTTCGCTACTGACTCGTCTAGCGACGACGACGA	NanoString probe

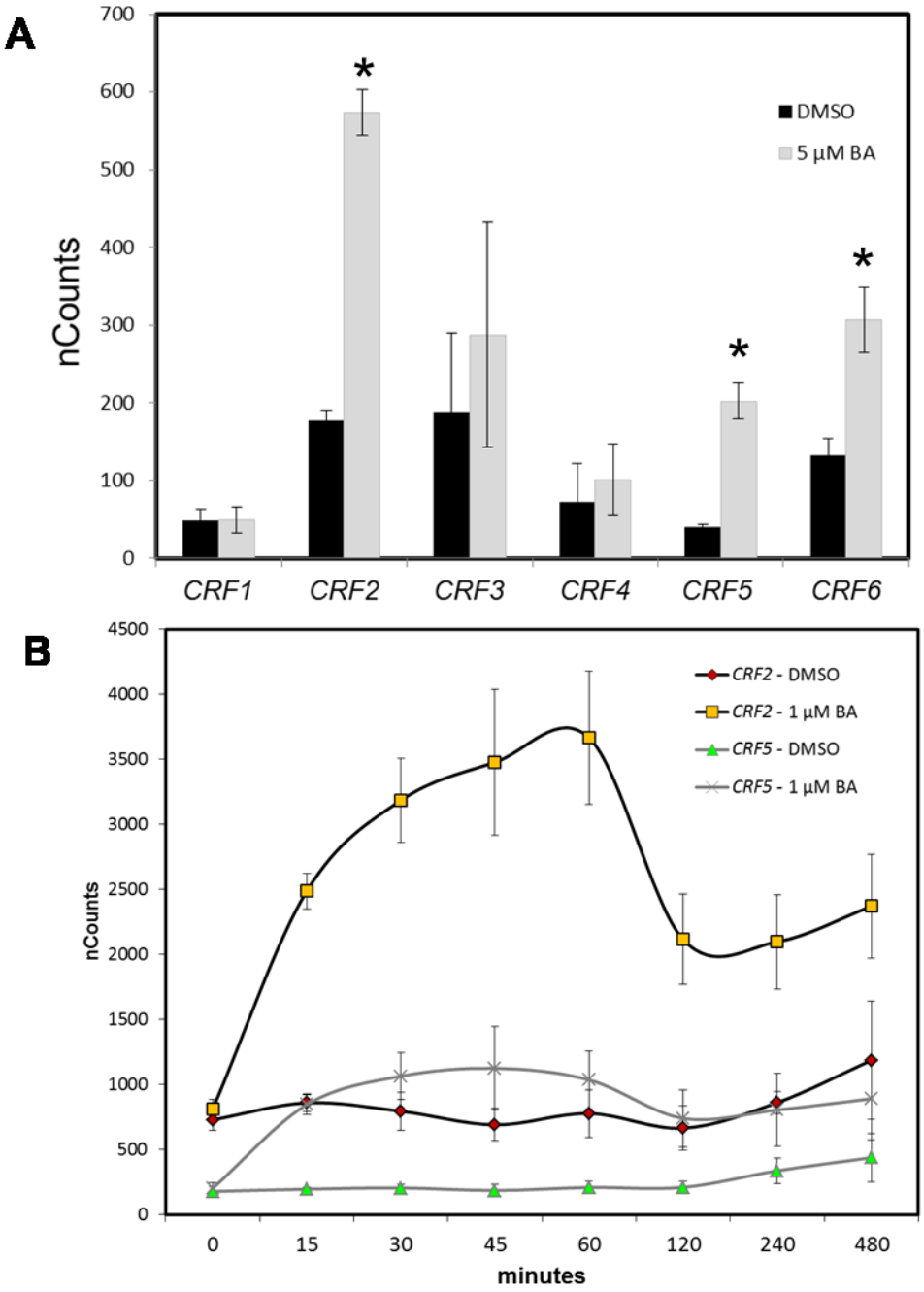


Figure 2.1: The *CRFs* are induced by cytokinin
(A) NanoString analysis shows *CRF2*, *CRF5* and *CRF6* are induced by cytokinin after 1 hour of treatment with 5 µM BA. **(B)** A time course of treatment with cytokinin shows induction of the *CRFs* as early as 15 minutes. (*= $p < 0.05$) (n=3)

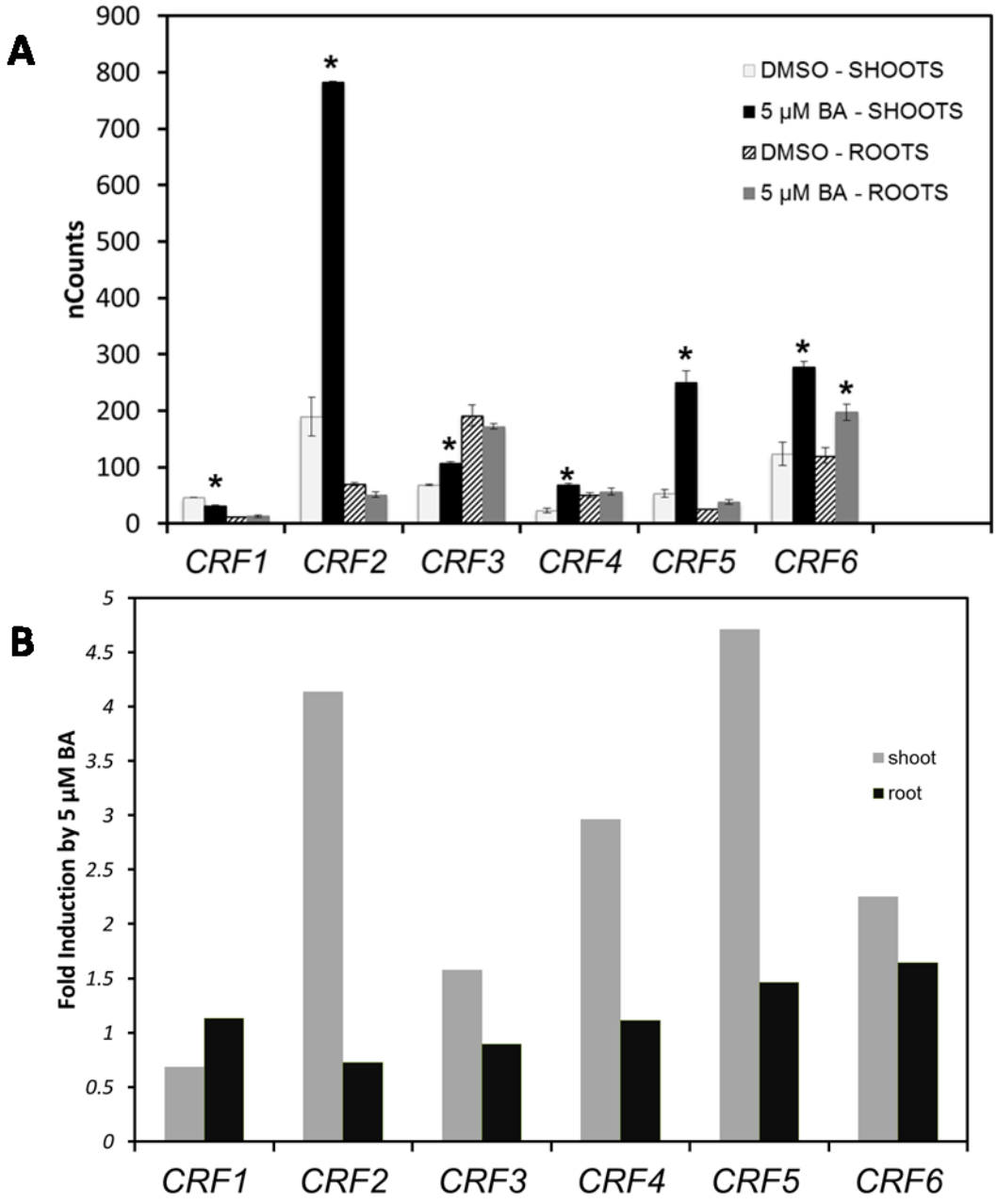


Figure 2.2: *CRF* regulation by cytokinin is prevalent in the shoot tissues for all *CRFs* (A) *CRF1* shows reduced expression while all other *CRFs* are induced in the shoot tissue by 5 µM BA while only CRF6 shows induction in the root tissue (*= pvalue ≤0.05) **(B)** Fold change of *CRF* expression treated/non-treated with cytokinin in both roots and shoots. (n=2)

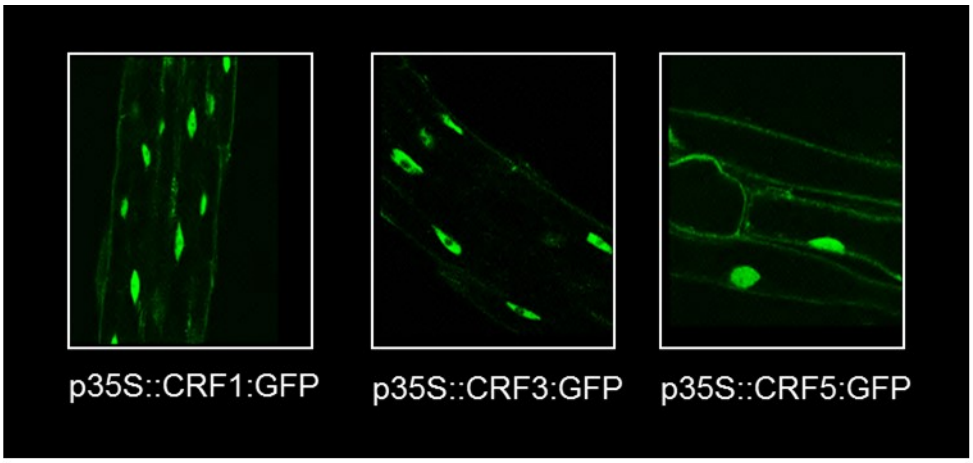


Figure 2.3: CRFs are localized primarily in the nucleus
Confocal microscopy of transgenic roots shows GFP expression throughout the cell with the majority localized within the nucleus.

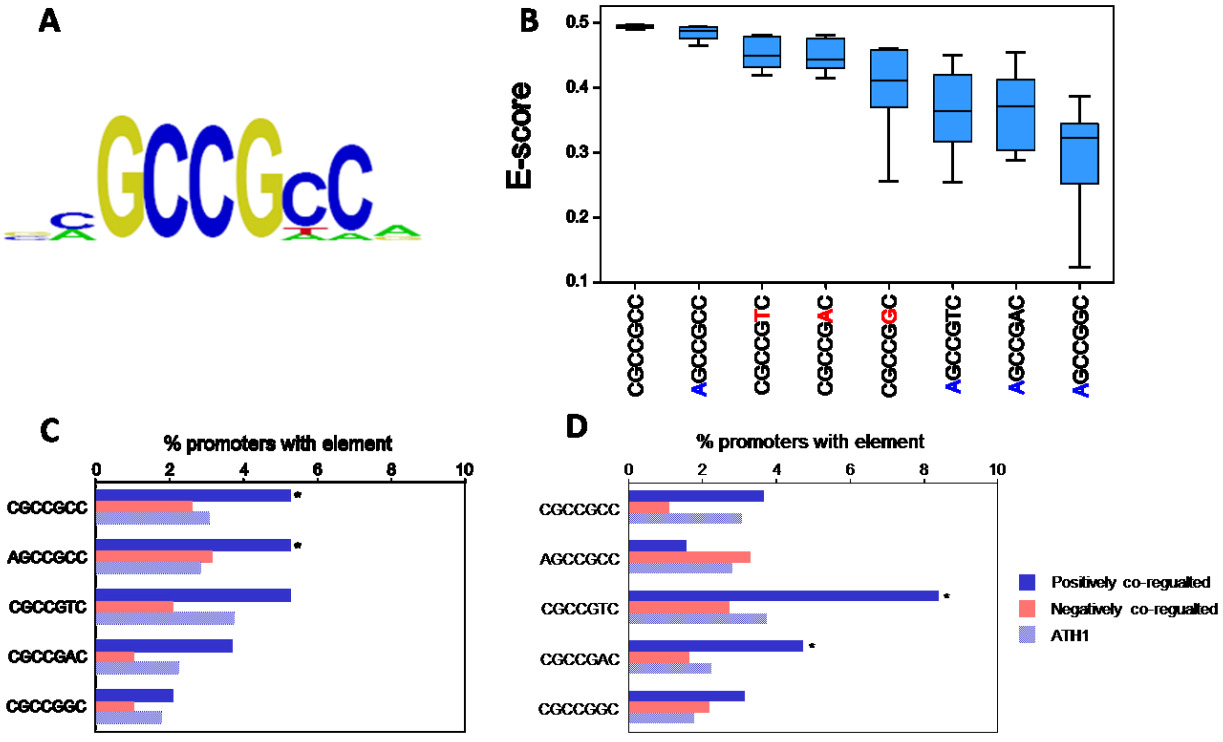


Figure 2.4 – CRF5 binds the GCC-box and is found in many co-expressed gene promoters Using protein binding microarray analysis, CRF5 was found to bind the GCC box with high affinity **(A)** with CGCCGCC being the 7-mer motif bound with the most affinity **(B)**. The top 200 genes found to be co-expressed with CRF5 using the "perturbations" series **(C)** and the "anatomy" series of the Genevestigator software **(D)** were enriched in these CRF5 7-mer binding motifs.

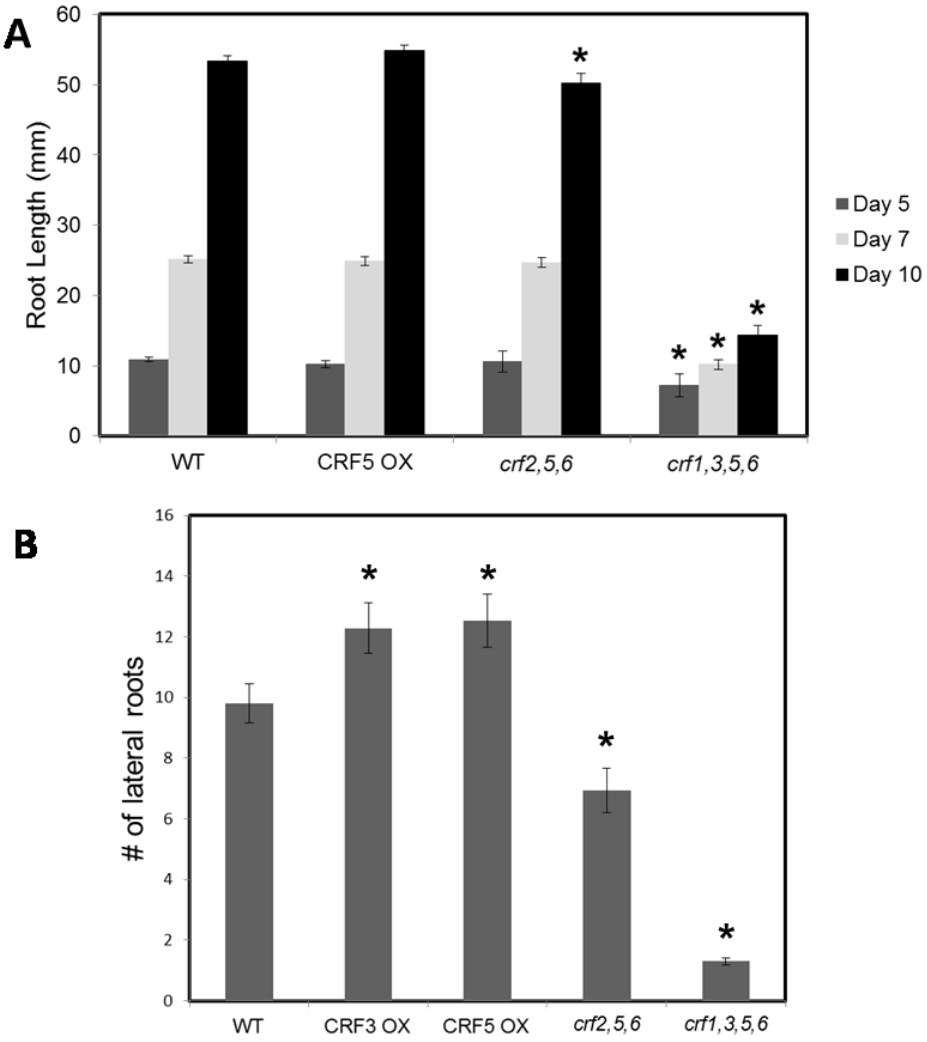


Figure 2.5: Multiple loss-of-function *crf*s display shorter roots and fewer lateral branches (A) The quadruple CRF quadruple mutant shows significantly smaller root length at 5-, 7-, and 10-dpg. **(B)** The quadruple CRF mutant displays fewer lateral roots at 10 dpg while the CRF3 OX and CRF5 OX line show an increase in lateral root formation. (*= pvalue ≤ 0.05)

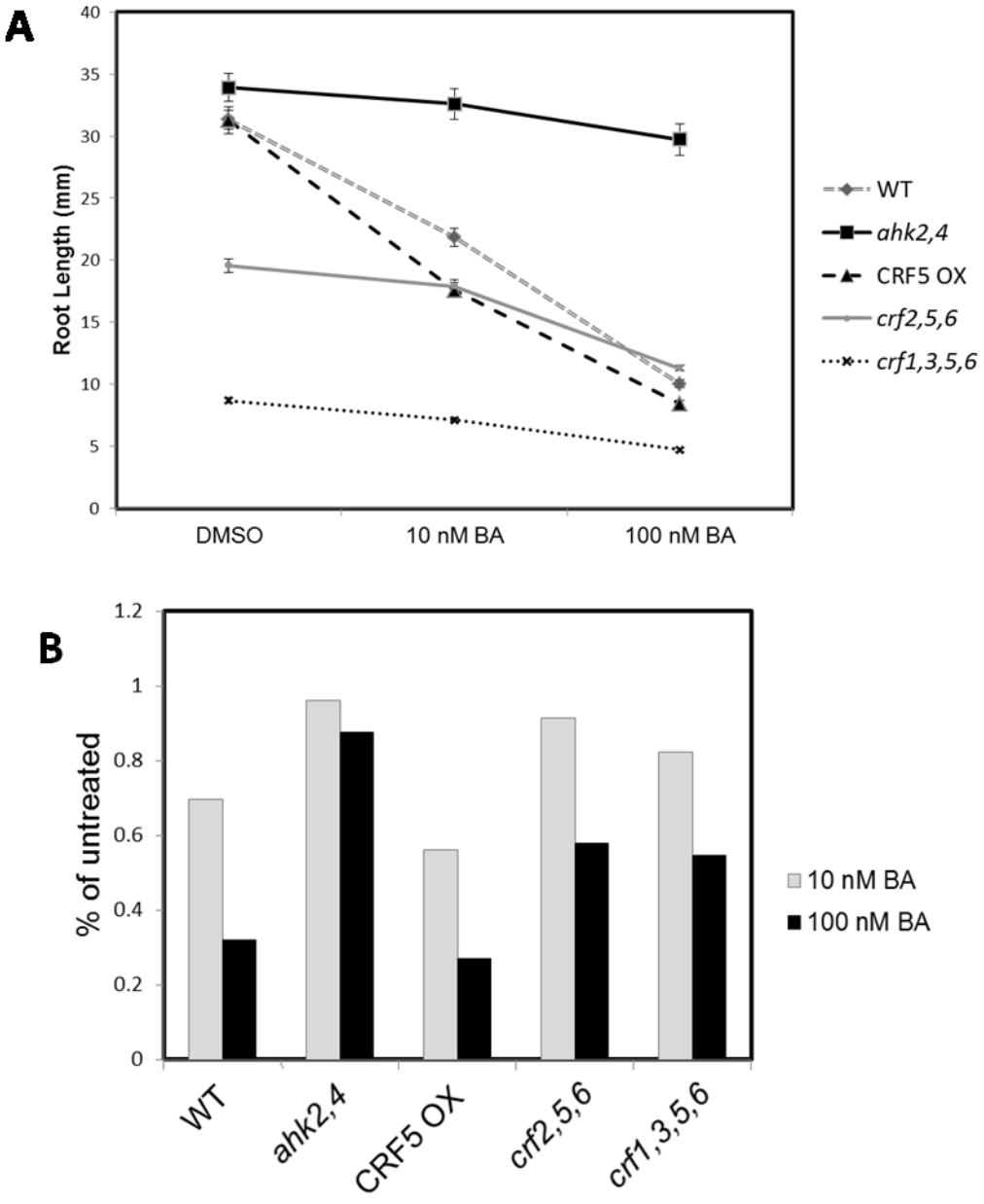


Figure 2.6: *crf* mutants are insensitive to cytokinin by root elongation assay (A) LOF *crf* mutants are insensitive to cytokinin by root elongation while GOF mutants are hypersensitive. **(B)** Percent root elongation of roots grown on increasing concentrations of cytokinin in comparison to DMSO control.

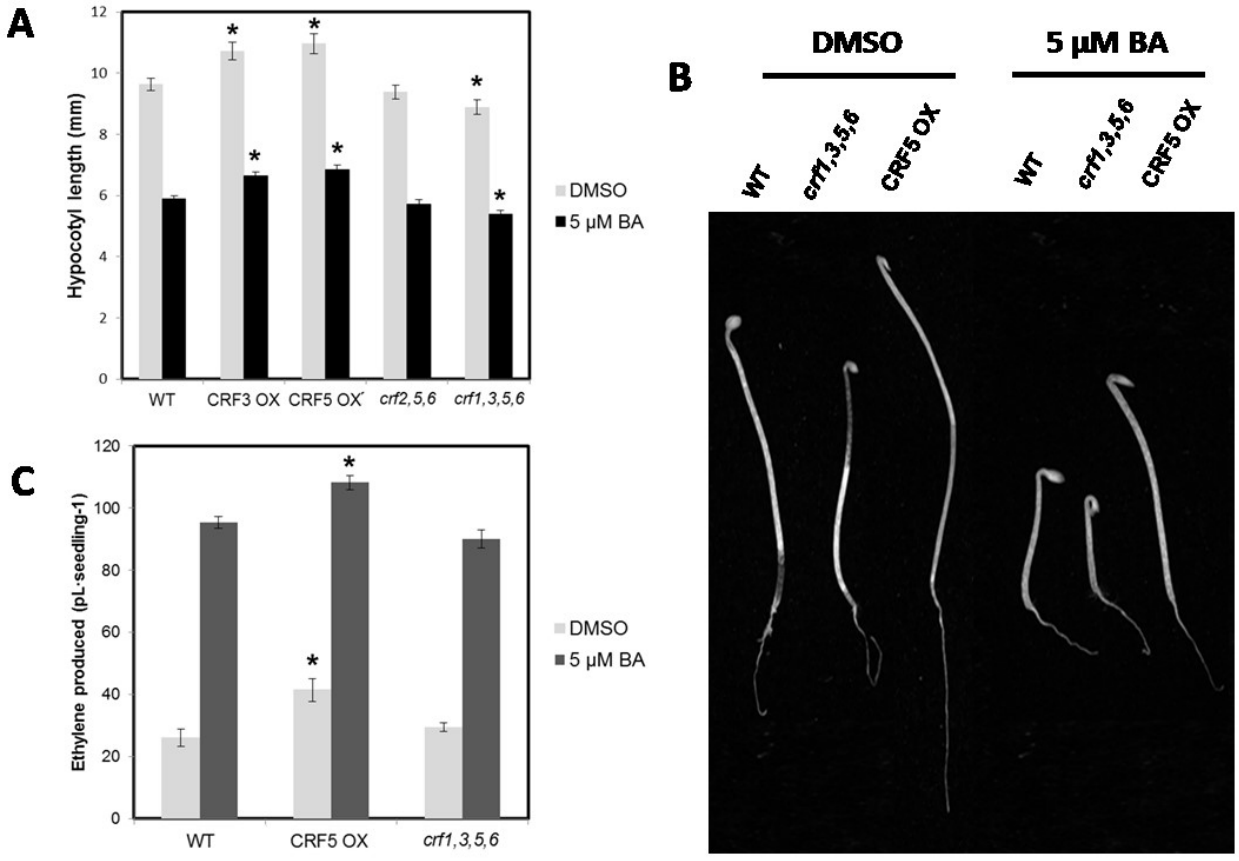


Figure 2.7: *CRF* overexpression results in elongated hypocotyl in the dark (A) Hypocotyl length of CRF mutants grown in the dark at 4-dpg on DMSO and 1 µM BA. **(B)** Dark grown mutants at 4- dpg. **(C)** The *CRF* overexpression mutant produces more ethylene than wild-type plants when grown in the dark (4-dpg). (*= pvalue ≤ 0.05)

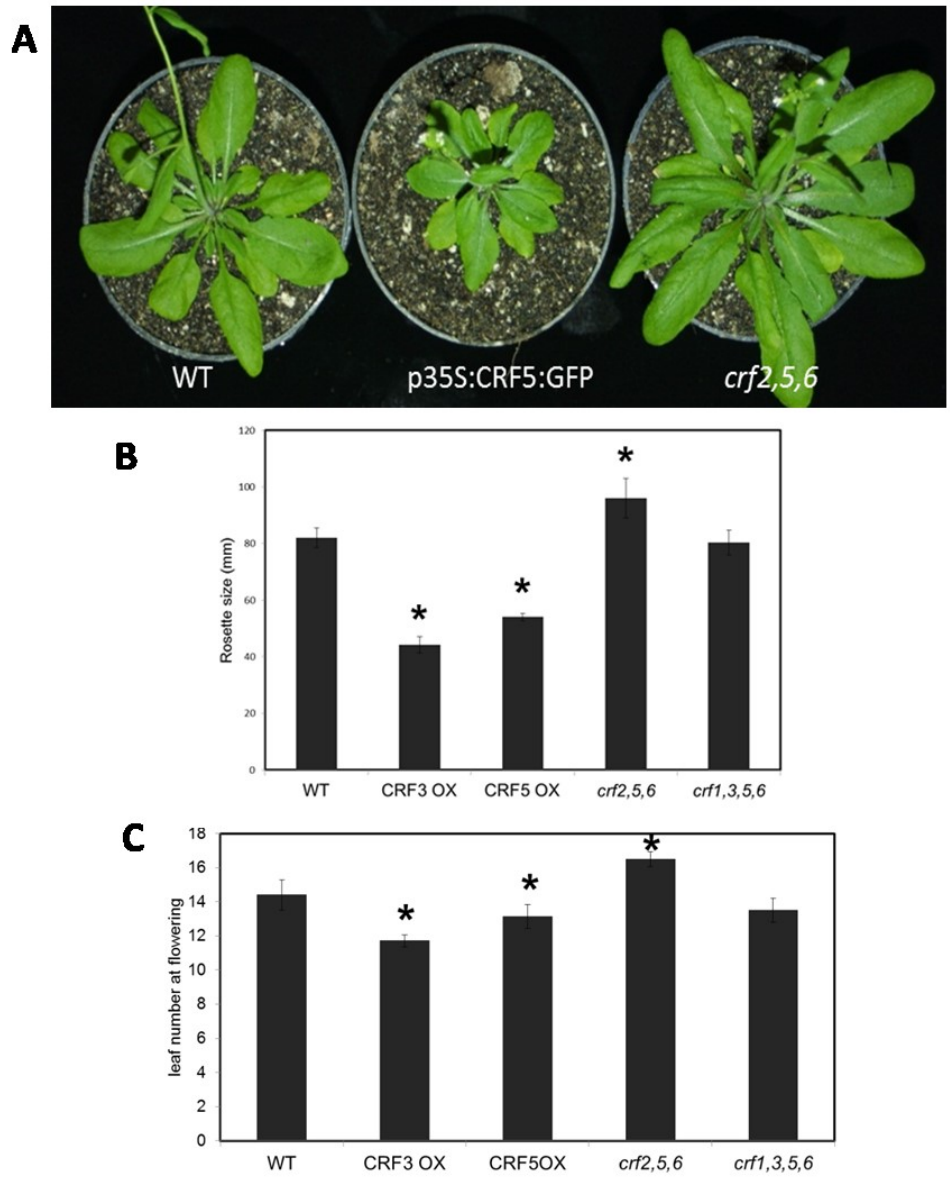


Figure 2.8: *crf* mutants display various shoot phenotypes
(A) Five week old CRF mutants and wild type plants grown in long day conditions. Overexpression of CRFs results in smaller rosette sizes while LOF lines display larger rosettes. **(B)** Average size of rosettes at 5 weeks **(C)** CRF mutants flower at different times as indicated by leaf number at first sight of bolting.(*= pvalue ≤ 0.05)

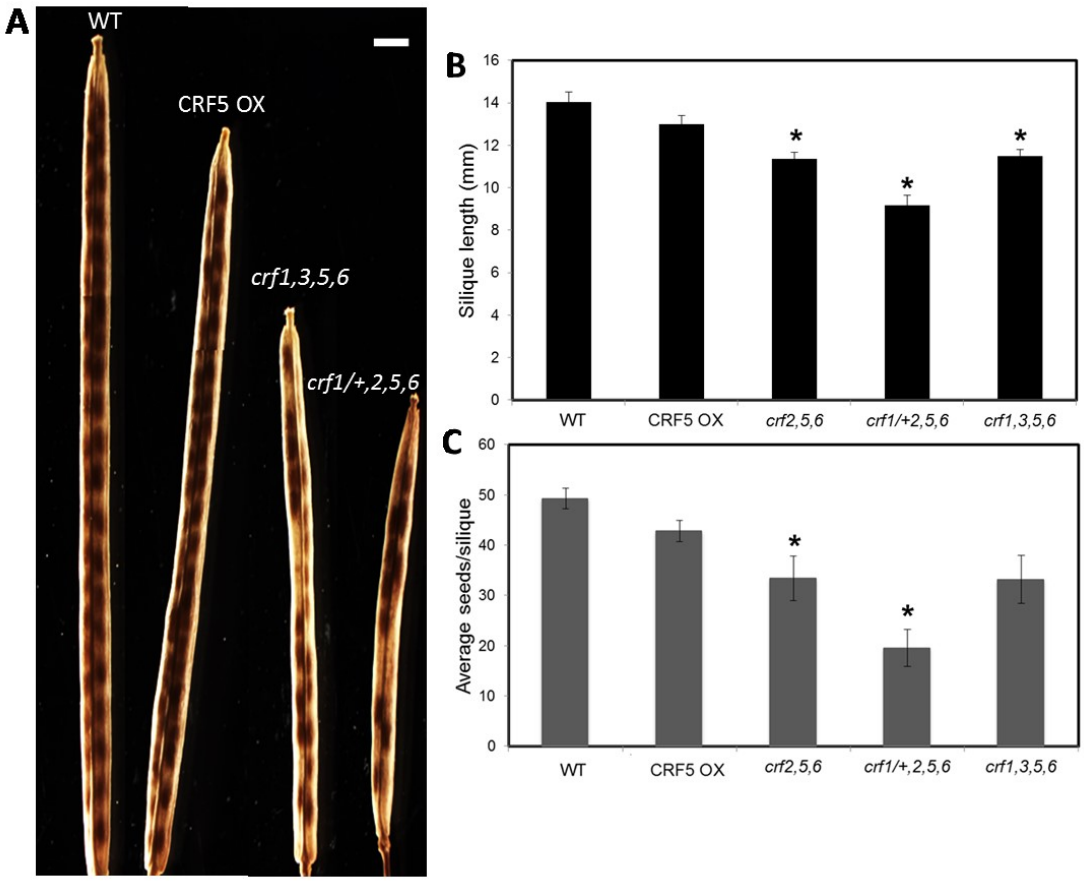


Figure 2.9: Higher order *crf* mutants aborted seeds and smaller siliques **(A)** Light microscopy through mature siliques show reduced seed number in *crf* mutants. The average length of mature siliques are shorter in *crf* mutants **(B)** and contain an average of fewer seeds per mature silique **(C)**. (*= pvalue ≤ 0.05) (scale bar = 1mm)

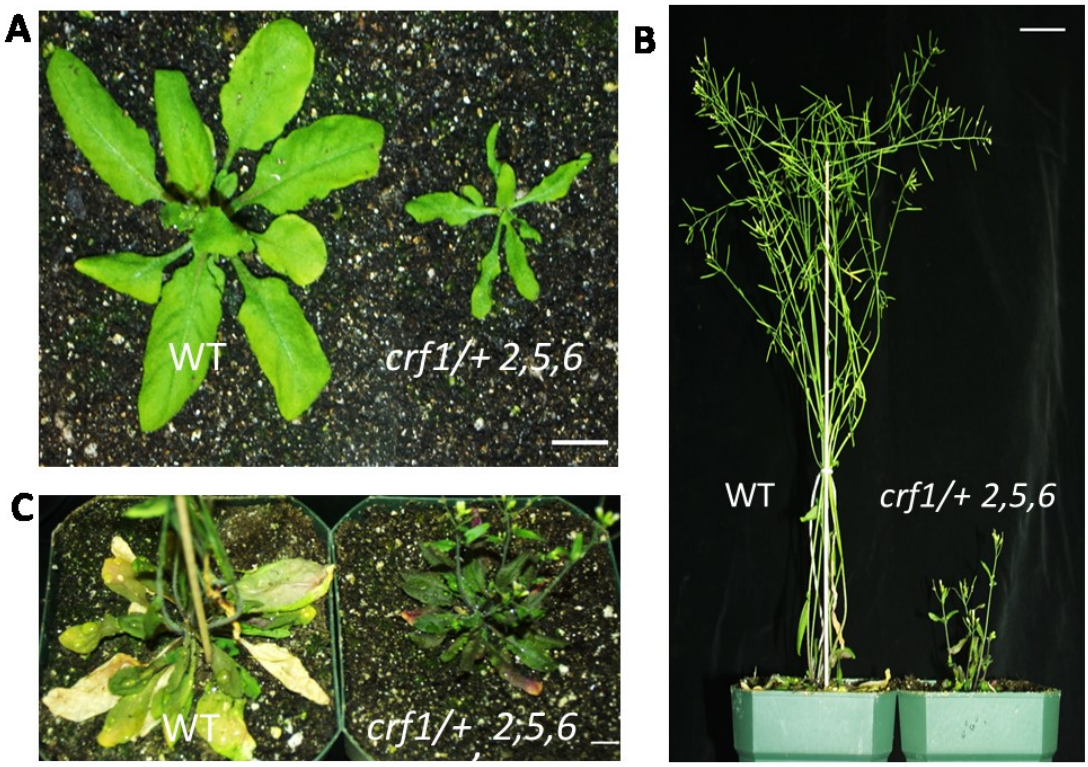


Figure 2.10: *crf* mutants display various shoot phenotypes
(A) Five-week-old *crf1/+,2,5,6* mutant and wild type plants grown in long day conditions. Mutant lines show smaller rosette with altered leaf phenotype. **(B)** and **(C)** eight-week-old *crf1/+,2,5,6* mutant and wild-type plants grown in long day conditions display smaller stature **(B)** and slowed leaf senescence **(C).** Scale bar = 12.5 mm **(A, C)** and 25 mm **(B).**

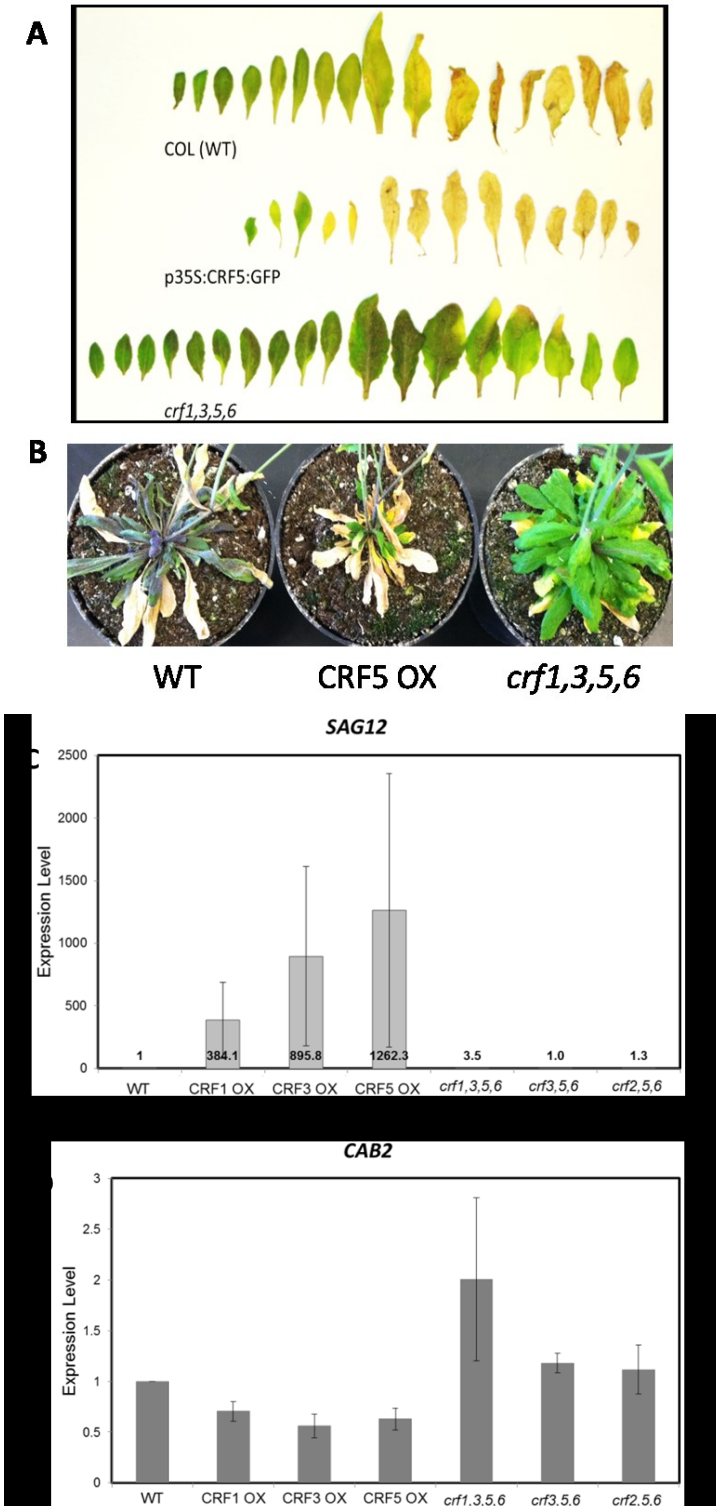


Figure 2.11: *crf* mutants show altered rate of leaf senescence (A) Leaves of wild type and *crf* mutants are arranged from newest to oldest with regard to emergence. *CRF* overexpression results in accelerated rate of leaf senescence. **(B)** 8 week old intact plants display altered rates of senescence. Real time analysis of senescence markers **(C)** *SAG12* and **(D)** *CAB2* in 6-week-old leaves of *crf* mutants grown in long day (14 hr light/10 hr dark) conditions.

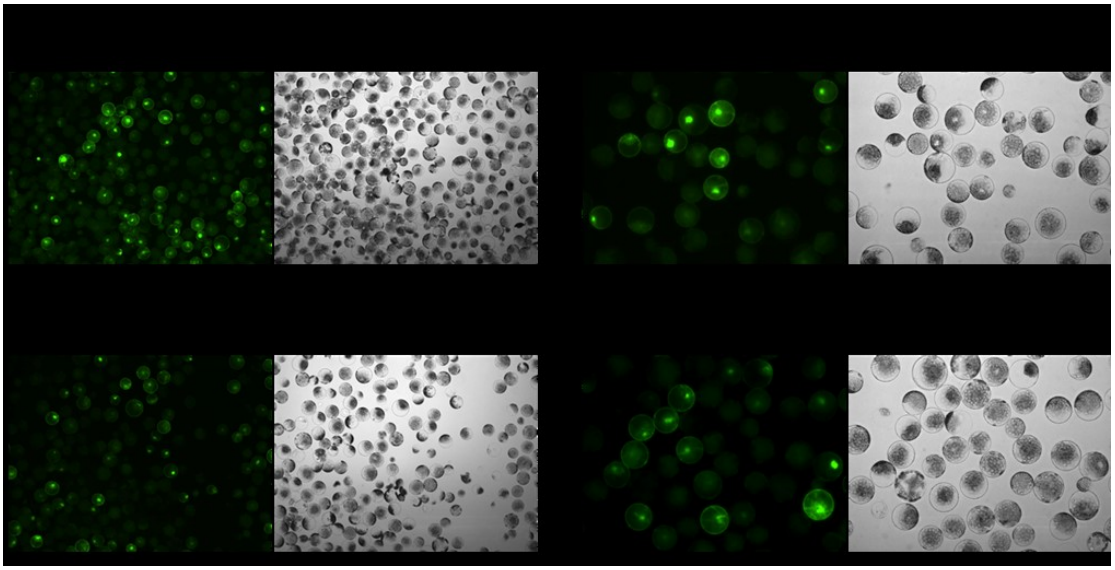


Figure 2.12: Mutation of the phospho-accepting residue of AHPs does not affect their interaction with the CRFs **(A)** AHP2 (D to E; activating mutation) interacts with CRF6, and **(B)** AHP2 (D to N; non-phosphorylatable version) also retains the ability to interact with CRF6, as well as ARR12 (not shown).

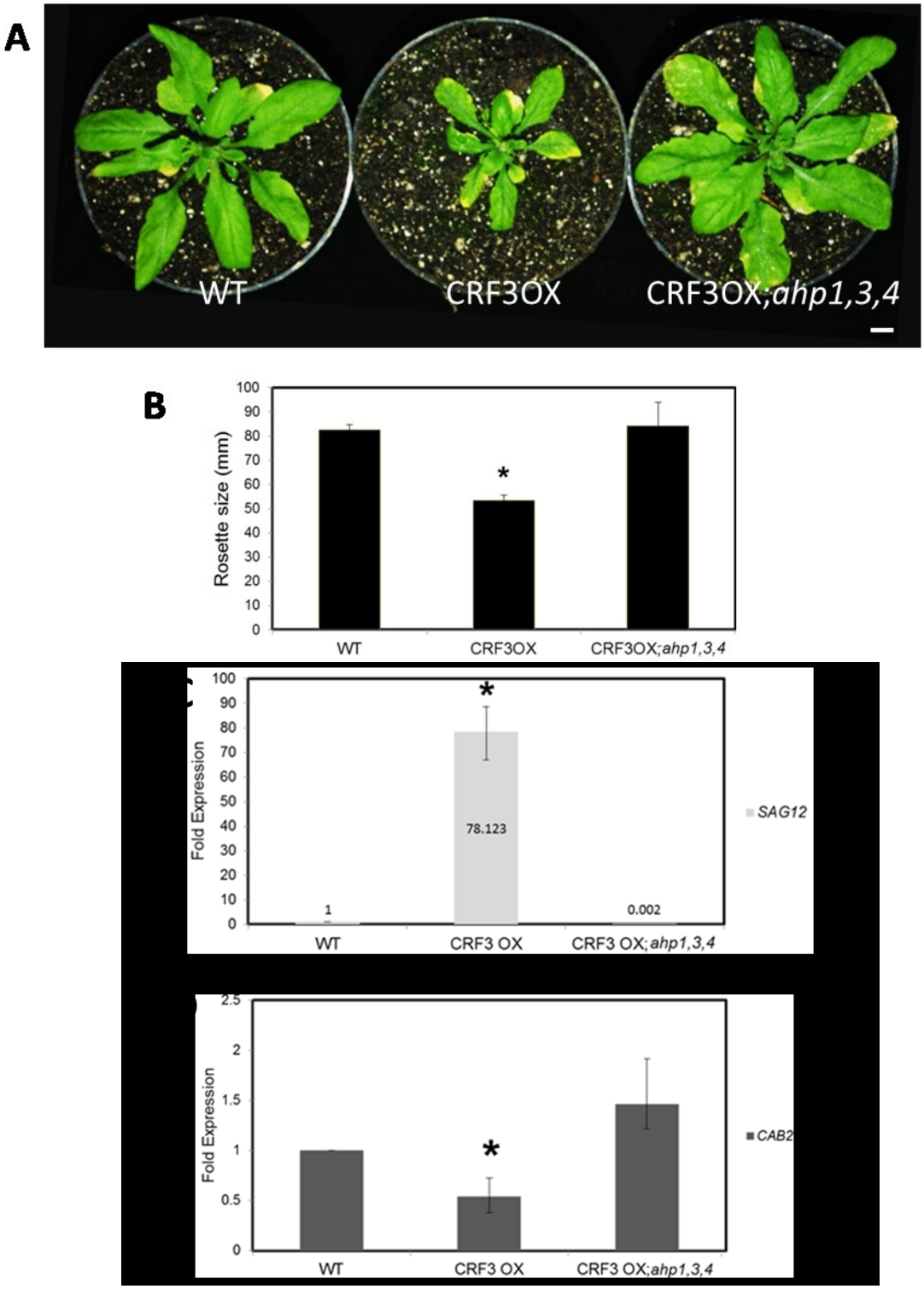
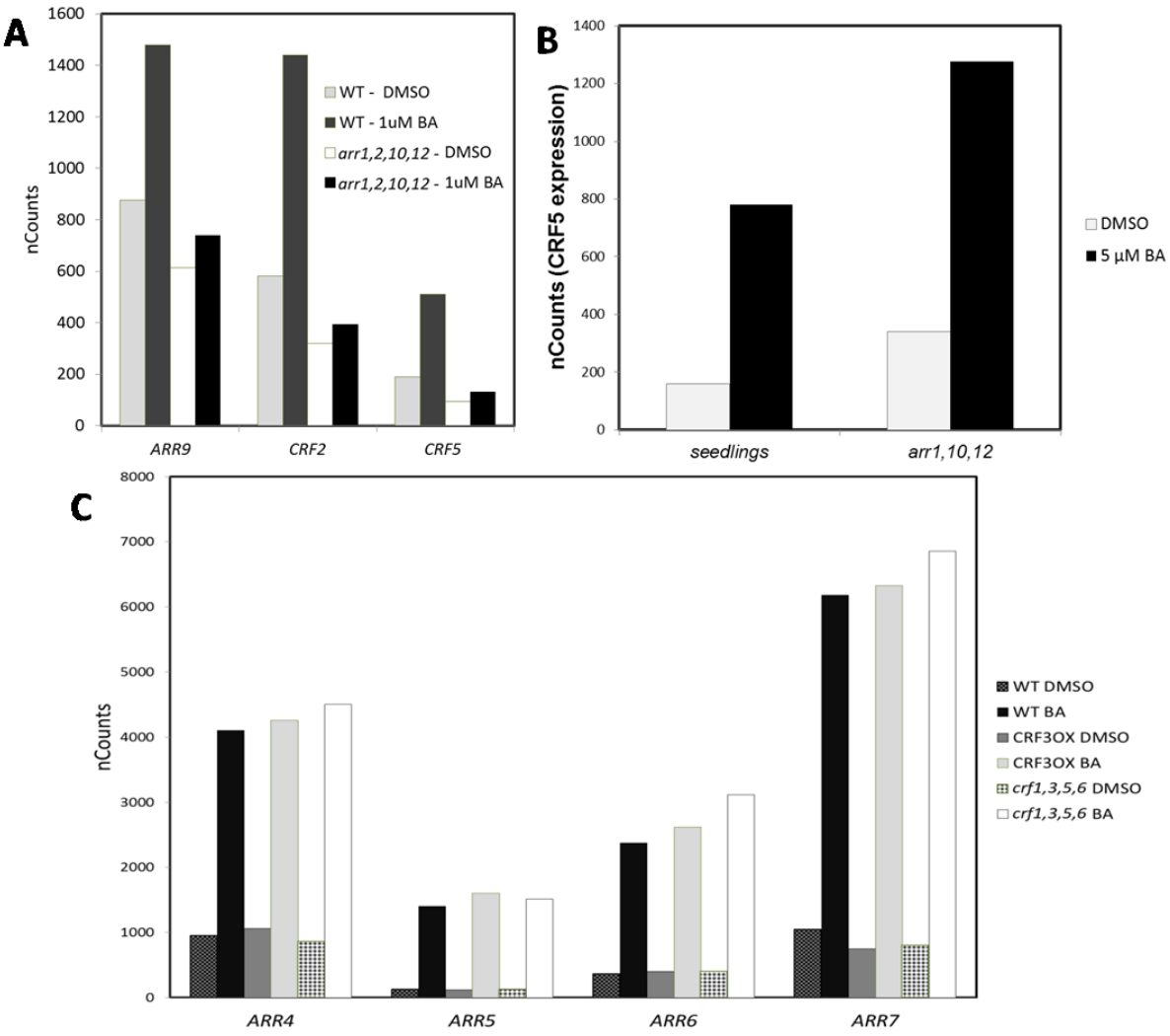
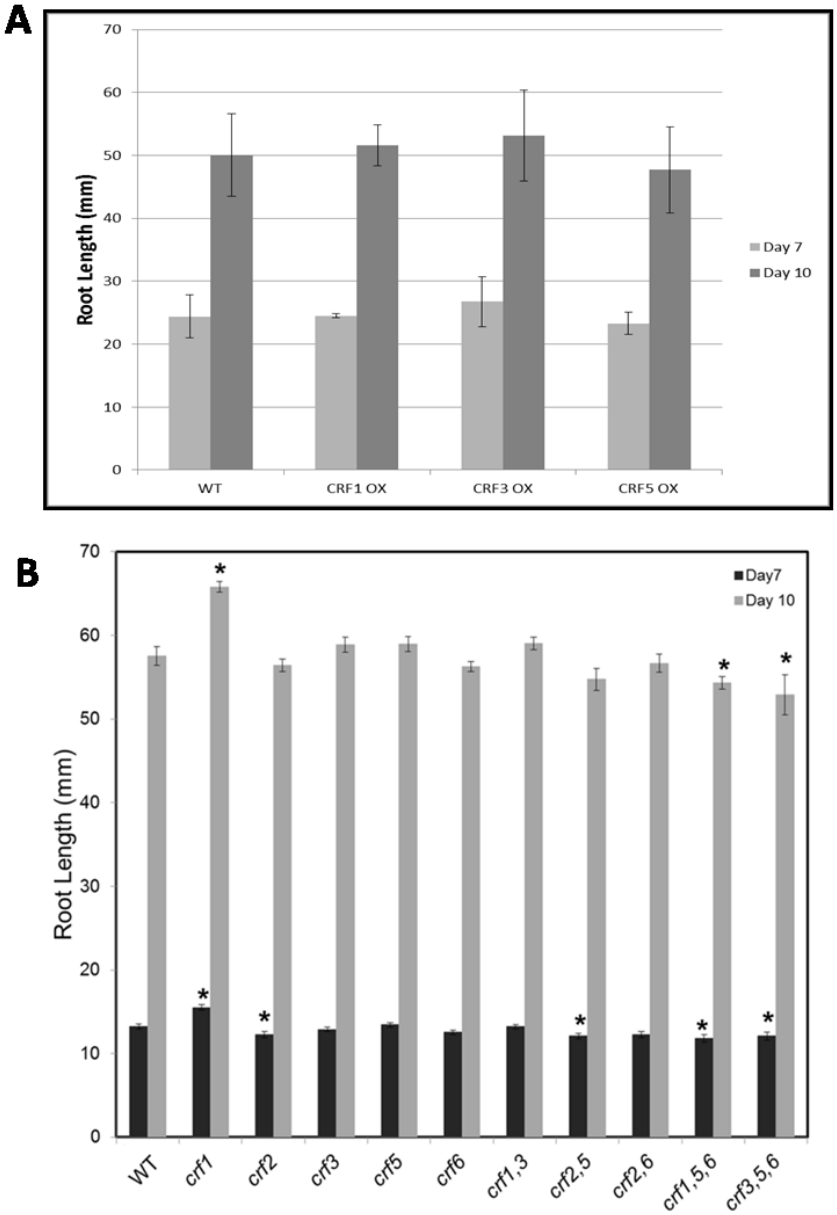


Figure 2.13: Phenotypes of CRF3OX are suppressed in *ahp1,3,4* mutant **(A)** Five-week-old plants show suppressed rosette phenotype. (scale = 10mm) **(B)** The small rosette size of the CRF3OX line is suppressed in the *ahp1,3,4* background. The *SAG12* levels are reduced **(C)** and *CAB2* levels increased **(D)** in the *ahp1,3,4* mutant, confirming the rate of senescence is suppressed.

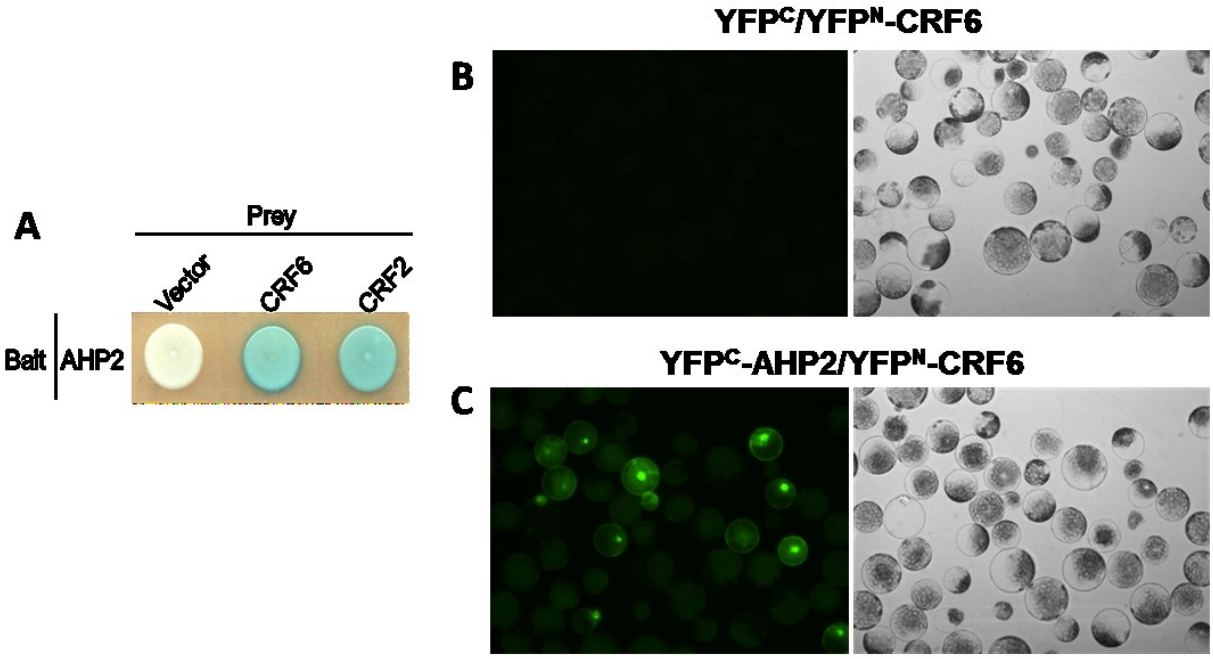


Supplementary Figure 2.1: *CRF*s are downstream of the Type-B *ARR*s and have no effect on Type-A *ARR* expression

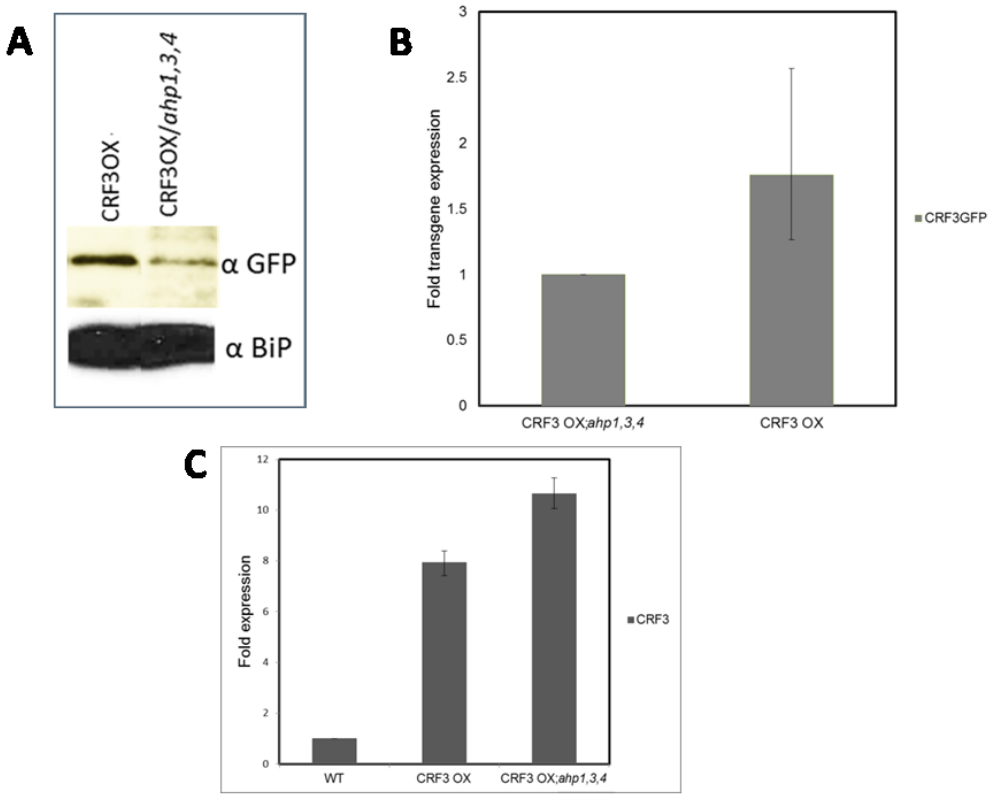
(A) NanoString® analysis confirms the induction of *CRF2* and *CRF5* is lost in a multiple type-B *arr* mutant, suggesting the CRFs play a role downstream of the primary cytokinin signaling pathway. **(B)** *CRF5* expression is greater in the type-A multiple mutant. **(C)** Induction of the type-A *ARR*s by cytokinin is not altered in the *CRF* overexpression or mutant lines (5 µM BA).



Supplementary Figure 2.2: Overexpression of *CRF* does not show altered root length and loss-of-function lines show variable primary root lengths (A) *CRF* overexpression lines at 7- and 10- dpg do not show significant alterations in root length. **(B)** Many single, double, and triple *crf* mutants do not show significant difference in root length as compared to wild type, however the triple mutants are significantly shorter. (*= pvalue ≤ 0.05)



Supplementary Figure 2.3: The CRFs interact with the AHPs
(A) Y2H shows positive interaction of AHP2 with CRF2 and CRF6. **(B)** Empty vector YFP^C does not interact with CRF6 fused to YFP^N. **(C)** BiFC shows AHP2 interaction with CRF5 in protoplasts.



Supplementary Figure 2.4: Levels of GFP tagged *CRF3* in the *ahp1,3,4* mutant are comparable to that of the *CRF3* overexpression line (A) Protein levels from plants expressing GFP tagged CRF3 **(B)** Transgene transcript levels from multiple mutant and overexpression line are comparable to one another. **(C)** Endogenous *CRF3* transcript levels compared to wild type show overexpression in both the CRF3 OX line as well as the CRF3 OX;*ahp1,3,4* line.

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CHAPTER 3: THE CRFs REGULATE ROOT APICAL MERISTEM SIZE

ABSTRACT

Cytokinin plays a role in maintaining the root apical meristem by promoting cell differentiation above the transition zone. Through members of the two-component signaling pathway, cytokinin function is restricted to the transition zone, which allows auxin to promote cell division. This results in a pool of cells that is available for new root growth. Plants harboring mutations in multiple *CRF*s have stunted root growth and a smaller root meristem. Conversely, overexpression of *CRF5* results in plants with a larger meristem compared to wild type. Analysis of gene expression in both *CRF* overexpression and loss-of-function lines revealed altered expression of several genes involved in regulated root apical meristem function. These results indicate that the CRFs are positive regulators of root meristem size in Arabidopsis, likely by negatively regulating cytokinin responsiveness which leads to alterations in the altering expression of genes involved in meristem function.

INTRODUCTION

The root apical meristem (RAM) is the source of all postembryonic root growth. The meristem must balance the rate of cell division with the rate of cell differentiation for proper root development. The maintenance of the root meristem

requires tight control of cytokinin and auxin signaling, which play antagonistic roles within the root tip [1, 2]. Cytokinin negatively regulates the size of the RAM. Lowering endogenous cytokinin levels leads to an increase in RAM size [2], while increased cytokinin levels or sensitivity causes a reduction in the size of the RAM [3]. Expression analyses of cytokinin oxidases in different domains of the RAM revealed that cytokinin acts primarily within the root transition zone, where it promotes cell differentiation and thus decreases the number of cells in the meristematic zone [3]. An auxin signaling repressor, *SHY2/IAA3*, controls the rate of cell division versus cell differentiation by regulating the balance between cytokinin and auxin function [3]. *SHY2* expression is induced by cytokinin through the type-B response regulators, *ARR12* (during the meristem growth phase) and *ARR1* (five days after germination to maintain the meristem size). The elevated *SHY2* levels in the transition zone of the meristem results in reduced expression of the PIN auxin transporters and a reduced amount of cell differentiation [2, 4]. Chromatin immunoprecipitation experiments show th*at SHY2* is a direct target of *ARR1*. The expression of an ARR1 protein with an inactive phospho-accepting domain does not enhance the large meristem size of *shy2*, indicating that *SHY2* acts downstream of *ARR1* [2]. Similarly, *ARR12* is required to activate *SHY2* during early meristem

growth, which was determined by the lack of SHY2:GUS expression for the first five days after germination in an *arr12* mutant line [4].

Here, we show that the CRFs are involved in regulating meristem size and maintenance. *CRF* overexpression lines have a larger meristem than wild type. Conversely, *crf* mutants have a smaller meristem and shorter roots. These data and the evidence that genes involved in regulating meristem size and function are altered within the root tip of *crf* mutants, suggest that the CRFs act to promote cell division and meristem size in the root tip.

RESULTS

CRFs regulate the size of the RAM

As shown in Chapter 2, the roots of *crf* mutants are shorter than wild type (Chapter 2, Figure 2.5A). The triple mutant *crf2,5,6* as well as the quadruple mutant *crf1,3,5,6* both displayed reduced primary root growth. In the case of the *crf1,3,5,6* mutant, the root length is severely stunted at 10 days-past-germination and the total length is merely 30% of wild type at the same stage (Chapter 2, Figure 2.5A). To assess whether the reduced length is attributed to a defect in meristem function, we measured the size of the meristematic zone at 5 days post-germination. To analyze the size, we counted the number of cortical cells in a file from the quiescent center to the first elongated cell in the transition zone [5]. In the quadruple *crf1,3,5,6* mutant, the meristem size was smaller than wild type at day five, as expected by the shorter root length. The *CRF5* overexpression line has a larger meristem (Figure 3.1A and B), but no significant difference was observed in the *CRF3* overexpression line.

Although not as severe as the quadruple *crf* mutant, the *crf2,5,6* mutant also had a reduced meristem size at day five (Figure 3.1 B).

Cytokinin treatment inhibits cell division, resulting in a smaller RAM size. We evaluated the root length of the *crf* LOF mutant and overexpression lines in response to exogenous cytokinin. As expected, wild-type roots were substantially shorter and had a smaller RAM when grown on cytokinin (Figure 3.1B). Interestingly, meristem size in the *CRF5* overexpression mutant line had the highest percentage decrease in response to cytokinin. Although the meristem was larger in the absence of exogenous cytokinin, the size of CRF5OX treated with cytokinin was 49% in comparison to the control, which was a larger reduction than observed in wild type (~40% reduction), suggesting CRFs are more sensitive to cytokinin treatment (Figure 3.1B and C). Conversely, the quadruple *crf* mutant was partially insensitive to cytokinin, showing a 30% reduction in RAM in response to cytokinin. This suggests that the CRFs are redundant positive regulators of cell division or negative regulators of cell differentiation in the meristem.

Gene expression changes in crf mutant root tips

Many genes that are important for controlling RAM function and maintenance have previously been identified. The CRFs play a role in this process, thus we analyzed gene expression changes for many of these genes in the root tips of *crf* mutants to assess if their expression levels are altered in response to changes in CRF function. As previously described in Chapter 2, we used NanoString® technology to determine transcript levels for a subset of RAM-associated genes in

the root tip. Specifically, members of the *CYCD3* family of genes showed expression changes in the *crf* mutants. In plants, the *CYCD* genes play an important role in the decision of mitotic cells to enter the cell cycle [6]. In late G1 phase, the CYCDs interact with CDKs which phosphorylate the retinoblastoma-related protein (pRBR). This phosphorylation inactivates it, releasing the E2F transcription factors and allowing transcription of genes required for entry to the S-phase [7]. In the *crf* mutants, the levels of *CYCD3;2* and *CYCD6;1* were surprisingly induced in comparison to wild-type root tips (Figure 3.2). There was no change in the expression of the *CYCD3;2* in the *CRF5* overexpression line. However, there was a correlative reduction of *CYCD6;1* transcript levels.

The expression of *WOX5*, a major regulator of stem cell activity, is known to be restricted to the quiescent center of the RAM. *WOX5* is responsible for maintaining the pluripotency of the surrounding cells. Auxin is also important in repressing *WOX5* expression outside of the QC [8]. In the *crf1,3,5,6* mutant, *WOX5* transcript levels were elevated more than 2-fold in comparison to wild type (Figure 3.3). Conversely, *WOX5* expression was lower in the *CRF5* overexpression line. WOX5 represses cell division through the regulation of the *PLETHORA (PLT)* family of genes in a cell non-autonomous manner. Among the *PLT* genes analyzed, *PLT3* was elevated 2.6-fold in the *crf* quadruple mutant line. By contrast, *PLT3* transcript levels were not increased (< 1.5-fold) in the CRF5OX line.

We examined *SHY2* expression in the root tips of *crf* mutants as it is known to play an important role in the regulation of auxin and cytokinin function in the root tip. In the quadruple *crf1,3,5,6* mutant, the transcript level of *SHY2* was decreased in

comparison to wild type. *SHY2* levels were also decreased in the *CRF5* overexpression line, but to a lesser extent as compared to the *crf1,3,5,6* mutant (Figure 3.3).

Proper root patterning depends upon the distribution of auxin within the appropriate root tissues. The polar auxin transport genes (*PIN*s) along with the auxin influx carriers, *AUX1* and *LAX2*, are primarily responsible for generating auxin gradients within the root tissues [9]. The *PINOID (PID)* genes are serine/threonine kinases that regulate subcellular distribution of PIN proteins [13]. We examined the expression levels of genes involved in auxin transport in the root tips in response to altered CRF function. No substantial change (> 1.5-fold) was seen in the expression level of the *PIN* transporters in either the mutant or overexpressing *CRF* plants (Figure 3.3). However, the levels of *PID* were reduced in the overexpression line and the level of *PID2* increased in the quadruple mutant (Figure 3.4). The *LAX2* gene also had altered expression, which was increased 1.7 fold over wild type in *crf1,3,5,6.*

DISCUSSION

Control of the root apical meristem (RAM) size is dependent on the tight control of auxin and cytokinin signaling in the appropriate zones of the root tip [2]. Cytokinin promotes cell differentiation while auxin promotes cell division [3]. Therefore, genes that control hormone signaling and transport must be highly regulated to maintain this balance. Cytokinin regulates the size of the meristem, at least in part, through induction of the *Aux/IAA* genes (known to be negative

regulators of auxin signaling) and *SHY2.* This induction occurs through the positive cytokinin signaling elements, *ARR1* and *ARR12* and subsequently causes reduced auxin transport in the transition zone [2]. Therefore, meristem size is altered by changes in gene expression that leads to altered auxin and cytokinin signaling.

Another hormone that plays a role in root meristem size and maintenance is giberellic acid (GA). Early in root development, high levels of gibberellin repress the type-B response regulator, *ARR1*, through repression of *REPRESSOR OF GA (RGA)*, but have no effect on the expression of *ARR12* [3]. This repression results in a lower cytokinin function in the root tip during early development, allowing the meristem to grow to the appropriate size. *ARR12* induces *SHY2* in early root development to promote meristem growth resulting in a higher high cell division to differentiation ratio [3]. Five days after germination, GA levels decrease and releases *ARR1* repression. This event allows for the appropriate ratio of cell division to cell differentiation during root growth. *CRF1* and *CRF2* both directly interact with ARR12 and weakly with ARR1, via yeast two hybrid and Bimolecular Fluorescent Complementation (BiFC) assays [10]. The interaction of the CRFs with ARR1 and ARR12, along with the altered meristem size, suggests that the CRFs also play a role in meristem size determination.

To analyze the effect of the CRFs on cell division, we examined expression of a subset of cyclin genes that are known to be responsive to cytokinin. In the *crf1,3,5,6* mutant, there were changes in the expression of *CYCD* family of cyclins (consisting of *CYCD1* to *CYCD4*). This family of cyclins is responsible for mediating cell division in response to external stimuli, along with the *CYCD3* genes that are

regulated by cytokinin [11]. Given the fact that the *crf1,3,5,6* mutant has a smaller meristem and reduced root length, it is surprising to find a subset of these cyclin genes up-regulated in the root tip. These results suggest either the levels of *CYCD* do not significantly impact cell division in the root tip, or the effects of the elevated *CYCD* levels are offset by other processes.

Loss of *WOX5*, a homeobox domain transcription factor that is a master regulator of stem cell function, causes differentiation of stem cells in the distal stem cell niche [12]. *WOX5* is repressed by the auxin response factors, *ARF10* and *ARF16*, which restrict *WOX5* expression to the distal stem cells (DSC) [8]. The AP2 transcription factor family of *PLETHORA* (*PLT*) genes is also involved in stem cell maintenance and is regulated by *WOX5* to inhibit differentiation of DSC [8]. *WOX5* is also induced by cytokinin. In the *crf1,3,5,6* mutant, *WOX5* and *PLT3* levels were increased, suggesting that *crf* LOF represses the expression of these genes either directly, or through altered auxin or cytokinin function.

SHY2 is a transcription factor that inhibits auxin response in the transition zone of the root. Cytokinin induces *SHY2* through the type-B ARRs, *ARR1* and *ARR12* [2]. It is probable that the CRFs promote *SHY2* expression by interacting with type-B ARRs. Therefore, removing the CRFs from the plant may release this repression of auxin signaling. The *crf1,3,5,6* mutant results in lower expression of *SHY2* in the root tip, which suggests that CRFs positively regulate *SHY2*. However, these results are contradictory given the short root phenotype of the quadruple mutant does not correlate with the lower *SHY2* expression observed. Additionally, we see a slight reduction in *SHY2* expression in the *CRF5* overexpression line. This

suggests that the effects on *SHY2* expression are secondary and downstream of CRF function.

Similarly, we looked at expression of auxin influx and efflux transport genes. The expression domains and levels of the PINs directly correlate with auxin function within the plant tissues [9, 32]. We did not observe significant change in the expression of *PIN* efflux carriers in either a gain or loss-of-function CRF lines. However, we saw a decrease in the amount of *PID* expression in the *CRF5* overexpression line. PID and PID2 promote apical recycling and endocytosis of the PINs [13]. Overexpression of *PID* increases PIN apicalization within the cells and results in frequent instances of root meristem collapse [14]. The decrease in PID expression observed in the CRF5OX line may also cause less apicalization of auxin efflux and higher auxin maximum in the root tip. This may explain the larger meristem observed in the CRF5OX lines. Similarly, the higher levels of PID2 in the *crf1,3,5,6* root tip may result in more apically localized PINs and increase the rate of PIN recycling. This would result in a decreased auxin maxima and increased cell differentiation. To confirm this hypothesis, we will need to visualize GFP tagged PINs and/or the *DR5:GFP* reporter in the *crf1,3,5,6* line to identify changes in localization within the root tip.

The *AUX1/LAX* gene family is important for proper auxin influx and has distinct expression and regulatory patterns [15]. *LAX1* and *LAX3* are induced by auxin, while *LAX2* is induced by cytokinin in the root tip [8]. LAX1 is not expressed within the root meristem zone. LAX2 is expressed primarily in the meristematic zone of the RAM while LAX3 is found in the QC and above the meristematic tissue in the

root [15]. LAX2 and LAX3 have complementary expression patterns surrounding the root primordia, and LAX3 promotes lateral root development [16]. In *crf1,3,5,6,* we observed a subtle increase in the levels of *LAX2* but a decrease in *LAX3*. As expected, we observed fewer lateral roots in the *crf1,3,5,6* line (Chapter 2, Figure 2.5A) which could be due to the reduction of *LAX3*.

Because many of the genes that are regulated by cytokinin in the root tip are highly induced in the *crf1,3,5,6* mutant, results from the gene expression studies suggest the CRFs may negatively regulate cytokinin function in the root tip. Additionally, auxin-related genes are reduced in loss-of-function *crf* lines. In many instances, the gene expression changes do not directly correlate with the meristem phenotype observed in the *crf* LOF mutants, for instance, higher *CYCD* expression, but smaller meristem size. However, the network controlling gene expression and hormonal balance in the root apical meristem is complex and therefore, alterations of CRFs may lead to complex changes. More studies will be needed to identify the direct targets of the CRFs that control meristem maintenance and whether they play a role in mediating the effects of cytokinin signaling.

MATERIALS AND METHODS

Plant growth

Seeds were sterilized as previously described in Chapter 2. All lines were grown on 1X MS agar with 1% sucrose, supplemented with the indicated level of the synthetic cytokinin, benzyladenine (BA) or a DMSO vehicle for control. Plates were grown vertically in 24-hour light regime at 22ºC.

Meristem size analysis

Seedlings were cleared by fixing in Carnoy's solution for 15 minutes, washed in 70% ethanol for 10 minutes, rinsed with water/ 0.1% Tween-20 for 10 minutes, then fixed on microscope slide with chloral hydrate solution (2g/750ul dissolved in water). Meristem size was calculated as described in [5].

Gene expression analysis

The 0.5 mm root tips from 20 plants were collected by dissection under a dissecting microscope. Total RNA was extracted using an RNeasy Plus kit (Qiagen, http://www.qiagen.com/) and was analyzed using NanoString® technology previously described in Chapter 2.

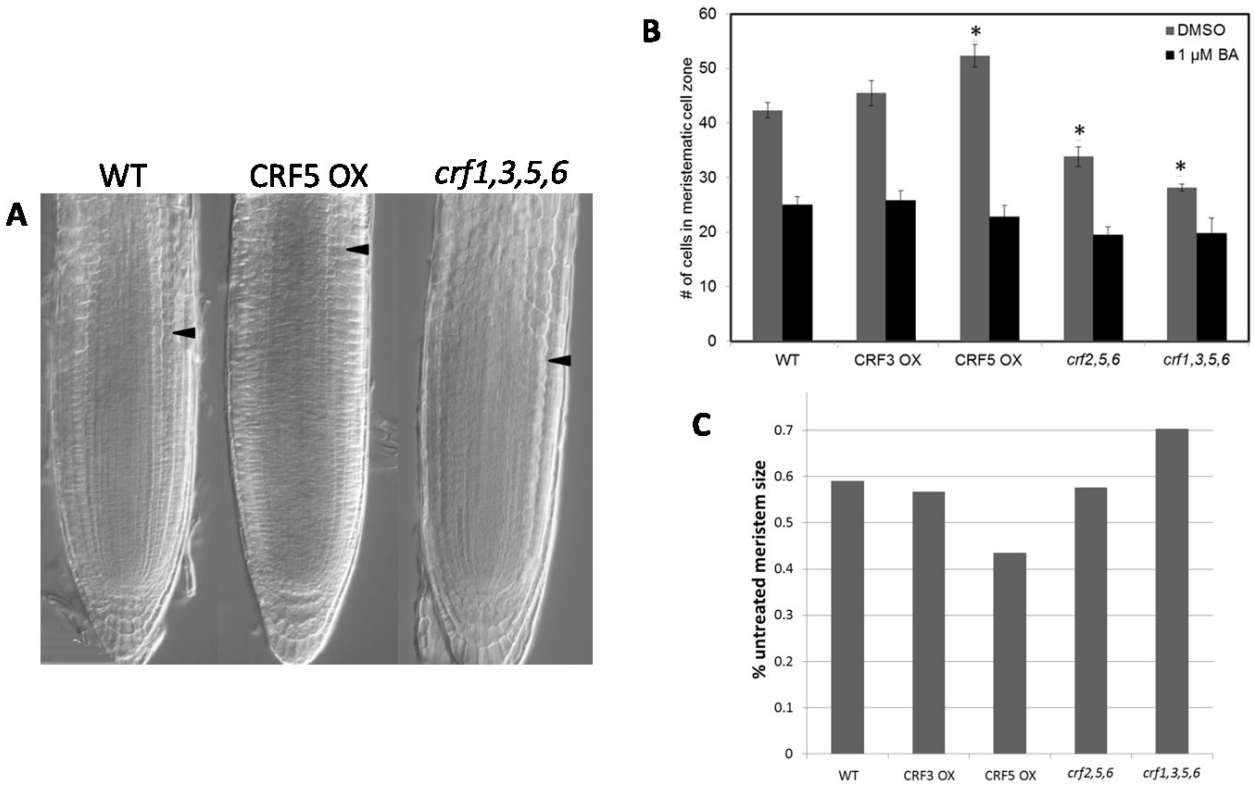

Figure 3.1: The *crf* mutants have smaller meristems that are partially insensitive to cytokinin (A) Meristems of 5- day old CRF mutant and overexpression lines. The arrowhead represents the last cell in the meristem cell file. **(B)** The number of cells in the meristematic zone at 5 dpg grown on both DMSO and 1 µM BA **(C)** Percent inhibition of cell division in the meristematic zone by growth on 1 µM BA. (*= pvalue ≤ 0.05)

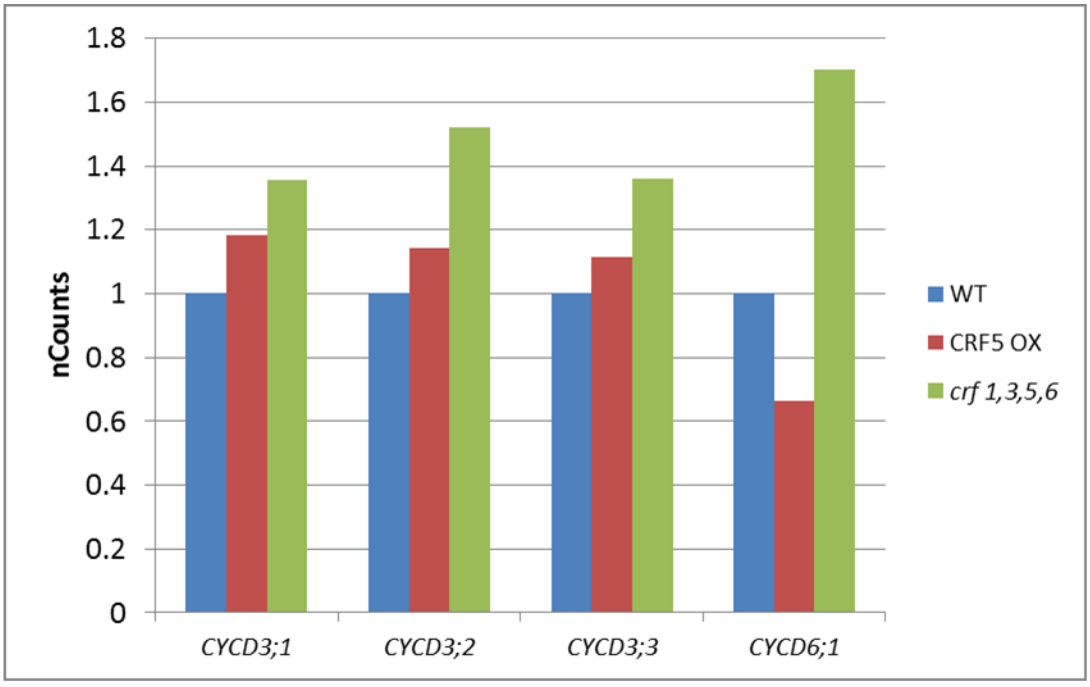

Figure 3.2 – Expression of cyclin genes in the root tip – NanoString® analysis was performed to assess cyclin related gene transcript levels in the *crf1,3,5,6* and *CRF5* overexpression line compared with wild-type root tips. Data are a representation of one replicate only.

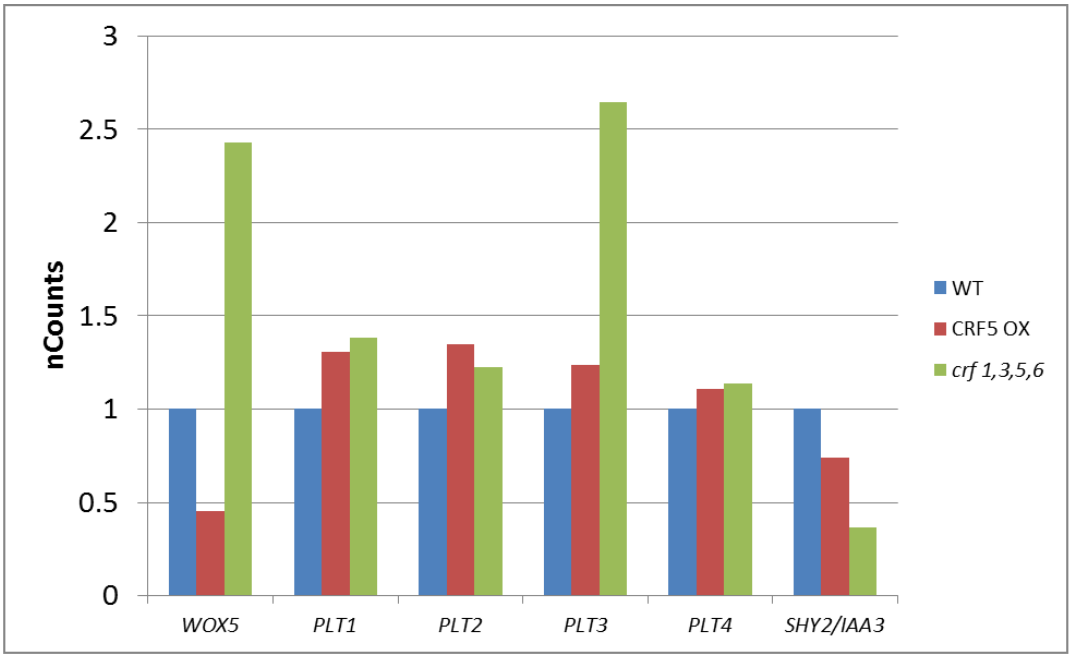

Figure 3.3 – Expression of genes involved in stem cell maintenance in the root tip – NanoString® analysis was performed to assess gene transcript levels of known regulators of stem cell function in the *crf1,3,5,6* and *CRF5* overexpression line compared to wild-type root tips. Data are a representation of one replicate only.

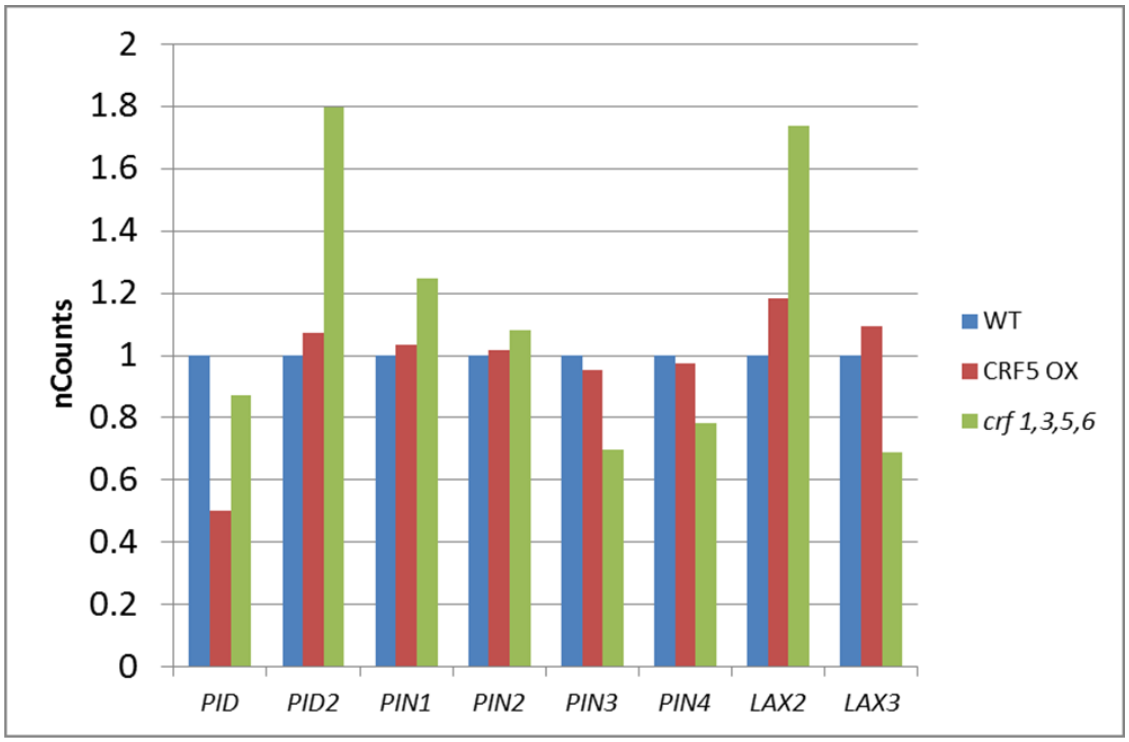

Figure 3.4 – Expression of auxin transport related genes in the root tip – NanoString® analysis was performed to assess auxin transport related gene expression in the *crf1,3,5,6* and *CRF5* overexpression line compared to wild-type root tips. Data are a representation of one replicate only.

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CHAPTER 4: IDENTIFYING DOWNSTREAM TARGETS AND BINDING SITES OF THE *CRF* FAMILY OF TRANSCRIPTION FACTORS

ABSTRACT

The *CRF*s are a family of transcription factors, of which a subset is induced in response to cytokinin. However, the direct targets of the CRFs and the specific processes which they regulate remain unknown. To help uncover possible targets and identify *cis*- elements preferentially bound by CRF5, we carried out a protein binding microarray. Additionally, we performed a microarray analysis on cytokinin−treated *crf1,3,5,6* seedlings and identified changes in gene regulation compared to wild-type seedlings. By surveying groups of regulated genes based on their gene ontology, we gained insight into the downstream responses that the CRFs influence both directly and indirectly.

INTRODUCTION

Transcription factors are responsible for the regulation of gene expression. Cytokinin plays a major role in regulating proper development by activating downstream genes and altering the function of transcription factors. While the type-B ARRs act as the primary response factors in the cytokinin signaling pathway, there

are also numerous genes induced by cytokinin which are classified as transcription factors. This suggests that the downstream response of cytokinin signaling consists of a complex network of gene activation and repression events. Several labs, including our own, have conducted microarray analyses of cytokinin-treated seedlings and identified multiple transcription factors that are regulated by cytokinin [1, 2, 3]. Among these, a subset of AP2 transcription factors known as the *Cytokinin Response Factors* (*CRF*s) is implicated in gene regulation through cytokinin signaling. AP2/ERF proteins comprise one of the largest families of transcription factors in plants and are defined by the presence of an AP2 DNA binding domain of approximately 68 amino acids [4,6]. Members possessing two AP2 domains are often involved in plant development. This includes APETALA2, which is involved in floral meristem maintenance, and AINTEGUMENTA, which plays a role in ovule development [4]. The ERF- like family of genes, which include the *CRF*s, contains only one AP2 domain, and is linked to biotic stress. The CRFs consist of six core family genes, three of which are transcriptionally induced by cytokinin [5]. Additionally, the cytokinin inducible expression of the CRF genes is compromised in an *arr1,12* mutant, placing this induction downstream of the type-B ARRs [5]. Together, these data suggest the CRFs play a role in controlling the transcriptional response through their interaction with the type-B ARRs. To unravel the complex network by which cytokinin regulates growth and development, we sought to uncover direct targets of the CRFs using transcriptome analyses and protein binding microarray technologies.

The regulation of transcription depends on the ability of transcription factors to recognize and bind to specific sequences in the regulatory regions of their target genes. These target genes commonly share 5-6 bp *cis*-regulatory sequences, making it possible to predict binding by scanning upstream regions of genes. Protein binding microarray 11 (PBM11) is a tool used to probe the binding sites for transcription factors. The array contains all possible 11-mer combinations, resulting in the occurrence of every palindromic 6-mer in 2000 oligonucleotide probes and every non-palindromic 6-mer in approximately 4000 probes [8]. Every palindromic 8-mer is found in 120 probes and non-palindromic 8-mers in 250 probes, thus allowing for greater statistical power by testing binding repetitively throughout the array.

Here we analyzed gene expression in a *crf* mutant using the Affymetrix microarray technology. We also defined the *cis*-regulatory elements associated with CRF binding, including both high and low affinity sequences. Together, these data shed light on CRF target genes.

RESULTS

Protein binding microarray analysis to determine CRF binding site

As discussed in Chapter 2, in order to identify the preferred promoter-binding motif recognized by the CRFs, we cloned the coding regions of *CRF5* into an *E. coli* expression construct with a C-terminal maltose binding protein tag. The protein was visualized on a 12% SDS/PAGE gel for expression level and correct size (Figure 4.1). To find the preferred binding elements, the pelleted cell culture was used for the analysis of binding to the PBM11 array. Similar to other AP2/ERF transcription

factors, CRF5 was found to bind the GCC box with high affinity (Chapter 2, Figure 2.4).

Microarray analysis of gene expression changes in crf1,3,5,6

To assess global changes of gene expression in response to lower levels of CRF proteins, we treated ten-day-old wild type and *crf1,3,5,6* seedlings with either BA (cytokinin) or DMSO (mock) in duplicate. The cDNA from the treated tissues was hybridized to an Affymetrix chip containing the most complete set of gene specific oligonucleotides available for the Arabidopsis genome. The chips were visualized using a scanning laser to excite the immobilized fluorescently labeled oligos and the intensity of the fluorescence directly correlated to the expression level. Using GeneSpring software, we analyzed the expression levels of genes from the microarray to identify genes whose expression was altered in the *crf* mutant.

We first compared gene expression in wild-type and *crf1,3,5,6* seedlings in the absence of cytokinin (Table 4.1 A and B). We found 111 genes that were differentially expressed in 10-day-old mutant seedlings compared to wild-type seedlings (p-value ≤ 0.05). We scanned the upstream regions of these uniquely regulated genes for the presence of the GCC motifs that are preferentially bound by CRF5. Compared to the expected frequency of the motifs in the entire genome by chance, we found an enrichment of the 6-mers, GCCGCC and GCCGGC, as well as the 7-mer, AGCCGCC (Figure 4.2).

In this list of basally regulated genes, we did not observe an overabundance of cytokinin responsive genes or genes encoding members of the cytokinin signaling

pathway. Using the TAIR Gene Ontology (GO) tool, we categorized the CRF-regulated genes into groups based upon their biological function. The percentage of genes in a group was compared to the percentage of the whole genome that appears in a particular group, to determine if a process is overrepresented in the *crf* mutant (Figure 4.3). Several biological processes differed in their representation in the mutant line. Perhaps most notable was the mis-regulation of defense and stress related response genes. There is also a reduction in the number of genes affiliated with transcription, suggesting the transcriptional cascade initiated by the CRFs might be interrupted in the mutant.

We also assessed gene expression of *crf* mutant seedlings treated with cytokinin compared with wild type. Out of the 295 genes found to be regulated by cytokinin in *crf1,3,5,6,* only 138 overlapped with those also regulated in wild type (Figure 4.4A). Of the 157 genes found to be uniquely induced or repressed by cytokinin in the *crf1,3,5,6*, we did not observe an overrepresentation of cytokinin-related genes (Table 4.2 A and B). However, there was a large fraction of genes found to be differentially expressed in wild type that were not found in *crf1,3,5,6,* suggesting the CRFs play a role in the regulation of these genes (Figure 4.4A). Similar to basally regulated genes in *crf1,3,5,6* seedlings (Figure 4.2), we also found an enrichment of the 6-mer, GCCGCC, as well as the 7-mer, AGCCGCC (Figure 4.4 B). By grouping the genes according to their biological function, we see the percentage of *crf1,3,5,6* regulated genes correlated to a particular biological process (Figure 4.5). However, in this case, we don't see an overrepresentation of genes belonging to a particular function mis-regulated in the *crf1,3,5,6*. There is a large

increase in the number of genes belonging to the “other biological processes” group, but further investigation revealed that these genes do not belong to one related group and therefore most likely do not represent a single particular process changed in the mutant line. The “response to stress” group remains higher in representation than in the whole genome, similar to the basal levels in *crf1,3,5,6,* further confirming the CRFs play a role in this process.

There were 144 genes regulated by cytokinin in the wild-type seedlings and not regulated in the mutant line (Figure 4.4A, Table 4.3 A and B). The fact that these genes are regulated in the wild-type plant but the regulation is absent in the *crf* mutant suggests that the CRFs may directly regulate the transcription of some of these genes in response to cytokinin. We scanned the upstream regions of these genes for GCC-box motifs and found high enrichment in many of the preferred CRF5 binding sites (Figure 4.6). These genes were also grouped according to their biological functions and once again, we saw a significant change in the number of genes related to response to stress (Figure 4.7).

Finally, we looked at expression level changes in genes that are regulated in both wild type and *crf1,3,5,6* seedlings in order to identify any changes in the degree of expression in the absence of *CRF*s. The majority of the shared genes were regulated to similar degrees in both wild-type and mutant lines compared to their DMSO treated tissues. The most highly induced genes are the type-A *ARR* genes. Several of these genes were induced to a slightly higher extent in the *crf1,3,5,6* mutant as compared to wild type, though the difference was subtle (Figure 4.8).

CRF5 induction was significantly compromised in the *crf1,3,5,6* mutant, confirming the presence of the T-DNA insertion at this locus (Figure 4.8).

Type-B ARR binding sites are located upstream of CRF genes

As it is known that cytokinin induces the expression of some of the *CRF* genes and that this induction is dependent on the presence of the type-B *ARR*s [5], we scanned the upstream regions of the *CRF*s to locate the presence of type-B ARR binding sites (Figure 4.9). We also used the AuxRE binding site (TGTCTC) as a negative control and the TATA box binding site (TTATTT), which is known to occur frequently in the upstream regions of genes, as a positive control. The *CRF*s which are most highly induced by cytokinin are *CRF2*, *CRF5* and *CRF6* (Chapter 2, Figure 2.1). These three genes have an overrepresentation of the type-B binding site upstream, confirming their regulation by the type-B response regulators. As expected, none of the genes have an overrepresentation of the AuxRE elements and most have an increased number of TATA box sites.

DISCUSSION

Using microarray data from cytokinin-treated *crf1,3,5,6* seedlings as well as results obtained by PBM11 identifying the preferential binding sites of CRF5, we obtained complementary datasets to identify potential genes regulated by the CRFs. The results of the PBM11 array for CRF5 show a preference for the classic GCC-box, GCCGCC. This is not surprising as the *CRF*s are members of the AP2/ERF transcription factor family of which several have been shown to bind the GCC-box

within the promoters of their targets [9,10]. CRF5 also binds with high affinity other variations of the GCC-box, both 6- and 7-mers.

Microarray analysis revealed the CRFs do not regulate the phosphorelay components of the cytokinin signaling pathway. In the absence of cytokinin, the CRFs show an abundance of stress-related genes altered in expression relative to wild-type seedlings, indicating that the CRFs play a role in the response to stress. Many of these genes also contained the GCC-box motifs within their upstream regions, indicating the CRFs may be directly regulating a subset of these mis-regulated genes. Because it has been shown that cytokinin is involved in responses to drought, salt stress, and pathogen infection [12, 13], it is not surprising to see the CRFs controlling gene expression related to stress response. The AP2/ERF family of transcription factors is also known to be closely involved in the response to many environmental stresses, including pathogen infection, salt stress, osmotic stress, wounding, drought, hypoxia, temperature stress and the stress-related hormones such as ethylene, jasmonic acid (JA) and abscisic acid (ABA) [9,10]. The CRFs have also been shown to be induced by salt treatment and are involved in leaf senescence (Chapter 2, Figure 2.11), further implicating their role in abiotic stress response [14, 15].

Treatment of the *crf* mutants with cytokinin results in regulation of three sets of genes: i) regulated by cytokinin only in the *crf* mutant, ii) regulated by cytokinin in both wild type and mutant and iii) genes not regulated in the *crf* mutant in response to cytokinin. The genes that were found to be regulated in the *crf1,3,5,6* contained an enrichment of the GCC-box motifs in their promoters, suggesting that CRFs

directly regulate their induction. The genes that are not regulated in *crf1,3,5,6* also contained an overrepresentation of the various GCC-box motifs bound by CRF5. As the induction of these genes is dependent on the CRFs, they are potential direct targets of the cytokinin-induced CRFs and when the CRFs are absent, their expression remains low.

There was some overlap in the number of genes regulated by cytokinin in the *crf1,3,5,6* mutant and wild-type seedlings and the fold-change in response to cytokinin of these shared genes was similar. All ten of the type-A ARRs, which are among the most highly induced genes by cytokinin [11], were induced in both wild type and mutant lines, but to a slightly higher degree in the *crf1,3,5,6* mutant line, suggesting the CRFs may suppress expression of the type-A *ARR*s. Moreover, we observed a significant increase in the induction of *ARR5* and decrease in the induction of ARR16 compared with wild type. Interestingly, the induction of the *AHK4* receptor in *crf1,3,5,6* is increased. The CRFs could play a negative feedback role in cytokinin signaling by inhibiting the action of AHK4, and removal of the CRFs could allow AHK4 to become more active (Figure 4.10). The research presented here suggests that the CRFs are acting downstream of cytokinin perception to regulate the response to biotic and abiotic stress. They may also play a role in a negative feedback of the signaling pathway through suppression of *AHK4* in response to cytokinin, thus creating a negative feedback loop similar to the type-A ARRs.

MATERIALS AND METHODS

Microarray analysis

Ten-day-old seedlings of wild type and *crf1,3,5,6* were treated in liquid 1X MS with 1% sucrose containing DMSO or 5 μM BA. The seedlings were frozen in liquid nitrogen and RNA was extracted using the Qiagen RNAeasy kit (www.qiagen.com). RNA at a concentration of 83.7 ng/μl was sent to the Genomics Core Facility at UNC along with ATH1 expression chips from Affymetrix. Two replicates were carried out on each genotype and treatment.

Data analysis

Using GeneSpring software, we imported the raw CEL files and parsed data lists with *p* values of less than 0.05. Using the TOUCAN 2 regulatory sequence analysis software we were able to search regulated genes for upstream CRF5 binding *cis*-elements (http://homes.esat.kuleuven.be/~saerts/software/toucan.php). Gene lists compiled by GeneSpring were then grouped by biological function using the TAIR website tool to retrieve bulk gene functions. The percent of genes belonging to a category was compared to the percent of genes in the whole genome belonging to the category.

TABLE 4.1A – Genes differentially up-regulated between *crf1,3,5,6* and wild-type seedlings			
AGI	**Gene Name**	**Gene Description**	**Fold Change relative to WT**
AT4G33720	-	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein)	6.52
AT5G42600	MRN1	oxidosqualene synthase, crucial for growth and development.	5.40
AT3G59930	-	defensin-like (DEFL) family protein.	5.26
AT1G64590	-	NAD(P)-binding Rossmann-fold superfamily with oxidoreductase activity	4.65
AT5G47990	THAD	CYP705A family of cytochrome P450 enzymes, thalianol metabolism	4.40
AT5G48010	THAS	oxidosqualene cyclase involved in the biosynthesis of thalianol	4.28
AT5G47450	ATTIP2;3	transports ammonium (NH3) and methylammonium across the tonoplast membrane	3.35
AT5G23840	-	MD-2-related lipid recognition domain-containing protein	3.24
AT1G66800	-	similar to alcohol dehydrogenase of unknown physiological function	3.15
AT5G48000	THAH	CYP705A family of cytochrome P450 enzymes, thalianol metabolism	3.03
AT2G16460	-	protein of unknown function	2.99
AT4G12550	AIR1	activated by auxin treatment, lateral root development	2.94
AT5G42580	CYP705A12	member of the cytochrome P450 family	2.78
AT1G14120	-	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	2.65
AT5G12030	HSP17.6A	heat shock protein with chaperone activity that is induced by heat and osmotic stress	2.63
AT5G38020	-	encodes a protein whose sequence is similar to SAM:salicylic acid carboxyl methyltransferase	2.53
AT2G16005	-	MD-2-related lipid recognition domain-containing protein	2.50
AT1G34510	-	peroxidase superfamily protein	2.49
AT3G25820	ATTPS-CIN	monoterpene 1,8-cineole synthase, atTPS-Cin	2.48
AT3G01420	DIOX1	alpha-dioxygenase involved in protection against oxidative stress and cell death, induced by SA and oxidative stress	2.46
AT5G38030	-	MATE efflux family protein, response to nematode	2.45
AT4G14060	-	polyketide cyclase/dehydrase and lipid transport superfamily protein	2.42
AT3G45680	-	major facilitator superfamily protein, response to nematode	2.41
AT1G01190	CYP78A8	member of CYP78A	2.41
AT3G06390	-	uncharacterized protein family	2.39
AT3G26330	CYP71B37	putative cytochrome P450	2.38
AT5G09520	PELPK2	hydroxyproline-rich glycoprotein family protein, unknown function	2.33
AT3G26460	-	polyketide cyclase/dehydrase and lipid transport superfamily protein	2.30
AT5G47980	-	HXXXD-type acyl-transferase family protein	2.30
AT4G11310	CP1	cysteine proteinase precursor-like protein	2.30

AT3G44540	FAR4	generates the fatty alcohols found in root, seed coat, and wound-induced leaf tissue	2.28
AT2G01880	PAP7	protein serine/threonine phosphatase activity	2.26
AT3G09220	LAC7	putative laccase, unknown function	2.24
AT1G47480 /// AT2G05440 /// AT2G05510	-	alpha/beta-Hydrolases superfamily protein	2.23
AT2G14610	PR1	PR1 gene expression is induced in response to a variety of pathogens	2.22
AT1G14960	-	polyketide cyclase/dehydrase and lipid transport superfamily protein,, defense response	2.20
AT4G22212	-	encodes a defensin-like (DEFL) family protein	2.19
AT5G59090	ATSBT4.12	serine-type endopeptidase activity; proteolysis, negative regulation of catalytic activity	2.18
AT5G43520	-	cysteine/Histidine-rich C1 domain family protein	2.18
AT1G17190	ATGSTU26	glutathione transferase belonging to the tau class of GSTs	2.16
AT4G11210	-	disease resistance-responsive (dirigent-like protein) family protein	2.13
AT1G73330	DR4	plant-specific protease inhibitor-like protein, repressed by drought	2.12
AT4G23700	ATCHX17	member of Putative Na+/H+ antiporter family	2.12
AT2G24850	TAT3	tyrosine aminotransferase that is responsive to treatment with jasmonic acid	2.11
AT4G37410	CYP81F4	member of CYP81F	2.11
AT5G63560	FACT	HXXXD-type acyl-transferase family protein	2.09
AT3G45710	-	major facilitator superfamily protein, oligonucleotide transport	2.09
AT5G48570	ATFKBP65	carboxylate clamp (CC)-tetratricopeptide repeat (TPR) proteins with potential to interact with Hsp90/Hsp70 as co-chaperones.	2.08
AT3G25830	TPS-CIN	monoterpene 1,8-cineole synthase, atTPS-Cin	2.08
AT3G57010	-	calcium-dependent phosphotriesterase superfamily protein	2.08
AT5G37990	-	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	2.07
AT3G22570	-	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	2.05
AT4G13280	ATTPS12	catalyzes the conversion of farnesyl diphosphate to (Z)-gamma-bisabolene and the additional minor products E-nerolidol and alpha-bisabolol.	2.05
AT3G58550	-	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	2.05
AT2G35380	-	peroxidase superfamily protein, response to oxidative stress	2.03
AT5G04120	-	cofactor-dependent phosphoglycerate mutase (dPGM) - like protein with phosphoserine phosphatase activity that may be responsible for serine anabolism	2.03
AT4G29270	-	HAD superfamily, subfamily IIIB acid phosphatase	2.02
AT2G47180	ATGOLS1	galactinol synthase that catalyzes the formation of galactinol from UDP-galactose and myo-inositol,	2.02

		promotes increased tolerance to salt, chilling, and high-light stress	
AT5G23830	-	MD-2-related lipid recognition domain-containing protein	2.00
AT1G31710	-	copper amine oxidase family protein; oxidation reduction, amine metabolic process	2.00

TABLE 4.1B – Genes differentially down-regulated between *crf1,3,5,6* and wild-type seedlings			
AGI	**Gene Name**	**Gene Description**	**Fold change relative to WT**
AT5G53290	CRF3	AP2/ERF superfamily of the transcriptional factors; cytokinin responsive	-10.67
AT2G21640	-	protein of unknown function that is a marker for oxidative stress response.	-10.09
AT5G09570	-	Cox19-like CHCH family protein	-10.06
AT2G04050	-	MATE efflux family protein; transmembrane transporter activity	-6.09
AT2G41730	-	unknown protein	-5.82
AT2G29870	-	aquaporin-like superfamily protein	-5.62
AT4G33070	ATPDC1	thiamine pyrophosphate dependent pyruvate decarboxylase family protein	-5.14
AT1G77120	ADH1	catalyzes the reduction of acetaldehyde using NADH as reductant	-4.72
AT1G17180	GSTU25	glutathione transferase belonging to the tau class of GSTs	-4.47
AT3G61630	CRF6	AP2/ERF superfamily of the transcriptional factors; cytokinin responsive	-4.46
AT5G10040	-	unknown protein	-4.35
AT4G10270	-	wound-responsive family protein	-4.25
AT3G43190	ATSUS4	sucrose synthase activity	-4.21
AT1G33055	-	unknown protein	-4.13
AT5G62520	SRO5	role for the protein in ADP ribosylation.; up-regulated by NaCl	-3.90
AT2G03760	ATSOT1	brassinosteroid sulfotransferase; response to salicylic acid and methyl jasmonate and bacterial pathogens	-3.48
AT1G05680	UGT74E2	UDP-glucosyltransferase, UGT74E2, that acts on IBA (indole-3-butyric acid) and affects auxin homeostasis	-3.32
AT3G02550	LBD41	lateral organ boundaries	-3.21
AT3G10040	-	sequence-specific DNA binding transcription factors	-3.15
AT2G32020	-	acyl-CoA N-acyltransferases (NAT) superfamily protein; response to abscisic acid stimulus	-3.12
AT2G47520	ATERF71	ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family	-3.09
AT5G15120	-	protein of unknown function (DUF1637); cysteamine dioxygenase activity	-3.01
AT5G44120	CRU1	12S seed storage protein; phosphorylation state is modulated in response to ABA	-2.92
AT3G02480	-	late embryogenesis abundant protein (LEA) family protein	-2.80
AT4G39675	-	unknown protein	-2.78
AT5G39890	-	unknown function (DUF1637); cysteamine dioxygenase activity	-2.66
AT1G19530	-	unknown protein; N-terminal protein myristoylation	-2.59
AT2G07671	-	ATP synthase subunit C family protein; hydrogen ion transmembrane transporter activity	-2.56

AT1G43800	FTM1	stearoyl-acyl-carrier-protein desaturase family protein	-2.51
AT2G19970	-	CAP (Cysteine-rich secretory proteins, Antigen 5, and Pathogenesis-related 1 protein) superfamily protein	-2.43
AT2G16060	ARATH GLB1	class 1 nonsymbiotic hemoglobin induced by low oxygen levels with very high oxygen affinity	-2.43
AT2G19990	PR-1-LIKE	PR-1-like protein homolog that is differentially expressed in resistant cultivars by powdery mildew infection	-2.41
AT1G52070	-	mannose-binding lectin superfamily protein	-2.39
AT1G48130	ATPER1	encodes a protein similar to the 1-cysteine (1-Cys) peroxiredoxin family of antioxidants; seed only	-2.38
AT1G52690	LEA7	late embryogenesis abundant protein (LEA) family protein	-2.32
AT4G33560	-	wound-responsive family protein	-2.31
AT3G23170	-	unknown protein	-2.27
AT2G29330	TRI	tropinone reductase (TRI); oxidoreductase activity,	-2.21
AT1G02520 /// AT1G02530	ABCB11	encodes an ATP-binding cassette (ABC) transporter	-2.21
AT4G24110	-	unknown protein	-2.17
AT3G27220	-	galactose oxidase/kelch repeat superfamily protein	-2.17
AT5G40420	OLE2	oleosin2, a protein found in oil bodies, involved in seed lipid accumulation	-2.16
AT5G42800	DFR	dihydroflavonol reductase; biosynthesis of anthocyanin	-2.16
AT4G28520	CRU3	12S seed storage protein; phosphorylation state is modulated in response to ABA	-2.15
AT5G44730	-	haloacid dehalogenase-like hydrolase (HAD) superfamily protein	-2.15
AT1G72360	ERF73	member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family	-2.14
AT5G39110	-	RmlC-like cupins superfamily protein; manganese ion binding, nutrient reservoir activity	-2.08
AT1G76650	CML38	calcium ion binding; response to wounding	-2.06
AT4G17260	-	lactate/malate dehydrogenase family protein; responds to ABA	-2.05
AT1G52050	-	mannose-binding lectin superfamily protein	-2.04
AT2G43610	-	chitinase family protein; carbohydrate metabolic process, cell wall macromolecule catabolic process	-2.02

TABLE 4.2A – Genes up-regulated by cytokinin in the *crf1,3,5,6* mutant			
AGI	**Gene Name**	**Gene Description**	**Fold change relative to non-treated**
AT5G47990	THAD	member of the CYP705A family of cytochrome P450; mutants have longer roots and altered gravitropism	4.01
AT1G64590	-	NAD(P)-binding Rossmann-fold superfamily protein; oxidation reduction, metabolic process	3.97
AT3G45700	-	major facilitator superfamily protein; xylan biosynthesis	3.19
AT3G45710	-	major facilitator superfamily protein; xylan biosynthesis	2.96
AT4G27970	SLAH2	protein with ten predicted transmembrane helices; transmembrane transport	2.72
AT3G13790	ATBFRUCT1	protein with invertase activity	2.48
AT5G19260	FAF3	member of the FANTASTIC FOUR (FAF) family that have the potential to regulate shoot meristem size in *Arabidopsis thaliana*	2.43
AT4G02850	-	phenazine biosynthesis PhzC/PhzF family protein; cytokinin signaling related	2.43
AT1G73300 /// AT5G36180	SCPL2	serine carboxypeptidase-like 2 (scpl2)	2.43
AT1G28130	GH3.17	encodes an IAA-amido synthase that conjugates Asp and other amino acids to auxin in vitro	2.43
AT4G11210	-	disease resistance-responsive (dirigent-like protein) family protein	2.39
AT1G14960	-	polyketide cyclase/dehydrase and lipid transport superfamily protein; biotic stimulus, defense response	2.39
AT4G19030	NLM1	an aquaporin whose expression level is reduced by ABA, NaCl, dark, and dessication; involved in arsenite transport and tolerance	2.28
AT3G29250	ATSDR4	NAD(P)-binding Rossmann-fold superfamily protein; oxidoreductase activity, copper ion binding	2.25
AT2G29490	ATGSTU1	glutathione transferase belonging to the tau class of GSTs.	2.19
AT1G03850	ATGRXS13	glutaredoxin required to facilitate *Botrytis cinerea* infection of *Arabidopsis thaliana* plants	2.17
AT3G50700	AtIDD2	zinc finger protein, similar to maize Indeterminate1 (ID1)	2.12
AT5G46230	-	protein of unknown function, DUF538	2.10
AT1G79460	GA2	ent-kaurene synthase B activity which catalyzes the second step in the gibberellins biosynthetic pathway	2.09
AT2G32680	AtRLP23	receptor like protein 23 (RLP23); defense response, JA signaling related	2.08
AT4G29700	-	alkaline-phosphatase-like family protein; metabolism	2.05
AT1G21120	IGMT2	O-methyltransferase family protein	2.00
AT1G04360	-	RING/U-box superfamily protein; zinc ion binding	2.00
AT3G06020	FAF4	member of the FANTASTIC FOUR (FAF) family that have the potential to regulate shoot meristem size in *Arabidopsis thaliana*	1.90
AT5G26260	-	TRAF-like family protein	1.89
AT5G14230	-	ankyrin repeat-containing domain	1.87
AT3G16870	GATA17	GATA factor family of zinc finger transcription factors	1.87
AT3G13360	WIP3	outer nuclear membrane protein that anchors	1.85

		RanGAP1 to the nuclear envelope; required for maintaining the nuclear shape of epidermal cells	
AT4G15290	ATCSLB05	gene similar to cellulose synthase; root hair elongation	1.82
AT2G29970	SMXL7	member of an eight-gene family (SMAX1 and SMAX1-like) that has weak similarity to a ClpB chaperonin required for thermotolerance	1.80
AT5G61010	ATEXO70E2	member of EXO70 gene family, putative exocyst subunits, conserved in land plants; salacylic acid biosynthesis	1.79
AT3G17120	-	unknown protein; response to brassinosteroid stimulus	1.76
AT3G19270	CYP707A4	protein with ABA 8'-hydroxylase activity, involved in ABA catabolism	1.75
AT3G26960	-	Pollen Ole e 1 allergen and extensin family protein	1.72
AT2G38180	-	SGNH hydrolase-type esterase superfamily protein	1.71
AT1G75620	-	glyoxal oxidase-related protein	1.70
AT3G54950	PLA IIIA	member of the Group 3 patatin-related phospholipases	1.70
AT4G35510	-	unknown protein	1.69
AT2G39220	PLP6	PATATIN-like protein 6 (PLP6); nutrient reservoir activity	1.69
AT1G65510	-	unknown protein; N-terminal protein myristoylation;	1.66
AT1G75450	CKX5	similar to cytokinin oxidase/dehydrogenase, which catalyzes the degradation of cytokinins	1.66
AT3G54720	ATAMP1	glutamate carboxypeptidase; mutants show increased cytokinin biosynthesis; involved with ethylene mediated hypocotyl elongation in light	1.65
AT5G63380		peroxisomal protein involved in the activation of fatty acids through esterification with CoA; JA biosynthesis	1.65
AT3G44320	NIT3	catalyzes the hydrolysis of indole-3-acetonitrile (IAN) to indole-3-acetic acid (IAA) and IAN to indole-3-acetamide (IAM) at lower levels	1.64
AT5G55050	-	GDSL-like Lipase/Acylhydrolase superfamily protein; proline transport	1.62
AT5G10970	-	C2H2 and C2HC zinc fingers superfamily protein; transcription	1.62
AT5G24990 /// AT5G25020	MEB2	vacuolar iron transporter (VIT) family protein	1.61
AT2G24570	ATWRKY17	WRKY Transcription Factor; Group II-d; negative regulator of basal resistance to Pseudomonas syringae	1.61
AT5G07450	CYCP4;3	cyclin p4;3 (CYCP4;3); cyclin-like	1.61
AT5G22980	scpl47	serine-type carboxypeptidase activity	1.60
AT1G76410	ATL8	zinc ion binding	1.60
AT1G56430	NAS4	encodes a protein with nicotianamine synthase activity.	1.59
AT2G03730	ACR5	member of a small family of ACT domain containing proteins thought to be involved in amino acid binding.	1.59
AT5G54510	DFL1	DWARF IN LIGHT 1; IAA-amido synthase that conjugates Ala, Asp, Phe, and Trp to auxin; overexpression leads to auxin hypersensitivity	1.58
AT2G28250	NCRK	erine/threonine-protein kinase	1.58
AT4G12440	APT4	adenine phosphoribosyl transferase 4	1.57
AT1G74790	-	membrane bound; catalytic activity	1.56

AT5G39785	-	structural constituent of ribosome	1.56
AT1G77740	PIP5K2	phosphatidylinositol-4-phosphate 5-kinase (PtdIns(4)P 5-kinase 2 involved in regulating lateral root formation and root gravity response	1.56
AT2G35990	-	putative lysine decarboxylase family protein	1.55
AT1G22880	CEL5	cellulase 5 (CEL5); carbohydrate metabolic process	1.55
AT5G13330	RAP2.6L	encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family	1.54
AT1G60560	-	SWIM zinc finger family protein; FUNCTIONS IN: zinc ion binding	1.54
AT2G37980	-	O-fucosyltransferase family protein	1.54
AT3G21230	4CL5	encodes a 4-coumarate coenzyme A ligase being able to use sinapate as substrate	1.53
AT1G30690	-	Sec14p-like phosphatidylinositol transfer family protein	1.53
AT1G24150	FH4	encodes a group I formin. Localized to cell junctions. Polymerizes actin. Binds profilin.	1.53
AT1G72840	-	disease resistance protein (TIR-NBS-LRR class)	1.53
AT2G35000	-	E3 ligase-like protein induced by chitin oligomers	1.53
AT1G80870	-	protein serine/threonine kinase activity	1.53
AT1G19450	-	carbohydrate transmembrane transporter activity, sugar:hydrogen symporter activity	1.53
AT3G45010	scpl48	serine carboxypeptidase-like 48 (scpl48); proteolysis	1.53
AT4G05410	YAOZHE	nucleolar protein with seven WD40-repeats that plays a role in embryo sac development and is critical for the correct positioning of the division plane of zygote and the apical cell lineage in Arabidopsis	1.52
AT2G39130	-	transmembrane amino acid transporter family protein	1.52
AT1G35330	-	RING/U-box superfamily protein; zinc ion binding	1.52
AT2G26980	CIPK3	serine-threonine protein kinase whose expression increases in response to abscisic acid, cold, drought, high salt, and wounding conditions	1.52
AT5G38210	-	protein serine/threonine kinase activity	1.51
AT5G19110	-	eukaryotic aspartyl protease family protein; N-terminal myristolation	1.51
AT3G20860	ATNEK5	member of the NIMA-related serine/threonine kinases (Neks) that have been linked to cell-cycle regulation	1.50

TABLE 4.2B – Genes down-regulated by cytokinin in the *crf1,3,5,6* mutant			
AGI	**Gene Name**	**Gene Description**	**Fold change relative to non-treated**
AT1G43160	RAP2.6	encodes a member of the ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family	-2.73
AT2G36690	-	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	-2.24
AT5G01210	-	HXXXD-type acyl-transferase family protein	-2.19
AT5G47220	ERF2	a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ATERF-2); positive regulator of JA defense	-2.14
AT1G09090	ATRBOHB	NADPH-oxidase plays a role in seed after-ripening; major producer of superoxide in germinating seeds.	-2.07
AT4G14130	XTR7	xyloglucan endotransglycosylase-related protein (XTR7)	-2.06
AT2G01900	-	DNAse I-like superfamily protein	-1.98
AT2G22880	-	VQ motif-containing protein; response to UV-B	-1.96
AT1G13670	-	unknown protein	-1.96
AT2G27690	CYP94C1	CYTOCHROME P450, induced in response to wounding and jasmonic acid treatment	-1.93
AT4G21410	CRK29	cysteine-rich receptor-like protein kinase; response to ABA	-1.88
AT1G14780	-	MAC/Perforin domain-containing protein; immunity related	-1.88
AT2G28960	-	protein serine/threonine kinase	-1.88
AT2G41180	SIB2	VQ motif-containing protein; regulation of defense response, systemic acquired resistance	-1.83
AT5G57760	-	unknown protein	-1.81
AT5G09440	EXL4	EXORDIUM like 4 (EXL4); defense response to fungus	-1.80
AT1G63840	-	RING/U-box superfamily protein; zinc ion binding; response to abscisic acid stimulus	-1.78
AT5G61160	AACT1	anthocyanin 5-aromatic acyltransferase 1 (AACT1)	-1.78
AT1G31885 /// AT2G21020	NIP3;1	NOD26-like intrinsic protein 3;1; transporter activity	-1.77
AT5G47240	atnudt8	nudix hydrolase homolog 8 (NUDT8); response to wounding	-1.76
AT5G58940	CRCK1	calmodulin-binding receptor-like kinase	-1.76
AT1G11450 /// AT1G11460	UMAMIT27	nodulin MtN21-like transporter family protein	-1.74
AT5G57785	-	unknown protein	-1.74
AT1G33800	ATGXMT1	glucuronoxylan(GX)-specific 4-O-methyltransferase responsible for methylating GlcA residues in GX	-1.74
AT3G26510	-	octicosapeptide/Phox/Bem1p family protein	-1.72
AT1G70230	TBL27	member of the TBL (TRICHOME BIREFRINGENCE-LIKE); involved in the synthesis and deposition of secondary wall cellulose	-1.71
AT5G38540 ///	-	mannose-binding lectin superfamily protein	-1.69

AT5G38550			
AT5G22890	-	C2H2 and C2HC zinc fingers superfamily protein; response to iron starvation	-1.69
AT4G39780	-	member of the DREB subfamily A-6 of ERF/AP2 transcription factor family	-1.68
AT5G24210	-	alpha/beta-Hydrolases superfamily protein; SA biosynthesis	-1.68
AT5G59780	MYB59	putative transcription factor	-1.68
AT4G20460	-	NAD(P)-binding Rossmann-fold superfamily protein; galactose metabolic process	-1.68
AT5G62360	-	plant invertase/pectin methylesterase inhibitor superfamily protein	-1.67
AT5G22460	-	alpha/beta-Hydrolases superfamily protein	-1.64
AT4G25810	XTR6	xyloglucan endotransglycosylase-related protein; carbohydrate metabolism	-1.63
AT5G07580	-	member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family	-1.63
AT2G27660	-	cysteine/Histidine-rich C1 domain family protein	-1.63
AT5G01740	-	nuclear transport factor 2 (NTF2) family protein; wound induced	-1.63
AT1G08180	-	unknown protein	-1.62
AT3G46280	-	protein kinase-related; ER to Golgi vesicle-mediated transport	-1.62
AT3G29410	-	terpenoid cyclases/Protein prenyltransferases superfamily protein; cytokinin signaling related	-1.62
AT5G46710	-	PLATZ transcription factor family protein	-1.61
AT1G13430	ATST4C	sulfotransferase; transcript levels rise in response to cytokinin treatment	-1.61
AT1G77920	TGA7	bZIP transcription factor family protein	-1.61
AT1G53510	ATMPK18	MAP Kinase	-1.60
AT3G22540	-	unknown function (DUF1677)	-1.58
AT3G54380	ATSAC3C	SAC3/GANP/Nin1/mts3/eIF-3 p25 family; photoperiodism, flowering	-1.58
AT5G14090	ATLAZY1	unknown protein involved in gravitropsim	-1.58
AT5G22500	FAR1	member of the eight-member gene family encoding alcohol-forming fatty acyl-CoA reductases (FARs)	-1.57
AT2G40000	HSPRO2	ortholog of sugar beet HS1 PRO-1 2; response to biotic stimuli	-1.57
AT2G20670	-	unknown protein	-1.57
AT1G19530	-	unknown protein	-1.57
AT4G29190	ATOZF2	zinc finger C-x8-C-x5-C-x3-H type family protein; transcription	-1.56
AT1G27290	-	unknown protein	-1.56
AT3G52480	-	unknown protein; response to fructose and sucrose	-1.56
AT5G44480	DUR	mutant has altered lateral root; UDP Glucose Epimerase	-1.56
AT3G61060	AtPP2-A13	phloem protein 2-A13; response to fructose and sucrose	-1.55
AT2G32030	-	Acyl-CoA N-acyltransferases (NAT) superfamily protein; response to ethylene	-1.54
AT2G31750	UGT74D1	UDP-GLUCOSYL TRANSFERASE 74D1	-1.54
AT2G15320	-	leucine-rich repeat (LRR) family protein	-1.54

AT5G24990 /// AT5G25020	-	leucine-rich repeat (LRR) family protein	-1.54
AT5G58900	-	homeodomain-like transcriptional regulator	-1.53
AT5G25350	EBF2	EIN3-binding F-box protein 2 (EBF2); part of the SCF complex, it is located in the nucleus and is involved in the ethylene-response pathway.	-1.53
AT5G07460	PMSR2	ubiquitous enzyme that repairs oxidatively damaged proteins	-1.53
AT1G75170 /// AT5G04780	-	Sec14p-like phosphatidylinositol transfer family protein	-1.53
AT2G04790	-	unknown protein	-1.53
AT1G51850	-	leucine-rich repeat protein kinase family protein; kinase activity	-1.52
AT5G54980	-	uncharacterised protein family (UPF0497)	-1.52
AT2G22800	HAT9	homeobox protein HAT9.	-1.52
AT5G44210	ERF9	member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family	-1.52
AT1G21910	DREB26	DREB subfamily A-5 of ERF/AP2 transcription factor family	-1.52
AT1G08430	ALMT1	Al-activated malate efflux transporter essential for aluminum tolerance	-1.52
AT5G53880	-	unknown protein	-1.51
AT5G53830	-	VQ motif-containing protein	-1.51
AT3G20340	-	gene is downregulated in the presence of paraquat, an inducer of photoxidative stress	-1.51
AT5G13750	ZIFL1	zinc induced facilitator-like 1; basipetal auxin transport	-1.51
AT4G24340 /// AT4G24350	-	phosphorylase superfamily protein; nucleoside metabolism	-1.51
AT1G05650 /// AT1G05660	-	pectin lyase-like superfamily protein; polygalacturonase activity	-1.50

TABLE 4.3A – Genes up-regulated by cytokinin in wild type but not the *crf1,3,5,6* mutant			
AGI	**Gene Name**	**Gene Description**	**Fold change relative to non-treated**
AT4G26150	CGA1	member of the GATA zinc finger transcription factors; modulates chlorophyll biosynthesis and glutamate synthase (GLU1/Fd-GOGAT) expression	3.41
AT4G28520	CRU3	12S seed storage protein that is tyrosine-phosphorylated and its phosphorylation state is modulated in response to ABA in seeds	2.91
AT1G69040	ACR4	ACT-domain containing protein involved in feedback regulation of amino acid metabolism	2.62
AT4G25410	-	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	2.43
AT4G39070	BBX20	BZS1 is a putative zinc finger transcription factor; brassinosteroids-regulated BZR1 target (BRBT) gene	2.35
AT2G38750	ANNAT4	calcium dependent membrane binding protein thought to be involved in Golgi mediated secretion	2.30
AT3G02610	-	stearoyl-acyl-carrier-protein desaturase family protein; fatty acid metabolism	2.20
AT2G47260	ATWRKY23	WRKY Transcription Factor; Group I involved in nematode feeding site establishment	2.15
AT4G39770	TPPH	haloacid dehalogenase-like hydrolase (HAD) superfamily protein	2.13
AT5G40390	RS5	a protein which might be involved in the formation of verbascose	2.12
AT5G04770	ATCAT6	a member of the cationic amino acid transporter (CAT) subfamily of amino acid polyamine choline transporters	2.00
AT1G31320	LBD4	LOB domain-containing protein 4; polarity and bilateral symmetry	1.99
AT2G36870	ATXTH32	xyloglucan endotransglycosylase/hydrolase	1.93
AT3G62930	-	thioredoxin superfamily protein; cell redox homeostasis	1.93
AT5G28640	AN3	protein with similarity to mammalian transcriptional coactivator that is involved in cell proliferation during leaf and flower development	1.91
AT5G11590	TINY2	member of the DREB subfamily A-4 of ERF/AP2 transcription factor family	1.88
AT1G78580	ATTPS1	enzyme putatively involved in trehalose biosynthesis; modulates cell growth but not differentiation by determining cell wall deposition and cell division	1.87
AT1G10480	ZFP5	zinc finger protein regulating trichome development by integrating GA and cytokinin signaling	1.87
AT2G46660	CYP78A6	CYP78A cytochrome P450 monooxygenase protein family that is required in the sporophytic tissue of the mother plant to promote seed growth	1.84
AT2G32930	ZFN2	zinc finger protein; transcription	1.84
AT2G36590	ATPROT3	proline transporter with affinity for gly betaine, proline, and GABA	1.81
AT3G51660	-	Tautomerase/MIF superfamily protein; IAA biosynthesis	1.80

AT1G48130	ATPER1	encodes a protein similar to the 1-cysteine (1-Cys) peroxiredoxin family of antioxidants	1.80
AT1G30040	ATGA2OX2	gibberellin 2-oxidase that is responsive to cytokinin and KNOX activities	1.78
AT3G13620	PUT4	POLYAMINE UPTAKE TRANSPORTER 4, an amino acid permease family protein	1.77
AT4G16000	-	unknown protein	1.77
AT1G68360	-	C2H2 and C2HC zinc fingers superfamily protein; transcription	1.72
AT3G45680	-	major facilitator superfamily protein;	1.70
AT1G21110 /// AT1G21120	IGMT3	O-methyltransferase family protein	1.70
AT1G64390	ATGH9C2	glycosyl hydrolase 9C2 ; carbohydrate binding, hydrolase activity	1.69
AT2G22930	-	UDP-Glycosyltransferase superfamily protein	1.68
AT5G59480	-	haloacid dehalogenase-like hydrolase (HAD) superfamily protein	1.68
AT3G56080	-	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	1.67
AT5G65860		ankyrin repeat family protein; methyltransferase activity	1.66
AT5G66985	-	unknown protein	1.66
AT5G65860	-	ankyrin repeat family protein	1.66
AT5G57150	-	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	1.65
AT4G15500	UGT84A4	protein that might have sinapic acid:UDP-glucose glucosyltransferase activity.	1.65
AT3G60390	HAT3	homeobox protein HAT3	1.64
AT4G38840	-	SAUR-like auxin-responsive protein family	1.64
AT1G04250	AXR3	transcription regulator acting as repressor of auxin-inducible gene expression	1.63
AT2G35300	LEA18	Late embryogenesis abundant 18 family protein; accumulate in response to low water availability conditions	1.62
AT1G49510	EMB1273	embryo defective 1273	1.60
AT5G06000	ATEIF3G2	one of the 2 genes that code for the G subunit of eukaryotic initiation factor 3 (EIF3)	1.59
AT3G44940	-	protein of unknown function (DUF1635)	1.57
AT5G56970	CKX3	protein whose sequence is similar to cytokinin oxidase/dehydrogenase, which catalyzes the degradation of cytokinins	1.57
AT4G27590	-	heavy metal transport/detoxification superfamily protein	1.56
AT3G21270	DOF2	Dof zinc finger protein	1.55
AT5G64620	C/VIF2	Plant cell wall (CWI) and vacuolar invertases (VI) play important roles in carbohydrate metabolism, stress responses and sugar signaling.	1.55
AT2G34510	-	unknown membrane bound protein	1.54
AT5G04330	CYP84A4	cytochrome P450 superfamily protein	1.53
AT3G26410	ATTRM11	protein involved in modification of nucleosides in tRNA	1.52
AT1G31770	ABCG14	ATP-binding cassette 14; coupled to transmembrane movement of substances	1.52

AT3G15810	-	protein of unknown function (DUF567)	1.51
AT1G44160	-	HSP40/DnaJ peptide-binding protein; protein folding	1.51
AT1G78120	TPR12	one of the 36 carboxylate clamp (CC)-tetratricopeptide repeat (TPR) proteins with potential to interact with Hsp90/Hsp70 as co-chaperones	1.51
AT2G03760	ATSOT1	brassinosteroid sulfotransferase	1.50
AT2G25930	ELF3	nuclear protein that is expressed rhythmically and interacts with phytochrome B to control plant development and flowering	1.50
AT5G62630	HIPL2	hipl2 protein precursor (HIPL2)	1.50

TABLE 4.3B– Genes down-regulated by cytokinin in wild type but not the *crf1,3,5,6* mutant			
AGI	**Gene Name**	**Gene Description**	**Fold change relative to non-treated**
AT1G21360	-	adenine nucleotide alpha hydrolases-like superfamily protein; response to iron starvation	-2.91
AT3G60520	ATSDI1	homologous to the wheat sulphate deficiency-induced gene sdi1; induced by sulfur starvation	-2.78
AT5G59540	-	protein of unknown function, DUF599	-2.44
AT2G45920	ATPP2-A8	phloem protein 2-A8 (PP2-A8); innate immune response	-2.34
AT5G59530	-	cysteine/Histidine-rich C1 domain family protein	-2.26
AT3G56710	-	uncharacterized protein family; ER to Golgi vesicle-mediated transport	-2.16
AT1G35670	ATTPPB	homologous to the C-terminal part of microbial trehalose-6-phosphate phosphatases	-2.14
AT1G23390	-	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein;	-2.13
AT1G80760	PRX37	putative apoplastic peroxidase Prx37	-2.05
AT5G63660	-	HCO3- transporter family	-2.04
AT4G33666	PROPEP2	elicitor peptide 2 precursor (PROPEP2)	-2.02
AT3G52340	-	unknown protein	-2.00
AT1G55850	-	calcium-binding EF-hand family protein	-1.99
AT1G24440	-	membrane bound hydroxyproline-rich glycoprotein family protein	-1.96
AT3G09020	ATBZIP	basic leucine-zipper 8 (bZIP); transcription	-1.96
AT1G03660	-	protein serine/threonine kinase	-1.95
AT5G57530	SCL-3	scarecrow-like protein (SCL3); responsive to GA	-1.90
AT1G80840	-	unknown protein	-1.90
AT3G23800	CRK12	CYSTEINE-RICH RLK (RECEPTOR-LIKE PROTEIN KINASE) 12	-1.88
AT5G43350	UGT76B1	glucosyltransferase that conjugates isoleucic acid and modulates plant defense and senescence	-1.86
AT5G20400	ATPRR2	a pinoresinol reductase involved in lignan biosynthesis	-1.86
AT5G26731	-	UDP-Glycosyltransferase superfamily protein	-1.84
AT5G14760	UMAMIT33	nodulin MtN21-like transporter family protein	-1.81
AT3G15450	UMAMIT31	nodulin MtN21-like transporter family protein	-1.81
AT1G71960	-	basic helix-loop-helix (bHLH) DNA-binding superfamily protein; lateral root and root hair development	-1.80
AT3G08860	-	unknown protein	-1.79
AT2G28270	MIOX2	myo-inositol oxygenase family gene	-1.79
AT5G04340	-	curculin-like (mannose-binding) lectin family protein	-1.77
AT1G72360	-	SAUR-like auxin-responsive protein family; auxin responsive	-1.77
AT3G23200	IOS1	putative member of the LRR-RLK protein family; contributes to interaction between Arabidopsis and Hyaloperonospora arabidopsidis	-1.76
AT3G49960	ATBZIP3	basic leucine-zipper 3 (bZIP3); DNA binding, sequence-specific DNA binding transcription factor activity	-1.74

AT4G28270	ATLEA3	Late embryogenesis abundant 3 (LEA3) family protein; accumulate in response to low water availability conditions	-1.74
AT5G12030	ATMYB56	member of the R2R3 factor gene family	-1.74
AT2G15390	ATC	protein that acts non-cell autonomously to inhibit floral initiation	-1.74
AT1G74940	CIPK25	member of AtCIPKs	-1.74
AT2G19570	EARLI1	putative lipid transfer protein, vernalization-responsive and cold-induced	-1.73
AT5G44420	ATGSTU24	glutathione transferase belonging to the tau class of GSTs	-1.73
AT1G29400	WAG1	PsPK3-type kinase; suppressors of root waving; root development	-1.72
AT3G47210	UMAMIT30	nodulin MtN21-like transporter family protein	-1.72
AT3G43190	FRA8	glycosyltransferase family 47 that is involved in secondary cell wall biosynthesis	-1.72
AT5G48430	-	peroxidase superfamily protein	-1.71
AT5G03380	ATSERAT2; 1	chloroplast/cytosol localized serine O-acetyltransferase involved in sulfur assimilation and cysteine biosynthesis	-1.69
AT4G30170	-	metal transport/detoxification superfamily protein	-1.69
AT4G00700	-	C2 calcium/lipid-binding plant phosphoribosyltransferase family protein	-1.69
AT2G36950	-	peroxidase family protein	-1.69
AT1G55920	-	heavy metal transport/detoxification superfamily protein	-1.66
AT5G58390	-	eukaryotic aspartyl protease family protein;	-1.66
AT2G28110	ATSUS4	protein with sucrose synthase activity (SUS4)	-1.66
AT4G01450	-	plant protein of unknown function (DUF247)	-1.65
AT1G53700	AML5	mei2-like gene family; positive regulation of meiosis	-1.64
AT1G17170	PDF1.2A	an ethylene- and jasmonate-responsive plant defensin	-1.64
AT4G12480	CDA1	cytidine deaminase	-1.64
AT5G25110	-	protein of unknown function (DUF581)	-1.64
AT2G27550	ATFUT4	predicted fucosyltransferase, based on similarity to FUT1, but not functionally redundant with FUT1.	-1.63
AT5G17800	HSP17.6A	cytosolic small heat shock protein with chaperone activity that is induced by heat and osmotic stress and is also expressed late in seed development.	-1.63
AT1G02820	ATRMA2	RING finger E3 ubiquitin ligase	-1.63
AT5G15830	-	enriched in root hair cells (compared to non-root hair cells)	-1.62
AT1G51800	-	uncharacterized protein family (UPF0497)	-1.62
AT2G46690	ATERF73	member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family	-1.61
AT5G18470	CZF2	putative c2h2 zinc finger transcription factor mRNA	-1.61
AT2G19800	-	Cysteine/Histidine-rich C1 domain family protein; oxidation reduction	-1.60
AT4G33960	PYD4	predicted to have beta-alanine aminotransferase activity	-1.60
AT1G31050	ABCG25	plasma membrane localized ABC transporter involved in abscisic acid transport and responses	-1.60

AT4G01440	-	aluminum induced protein with YGL and LRDR motifs	-1.60
AT4G28040	FIN4	encodes for L-aspartate oxidase involved in the early steps of NAD biosynthesis	-1.59
AT2G36970	-	unknown protein	-1.59
AT4G13660	-	protein whose sequence is similar to flavanone 3 hydroxylase from Malus	-1.58
AT3G11340	ATPT1	an inorganic phosphate transporter Pht1;1	-1.58
AT4G23200	SBP3	selenium-binding protein 3 (SBP3)	-1.58
AT5G66985	ATWRKY40	pathogen-induced transcription factor	-1.58
AT1G50420	XTH12	xyloglucan endotransglucosylase/hydrolase 12 (XTH12)	-1.58
AT1G61590	-	ankyrin-repeat containing protein; transcription factor import into the nucleus	-1.57
AT1G68880	-	alpha 1,4-glycosyltransferase family protein	-1.57
AT1G23040	-	RING/U-box superfamily protein; zinc binding	-1.56
AT1G29020	ATCSLE1	similar to cellulose synthase	-1.56
AT3G14280	ATSPP2	sucrose-phosphatase (SPP2)	-1.56
AT5G64890	-	unknown protein	-1.55
AT3G62270	LCR74	predicted to encode a PR (pathogenesis-related) protein	-1.54
AT4G08770	NIP6	protein with boron transporter activity; directs boron to young developing tissues in the shoot	-1.54
AT4G12490	-	kelch repeat-containing F-box family protein; cyclin like	-1.53
AT1G78090	ATCDPK2	Ca(2+)-dependent, calmodulin-independent protein kinase; positive regulator of ABA signaling	-1.52
AT2G39530	SIB1	Sig1 binding protein; interacts with Sig1R4. As well as Sig1, SibI is imported into chloroplasts and its expression is light-dependent in mature chloroplasts.	-1.52
AT5G43520	-	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	-1.52
AT5G45070	-	ubiquitin-protein ligase	-1.51
AT5G43180	-	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	-1.51
AT5G48850	-	unknown protein	-1.50
AT3G25930	GLTP2	glycolipid transfer protein 2	-1.50

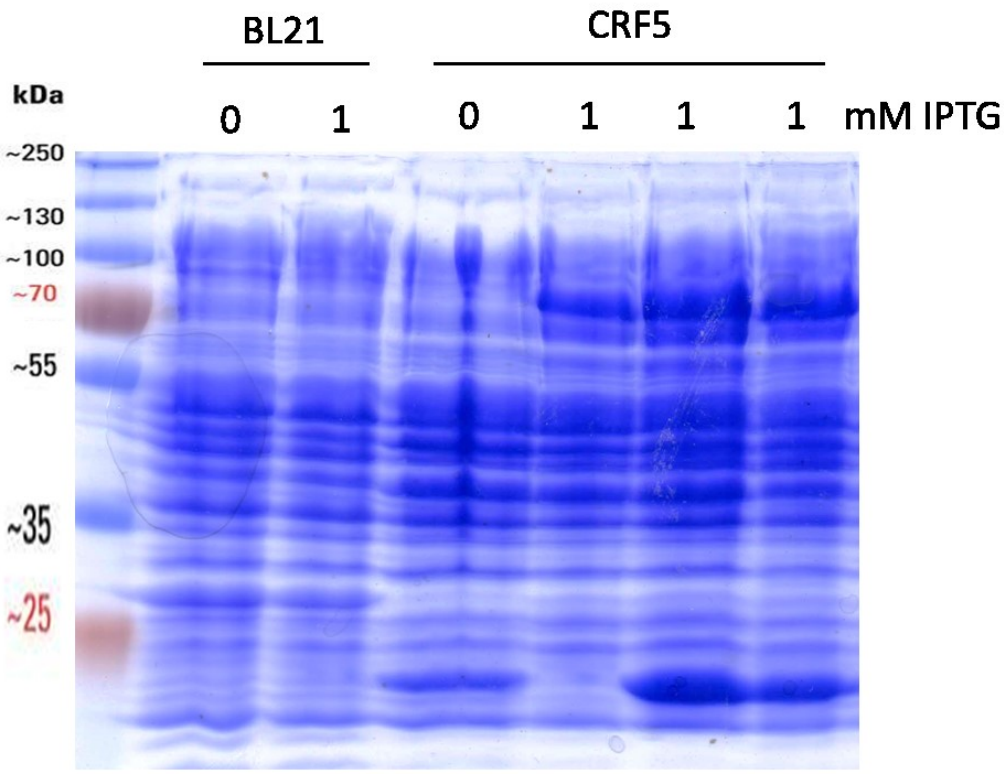

Figure 4.1 – Expression of CRF5 in *E. coli*
Sonicated cell culture of CRF5 expressed in BL21 shows appropriate expression and size of the MBP tagged protein (expected size 75 kDa)

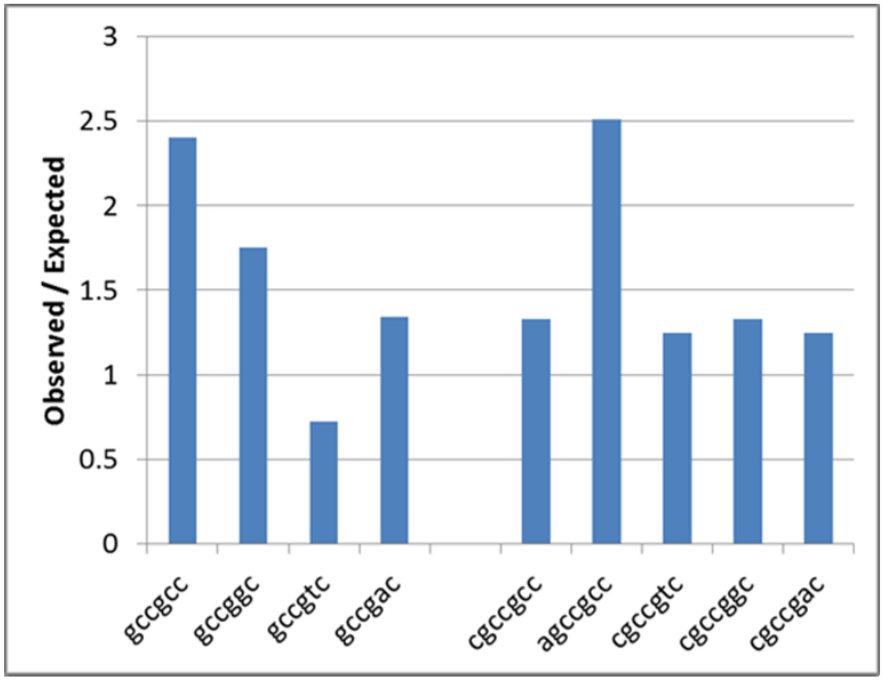

Figure 4.2 – The GCC box motifs are enriched in genes differentially regulated in untreated *crf1,3,5,6* seedlings – Upstream regions of genes found to be mis-expressed in *crf1,3,5,6* were scanned for the GCC box motifs previously identified to be potential CRF5 binding sites.

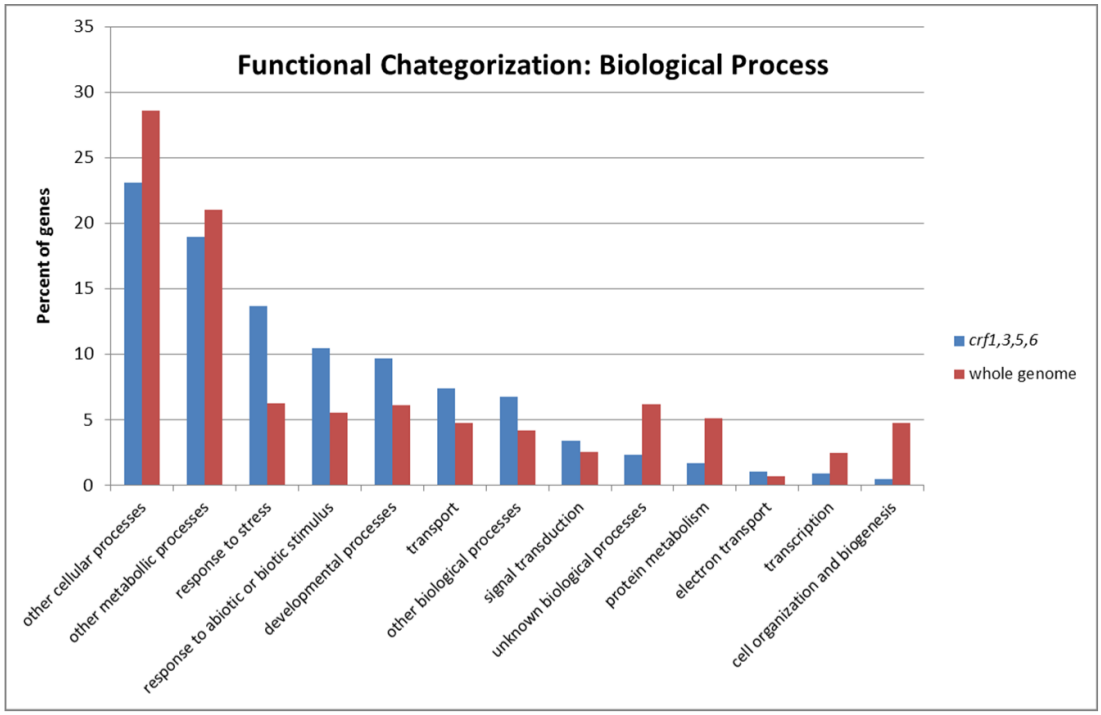

Figure 4.3 – Biological functions of genes regulated basally in the *crf1,3,5,6* mutant only – Genes which expression levels changed 2-fold in the mutant compared to wild type expression were categorized by their biological processes using the TAIR GO annotation tool. (www.arabidopsis.org) The percent of genes in a category was compared to the percent of genes in the whole genome belonging to the category.

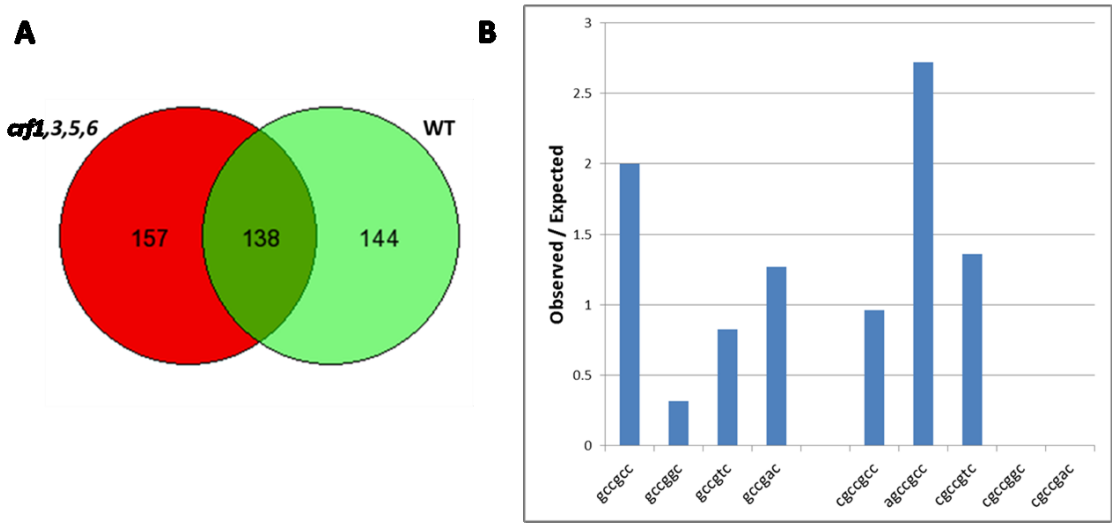

Figure 4.4 - Genes regulated by cytokinin in the *crf1,3,5,6* mutant background show an enrichment of GCC box motifs in their promoters – Microarray analysis revealed 157 genes uniquely regulated by cytokinin in the mutant background (A) and upstream regions of these genes contain an enrichment of the GCC box motifs potentially bound by CRF5 (B).

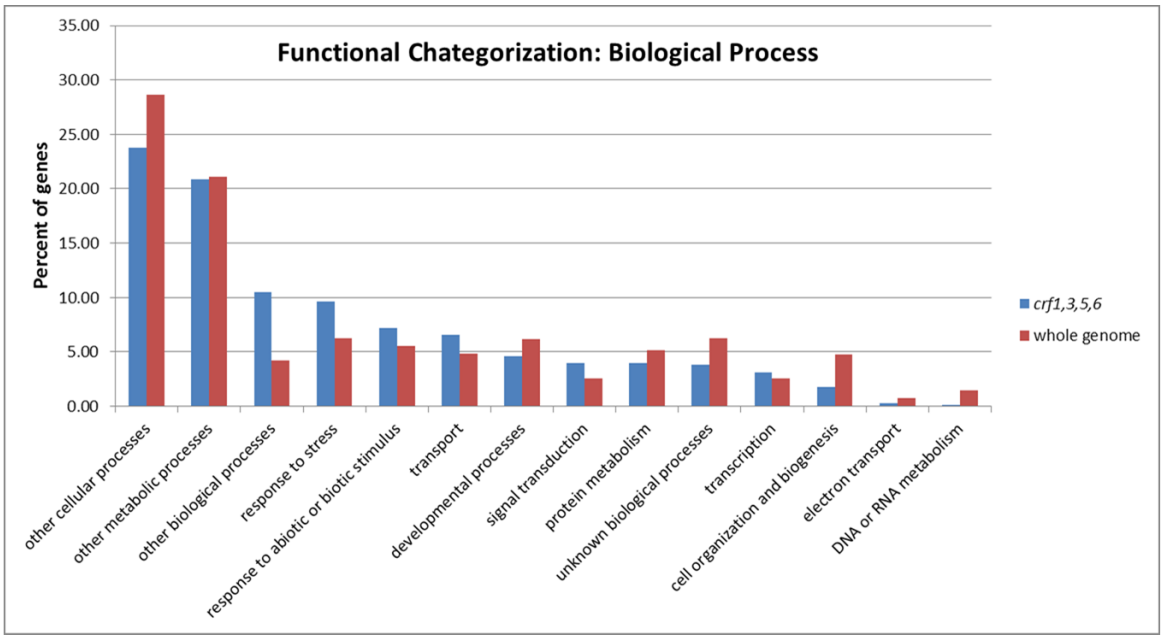

Figure 4.5 – Biological functions of the genes regulated by cytokinin in *crf1,3,5,6*, but not wild type seedlings – Genes in which the expression levels changed 1.5-fold in the mutant but not wild type after cytokinin treatment are grouped by biological processes using the TAIR GO annotation tool. (www.arabidopsis.org) . The percent of genes in a category is show as compared to the percent of the whole genome belonging to the group.

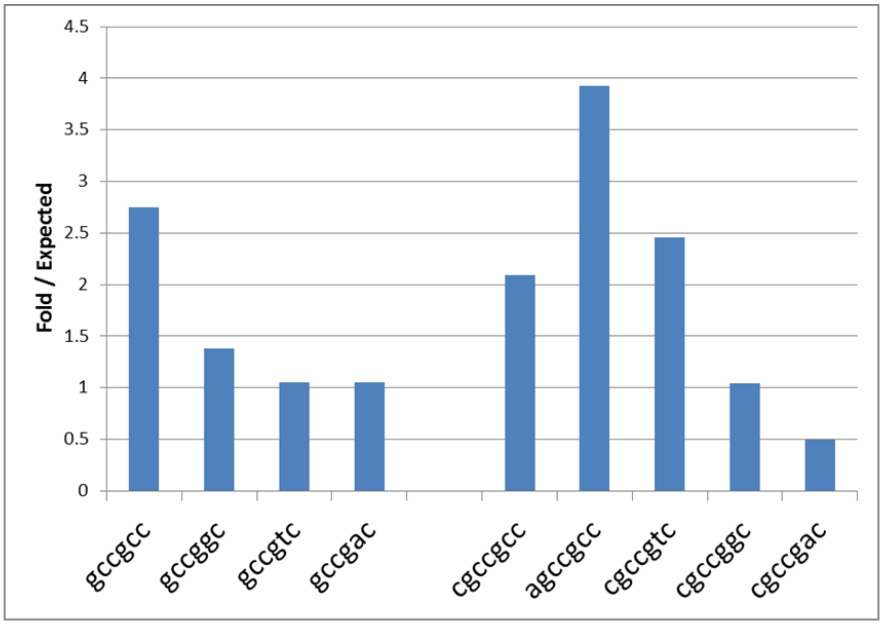

Figure 4.6 – GCC box motifs are enriched in upstream regions of genes regulated by cytokinin in wild type but not *crf1,3,5,6* – The GCC-box motifs are over-represented in the wild-type regulated genes, suggesting they may be regulated by CRFs and loss of CRFs removes their induction.

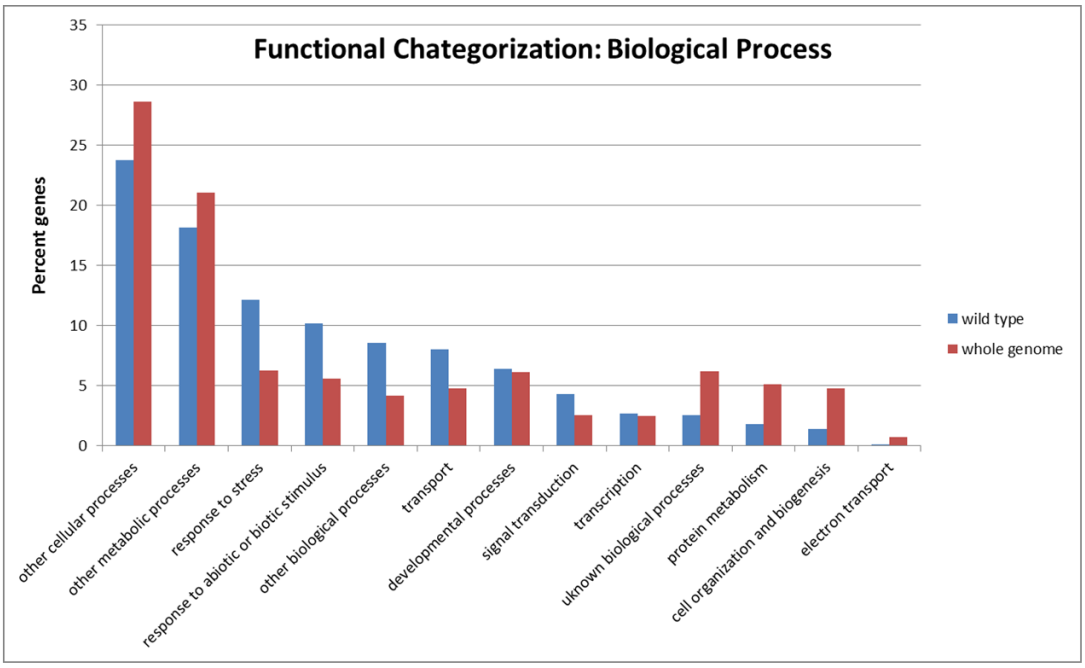

Figure 4.7 – Biological functions of the genes regulated by cytokinin in wild type but not *crf1,3,5,6*– Genes in which the expression levels changed 1.5-fold in wild type but not *crf1,3,5,6* after cytokinin treatment are grouped by biological processes using the TAIR GO annotation tool. (www.arabidopsis.org) . The percent of genes in a category is show as compared to the percent of the whole genome belonging to the group.

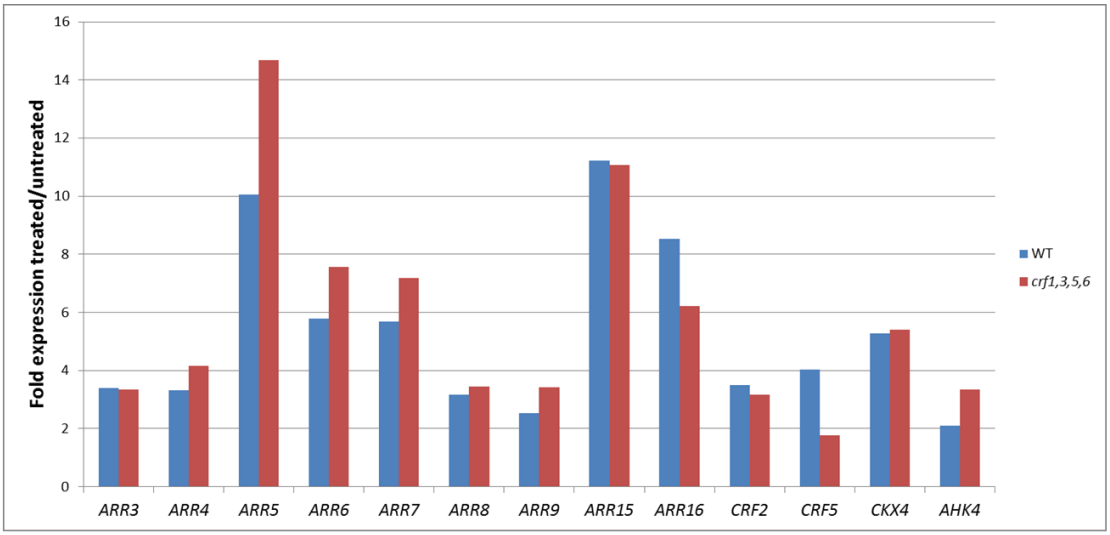

Figure 4.8– Cytokinin induced gene expression found in *crf1,3,5,6* and wild type – Microarray analysis shows the type-A *ARR* genes to be similarly induced by cytokinin in *crf1,3,5,6* and wild-type treated seedlings. Differences observed were higher induction of *ARR5* and the reduction of *ARR16*, as well as the increased induction of the *AHK4* receptor.

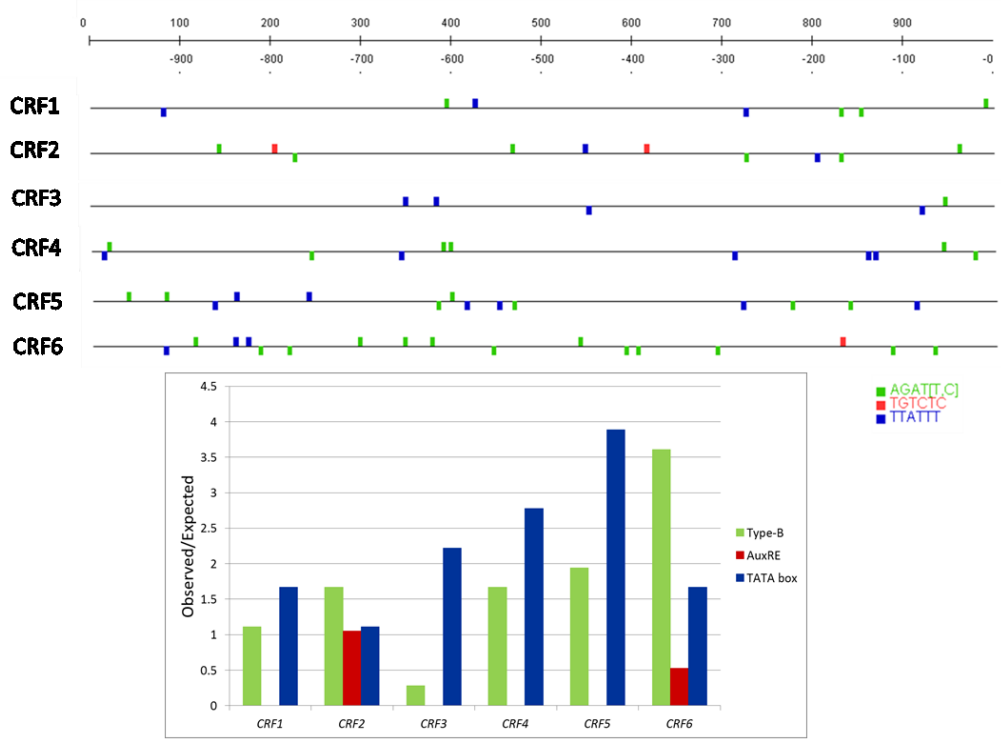

Figure 4.9– *CRF*s contain the Type-B binding site in their upstream regions – Using the TOUCAN workbench for regulatory sequence analysis, we scanned 1 kb upstream of the *CRF*s for the short, type-B ARR binding site, AGAT [T/C].

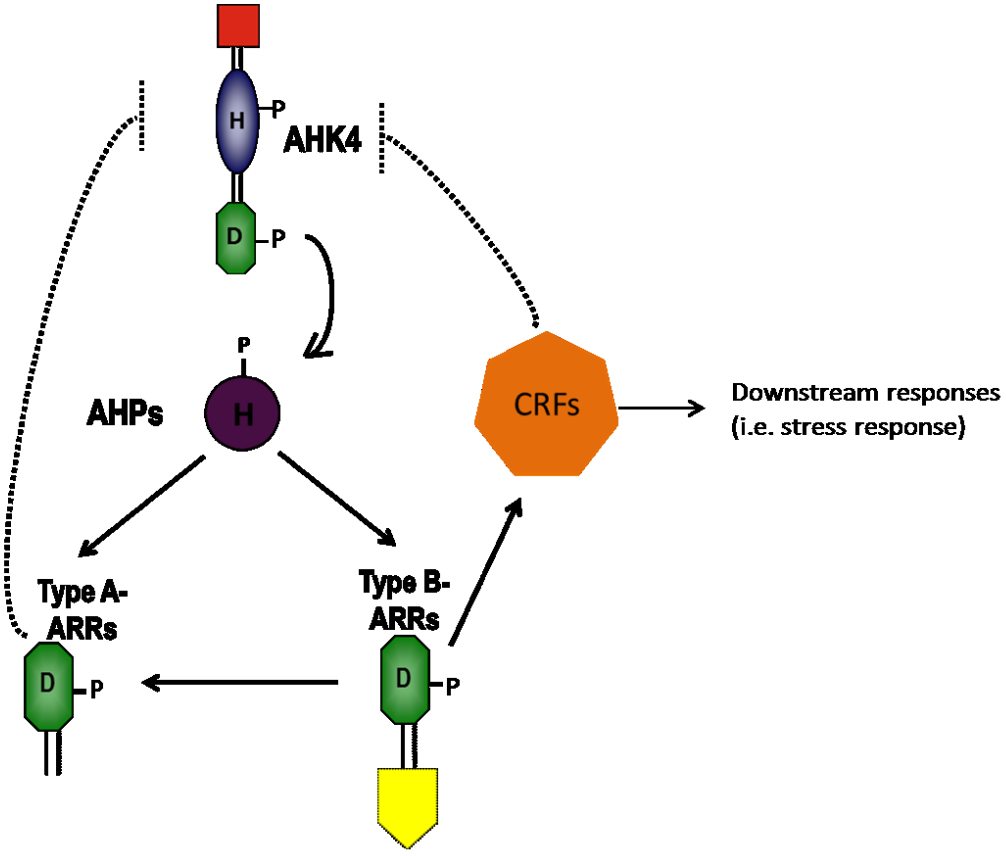

Figure 4.10 – Model of cytokinin signaling including the CRFs – The proposed model shows CRFs negatively regulating *AHK4* and driven by the type-B ARRs.

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CHAPTER 5: FUTURE DIRECTIONS

OVERVIEW

This body of work shows that the CRFs are a family of transcription factors that are regulated by cytokinin and are a part of the complex transcriptional cascade occurring downstream of cytokinin perception in plants. The processes that are regulated by the CRFs include senescence, meristem size, primary and lateral root growth, flowering time and rosette size. More studies are needed to uncover the mechanisms by which the CRFs regulate these processes. Below, we highlight the future work that will need to be done to better understand the role of the CRFs in these developmental processes and in cytokinin signaling.

Identify direct targets that are activated or repressed by the CRFs

Chromatin immunoprecipitation combined with sequencing (ChIP:seq) is useful to identify *in vivo* targets of transcription factors [1, 2]. The primary goal of ChIP:seq of the CRFs is to determine their direct targets and the upstream sequences to which they bind. By identifying genes regulated by the CRFs, we can begin to build a more complete transcriptional network downstream of cytokinin signaling. Secondly, we can compare targets of the individual CRFs to see if there is an overlap or specificity among these genes. As well as uncovering direct targets,

we can look at the effects of cytokinin on binding of CRFs to these targets. If, after addition of cytokinin, we see enrichment of a particular gene compared to untreated samples, we can conclude cytokinin drives the response through CRF activation.

These analyses, together with the previous expression profiling of *crf* LOF mutants and CRF overexpressing lines as well as the previous phenotypic analysis of these mutants, will help us to develop a model that describes the regulatory circuits controlled by CRFs. As we also know that CRFs interact with a subset of type-B ARRs [3], we can also look at the overlap seen in CRF targets compared with known type-B ARR targets to further what roles the CRFs play in the transcriptional cascade downstream of cytokinin signaling.

Further determine the role of the CRFs in root meristem maintenance

Regulation of root apical meristem (RAM) size is dependent on the tight control of auxin and cytokinin signaling in the appropriate zones of the root tip [4]. The meristems of the CRF overexpression lines are larger while the *crf* LOF mutants are smaller (Chapter 3, Figure 3.1), indicating the CRFs play a role in the control of cell division and differentiation in the root tip. Phenotypic analyses as well as preliminary gene expression studies of the CRFs have suggested they may play a negative role in some of the processes controlled by cytokinin signaling. Removing this putative negative regulation, as in the *crf* LOF lines, may result in elevated cytokinin function, producing a smaller meristem [5] Cytokinin regulates the size of the meristem, at least in part, through the induction of the Aux/IAA gene (known to be negative regulators of auxin signaling), *SHY2*, through ARR1 and ARR12,

causing inhibition of auxin transport in the transition zone [4]. Yeast two hybrid and BiFC experiments have shown that CRF1 and CRF2 directly interact with the ARR12 protein [3]. Perturbation of this interaction may possibly cause changes in auxin and cytokinin function and thus alteration of meristem size. It would be interesting to determine if the type-B response regulators are directly regulating the CRFs in the meristematic zone to repress *SHY2,* and subsequently restricting cytokinin signaling in order to promote cell division.

Visualize the localization of auxin and cytokinin signaling in the meristem of crf LOF lines

Because the *crf* LOF and CRF overexpression lines show altered meristem size, we could determine if this regulation is primarily through auxin signaling, cytokinin signaling or both in the root tip. The proper function of the root apical meristem depends on the appropriate balance of auxin and cytokinin function as well as the appropriate expression of genes controlling appropriate root development. There are several reporters for cytokinin and auxin function that use fluorescent tags in specific tissues. PINs are efflux carriers responsible for creating and controlling auxin gradients within plant tissues [6]. Mutant lines containing GFP tagged PIN proteins driven by their endogenous promoters were used to estimate auxin transport within specific tissues and treating these tissues with auxin or cytokinin affects and redistributes these gradients [6, 7, 8]. Localization of PIN proteins is altered in type-A ARR mutants as well as in *shy2* loss and gain-of-function lines [4, 8] and PINs are found more broadly distributed in the type-B *arr12* loss-of-function line [7]. Therefore, we can compare PIN:GFP, DR5:GFP (synthetic auxin reporter)

and TCS:GFP (synthetic cytokinin reporter) in *crf* LOF backgrounds with previously reported results in other cytokinin signaling mutants as well as with wild-type plants. To determine if the CRFs control division or differentiation in the RAM, we can look at markers of cell division in the root tip, such as pCYCD6;1::CYCD6;1::GUS. In the same manner, we will look at the expression levels and patterns of other genes previously shown to play a role in meristem development and maintenance, such as *WOX5*, *PLT*, *ARR1* and *ARR12*, by creating GFP tagged fusions of these genes in the *crf* mutant background. These genes play pivotal roles in meristem formation and maintenance and we have seen expression changes in several of the genes by NanoString® analysis of the *crf1,3,5,6* mutant and *CRF5* overexpression lines (Chapter 3, Figure 3.2, 3.3 and 3.4). Looking at alterations in the patterns of expression of these genes in a *crf* LOF line will allow us to develop hypotheses of how the CRFs are acting in the control of meristem growth.

By using visual markers, we can examine not only the level of expression of these genes, but any ectopic patterns of expression in *crf1,3,5,6*. Comparing the expression patterns of these genes in *crf1,3,5,6* to the expression seen in wild-type plants will help us better understand the processes and pathways in which the CRFs are involved in the root apical meristem. If the CRFs are acting as negative regulators of cytokinin signaling in the meristem, in a *crf* multiple LOF mutant carrying pPIN::PIN:GFP proteins driven by their endogenous promoters, we would expect the areas of PIN expression, as well as DR5:GFP, to be reduced and more restricted, similar to what is observed when cytokinin is added exogenously [4]. Conversely, the patterns of expression of pARR1::ARR1:GFP,

pARR12::ARR12:GFP and pSHY2::SHY2:GFP would be expected to be broader, extending further toward the root tip, if the inhibition of cytokinin signaling has been removed in the LOF mutant. Cytokinin induces *SHY2* through ARR1, but removal of a putative negative regulation of this induction, and perhaps direct repression by the CRFs on ARR1, would result in a larger area of expression and thus a larger area of cell differentiation. Alternatively, the CRFs may be acting as negative regulators of auxin signaling in the root, independent of cytokinin signaling. If this is the case, we may see the expression patterns of ARR1, ARR2 and SHY2 to be restricted, and areas of TCS expression broader throughout the meristem. It is also possible that the CRFs are acting independently of the two hormone pathways. If this is the case, we may or may not observe changes in hormone signaling related gene expression and patterns.

Our preliminary results show that the expression levels of *WOX5*, *PLT1* and *CYCD6;1* are higher in the *crf1,3,5,6* than in wild-type root tips (Chapter 3, Figure 3.2 and 3.3). *WOX5* activity is repressed by auxin signaling and restricted to the quiescent center cells; expression of *PLT1* is reliant on *WOX5* and thus also restricted to the quiescent center [9]. If auxin signaling is restricted and reduced in *crf1,3,5,6*, we would expect the spatial pattern of expression of *WOX5* and *CYCD6;1* to be larger in the meristem. However, if the CRFs are negatively regulating auxin signaling, the areas of expression would be more restricted and at levels lower than in wild type. Because their expression is so low and distinct, it will be more reliable to analyze the effects of *crf* mutation using reporter GFP than by qPCR or NanoString analysis. Again, it is possible that the CRFs are acting independently of these genes

in the control of meristem size. If this is the case then we may see no change in gene expression or patterns for these genes.

Analyze meristem size and architecture in crf LOF lines crossed to known meristem related LOF lines

To determine the genetic interaction of the CRFs with other players in the control of meristem size, we will construct and analyze *crf* LOF lines and CRF overexpression lines combined with mutations in other genes that affect RAM size and function. If *crf* LOF mutants, which display a small root apical meristem, are crossed to a mutant also with an enlarged RAM, such as *arr1,12* [7], the meristem will be larger than wild type if *ARR1* is acting epistatically to *CRF5* and will be smaller than wild type if *CRF5* is acting epistatically to *ARR1*. Likewise, if the CRF overexpression lines are crossed to a mutant with a small meristem, such as *shy2*, the meristem size phenotype will be that of the gene that is acting epistatically to the other.

The distal end of the root containing the quiescent center and stem cell pool is known as the stem cell niche. The maintenance and organization of this area is controlled by the expression of several genes and by the correct balance of hormones in these tissues. *WOX5*, a homeobox domain transcription factor, is a master regulator of stem cell function. Expressed in the mitotically inactive quiescent center, WOX5 acts non-cell autonomously to maintain the stem cell pool and restrict their differentiation. Removal of WOX5 from the system causes premature differentiation of the columella stem cells [9]. WOX5 is repressed by auxin through

the action of ARF10 and ARF16 to restrict its expression specifically to the distal stem cells (DSC) [9]. The AP2 transcription factor family of *PLETHORA* genes also plays a crucial role in stem cell maintenance and is regulated by WOX5 to inhibit differentiation of DSC [9]. In the *crf1,3,5,6* mutant, there was an increase in the expression of *WOX5* and several *PLT* genes (Chapter 3, Figure 3.3). This suggests that the organization of the DSC in *crf* mutants, as well as mutants crossed to *wox5* and *plt1* LOF lines should be further analyzed.

Analyze the embryo lethality phenotype of *crf1,2,5,6*

In early embryonic development, auxin and cytokinin play antagonistic roles in cell patterning; ectopic expression of cytokinin signaling results in embryonic arrest [10]. Disruption of the genes encoding the cytokinin receptors results in a larger embryo size than wild type, but no gross defects in cellular organization, indicating that cytokinin is not essential for proper embryo development [11]. The TCS:GFP (indicative of cytokinin function) reporter is not detected in the embryo until the 16 cell stage, when it is localized in the area of the founder cells [10]. After the hypophysis has undergone division in the transition stage, TCS:GFP is repressed in the basal cell and its descendants, but is still present in the apical lens shaped cell [10]. At the heart stage of embryogenesis, a second area of TCS:GFP is then initiated at the shoot stem cell primordium [10]. Additionally, the expression patterns of *ARR7* and *ARR15*, negative regulators of cytokinin signaling, as well as auxin signaling are conversely related to that of cytokinin signaling, suggesting that ARR7 and ARR15 repress cytokinin signaling in the area that is to become the quiescent center and root stem cell niche [10]. Disruption of both ARR7 and ARR15 by an

inducible amiRNA results in defective morphology [10] and the loss of expression of *WOX5*, *PLT1*, and *SCARECROW* (*SCR*), all known to be key transcription factors driving the root stem cell specification [9, 12]. These data indicate that the restriction of cytokinin signaling in early embryogenesis is important for proper cell division and differentiation. It is possible that the CRFs play a part in restricting cytokinin function to specific tissues in the embryo through their transcriptional regulation by the auxin response factor, *MONOPTEROS (MP*), and the subsequent activation of type-A ARRs [13]. The role of the CRFs in embryo development may be tightly related to cytokinin and auxin signaling or may be independent of both and it will be important to analyze their relationship to these pathways.

Determine the stage of embryonic arrest in the crf1,2,5,6 mutant

We were unable to obtain a homozygous *crf1,2,5,6*, mutant, which suggests that this combination results in embryonic arrest. By observing the embryo at different developmental stages, we can determine the earliest point at which the development of the *crf1,2,5,6* mutant deviates from the wild type. From our knowledge of cytokinin signaling within the embryo, we can posit whether the CRFs regulate embryo development through cytokinin signaling or a separate pathway. As this mutant contains mutations in the CRFs known to be induced by cytokinin, we hypothesize that they may play a role in the control of cytokinin signaling in the embryo. Another *crf* quadruple mutant, *crf1,3,5,6,* does not show embryonic lethality, suggesting that *crf1,2,5,6* contains the CRFs that play the most important role in embryo development. Because both CRF1 and CRF2 have been shown to interact

directly with ARR7 [3], these two CRFs may play an important role in proper cytokinin function within specific cells for appropriate embryo development.

If CRFs restrict cytokinin function in specific tissues of the embryo, loss-of-function mutants could result in ectopic cytokinin action and disruption of embryo organization. Cytokinin signaling, as revealed by the pattern of the TCS reporter [9], is first detected at the 16 cell embryonic stage and is then restricted from specific cells forming the future root stem cell niche by ARR7 and ARR15 [10]. The CRFs could also be acting to restrict signaling either in this early stage through their interaction with ARR7, or later when the second area of cytokinin function appears at the future shoot meristem zone [10]. If the CRFs are only involved in restriction of cytokinin at the later stage, we may see altered embryonic cell patterns at the heart stage, or possibly earlier. If CRFs are involved at the early stage of patterning, the embryos would show serious patterning defects and failed differentiation. It is possible that the CRFs are working to restrict cytokinin function throughout embryogenesis, but this result would be indistinguishable from the previous phenotype as it would terminate at a very early stage as well.

Evaluate patterns of expression of cytokinin and auxin reporters within the developing crf1,2,5,6 embryo

Cytokinin and auxin play important roles in the developing embryo. To determine if the CRFs are altering cytokinin signaling or auxin signaling in the embryo, we will examine expression patterns of DR5:GFP and TCS:GFP in *crf1,2,5,6*. Comparing the expression patterns to the triple mutant siblings in this line,

as well as to wild type, we can see if LOF of these four CRFs alter the spatial pattern of expression of cytokinin and auxin function.

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