THE EFFECT OF CYTOKININ ON THE CHROMATIN LANDSCAPE OF ARABIDOPSIS

Duncan Alexander McPherson

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Approved by:

Joe Kieber

Greg Copenhaver

Dan McKay

ABSTRACT

Duncan Alexander McPherson: The Effect of Cytokinin on the Availability of Chromatin at Regulatory Elements
(Under the direction of Joseph J. Kieber)

Cytokinin, a N^6 substituted adenine, is a phytohormone with pleiotropic effects, including shoot activation, root inhibition, delay of leaf senescence, and tissue regeneration. Cytokinin is perceived by a histidine kinase and ultimately results in the activation of a response regulator that controls transcription of cytokinin-dependent genes. How a single signaling molecule is involved in numerous distinct developmental processes and how it integrates with other signaling pathways remain fundamental questions in plant biology.

Formaldehyde-assisted isolation of regulatory elements (FAIRE) is a technique used to isolate nucleosome-depleted regions of the genome. Nucleosome depletion is characteristic of open chromatin and such sites often contain regulatory elements that control the expression of nearby genes.

In this thesis, I demonstrate that FAIRE can be used to isolate cytokinin-induced open chromatin in Arabidopsis seedlings. After sequencing and peak calling, the called peaks identified in the cytokinin treated samples were found to be enriched for the motif TAGATT, which contains the known type-B RR binding motif AGAT. These peaks were additionally enriched for binding motifs of other transcription factors, suggesting that the cytokinin response can be moderated by other signals and likely act differently in different cellular contexts. Thus, the work described in this thesis has helped refine the transcriptional model of the cytokinin response in plants and provides the foundation and tools to investigate and dissect the dynamics of the cytokinin response and how it integrates with other signaling pathways.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABI5	Abscisic acid insensitive 5
ABRE	Abscisic acid response element
BA	Benzyladenine
BAM	Binary Alignment Map
BME	Beta mercaptoethanol
BPC	Basic pentacysteine
CHIP	Chromatin immunoprecipitation
Col-o	Columbia
CRF	cytokinin response factor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DREME	Discriminative Regular Expression Motif Elicitation
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methanesulfonate
FAIRE	Formaldehyde assisted isolation of regulatory elements
FASTA	Fast Adaptive Shrinkage Thresholding Algorithm
GO	Gene ontology
HK	Histidine kinase
HPt	Histidine phosphotransfer protein
MEME	Multiple Em for Motif Elicitation
MS	Murashige and Skoog
PBS	Phosphate buffered saline solution
PCR	Polymerase chain reaction

PMSF	Phenylmethanesulfonyl fluoride
RR	Response regulator
SDS	Sodium dodecyl sulfate
SNP	Single Nucleotide Polymorphism
TAIR	The Arabidopsis Information Resource
TCP	Teosinte branched/cycloidea/PCF
T-DNA	Transfer DNA
UNC HTSF	University of North Carolina's High Throughput Sequencing Facility

CHAPTER 1: CYTOKININS AND THE CYTOKININ PERCEPTION PATHWAY

Introduction

Cytokinins are plant hormones formed by biochemical substitutions on the N^6 amine group of adenine. Cytokinins have been shown to promote shoot formation, delay leaf senescence, regulate female gametophyte and vascular development, and regulate biotic and abiotic interactions (Skoog and Miller, 1957; Smart et al., 1991; Kant et al., 2015; Cheng et al., 2013; Schaefer et al. 2015). Genes that are consistently regulated by cytokinin in Arabidopsis have been identified by meta-analysis of microarray experiments (Bhargava et al., 2013), but how the core cytokinin pathway regulates all these genes is still unknown.

Cytokinins use a phosphorelay for signal transduction functionally similar to two-component systems in bacteria (Mizuno, 2005). In two-component systems, a histidine residue on a histidine kinase (HK) is phosphorylated in response to activation by a signal. When activated, the HK phosphorylates an aspartic acid residue on a response regulator (RR) protein, which triggers downstream effects. Cytokinin signaling is structured like a phosphorelay, which is slightly more complicated. The initial step is the perception of cytokinin in the lumen of the endoplasmic reticulum by binding to the CHASE domain of an HK (Higuchi et al., 2004). The activated HK passes a phosphate from a histidine residue in its kinase

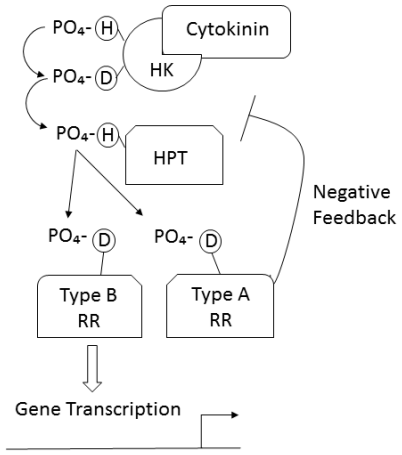


Figure 1.1. Diagram of the Cytokinin Perception Pathway.

domain to an aspartic acid residue in its receiver domain. HKs then transfer the phosphates to histidine residues on histidine phosphotransfer (HPt) proteins (Suzuki et al., 2000), and finally to an aspartic acid residue on an RR (Figure 1.1). There are two types of RRs that are the final acceptors of this phosphate: type-A RRs, which negatively regulate the cytokinin response pathway, and type-B RRs, which are transcription factors that mediate the transcriptional cascade downstream of cytokinin perception, including type-A RRs (Rashotte et al., 2006; To et al., 2004). In Arabidopsis, transcriptional studies have identified thousands of cytokinin-responsive genes (Rashotte et al., 2003). The consensus type-B binding sequence AGAT(T/C) has been identified as a core binding motif, and a longer version AAGAT(T/C)TT has also been defined (Muller and Sheen, 2008; Taniguchi et al., 2007; Franco-Zorilla et al., 2014). Despite the growing knowledge on the cytokinin signaling pathway, other components involved in the response to cytokinin remain unknown. For example, how cytokinins are transported into the cell to be perceived at the lumen of the ER and how type-A RRs are downregulated to allow the next pulse of cytokinin perception are unknown. In my graduate studies, I explored how cytokinin altered the chromatin landscape and developed a genetic screen to identify novel elements involved in the response to cytokinin.

CHAPTER 2: USING FAIRE-SEQ TO DETERMINE CYTOKININ-RESPONSIVE OPEN CHROMTAIN REGIONS AND REGULATORY ELEMENTS

Introduction

Cytokinin's pleiotropic effects suggest that the transcriptional cascade is highly regulated in such a way that particular genes can be activated at different times and in different organs. The regulatory elements involved would be targets of type-B RRs and other transcription factors that mediate cytokinin perception. These other transcription factors could be the output from other hormone signals or factors that are activated downstream of the cytokinin perception pathway. Characteristically the binding of transcription factors causes chromatin to open by shifting and removing nucleosomes from the DNA (Sullivan et al., 2014), changing the chromatin landscape and providing a detectable marker for regulatory elements.

Measuring nucleosome depletion is an effective way to identify open chromatin and analyze regulatory elements. First, nucleosome depletion is correlated with transcriptional regulation because nucleosome binding to regulatory elements is inhibited by occupation of these elements by transcription factors (Boeger et al., 2003). Second, nucleosome depletion can promote transcriptional regulation because remodeling chromatin can be a mechanism to facilitate transcription factor binding (Li et al., 2011). Third, nucleosome depletion can follow transcriptional regulation because transcriptional factors can recruit chromatin remodelers to the regulatory element (Furuta et al., 2011). Therefore, isolating and identifying nucleosome depleted regions, a mark of open chromatin, provides detailed information about how genes are regulated in the cell.

Formaldehyde Assisted Isolation of Regulatory Elements (FAIRE) is a method to identify open chromatin (Giresi et al., 2007). Briefly, histones are crosslinked to DNA *in vivo*. The DNA is then isolated and sheared. Open chromatin is then separated from DNA-histone complexes using phenol-chloroform extraction, wherein DNA-histone complexes are found in the interphase, while open chromatin is found in the aqueous phase (Giresi et al., 2007). FAIRE-seq is an elaboration of this method in which isolated open chromatin segments are subsequently sequenced so that the active regulatory elements throughout the genome can be identified in a high throughput manner. FAIRE-seq in Drosophila detailed both the activity and the precise length of previously unknown motifs (McKay et al., 2013). While FAIRE has been reported in Arabidopsis (Omidbakhshfard et al., 2014), the resultant data set had effectively no regions enriched for open chromatin. In this report, I successfully modified this FAIRE-Seq protocol to determine open chromatin regions and regulatory elements that are responsive to cytokinin in 10-day-old Arabidopsis seedlings. Moreover, I demonstrate that cytokinin induces extensive remodeling of the Arabidopsis genome and identified several cytokinin-responsive regulatory elements. Thus, this work has helped refine the transcriptional model of the cytokinin response in plants.

Methods

Seedlings were grown on MS plates for 10 days in constant light. After 10 days, they were immersed in MS media containing either 5 uM BA in DMSO or a DMSO control for one hour. Roots and shoots were then separated to yield four samples; Roots +BA, Roots –BA, Shoots +BA, and Shoots –BA. These samples were then fixed in 1% formaldehyde in PBS buffer for 10 minutes. Shoots were fixed under vacuum, roots were not. The fixations were then

quenched with 2.5 M Glycine and left for five more minutes in the same conditions. The tissues were then blotted dry and frozen in liquid nitrogen. Each tissue was ground finely and transferred to 20 mL Buffer 1 (100 mL: 10 mM Tris-HCl pH 8, 400 mM sucrose, 5 mM BME, 0.1 mM PMSF, and one Roche complete protease inhibitor cocktail tablet). The solution was filtered through miracloth and then centrifuged at 11000 g for 20 minutes at 4 °C. The supernatant was removed and the pellet was resuspended in 1 mL of Buffer 2 (10 mL: 10 mM Tris-HCl pH 8, 250 mM sucrose, 10 mM $MgCl_2$, 1% Triton X-100, 5 mM BME, 0.1 mM PMSF, and one-half a Roche complete protease inhibitor cocktail tablet). These were then centrifuged at 14000 rpm at 4 °C for 10 minutes, decanted, and the wash repeated two more times. After the third wash, the pellet was resuspended in 300 uL Buffer 3 (5 mL: 10 mM Tris-HCl pH 8, 1.7 M sucrose, 2 mM $MgCl_2$, 0.15% Triton X-100, 5 mM BME, 0.1 mM PMSF, and one-quarter a Roche complete protease inhibitor cocktail tablet) and then layered on another 300 uL of Buffer 3. These were then centrifuged at14000 rpm at 4 °C for 70 minutes, decanted, and resuspended in 1 mL of Nuclei Lysis Buffer (5 mL: 50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, 0.1 mM PMSF, and one-quarter a Roche complete protease inhibitor cocktail tablet). These were then sonicated in a Bioruptor to a fragment length ranging from 200-500 bp. The lysate was spun down and the supernatant was split into three portions; two aliquots of 450 uL that were labelled FAIRE and one aliquot of 100 uL that was labelled INPUT. The FAIRE aliquots were extracted with one volume of phenol:chloroform:isoamyl alcohol, spun for five minutes at 14000 rpm, and then the aqueous layer was moved to a fresh tube. This was repeated with a new volume of phenol:chloroform:isoamyl alcohol. Then each aqueous portion was washed with one volume of chloroform, spun for five minutes at top speed, and the aqueous layer moved to a fresh tube. The DNA was then precipitated with 2 volumes of ethanol and 0.1 volumes of sodium acetate, spun

down, washed with 70% ethanol, and then resuspended in water. Both the FAIRE and the INPUT samples were incubated in a 65 °C heat block overnight to decrosslink the samples. The next day, FAIRE and INPUT were treated with RNAse A for 30 minutes. The INPUT aliquots were treated with proteinase K for an hour and then taken through the same phenol:chloroform:isoamyl alcohol, chloroform, and ethanol precipitation steps that the FAIRE aliquots were. The two FAIRE aliquots for each sample were combined and then the FAIRE and the INPUT samples were quantified by Qubit. If the FAIRE samples had <10% as much DNA as an equivalently sized INPUT sample would have, I concluded that crosslinking was successful and the FAIRE samples were sent to the UNC High-Throughput Sequencing Facility (HTSF) to be sequenced on an Illumina 2500, returning 50-bp single-end reads.

The reads were filtered for quality by bbduk.sh and mapped to the TAIR10 genome using bbmap.sh (Bushnell, 2015). Peaks were called from the resultant BAM files with macs2 callpeak (Zhang et al., 2008). The optimal p-values were found using macs2 callpeak (Zhang et al., 2008). Cytokinin-specific peaks were found by subtracting peaks in the DMSO control data set from the BA data set in the same tissue using bedtools subtract (Quinlan and Hall, 2010). Common peaks were found by intersecting these datasets with bedtools intersect (Quinlan and Hall, 2010). In order to find which peaks were behaving consistently, cytokinin-specific peak files from each replicate were intersected as were the common peak files. Shoot specific peaks were found by subtracting the raw root minus peak file from the raw shoot minus peak file. Intersection was performed with a minimum overlap required of 0.8. Subtraction was performed with a minimum overlap of 0.5. The corresponding genes were found using bedtools to identify genes with FAIRE peaks between 2000 bp upstream of the transcriptional start site and 1000 bp downstream of the transcriptional stop site. The corresponding sequences were found using bedtools getfasta

(Quinlan and Hall, 2010). GO analysis generated BioMaps through the Virtual Plant Web Tool (Katari et al., 2010). Multiple Em for Motif Elicitation (MEME-chip) (Machanick and Bailey, 2011) identified motifs significantly enriched in the cytokinin specific peak FASTA files. Its subsidiary program, Discriminative Regular Expression Motif Elicitation (DREME) (Bailey, 2011), identified short motifs ranging from five to ten base pairs long. DREME was performed first using shuffled sequences as a control, and then using the common FAIRE peak FASTA dataset as a control. The shuffled sequences were derived by randomly generating strands of equal length to those in the dataset and with equal frequency of each nucleotide. The common FAIRE peak FASTA dataset was the list of peaks shared between the BA treatment and DMSO control found using bedtools intersect (Quinlan and Hall, 2010).

Results

1. Cytokinin induces different chromatin profiles in different tissues

Cytokinin produces distinct effects depending on tissue type, implying that the hormone acts in a context-specific manner (Brenner and Schmulling, 2012). I have found in these FAIRE experiments that cytokinin regulates genes in a tissue-specific manner, suggesting a mechanism for how a single hormone can affect different pathways in different tissue types.

I used FAIRE-seq to compare the chromatin profiles of Arabidopsis seedlings treated with cytokinin to the profiles of seedlings that were not, analyzing roots and shoots separately. The reads gathered from this experiment were mapped to the Arabidopsis genome (Kersey et al., 2015) and sequence coverage was found using macs2 (Zhang et al., 2008). The sequence coverage was highly correlated between replicates. Pearson correlation, a measure of linear relationship between replicates, indicated a strong positive correlation between the two

replicates, suggesting that the replicates commonly peaked and troughed at the same loci. This was reflected in the frequency at which peaks called from the two replicates intersected (Table 2.1, Figure 2.1). These files were compared using bedtools (Quinlan and Hall, 2010) to derive the common peak files and the cytokinin-specific peak files. 9725 peaks were identified in both replicates in roots in response to cytokinin and 3354 peaks in shoots. This suggests there may be differences in strength of the response in roots and shoots, though this may reflect how cytokinin was delivered to these tissues.

Table 2.1. Pearson correlation of bedgraph files between replicates calculated with Wigcorrelate

Shoot +BA	Shoot-BA	Root +BA	Root -BA
0.850	0.924	0.968	0.976

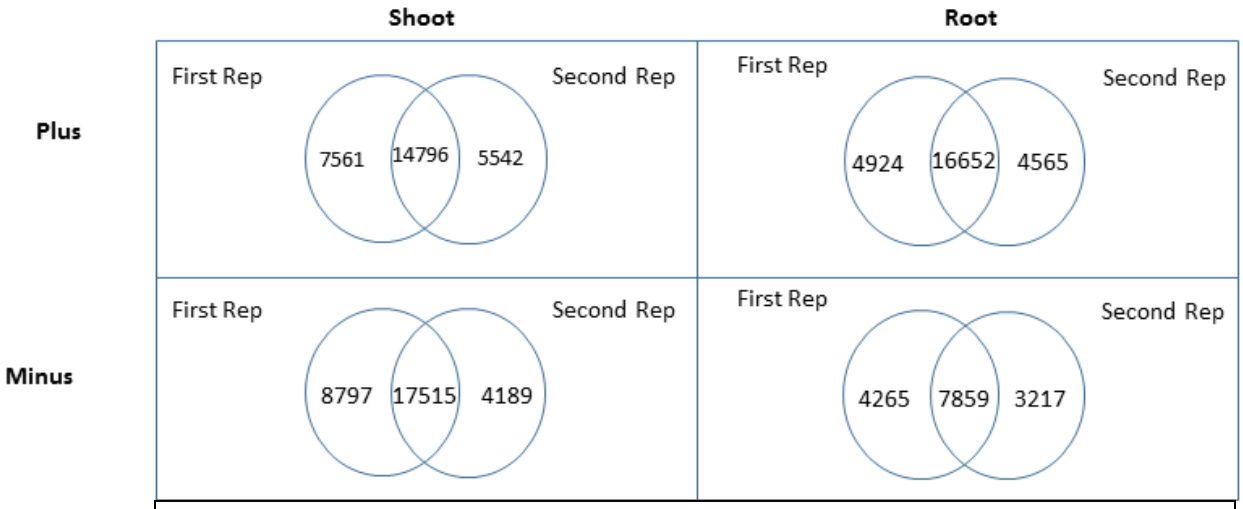


Figure 2.1. Venn Diagrams of replicate peak overlap. Each circle counts the number of peaks called in that sample in that replicate. The intersection represents the peaks that appear in both replicates (required overlap >80% of the original peak).

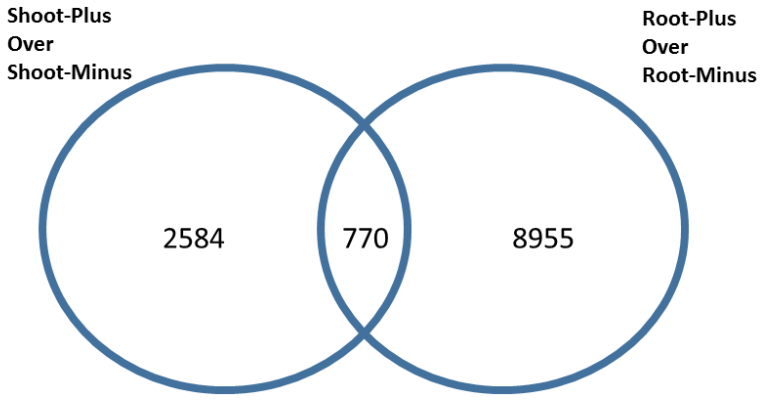


Figure 2.2. Overlap in cytokinin-specific peaks between the two tissues. Minimum overlap was set to 0.8.

When the seedlings were immersed in control or cytokinin-containing media, the media was taken up by the roots and moved by transpiration to the shoots. Therefore the rapid and large response in roots may reflect more efficient treatment of cytokinin.

However, in addition to the difference in quantity, the different tissues also display distinct open

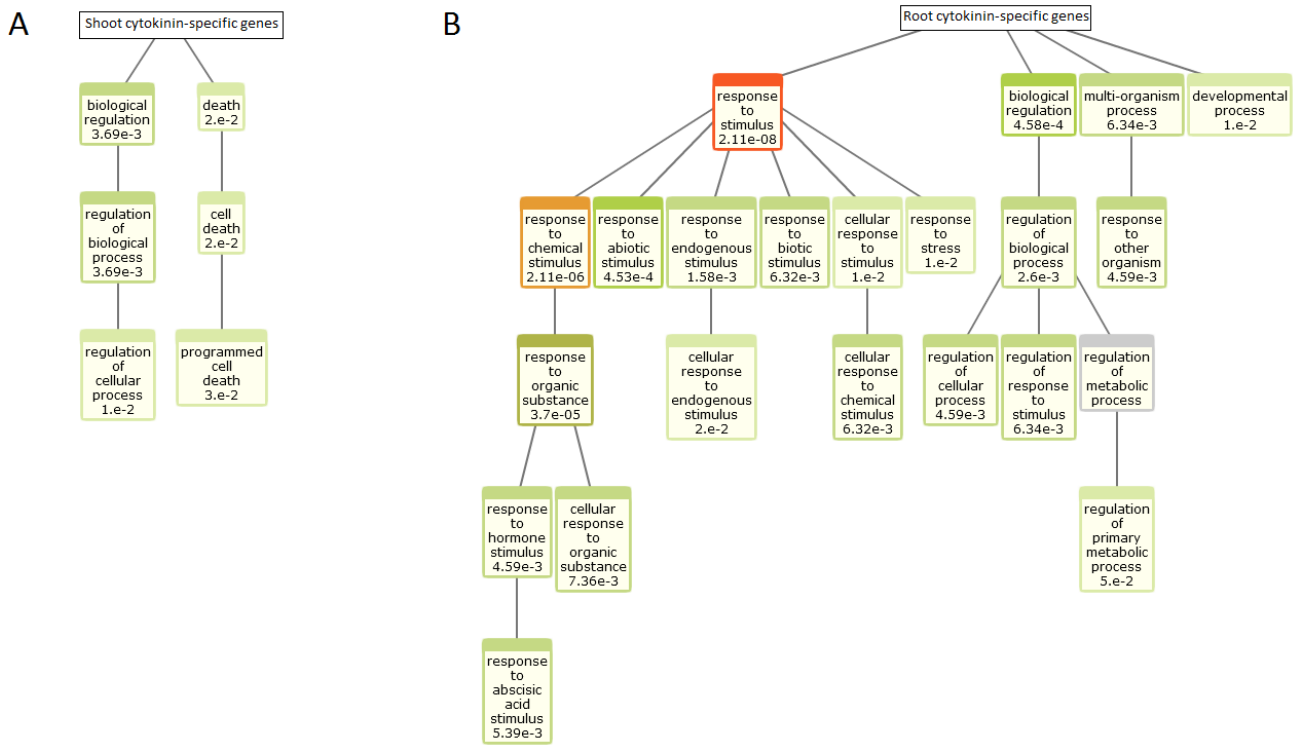


Figure 2.3. Biomaps displaying GO terms enriched in cytokinin response. GO terms enriched in genes associated with cytokinin-responsive peaks in shoots (A) and roots (B). P-value ≤ 0.01

chromatin profiles. The overlap between cytokinin-specific peaks in the roots and cytokinin-specific peaks in the shoots is relatively low (Figure 2.2), suggesting that the two

tissue types have distinct sets of genes activated by cytokinin. The genes associated with cytokinin-specific peaks were functionally annotated through GO analysis using VirtualPlant (Katari et al., 2011). This analysis revealed that the cytokinin-specific peaks in each tissue are associated with different pathways (Figure 2.3). Genes associated with cytokinin-specific peaks in the root are enriched for various terms, including stimulus by abscisic acid. This suggests interplay between abscisic acid and cytokinin response pathways. Other terms that were also enriched suggest interplay between cytokinin and development, metabolism, and abiotic stimulus. Interestingly, genes associated with shoot cytokinin responsive FAIRE peaks are enriched for only one term that is not in root response, and that is programmed cell death. This may be illustrative of cytokinin's role in leaf senescence (Kant et al., 2015).

2. Cytokinin induces a shoot-like chromatin profile in roots

Cytokinin induces transcription of multiple shoot-specific genes in roots (Brenner and Schmulling, 2012). This is reflected in the chromatin profiles as well. Shoot-specific peaks were found by subtracting the root peaks without cytokinin from the shoot peaks without cytokinin. This dataset represents the peaks that are basally present in shoots but not in roots. The genes associated with these peaks are enriched for GO terms such as abscisic acid response

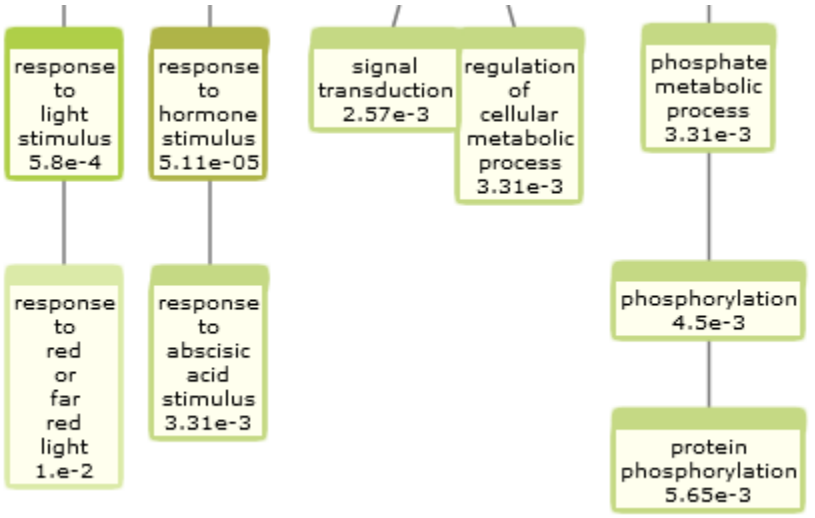


Figure 2.4. Terminal nodes for GO terms enriched in genes associated with shoot-specific peaks. P-value ≤ 0.01

and response to light stimulus, which are consistent with shoot-related data (Figure 2.4). Taking the intersection of this peak file and the file of peaks in roots that are specific to cytokinin reveals

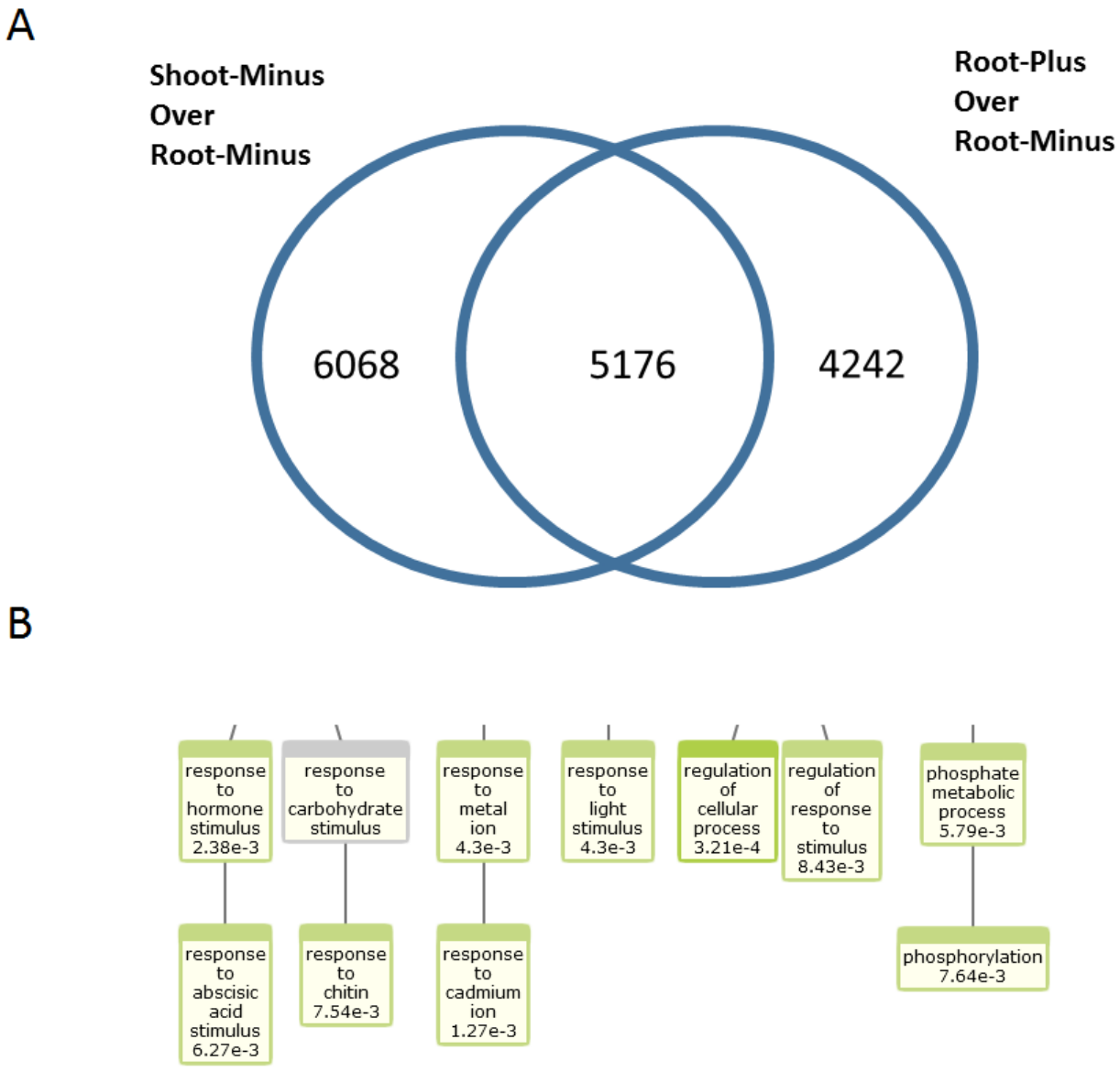


Figure 2.5. Cytokinin treated roots have a shoot-like profile. Overlap between Shoot specific peaks and Cytokinin induced root peaks. (A) Terminal nodes of the Biomap of GO terms enriched in the 5176 genes from the intersection (B)

a significant overlap (p-value < 2.2e-16 with Fisher's exact test) (Figure 2.5a). Roughly 53% of the peaks induced by cytokinin in roots are peaks that are otherwise specific to shoots. As expected, the overlap is enriched for stimulus terms common to both sets, including abscisic acid

(Figure 2.5B). This data suggests that promotion of shoot development in tissue culture by cytokinin (Skoog and Miller, 1957) may be mediated by cytokinin-controlled changes in the chromatin profile.

3. *Cytokinin-specific peaks are associated with genes involved in primary response to cytokinin.*

Since the ultimate output of cytokinin perception is transcriptional regulation, it is reasonable

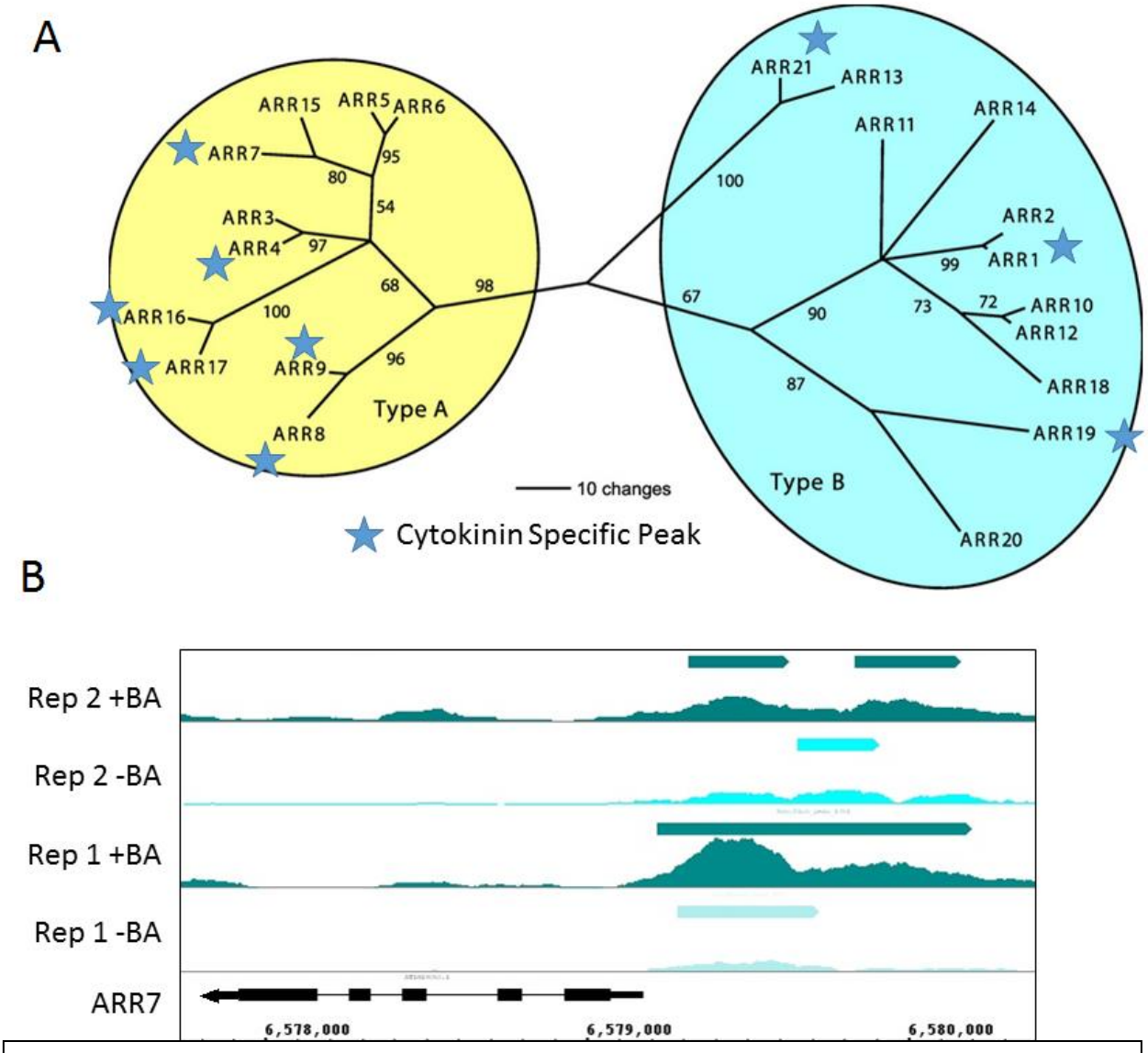


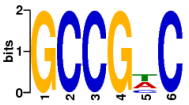
Figure 2.6. Peaks upstream of cytokinin response genes. Phylogenetic tree of the type-A RRs (left) and the type B RRs (right) with the RRs associated with a cytokinin specific FAIRE peak marked with a blue star (A). *ARR7* with cytokinin specific peaks 5' to the transcriptional start site. Two replicates in root with and without cytokinin are shown.

to surmise that many of the cytokinin-specific peaks would be related to that effect. Type-A response regulators in particular are cytokinin primary response genes (D'Agostino et al., 2000). Several type-A response regulators have cytokinin-specific FAIRE peaks associated with their 5' regulatory regions (Figure 2.6). The identification of known cytokinin-regulated genes from our FAIRE analysis provides confidence that the other peaks identified, though function yet unknown, are likely cytokinin-responsive.

4. Motifs enriched in peaks

MEME-chip was used to identify potential cis-acing motifs that are enriched in cytokinin-specific FAIRE peaks (Machanick and Bailey, 2011). DREME (Bailey, 2011), a component of MEME chip, identified several known motifs in the root data set (Table 2.2, middle-right column). Two of the motifs identified, the poly-A and the TATA box, may simply reflect basal transcriptional functions such as terminators and promoters. However, many of the motifs had predicted binding partners (Franco-Zorilla et al., 2014) that were supported by other pieces of evidence. The motif TGGGC was first identified as the Site II element enriched ahead of cytochrome C and members of the oxidative phosphorelay pathway (Welchen and Gonzalez, 2005; Welchen and Gonzalez, 2006). Subsequent analysis found it could be bound by the *TEOSINTE BRANCHED/CYCLOIDEA/PCF* (TCP) protein family in Arabidopsis (Giraud et al., 2010). TCP transcription factors are activated by the cytokinin response pathway both transcriptionally (Lucero et al., 2015) and post-translationally (Steiner et al., 2012). The palindromic motif CACGTG is known as the Abscisic acid responsive element (ABRE) and is targeted by *ABSCISIC ACID INSENSITIVE*(ABI5) (Reeves et al., 2011). Both cytokinin and abscisic acid have been shown to play an important role in the response to abiotic stress

(Argueso et al., 2009), and this may reflect that overlap. TGACY, the W-box, is recognized by the WRKY family of proteins which are involved in immune response (Yu et al., 2001), a function also linked to the cytokinin response pathway (Hwang et al., 2012). The GAGA box can be found as a k-mer of any length of repeating GA sequences, and is bound by *BASIC PENTACYSTEINE* proteins (BPCs) in CHIP experiments (Simonini et al., 2012). The link between BPCs and cytokinin response is currently being investigated (see below). *CYTOKININ RESPONSE FACTORS* (CRFs) are transcription factors in the *APETALLA2* (AP2) family upregulated by cytokinin (Rashotte et al., 2006). A protein-binding microarray has shown their binding motif to be GCCGNC. Of special note was the isolation of a motif containing the AGAT core motif that is bound by type-B RRs. The core motif of the Type-B RRs is AGAT (Sakai et al., 2000), with a variety of elongated versions also identified, including AGATT and AAGATCTT (Hosoda et al., 2002; Taniguchi et al., 2007). Treatment with cytokinin is expected to cause binding of transcription factors at this motif. FAIRE provides evidence that this binding displaces nucleosomes in some fashion. FAIRE showed binding of the RRs to the DNA which could be used to study the dynamics of this binding. The dataset of cytokinin-specific FAIRE peaks in roots was submitted to DREME a second time, but this time the control sequences were FAIRE peaks that were present regardless of cytokinin treatment. An interesting observation is that of the transcription factor-related motifs, only the Type-B RR motif and the BPC-related motif, the GAGA box, were significantly enriched (Table 2.2, right column). This suggests that the other transcription factors function upstream of other genes that are not regulated by cytokinin.

Table 2.2. Motifs enriched in DREME in the Root cytokinin-specific FAIRE peaks file			
Enriched Motif	Transcription Factor	Shuffled sequences as control (E-value)	Non-specific FAIRE peaks as control (E-value)
	TCP15	5.1e-152	---
	---	2.3e-57	1.19e-220
	ABI5	6.8e-45	---
	BPC	2.8e-22	7.00e-8
	---	1.2e-21	4.49e-13
	ARR	1.0e-9	4.90e-7
	CRF	1.5e-8	---
	WRKY	2.3e-7	---

5. Further analysis of identified motifs

In order to confirm the relationship between Type-B RR binding and change in chromatin conformation, the FAIRE peaks datasets from both roots and shoots were overlapped with a dataset *ARR10* specific CHIP peaks generated from 10 day old whole seedlings (Eric Schaller, unpublished). 1288 of the top 4992 CHIP peaks overlapped a FAIRE peak (Figure 3.1A), 1284 (99.7%) of these overlaps in peaks contained the core AGAT motif and 469 of these were the TAGATT motif found through DREME analysis. To determine that these CHIP peaks and FAIRE peaks coincided, the distances between each CHIP peak and the closest FAIRE peak were determined. The histogram of these distances was normally distributed around zero (Figure 3.1B), suggesting that *ARR10* binding occurs at or near the same position at which the chromatin is opening in response to cytokinin.

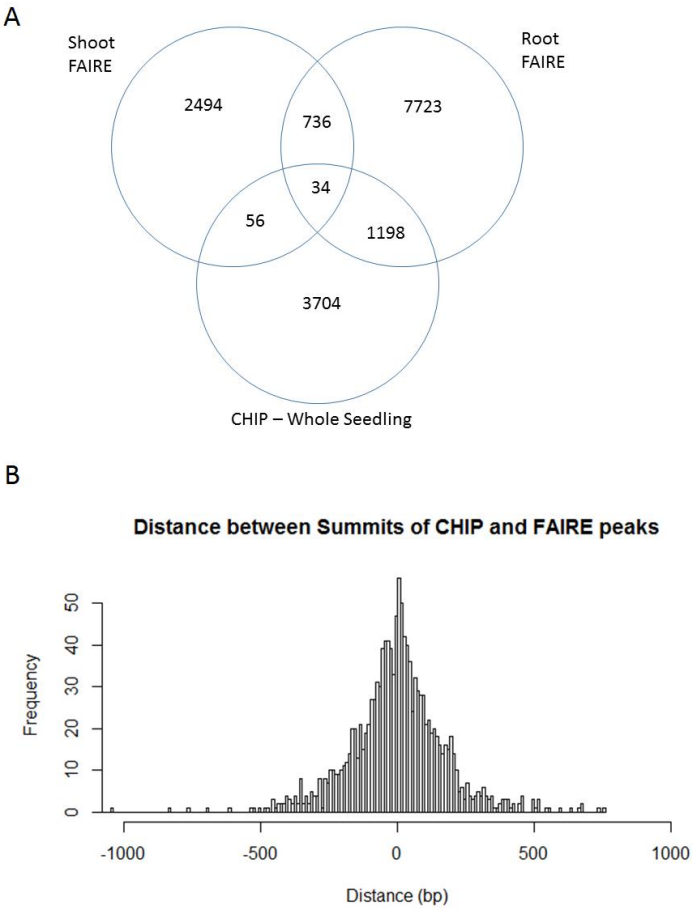


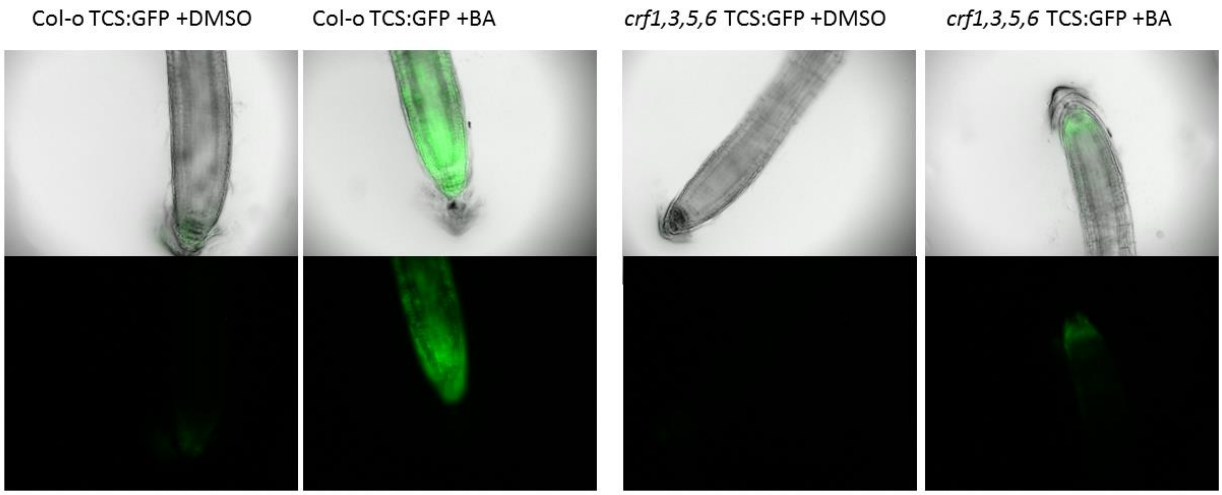
Figure 2.7. Comparison of *ARR10* CHIP peaks and cytokinin specific FAIRE peaks. Venn diagram depicting peak overlap. (A) Histogram representing summit-to-summit distances limited to summits less than 1000 bp apart. (B)

The GAGA box, one of the few motifs enriched in cytokinin-specific peaks relative to other FAIRE peaks, is the binding motif for the BPC transcription factors. BPCs interact with type-A RRs in yeast-two-hybrid assays (Carly Shanks, unpublished), which is interesting because type-A RRs negatively regulate cytokinin signaling through an unknown mechanism (To et al., 2004). These data suggest that the type-A RRs may regulate the ability of the BPCs to co-regulate target genes with the type-B RRs.

The CRFs are transcriptionally regulated by cytokinin (Rashotte et al., 2006) but, unlike the BPCs, their binding motif is enriched at a similar level in non-cytokinin-specific FAIRE peaks, suggesting they act independently of cytokinin signaling in other gene promoters. To test the interplay of cytokinin signaling and CRF signaling, a TCS:GFP transgene was crossed into the *crf1,3,5,6* mutant background. TCS utilizes the type-B RR binding motif to drive expression of the conjugated reporter gene (Zurcher et al., 2013) and as such acts as a reporter in the activity of type-B RRs. Wildtype plants with the TCS:GFP construct have low GFP expression in the absence of cytokinin and much higher expression after cytokinin treatment (Figure 3.2A). *crf1,3,5,6* TCS:GFP plants have almost no expression of GFP in the absence of cytokinin, suggesting a role of CRFs in the basal expression of the type-B RR motif. However, in the presence of cytokinin GFP expression increases in the *crf* mutant (Figure 3.2B), indicating that the cytokinin perception pathway is active even in the absence of CRFs. This suggests that the binding and activity of the CRFs may be independent of type-B binding and activity. GFP signal increases during cytokinin perception in tissues where the CRFs are expressed and active, but the CRFs are not necessary for signaling, so cytokinin can work in a different degree or a different manner in other tissues. This may reflect the mechanism by which one signal like cytokinin acts to affect multiple distinct pathways in various cellular contexts.

In summary, the power of FAIRE in regards to studying the cytokinin perception pathway is threefold. First, FAIRE provides insight into the *in vivo* sequence of the Type-B RR motif and an ability to measure its activation state. Second, FAIRE implicates other motifs such as the GAGA box as a possible mechanism for the BPCs to mediate cytokinin induced transcription. Third, FAIRE identifies motifs, like the CRF binding motif GCCGNC, which provide context for the cytokinin signal and may alter how cytokinin is perceived in different tissue types.

A



B

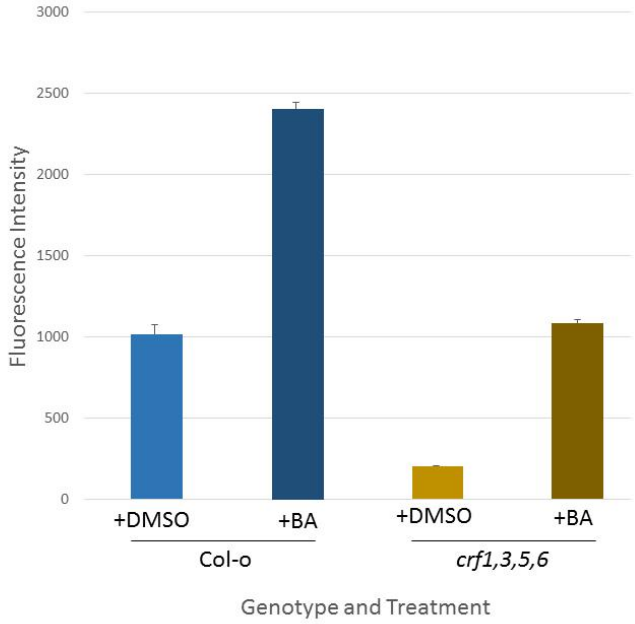


Figure 2.8. Activation of a TCS promoter by cytokinin in *crf1,3,5,6*. Columbia TCS::GFP (Col-0) and *crf1,3,5,6* TCS::GFP seedlings were grown for 9 days on duplicate vertical plates and treated by immersion in MS media with benzyladenine (BA) or DMSO control for 24 hours. Fluorescence was measured in the meristematic zone using a Nikon microscope. (A) Average fluorescence intensity in the root apical meristems of 20 seedlings was found using ImageJ. (B)

CHAPTER 3. SENSITIZED GENETIC SCREEN FOR COMPONENTS OF CYTOKININ RESPONSE PATHWAY

Introduction

Genetic screens have been used to find core components of the cytokinin response pathway (Inoue et al., 2001). However, it is likely that there are components of the cytokinin pathway that have not been identified. For example, although cytokinin is transported through the plant by means of the vascular tissue (Kudo et al., 2010), it is unknown what proteins are involved with the transport of cytokinin from the vasculature into the cell. Many proteins that are not found may simply have small effects on cytokinin sensitivity when mutated so that they would not show up in conventional genetic screens for components of the cytokinin perception pathway. This can be circumvented using sensitized Arabidopsis lines that are on the verge of a phenotypic response. An unbiased genetic screen in a cytokinin-sensitized background is currently underway to identify genes of small effect in the cytokinin perception pathway.

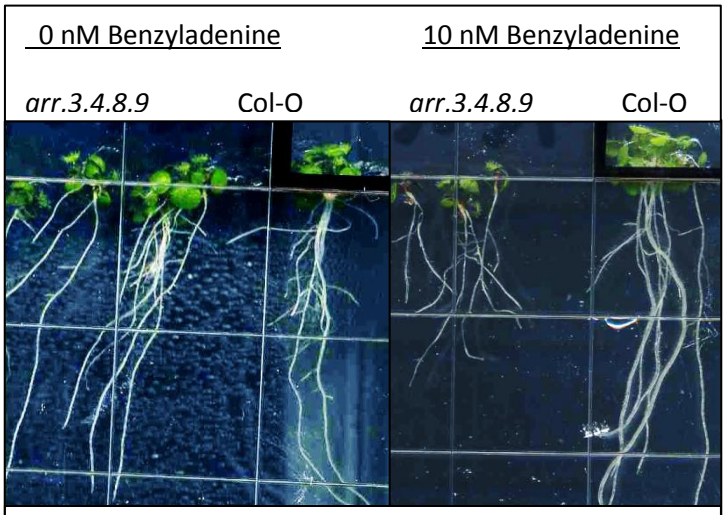


Figure 3.1. Root length in Type-A RR mutant seedlings (*arr3,4,8,9*) and wildtype Columbia seedlings (Col-O). Seedlings were sown on MS plates with 10 nM benzyladenine or DMSO control and grown upright in 24 hr light for 10 days.

Methods

Single T-DNA insertion alleles of the type-A RRs 3, 4, 8, and 9 were crossed to generate the quadruple knockout line. With all four alleles, the

seedlings are hypersensitive to cytokinin (To et al., 2004). *arr3,4,8,9* seedlings have a dramatically shorter root in the presence of 10 nM cytokinin than wild type Arabidopsis seedlings, but have roots of a similar length in the absence of cytokinin (Figure 1.2). Therefore a screen in *arr3,4,8,9* background for wildtype-length roots in the presence of cytokinin would identify positive regulators of the cytokinin pathway, including those having only a small effect on cytokinin sensitivity. Briefly, *arr3,4,8,9* seedlings were mutagenized with 0.4% EMS for 8 hours. The mutagenized seeds (M1) were grown and their progeny (M2) was harvested. The M2 seeds were screened for suppressors of hypersensitivity on MS plates containing 10 nM benzyladenine (BA). After 10 days, putative suppressor mutants were selected by eye and transferred to soil. Ninety-five putative mutants have been grown and genotyped for the T-DNA insertions in the response regulators. They were genotyped by PCR using a three primer strategy where two primers flanking the response regulators and one T-DNA-specific border primer were used to amplify the genomic DNA. Wildtype plants would have a band the length of the gene. Mutant plants would have a shorter band the length of the distance from the T-DNA's border and the gene border. When harvested, their progeny (M3) will be plated in similar conditions to confirm the suppressor phenotype is heritable and also on plates with no cytokinin to confirm the suppressor phenotype is cytokinin-specific. The M3 will be backcrossed to unmutagenized *arr3,4,8,9* to generate the F1, which will be selfcrossed to create the F2. The F2 should be segregating for the suppressor phenotype. F2 plants with the suppressor phenotype will be selected and sequenced to identify chromosomal segments that are homozygous for EMS-induced SNPs using next-generation mapping (Austin et al., 2011). These identified genomic intervals will be searched for SNPs most strongly correlated with the suppressor phenotype and located inside gene bodies to find the putative causal SNP and its associated gene.

Results

Ninety-five putative mutants have been indentified in the M2 generation (Figure 1.3) . All screened mutants have been genotyped for the T-DNA insertions. All ninety-five lanes

Figure 3.2. Sampling of screen plates where putative mutants are easily discerned. Seedlings were sown on 10 nM benzyladenine MS plates and grown upright in 24 hr light for 10 days.

contain the PCR bands characteristic of their mutant parent indicating the putative mutants contain the original T-DNA insertions. The progeny of these mutant lines are being tested in the presence and absence of cytokinin to determine that the phenotype is heritable and cytokinin specific. One of these mutant lines with strong, heritable, cytokinin-specific phenotypes may contain a mutation in a gene not previously known to be a component of the cytokinin perception pathway. The powerful sequencing approaches our lab is using will drastically decrease the number of generations required before the causative SNP is identified by scanning the genome at a resolution far more fine than any PCR mapping strategy. Thus our lab is well-positioned to quickly and effectively discover previously unknown components of the cytokinin perception pathway in Arabidopsis.

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