THE MECHANISM OF TYPE-A ARABIDOPSIS RESPONSE REGULATORS IN CYTOKININ SIGNALING IN *ARABIDOPSIS THALIANA*

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ABSTRACT

Carly Michelle Shanks: The mechanism of type-A *Arabidopsis* response regulators in cytokinin signaling in *Arabidopsis thaliana*
(Under the direction of Joseph Kieber)

Cytokinin is a phytohormone that regulates numerous processes in plant growth and development, including cell division, meristem maintenance, sink/source relationships, nutrient up-take, vascular development, and biotic and abiotic stress responses. The cytokinin signal is relayed through a two-component signaling system and ultimately leads to changes in gene expression. The type-A Arabidopsis response regulators (ARRs) are transcriptionally up-regulated in response to cytokinin and are stabilized by phosphorylation of their receiver domain. The ten type-A ARRs act as redundant negative regulators of cytokinin signaling and participate in a negative feedback loop to reduce cytokinin responsiveness. Previous studies have suggested that the type-A ARRs interact with other target proteins to negatively regulate the pathway, however, the mechanism has remained unclear. Here we explore how the type-A ARRs regulate cytokinin signaling.

In this study, the type-A ARRs are implicated in multiple plant processes, including nematode infection, transcription factor regulation, and interaction with the exocyst complex. For example, we find defense response genes are basally up-regulated in the type-A *arr3,4,5,6,7,8,9,15* loss-of-function mutant, and upon nematode infection these genes are hyper-induced, which leads to decreased pathogen success. To further examine type-A ARR function, we conducted a yeast two-hybrid screen for type-A ARR binding partners and found that the type-A ARRs interact with a member of the BASIC PENTACYSTEINE (BPC6) transcription factor family and a subunit of the exocyst complex, Exo70D3. Our research suggests that the BPC proteins are part of a network of transcription factors that regulates

cytokinin response genes, and the type-A ARRs interact with BPC proteins to modify their activity. Furthermore, we find that the Exo70D proteins are positive regulators of cytokinin signaling and our data suggest that the Exo70D proteins regulate type-A ARR protein levels. Overall, we provide some mechanistic insight into the multiple roles of the type-A ARRs and how they regulate cytokinin responsiveness.

To my husband, Benjamin. Thank you for letting me be the best version of myself and for being my best friend. I could not have done this without you.

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PREFACE

In chapter 1, the first section of this chapter was published in the eLS Wiley Online Library, a peer-reviewed publication for online review articles. Co-first authorship is shared between Carly Shanks and Christian Burr, both members of Joesph Kieber's laboratory. Reference: "Burr, C.A. Sacks, C.M., and Joseph, J.J. (2014). Two-component signaling in plants. In: eLS. John Wiley & Sons, LtD: Chichester. DOI:10.1002/979780470015902.a0025266."

Chapter 2 was published in the journal Molecular plant-microbe interactions. Co-first authorship is shared between Carly M. Shanks and John Hollis Rice. This publication was a collaboration between Joseph Kieber's and laboratory and Tarek Hewezi's laboratory at the University of Tennessee, Knoxville. The supplemental gene list files are not shown, but can be found online. Reference: Shanks, C.M., Rice, J.H., Zubo, Y., Schaller, G.E., Hewezi, T., and Kieber, J.J. (2016). The role of cytokinin during infection of *Arabidopsis thaliana* by the Cyst Nematode
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Chapter 3 will be submitted to the journal The Plant Cell in the next month. For my contributions, I conducted and designed the experiments and wrote the manuscript with the exception of the following, which was done by other members of the Kieber lab or our collaborators. A yeast two-hybrid screen was conducted previously in the Kieber lab and identified the interaction between *BASIC PENTACYSTEINE 6* (BPC6) and type-A ARR4. We received the pACT2 prey destination vector and yeast train from Alexander Heyl at Adelphi University. The coding sequences for *BPC1*, *BPC2*, *BPC3*, *BPC4*, and *BPC6* were cloned into entry vectors by Chia-Yi Cheng, Jason Punwani and Jamie Winshell. Seeds for the multiple *bpc1-1,2,3, bpc4,6*, *bpc1-1,2,4,6*, and *bpc1-1,2,3-1,4,6* mutants were received from the lab of Charles S. Gasser from University of California Davis. Chia-Yi Cheng worked to make these lines homozygous after we received segregating lines. We received T-DNA insertional mutants for *bpc1-1,2* and *bpc1-1,2,3-1,4,6,7* from the Arabidopsis Biological Resource. The BPC6 ChIP-sequencing experiments, X-ChIP PCR, and DREME motif analysis was conducted by members of Klaus

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In chapter 4, I designed and conducted the experiments with the following exceptions. The *Exo70D3* coding sequence was cloned by Jason Punwani. Joe Kieber conducted the original yeast two-hybrid screen. We received T-DNA insertional mutants for single *exo70D1-1*, *exo70D1-2*, *exo70D2-1*, *exo70D2-2* mutants from the ABRC stock center.

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LIST OF ABREVIATIONS

AHK - Arabidopsis histidine kinase

AHP - Arabidopsis histidine-containing phosphotransfer protein

AP2/ERF - APETALA2/Ethylene Responsive Factor

ARR - Arabidiopsis Response Regulator

BA - Benzyladenine

BPC - BASIC PENTACYSTIENE

BiFC - Bimolecular fluorescence complementation

ChIP-seq - Chromatin immunoprecipitation followed by sequencing

CRF - Cytokinin response factor

DR5 - auxin responsive promoter reporter system

GFP - Green fluorescent protein

PIN1 - PIN-FORMED1

QC - quiescent center

RAM - Root apical meristem

RNA-seq - RNA extraction and library synthesis followed by next-generation sequencing
SAM - Shoot apical meristem

SHY2 - SHORT HYPOCOTYL 2

TCS - Two-component signaling system

CHAPTER 1: INTRODUCTION

Section 1: Two-component signaling in plants

Summary

Two-component signaling pathways are used by bacterial cells to perceive and respond to a wide variety of environmental signals. In their simplest form, these are comprised of a histidine kinase receptor whose activity is regulated by a signal and a response regulator, whose activity is controlled by phosphorylation on an Asp residue that is mediated by the histidine kinase. Plants use two-component signaling elements to respond to endogenous and environmental signals. The plant pathways are either an extended version of the two-component pathway called a phosphorelay, or include degenerated elements that no longer function as histidine kinases. These elements are conserved throughout the plant kingdom, though they are best understood in the model plant species *Arabidopsis thaliana*. The most complete and best understood of these is the signaling pathway for the phytohormone cytokinin, which is perceived by ER-localized histidine kinase receptors, and ultimately regulate the phosphorylation of a set of response regulators that mediate the transcriptional response to cytokinin.

Introduction

The two-component signal transduction system (TCS), first characterized in prokaryotes, is used by bacteria, fungi, and plants to sense and respond rapidly to changes in the environment or to endogenous cues (Stock et al., 2000; Schaller et al., 2011). The prototypical TCS includes a transmembrane sensor histidine protein kinase (HK) and soluble response regulator (RR) protein (Stock et al., 2000) (Figure 1.1). Typically, the sensor HK contains an input domain that perceives a signal, which regulates its autophosphorylation activity via transfer of a phosphate from adenosine triphosphate (ATP) to a conserved histidine (His) residue within its transmitter domain. This phosphate is transferred from the sensor HK to an aspartic acid (Asp) residue within the receiver domain of a RR (Stock et al., 2000). Phosphorylation of the Asp residue induces a conformational change within the receiver domain

that most often activates an output domain of the RR. While the receiver domains of RRs are highly conserved, there are diverse types of output domains, the most common of which are DNA-binding domains (Bourret, 2010), though a subset of RRs lack an output domain altogether.

Multiple permutations of TCS pathways exist, including an extended version with multiple phosphotransfers referred to as a phosphorelay, which is the most common pathway architecture for plant two-component signaling (Schaller et al., 2008a). A phosphorelay includes hybrid HKs, which contain an input domain, a transmitter HK domain, and a receiver domain, a histidine-containing phosphotransfer (HPt) protein and a RR (Schaller et al., 2008a). Upon activation of the HK, the phosphate group is transferred from the His residue within the transmitter domain to the Asp residue of the fused receiver domain. The phosphate is then shuttled by an HPt protein on their conserved His residue to the receiver domain of the RR (Figure 1.1).

Plants use TCS elements for a variety of cellular processes, including the regulation of the circadian rhythm and the response to cytokinin, ethylene, red light, and osmosensing (Schaller et *al.*, 2011). In plants, TCS components have been studied extensively in the dicot model species *Arabidopsis thaliana*. Most notably, the perception of the phytohormone cytokinin occurs via a well-characterized signaling pathway that makes use of a canonical multi-step phosphorelay (To and Kieber, 2008). The TCS components are also found in monocot species, including rice and maize, and in early land plants including *Selaginella moellendorfi* and *Physcomitrella patens* (Table 1.1). While there are many conserved functions of TCS components across plant species, there are also species-specific adaptations that have developed throughout evolution (Pils and Heyl, 2009).
Here, we will discuss in turn the various classes of two-component elements found in plants and the roles uncovered so far for these signaling proteins.

Histidine Kinases in Plants

Histidine kinases perceive various signals and act at the beginning of the TCS phosphorylation cascade. In plants, there are both functional HKs, which have intrinsic histidine kinase activity, and degenerate HKs that are evolutionarily derived from HKs, but which have lost residues essential for histidine kinase activity. The functional HKs are generally present as hybrid sensor HKs, which are

comprised of a sensor domain that binds to or perceives the presence of a signal, a histidine kinase domain, which autophosphorylates in response to the signal, and a fused receiver domain, to which the phosphate is transferred before moving to the downstream Hpt proteins. In this section, what is known about the structure and function of histidine kinases in plants will be reviewed.

Cytokinin Signaling

The best characterized example of phosphorelay signaling in plants is the cytokinin response pathway. The plant hormone cytokinin is an N^6-substituted adenine derivative that is involved in many aspects of plant development. The receptors have two transmembrane domains, with the cytosolic portion including a histidine kinase and a C-terminal receiver domain that contain all the highly conserved residues required for enzymatic function (Figure 1.1). These receptor HKs employ an evolutionarily ancient CHASE domain for cytokinin binding (Caesar et al., 2011). CHASE domain containing HKs are found in mosses (*Physcomitrella patens*), early vascular plants (*Selaginella moellendorffi*), monocots and dicots (Pils and Heyl, 2009) (Table 1.1). In higher plants, the cytokinin receptor HKs are present as a small family of 3-4 genes that have partially overlapping functions. These cytokinin HK receptors bind various species of biologically active cytokinins with different affinities (Romanov et al., 2006; Lomin et al., 2011), with K_D values in the range of 1-10 nM, which is consistent with the endogenous concentrations of cytokinin. The binding of cytokinin to these receptors results in autophosphorylation and in some cases also shuts off a phosphatase activity (Mähönen *et al.*, 2006a). The cytokinin-binding site of AHK4 is small and involves approximately 20 amino acids (Hothorn et al., 2011). This small size likely explains the inactivity of the cytokinin conjugates, which are too large to fit into the site.

The cytokinin receptors are found in the endoplasmic reticulum (ER) membrane, with the cytokinin-binding domain occurring in the lumen of the ER (Wulfetange et al., 2011). By analogy to prokaryotic HKs, signal transmission across the ER membrane by the cytokinin receptors likely occurs by conformational changes brought about by ligand binding, which facilitate *trans*-phosphorylation of the histidine kinase domains. Genetic analysis indicated that the cytokinin receptors have overlapping roles in cytokinin signaling, though subsets play predominant roles for certain responses (Higuchi et al., 2004; Nishimura et al., 2004).

CKI1

The CKI1 HK was first identified by its ability, when overexpressed, to promote cell proliferation in culture independent of cytokinin (Kakimoto, 1996). CKI1 is an active histidine kinase, but lacks a CHASE domain and thus does not bind to cytokinin. CKI1 is essential for the development of the female gametophyte (Pischke et al., 2002), and also plays a role in cambial development, likely feeding into the cytokinin response pathway (Deng et al., 2010). In both roles, it likely acts by signaling through the downstream AHP proteins in a phosphorelay pathway.

Osmosensing

Interestingly, bacteria, yeast and plants all use TCS signaling to respond to changes in the osmolarity of their environment. In Arabidopsis the hybrid sensor ATHK1 senses high osmolarity and signals through HPt and RR proteins to mediate the response to water stress (Urao *et al.*, 1999; Wohlbach *et al.*, 2008). Two-component systems have also been implicated in osmosensing in poplar and rice (Chefdor et al., 2006; Kushwaha et al., 2013).

Soluble HKs

The Arabidopsis *AHK5* and maize *ZmHK9* genes encode functional histidine kinases that lack a transmembrane domain and thus are unlikely to bind to cytokinin. Both *AHK5* and *ZmHK9* have been linked to abscisic acid and ethylene responsiveness, as well as to drought and stress responses (Desikan et al., 2008; Wang et al., 2012). *ZmHK9* expression is induced in response to drought and ABA, and overexpression of *ZmHK9* results in increased drought tolerance in part by regulating the number and size of stomata (Wang et al., 2012). Likewise, AHK5 responds to hydrogen peroxide levels and regulates stomatal closure (Desikan et al., 2008). Furthermore, AHK5 positively regulates salt sensitivity and contributes to resistance to both bacterial and fungal pathogens. AHK5 was shown to act through the HPt proteins AHP2 and AHP5 to activate the ARR4 and ARR7 RRs (Mira-Rodado et al., 2012).

Ethylene Receptors

Ethylene is a gaseous plant hormone that plays a role in a diverse array of growth and development processes, including fruit ripening, biotic and abiotic stress and leaf and floral senescence

and abscission. The ethylene receptors are encoded by a small gene family that includes both functional and degenerate HKs (Schaller and Kieber, 2002). The ethylene-binding site includes a copper cofactor and is localized within the transmembrane spanning domains of the receptors (Schaller and Bleecker, 1995), consistent with the hydrophobic nature of ethylene. In *Arabidopsis*, the five ethylene receptors (ETR1, ETR2, EIN4, ERS1 and ERS2) are primarily localized at the ER membrane where they form large multisubunit heteromeric complexes containing multiple receptor subtypes. The ETR1 and ERS1 both possess histidine kinase activity, but the remainder of the ethylene receptors are missing amino acids essential for histidine kinase activity. The kinase activity of ETR1/ERS1 does not play a primary role in signaling, but rather plays a role in modulating the initial kinetics of the response and the recovery from ethylene exposure, possibly through downstream Hpt and RR proteins.

Phytochromes

The phytochrome receptors sense red and far red light through a bound bilin chromophore. Perception of red light activates phyochromes, and this is reversed by absorption of far-red light. Phytochromes are found in bacteria and fungi where they resemble canonical HKs, but in higher plants the phytochrome receptors have lost multiple conserved amino acids that are essential for histidine kinase activity, rather some appear to possess serine/threonine kinase activity (Rockwell et al., 2006). There are five phytochromes in Arabidopsis that have partially overlapping roles in light perception. While phytochromes do not signal through TCS elements, the activity of at least one, PhyB, is modulated by interaction with the ARR4 RR in *Arabidopsis*, which likely mediates crosstalk between cytokinin and light signaling (Sweere et al., 2001).

Histidine-Containing Phosphotransfer Proteins

The His-containing phosphotransfer proteins (HPts) act downstream of the membrane-bound HKs to shuttle phosphate groups to the primarily nuclear localized RRs (Stock et al., 2000). The phosphate group is transported on a conserved His residue within the conserved sequence, XHQXKGSSX (Stock et al., 2000; Dortay et al., 2006). The Arabidopsis genome includes five phosphotransfer proteins (AHPs) that contain the canonical His residue in their HPt domain (Suzuki et al.,

1998; Suzuki et al., 2000), as well as one "pseudo" AHP (PHP) protein that lacks the conserved His residue (Mähönen et al., 2006b). The Hpts are also encoded by small gene families in other plant species, though in some the total and relative number of pseudo and functional Hpts is different (Pareek et al., 2006; Tsai et al., 2012) (Table 1.1). Phylogenetic analysis suggests that the HP proteins expanded after the split between monocots and dicots from a single ancestral gene (Chu et al., 2011).

The AHPs interact with both hybrid HKs and RRs based on yeast two-hybrid analysis (Imamura et al., 1999; Urao et al., 2000; Tanaka et al., 2004; Dortay et al., 2006), and have been demonstrated to be capable of acquiring a phosphate from HKs and donating that phosphate to RRs *in vitro* (Suzuki et al., 1998; Asakura et al., 2003), consistent with their hypothesized role in a multi-step phosphorelay. In Arabidopsis, the five AHP proteins act as partially redundant positive regulators of cytokinin signaling (Hutchison et al., 2006), with the pseudo HP playing a negative role in cytokinin signaling, likely through a dominant negative mechanism (Mähönen et al., 2006b). The AHPs also act downstream of CKI1 in female gametophyte development, AHK1 in osmosensing, and AHK5 in stomatal function.

AHP function is the target of crosstalk nitric oxide signaling, which plays a role in the response to biotic and abiotic stress. Nitric oxide directly modifies the cysteine thiol of proteins as a redox-based posttranslational modification, which is known as S-nitrosylation. AHP function is negatively regulated by S-nitrosylation which reduces their ability to act as phosphotransfer proteins (Feng et al., 2013). Thus, elevated levels of nitric oxide reduce the sensitivity of plant tissues to cytokinin.

Response Regulators in Plants

Response regulators (RR) are characterized by having a conserved DDK motif in their receiver domain (Mizuno, 1997; Stock et al., 2000). RRs were first identified in plants as cytokinin primary response genes with sequence similarity to bacterial RRs (Brandstatter and Kieber, 1998; Urao et al., 1998; Imamura et al., 1999). The RRs catalyze their own phosphorylation of the Asp residue in their receiver domain, but rely on the HP proteins to provide a high energy phosphodonor (Mizuno, 1997; Stock et al., 2000; Bourret, 2010). The RRs can also possess intrinsic phosphatase activity that catalyzes the hydrolysis of the phosphate group at the Asp residue (Imamura et al., 1999; Bourret, 2010).

The RRs found in plants fall into four groups based primarily on their domain structure and sequence similarity: type-A, type-B, type-C, and pseudo/clock-related RRs (To and Kieber, 2008). In *Arabidopsis*, these are referred to as the *Arabidopsis* RRs (ARRs), and in other species the first two letters refer to the species of origin (e.g. OsRRs refer to *Oryza sativa*, or rice RRs). Altogether, there are 23 functional ARRs in Arabidopsis, which contain a receiver domain at their N-terminus, but differ in their C-terminal extensions (Figure 1.2).

In higher plants, the RR gene family has expanded more than other TCS signaling elements (Table 1.1). Further, phylogenetic analysis of RRs indicates that gene family has expanded substantially after the monocot and dicot lineages evolutionarily diverged (Tsai et al., 2012), suggesting that the RRs may have diverse roles in higher plants, and that some of these roles may be distinct in monocots and dicots.

Type-A Response Regulators

The type-A RRs are characterized by having a short C-terminal extension following their N-terminal receiver domain and as being transcriptionally induced in response to cytokinin (Imamura et al., 1999; Asakura et al., 2003; Jain et al., 2006). The N-terminal receiver domain is highly conserved, while the amino acid sequences in the C-terminal extensions are the most variable (D'Agostino et al., 2000; Jain et al., 2006).

The type-A RRs are not found in the genomes of green algae, but are found in all land plants, suggesting that they appeared during the transition to land plants (Pils and Heyl, 2009). Interestingly, the type-A ARRs first appear together with the cytokinin receptors, as neither are present in the genomes of any green algae, which suggests that the type-A RRs are important for the function of cytokinin receptors (Pils and Heyl, 2009). Phylogenetic analysis of rice, maize, and *Arabidopsis* type-A RRs shows that the monocot and dicot type-A RRs generally fall into distinct clades, which suggests that the most recent common ancestor of monocots and dicots had a small family of type-A RRs that expanded through species-specific gene duplication events (Jain et al., 2006; Chu et al., 2011; Tsai et al., 2012). In Arabidopsis, there are ten type-A ARRs and each of them fall into five pairs of related sequences, which

suggests that these pairs arose from the most recent genome duplication event during the evolution of Arabidopsis (D'Agostino et al., 2000; Vision et al., 2000).

Cytokinin Signaling

The type-A RRs are cytokinin primary response genes as they are transcriptionally up regulated in response to cytokinin in the absence of *de novo* protein synthesis (Brandstatter and Kieber, 1998; D'Agostino et al., 2000). In Arabidopsis, the type-A ARRs are partially redundant, negative regulators of cytokinin signaling (To et al., 2004). In addition to the transcriptional induction of type-A ARRs, cytokinin treatment also enhances the stability of a subset of the type-A ARR proteins in a manner dependent upon phosphorylation of the Asp residue in the receiver domain (To et al., 2007). The function of the type-A RRs as primary cytokinin response genes is conserved in monocots (Asakura et al., 2003; Jain et al., 2006; Tsai et al., 2012).

A primary role of the type-A ARRs is to act as negative feedback regulators of cytokinin signaling, dampening the response to cytokinin in response to elevated or prolonged levels of the signal. Phosphorylation of the target Asp residue on the type-A ARRs is essential for their function as negative regulators of cytokinin signaling (To et al., 2007). The type-A ARRs likely inhibit cytokinin responses by interacting with other proteins in a manner dependent on the phosphorylation state of the receiver domain to regulate their function.

The type-A ARRs are also an important target for other signaling pathways to modulate the sensitivity of cells to cytokinin through modulation of type-A ARRs expression. For example, auxin induces expression of a subset of type-A ARRs in the developing root apical meristem, and represses expression of several type-A ARRs in the shoot apical meristem, thus altering the sensitivity of the target tissue to cytokinin. Further, type-A ARRs are directly induced by WUSCHEL, a transcription factor that plays a key role in regulating shoot apical meristem function (Leibfried et al., 2005).

Additional Roles for Type-A ARRs

Disruption of type-A ARR function leads to a plethora of effects on plant growth and development, many of which reflect enhanced sensitivity to cytokinin. However, there are additional, cytokinin-

independent roles for type-A ARRs. One clear example is the role of a subset of type-A ARRs in the regulation of the circadian rhythm. Disruption of the *ARR3* and *ARR4* type-A ARRs in *Arabidopsis* leads to a longer circadian period as compared to the wild type, but this is not a result of increased sensitivity to cytokinin (Salomé et al., 2005). Further, disruption of *ARR8* and *ARR9*, two other type-A ARRs, suppresses this elongated circadian period in *arr3 arr4*, suggesting that these pairs of type-A ARRs play antagonistic role in regulating the circadian clock, possibly via regulation of the pseudoresponse regulators (see below).

Type-B Response Regulators

While the type-A RRs have a short C-terminal extension, the type-B RRs have longer C-terminal extensions that contains a Myb-like DNA binding domains referred to as the GARP domain (Figure 1.2). The C-terminal region of type-B ARRs are variable outside of the conserved GARP domain, but often contain activation regions and potential nuclear localization signals (Imamura et al., 1999; Sakai et al., 2000; Schaller et al., 2008a). The type-B RRs are encoded by multigene families in both monocots and dicots, and are found in lower plants, including basal green algae (Pils and Heyl, 2009). Phylogenetic analysis of RRs in plants suggests an expansion of the type-B OsRRs after the divergence of monocots and dicots, suggesting that there may be specific functions of type-B RRs in monocots (Kim et al., 2012; Tsai et al., 2012). One example of this is the *EARLY HEADING DATE 1* (*EHD1*) gene in rice, which is necessary to promote short day flowering and encodes a rice type-B RR (OsRR30) (Doi *et al.*, 2004). Consistent with the notion that this is a monocot-specific type-B RR, no ortholog of OsRR30 is found in *Arabidopsis*, and there are no *Arabidopsis* RR mutants with a similar phenotype to OsRR30 (Doi et al., 2004; Tsai et al., 2012).

Cytokinin Signaling

Unlike the type-A ARRs, the transcription of the type-B ARRs is not induced by cytokinin (Imamura et al., 1999) and they act as redundant positive elements in cytokinin signaling (Mason et al., 2005). The type-B ARRs are nuclear-localized transcription factors that bind DNA in a sequence-specific manner (Sakai et al., 2000). They induce transcription of cytokinin regulated genes, including the type-A

ARRs (Sakai et al., 2001), binding to a common sequence motif found enriched upstream of cytokinin response genes (Rashotte et al., 2003). For example, ARR1 and ARR2 bind preferentially to 5'-AGATT-3' nucleotide sequences (Sakai et al., 2000). Disruption of the type-B ARRs blocks nearly all cytokinin-induced changes in gene expression (Argyros et al., 2008), indicating that they are essential for the transcriptional changes induced by cytokinin. The type-B RRs are phosphorylated on an Asp residue in their receiver domain in response to cytokinin activation of the cytokinin receptor HKs through the HP intermediates (Figure 1.2). Phosphorylation of the receiver domain is predicted to cause a conformational change that releases the suppression of the DNA-binding domain by the receiver domain.

Type-C Response Regulators

Of the 23 functional ARRs in *Arabidopsis* that have a conserved receiver domain, two do not fit into the type-A or type-B ARRs class, and these have been named type-C ARRs (Kiba et al., 2004). The type-C ARRs have a similar structure to the type-A ARRs (Figure 1.2), but unlike the type- A ARRs, these are not transcriptionally upregulated in response to cytokinin. It is likely that the type-A RRs evolved from the type-C RRs because the type-C RRs are found in the genomes of green algae, while the type-A RRs are not (Pils and Heyl, 2009).

The type-C ARRs interact with the AHP proteins and can receive a phosphate from them on a conserved Asp residue (Kiba et al., 2004; Horak et al., 2008). It has been proposed that the type-C ARRs may interfere with cytokinin signaling by acting as a phosphate sink to compete with type-B ARRs, thus negatively regulating cytokinin responses, but as yet this is not supported by analysis of loss-of-function alleles (Horak et al., 2008).

Pseudo Response Regulators

The main characteristic of the pseudo RRs (PRRs) is that the conserved Asp residues in the receiver domain that is the target of phosphorylation is altered to a glutamic acid (Glu) (Makino et al., 2000; Matsushika et al., 2000), though the receiver domain at the N-terminus shares high sequence similarity with the other RRs (Makino et al., 2000; Matsushika et al., 2000). The Asp to Glu change in the receiver domain of the APRRs may serve as a phosphomimic form of the RR, resulting in a constitutively

active form of the protein (Schaller et al., 2008a). Rather than phosphorylation at an Asp residue, the PRRs are regulated by Ser/Thr phosphorylation, which enhances their interaction with an F-box protein and thus their degradation, and can also promote interactions among the PRR family members (Fujiwara et al., 2008).

The nine APRRs are divided into two groups, one with a C-motif and the other with a B-motif (Makino et al., 2000; To and Kieber, 2008) (Figure 1.2). There are five APRRs with the C-motif at the C-terminus, which is a conserved Constans/Constans-like/TOC1 domain (CCT) domain (Mizuno, 2005). The other APRRs have the B-motif, or Myb-like motif, which is also found in the type-B ARRs (Schaller et al., 2008a). Phylogenetic analysis suggests that the ancestral PRRs had a fully functional receiver domain that was lost during the evolution of higher land plants (Satbhai et al., 2011).

Similarly to the type-C ARRs, transcription of the APRRs is not induced by cytokinin (Makino et al., 2000). However, the *APRR* levels oscillate in a 24-hour period, with expression patterns that are subject to the circadian rhythm (Makino et al., 2000; Matsushika et al., 2000). APRR1/TOC1 was one of the first APRR proteins to be characterized. The *toc1* mutants have major defects in circadian rhythm, and it has been proposed that APRR1/TOC1 is the main component of the central oscillator in plants, controlling leaf movements, and flowering (Makino et al., 2000; Strayer et al., 2000). The other APRRs are also involved in circadian rhythm as loss-of-function mutations cause circadian clock-related phenotypes in plants (Mizuno, 2005). APRR1/TOC1 acts as a positive regulator of clock-regulated gene expression and other C-motif containing family members (PRR5, PRR7, and PRR9) act as negative regulators.

Conclusions

TCS signaling elements in plants are derived from bacterial signaling systems, perhaps through endosymbiont partners, and they function either in the context of an intact phosphorelay, or more commonly the elements have lost their ancestral His/Asp phosphorylation mechanisms of activation. These elements play diverse roles in growth and development, and an important unresolved issue of how specificity is achieved among the various pathways. While TCS signaling is important in plants, it clearly represents a minority of signaling mechanisms as Ser/Thr phosphorylation is the predominant player in

plant signaling. It remains to be seen what features of TCS signaling are advantageous for cytokinin signaling such that it retained a functional phosphorelay pathway.

Section II: The transcriptional response of cytokinin signaling

The final step of the cytokinin signaling cascade is the activation of the type-B ARRs, which are the primary transcription factors that regulate cytokinin response genes (Mason et al., 2005; Yokoyama et al., 2006; Argyros et al., 2008). The type-B ARRs evolved in plant species before the other two-component signaling elements like the AHK receptors, type-A ARRs, suggesting that they had a plant-specific function that was the pre-cursor to cytokinin signaling (Pils and Heyl, 2009). Phosphorylation of the type-B ARR receiver domain leads to activation of the C-terminal DNA-binding domain (Taniguchi et al., 2007). Upon activation, the type-B ARRs will bind to their target genes and regulate transcription. The type-B ARRs bind to the core binding motif is 5'-AGAT-3' *in vitro* (Sakai et al., 2001; 2000). This sequence is found in the promoters of cytokinin-regulated genes. There are 11 type-B ARRs in Arabidopsis that fall into 3 subfamilies based upon phylogenetic analysis. Genetic studies show that type-B ARR proteins ARR1, ARR2, ARR10, ARR11, ARR12 of subfamily-1 have overlapping and redundant roles in cytokinin signaling and are responsible for most cytokinin gene regulation (Argyros et al., 2008; Yokoyama et al., 2006; Mason et al., 2005). The *arr1,10,12* mutant is almost completely insensitive to cytokinin signaling (Argyros et al., 2008), indicating that these genes are the responsible for the majority of cytokinin response gene regulation. There are many genes that have been identified as being regulated by cytokinin signaling.

Primary cytokinin response genes were identified in a meta-analysis that examined genes consistently and robustly differentially regulated in response to cytokinin (Bhargava et al., 2013). In this list termed, the 'golden' list, there are 226 genes identified that are either up-regulated or down-regulated in response to cytokinin. Included in this list are the ten type-A ARR proteins, that are negative regulators of cytokinin signaling (Branstatter and Kieber, 1998; To, 2004). Also, regulated by cytokinin are the cytokinin oxidase/dehydrogenase *(CKXs*) that degrade cytokinin, the Cytokinin Response Factors (*CRFs*) that are transcription factors, and the transcriptional regulator *SHY2/IAA3*, which inhibits auxin signaling. In addition to these specific genes there is an overall enrichment for genes involved in nutrient transport,

abiotic stress, pathogen defense and redox regulation (Brenner and Schmülling, 2012; Bhargava et al., 2013). Furthermore, the type-B ARRs also regulate other transcription factors (Brenner and Schmülling, 2012).

The type-B ARRs are at the head of the cytokinin transcriptional cascade as they regulate other transcription factors in response to cytokinin. One example are the CRFs, which are AP2/ERF transcription factors and their function has also been linked to cytokinin (Raines et al., 2015). CRF mutants display cytokinin response phenotypes, and multiple genes differentially regulated in these mutants are cytokinin-regulated genes. For example, the type-A ARRs are down-regulated in the *crf1,3,5,6* loss-of-function mutants (Raines et al., 2015). This regulation of the type-B ARRs on the CRFs, represents a positive feed-forward regulation, in which type-B ARRs up-regulate the CRFs in response to cytokinin and then the CRFs will regulate another set of genes, potentially as co-regulators with the type-B ARRs (MacQuarrie et al., 2011). The type-B ARRs also regulate signaling molecules that are not transcription factors like the DELLA proteins that regulate signaling of the plant hormone gibberellic acid. The DELLA proteins lack the ability to bind to DNA, but act as transcriptional co-activators. The type-B ARRs interact with DELLA proteins and recruit them to the promoters of cytokinin response genes (Nora Marín-de la Rosa, 2015). This interaction represents a mechanism of hormonal cross/talk between cytokinin and gibberellic acid signaling.

There are also mechanisms in place to fine-tune or turn off the cytokinin signal. At the level of the receptor in the absence of cytokinin signaling the AHK4 receptor acts as a phosphatase to remove phosphates from the AHP proteins and dampen the cytokinin response (Mahönen et al., 2006b). The pseudo AHP protein, AHP6 lacks the conserved histidine residue and negatively regulates the pathway, likely through interaction with the receptors (Mahönen et al., 2006a). The type-A ARRs negatively regulate cytokinin signaling and evolved in land plants at the same time as the AHK cytokinin receptors, suggesting that perceiving the signal is just as important as is a way to turn off the signal, so it can be turned on again at the correct time (Pils and Heyl, 2009). The *CKX* genes are also up-regulated in response to cytokinin and to degrade cytokinin and regulate the amount of active cytokinin in the cell (Werner, 2003). Furthermore, activation of cytokinin response genes is regulated through degradation of the type-B ARRs. The F-box protein KISS ME DEADLY (KMD) target type-B ARRs and form a complex

with the S-PHASE KINASE-ASSOCIATED PROTEIN1 (SKP1)/Cullin/F-box (SCF) E3-ubiquitin ligase complex to recruit type-B ARRs for degradation (Kim et al., 2013).

The type-B ARRs regulate transcription of cytokinin response genes. The genes differentially regulated in response to cytokinin are involved in regulation of cytokinin signaling and metabolism, auxin signaling, stress responses, redox stress, secondary metabolism, and development. Cytokinin also regulates auxin signaling genes, which represents a mechanism of cytokinin/auxin regulation. Furthermore, other transcription factors are regulated in response to cytokinin like the CRFs. The CRFs are up-regulated in response to cytokinin and regulate a sub-set of cytokinin signaling responses. Finally, negative regulators of the signaling pathway are employed to fine-tune and turn off the signal. The type-B ARRs have been identified as primary transcriptional regulators of cytokinin signaling and multiple cytokinin response genes have been identified. Future studies will focus on determining the transcriptional network that results in the regulation of the variety of growth and developmental processes that cytokinin is known to control.

Table 1.1 The number of known two-component signaling elements in selected plant species.

Species	HK	PHK	HP	PHP	Type-A RR	Type-B RR	Type-C RR	PRR / clock	Total RRs	Reference
Arabidopsis thaliana	8	9	5	1	10	11	2	5	23	(Schaller et al., 2008b)
Oryza sativa	4	1	2	3	13	13	2	8	36	(Du et al., 2007)
Zea mays	8	3	7	2	21	7			28	(Chu et al., 2011)
Selaginella moellendorfi	2		2		2	5			7	(Pils and Heyl, 2009)
Physcomitrella patens	3		2		7	5	2		14	(Pils and Heyl, 2009)

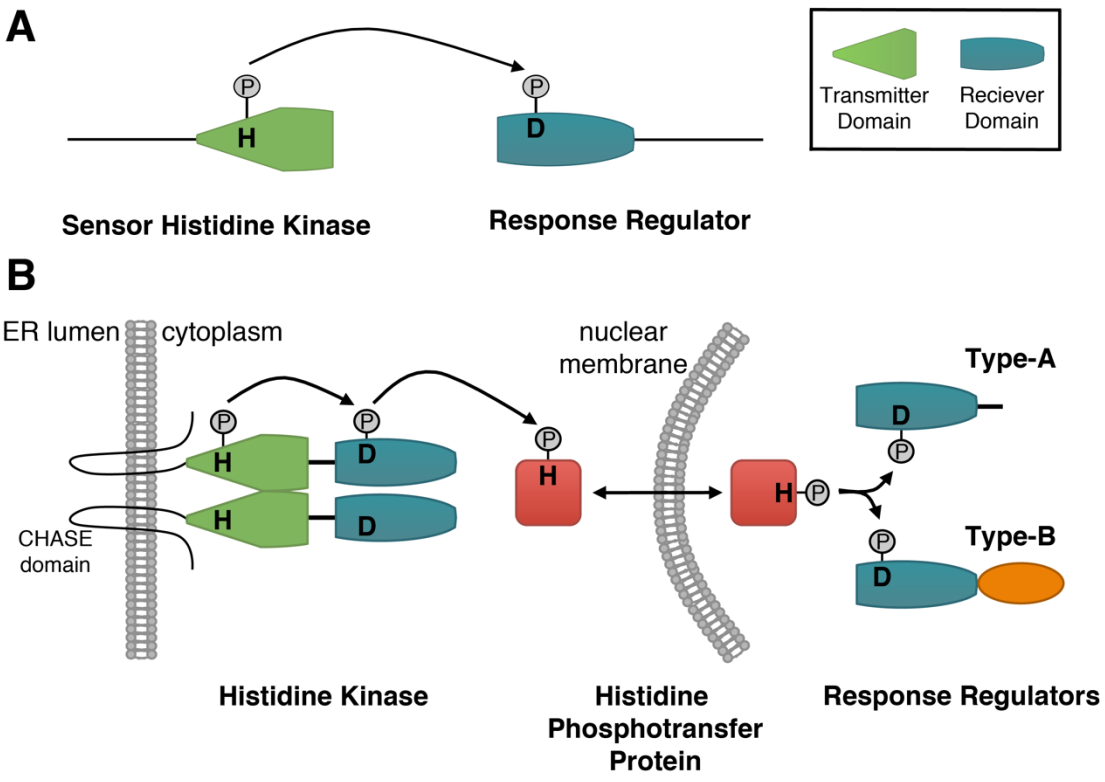

Figure 1.1 The prototypical two-component system and the cytokinin signaling pathway in *Arabidopsis.* **(A)** In the prototypical two-component system, the sensor His kinase receives a signal, triggering autophosphorylation on a conserved His (H) residue in the transmitter domain. The phosphate group is then transferred to an aspartate (D) residue in the receiver domain of the response regulator protein, which regulates its activity **(B)** The cytokinin signaling pathway is an example of a multi-step phosphorelay system. The CHASE domain in the His kinase receptor binds to cytokinin, triggering autophosphorylation of a conserved His residue in the transmitter domain, which then transfers the phosphate group to the receiver domain within the receptor. The phosphate is transferred from the receiver domain to the Hpt, which shuttle the phosphate to the receiver domain of either the type-B or type-A response regulator proteins, most of which are in the nucleus.

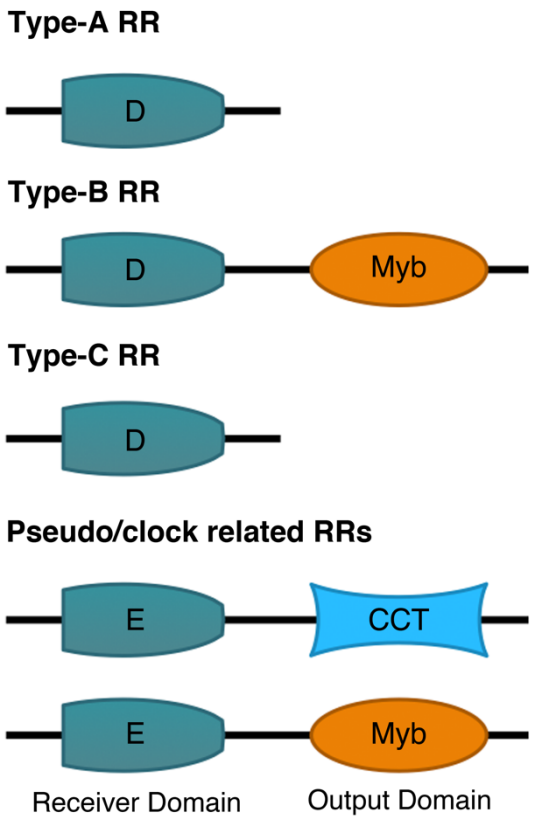

Figure 1.2 Domain architecture of the response regulator proteins in plants. Plant response regulators fall into four groups: Type-A, Type-B, Type-C and Pseudo/clock-related response regulators (RR). Typically, response regulator proteins consist of two domains, a receiver domain and an output domain. Type-B RRs have a Myb-like DNA-binding output domain C-terminal to the receiver domain. The type-A and type-C RRs have a short C-terminal extension and lack an output domain. The pseudo/clock related RRs are split into two groups, containing either a Myb-like motif or CCT motif in their output domain. The type-A, type-B and type-C response regulators contain a conserved aspartate residue in the receiver domain that is the target of phosphorylation, but the Pseudo/clock related RRs lack this Asp residue, and it is often replaced with a glutamate residue.

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CHAPTER 2: THE ROLE OF CYTOKININ DURING INFECTION OF *ARABIDOPSIS THALIANA* BY THE CYST NEMATODE *HETERODERA SCHACHTII*

Summary

Plant-parasitic cyst nematodes induce the formation of hypermetabolic feeding sites, termed syncytia, as their sole source of nutrients. The formation of the syncytium is orchestrated by the nematode in part by modulation of phytohormone responses, including cytokinin. In response to infection by the nematode *Heterodera schachtii*, cytokinin signaling is transiently induced at the site of infection and in the developing syncytium. Arabidopsis lines with reduced cytokinin sensitivity show reduced susceptibility to nematode infection. e infection, indicating that cytokinin signaling is required for optimal nematode development. Furthermore, lines with increased cytokinin sensitivity also exhibit reduced nematode susceptibility. To ascertain why cytokinin hypersensitivity reduces nematode parasitism, we examined the transcriptomes in wild-type and a cytokinin-hypersensitive type-A *arr* Arabidopsis mutant in response to *H. schachtii* infection. Genes involved in the response to biotic stress and defense response were elevated in the type-A *arr* mutant in the absence of nematodes and were hyper-induced following *H. schachtii* infection, which suggests that the Arabidopsis type-A *arr* mutants impede nematode development because they are primed to respond to pathogen infection. These results suggest that cytokinin signaling is required for optimal *H. schachtii* parasitism of Arabidopsis, but that elevated cytokinin signaling triggers a heightened immune response to nematode infection.

Introduction

Plant-parasitic cyst nematodes, *Heterodera* spp, are sedentary endoparasites of plant roots in many economically important plant species in which they cause tremendous yield losses (Barker and Koenning, 1998; Chitwood, 2003). These obligate biotrophs establish an intimate association with their host plants to maintain their sedentary lifestyle. After hatching, the infective second-stage juvenile (J2) of cyst nematodes penetrates the plant root and migrates towards the vascular tissues, where it selects a single cell as an initial feeding cell. Soon after this initial selection, hundreds of neighboring cells are fused with the initial feeding cell through cell-to-cell fusion, resulting in the formation of a specialized multinucleated feeding site called a syncytium. The mechanisms through which cyst nematodes induce the re-differentiation of normal root cells into metabolically active syncytium cells are unclear, but appear to involve proteinaceous stylet secretion of nematode effector proteins, which are considered the genetic determinants of nematode parasitism (Haegeman et al., 2012; Hewezi and Baum, 2013). Genome-wide gene expression analysis of nematode-induced syncytia in Arabidopsis pointed to key roles of phytohormones and their downstream signaling pathways in the transition of infected root cells into metabolically active sinks (Szakasits et al., 2009). While molecular and genetic studies have supported a role of auxin and ethylene in the development of syncytia (Quentin et al., 2013; Cabrera et al., 2015), direct evidence supporting a functional role of cytokinins in syncytium formation and function is lacking.

Cytokinins are N^6-substituted adenine-derived plant hormones that regulate numerous plant growth and developmental processes, including shoot and root growth, stem cell maintenance and differentiation, as well as the response to biotic and abiotic factors (Argueso et al., 2009; Kieber and Schaller, 2014). The first committed step in cytokinin biosynthesis, catalyzed by isopentenyltransferases (IPT), is the formation of cytokinin ribotides/ribosides (Hirose et al., 2008), which are subsequently converted to the active, free base forms of cytokinins by the LONELY GUY (LOG) family of enzymes (Kuroha et al., 2009). Cytokinin signaling occurs via a multi-step phosphorelay system that modulates gene transcription (Kieber and Schaller, 2014)(Figure 2.1A). Cytokinin binding to the Arabidopsis histidine kinase (AHK) receptors results in autophosphorylation of a conserved histidine residue (Inoue et al., 2001; Suzuki et al., 2001; Ueguchi et al., 2001; Yamada et al., 2001). This phosphate is subsequently transferred to a conserved aspartic acid residue within the AHK receiver domain and then to an

Arabidopsis histidine-containing phosphotransfer protein (AHPs) (Hutchison et al., 2006). The AHPs finally transfer the phosphate to an aspartic acid residue within the receiver domain of the Arabidopsis response regulators (ARRs). The ARRs fall into two classes: the type-A ARRs, which are negative elements in cytokinin signaling (To et al., 2004) and the type-B ARRs that are positive regulators of cytokinin signaling. Phosphorylation of the type-B ARRs activates their DNA-binding domain and leads to transcription of cytokinin primary response genes, which include the type-A *ARR*s (Hwang and Sheen, 2001; Argyros et al., 2008).

Cytokinins have been implicated in multiple host-pathogen interactions and defense responses (Argueso et al., 2009; Choi et al., 2011; Robert-Seilaniantz et al., 2011; Naseem and Dandekar, 2012). Some pathogens are capable of synthesizing cytokinins and/or elevating endogenous cytokinin levels, which is often linked to the success of the pathogen (Walters and McRoberts, 2006; Robert-Seilaniantz et al., 2007). These include gall-forming pathogenic bacteria such as Agrobacterium (Claeys et al., 1978; Hwang et al., 2010), the biotrophic actinomycete *Rhodococcus fascians* (Pertry et al., 2009), and biotrophic fungal and bacterial pathogens that form green bionissia or green islands (López-Carbonell et al., 1998; Walters et al., 2008). In addition to altering plant development, pathogen-derived cytokinins may also act to delay senescence and/or increase sink activity. In some cases, increased cytokinin signaling is associated with increased pathogen success. For example, *Rhodococcus fascians* secretes a mix of cytokinins that cannot be degraded by the cytokinin oxidases (CKXs), which are enzymes that degraded cytokinin, to induce the production of leafy galls (Depuydt et al., 2008; Pertry et al., 2009). Pre-treatment of Arabidopsis with low levels of cytokinin enhances the number of pathogen spores produced by the oomycete *Hyaloperonospora arabidopsis* (Argueso et al., 2012). However, elevated cytokinins have also been linked to decreased pathogen success. For example, cytokinins promote resistance of Arabidopsis to *Pseudomonas syringae*, mediated in part via the direct interaction of the salicylic acid (SA) response factor TGA3 with ARR2, a type-B ARR (Choi et al., 2010). Further, mutants that are hypersensitive to cytokinin (e.g. type-A *arr* mutants) support reduced growth of *H. arabidopsis*, likely a result of a heightened defense response (Argueso et al., 2012).

Cytokinins have been linked to the interaction of nematodes with plants. Both *H. schachtii* and *Meloidogyne incognita* (a root knot nematode) produce cytokinins, predominantly benzyladenine and

zeatin-type varieties (Dimalla and van Staden, 1977; Bird and Loveys, 1980; De Meutter et al., 2003). Infection of either *Lotus japonicus* or tomato with *M. incognita* induced expression of a cytokinin-responsive *ARR5* reporter gene (Lohar et al., 2004). This induction was first observed when the J2 nematode reached the vascular bundles and persisted through the early development of the gall, but then declined as the galls matured. Consistent with this, infection of rice with the root knot nematode *M. graminicola* resulted in altered expression of many genes involved in cytokinin function (Kyndt et al., 2012). Overexpression of a cytokinin oxidase in transgenic hairy roots of *L. japonicus* resulted in a decrease in the number of galls produced by *M. incognita* (Lohar et al., 2004). Further, infection of Arabidopsis with *H. schachtii* resulted in the induction of the cytokinin-responsive P_{TCS}:ER-GFP reporter 14 days after infection (Absmanner et al., 2013).

Here we examine the role of cytokinin signaling in the infection of Arabidopsis roots by the cyst nematode *H. schachtii*. Cytokinin signaling is up-regulated at the site of infection in response to *H. schachtii*. Several cytokinin-insensitive lines display reduced nematode susceptibility as measured by a reduced number of juvenile stage 4 (J4) females developing per root system. Interestingly, cytokinin-hypersensitive lines, such as the *arr3,4,5,6,7,8,9,15* type-A ARR octuple mutant, are also less susceptible to nematode infection. These results demonstrate that precise modulation of cytokinin signaling is important for successful infection of Arabidopsis roots by *H. schachtii*.

Results

Cytokinin signaling is induced at the site of nematode infection

Syncytium formation is associated with extensive gene expression changes. Examination of 7,225 genes identified as differentially expressed in isolated syncytium cells induced by *H. schachtii* in Arabidopsis (Szakasits et al., 2009) revealed significant overlap (hypergeometric probability $P(X \geq 128) = 8.5e^{-26}$) with a set of 226 robustly cytokinin-regulated genes, termed the "Golden" list (Bhargava et al., 2013) (Figure 2.1B and data not shown). 64% of the 128 overlapping genes are regulated in the same manner, either up or down, in response to nematode infection and exogenous cytokinin, indicating that nematode infection likely increases cytokinin function in the host root (data not shown). The similarly regulated genes include the type-A gene *ARR7*, which is a highly induced cytokinin primary response

gene, that is up-regulated by both cytokinin and nematode infection. The remaining 36% of the 128 overlapping genes that are regulated in opposite directions could be due to the fact that the syncytium data is pooled between 5 days post infection (dpi) and 15 dpi and there may be differing cytokinin-regulated responses in syncytium formation at these time-points. The significant overlap between genes robustly regulated by cytokinin and genes differentially expressed in response to nematode infection in the root suggest that nematodes alter cytokinin signaling as part of the infection process in Arabidopsis.

To explore the temporal and spatial changes in cytokinin signaling that take place in response to nematode infection, we inoculated ten-day-old seedlings of an Arabidopsis line that harbors the TCSn::GFP synthetic reporter, which reflects cytokinin-regulated type-B ARR activity (Zürcher et al., 2013), with *H. schachtii* J2 nematodes (Figure 2.1C-G). A low level of GFP fluorescence was observed in the vascular tissues of non-infected roots, while at 2-3 dpi by *H. schachtii* during the parasitic J2 stage a substantially elevated GFP fluorescence signal was observed both in the root tissues surrounding the nematode and at the site of the developing syncytium (Figure 2.1D). The TCSn::GFP signal peaked in well-developed syncytia during the early J3 infective stage (5-6 dpi) and in the late J3 stage (9-10 dpi), and then declined in the mature syncytium of J4 nematodes (14 dpi) to an intensity similar to the background levels observed in non-infected roots.

Effects of cytokinin mutants on nematode infection

To further explore the role of cytokinin in plant susceptibility to *H. schachtii* infection, we examined mutants with altered cytokinin signaling. We first examined the effects of various cytokinin-insensitive mutants, including the cytokinin receptor mutants *ahk4*, *ahk2,4*, *ahk2,3,* and *ahk3,4* (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006; Cheng and Kieber, 2013), the *ahp1,2,3* triple mutant (Hutchison et al., 2006), and the type-B ARR mutants *arr1,12,* and *arr1,10,12* (Mason et al., 2005). All of these cytokinin-insensitive lines exhibit reduced susceptibility to nematode infection compared to wild-type plants (Figure 2.2). These results suggest that the canonical cytokinin signaling pathway is necessary for optimal nematode development.

We next examined nematode susceptibility in lines disrupted for various type-A ARR genes, including the *arr3,4,5,6*, *arr3,4,5,6,7,8,9* and *arr3,4,5,6,7,8,9,15* multiple mutants, which are

hypersensitive cytokinin mutants (Figure 2.3A). Previous studies have shown that the *arr3,4,5,6,7,8,9,15* octuple mutant is the most cytokinin hypersensitive line, while the *arr3,4,5,6* is the least hypersensitive line (To et al., 2004; Zhang et al., 2011). These mutants have either no (*arr3,4,5,6*) or a modest effect on root growth and development in the absence of exogenous cytokinin (To et al., 2004; Zhang et al., 2011). We observed a significant decrease in nematode susceptibility as the hypersensitivity of the host line to cytokinin increased (Figure 2.3B-D). The *arr3,4,5,6* mutant showed a 24% decrease in the number of J4 nematodes after three weeks of growth and the *arr3,4,5,6,7,8,9* a 46% decrease, and the *arr3,4,5,6,7,8,9,15* a 51% decrease.

To further test if cytokinin hypersensitivity affected nematode parasitism, we examined lines that exhibit hypersensitivity due to overexpression of *ARR10*, a type-B ARR. Two independent transformants for *ARR10* overexpressed in a type-B *arr1,10,12* mutant background were examined for cytokinin responsiveness and nematode susceptibility. Both lines (*35S::ARR10#A1* and *35S::ARR10#A4*) were hyper-responsive to exogenous cytokinin as determined by root elongation assays (Figure 2.3A). The parental *arr1,10,12* line has a greatly reduced root system as a result of strong cytokinin insensitivity (Hill et al., 2013), which precludes analysis of nematode infection for this line. Similar to the type-A ARR mutants, both of these cytokinin-hypersensitive lines displayed a significant decrease in nematode susceptibility relative to wild-type plants (Figure 2.3E). Taken together, these results indicate that elevated cytokinin signaling is detrimental to nematode parasitism of Arabidopsis plants.

RNA-Seq analysis of wild-type and *arr3,4,5,6,7,8,9,15* mutant roots in response to nematode infection.

To address the mechanisms underlying the impaired nematode parasitism in the cytokinin hypersensitive mutants, we examined the transcriptional profile of wild-type and *arr3,4,5,6,7,8,9,15* octuple mutant roots in the absence and presence of nematodes using RNA-sequencing during the syncytium formation and maintenance phases. Ten-day-old seedlings were infected with J2 *H. schachtii* nematodes and three biological replicates of root tissue were collected at 4 dpi (syncytium formation phase) and 10 dpi (syncytium maintenance phase) along with the corresponding uninfected controls. There are 1,239 genes differentially regulated at 4 dpi in wild-type roots in response to infection, and 1,311 genes differentially regulated at 10 dpi (FDR < 0.05). *ARR7* is up-regulated at 4 dpi in wild-type

roots in response to nematode infection, which is consistent with the previous microarray study of nematode-infected Arabidopsis roots (Table S2.1) (Szakasits et al., 2009). Combining the two data sets yielded 2,067 genes differentially regulated in infected wild-type roots at 4 dpi and/or 10 dpi. These 2,067 genes were compared to those identified in a previous study of isolated syncytium cells induced by *H. schachtii* in Arabidopsis (Szakasits et al., 2009), which identified 7,225 genes differentially regulated in syncytia at 5 dpi and/or 15 dpi (Szakasits et al., 2009). There was significant overlap (43% of the genes identified as differentially expressed in response to infection in the current analysis were also identified in the prior study) between the two data sets (hypergeometric probability P (X>=888) $1.29e^{-85}$) (Figure S2.1). However, the analysis here identified fewer differentially expressed genes, which likely reflects the use of entire roots rather than isolated syncytium tissue (Szakasits et al., 2009). A set of 1,179 genes were identified in the current study that were not identified in the isolated syncytium tissue, which could be due to identification of genes that were differentially regulated distal to the syncytium.

We next examined gene expression in the type-A *arr3,4,5,6,7,8,9,15* mutant. In the absence of nematode infection, there were 4,822 genes differentially expressed in the 14 day-old (i.e. 4 days after mock inoculation) *arr3,4,5,6,7,8,9,15* mutant roots compared to control wild-type roots (2,224 up-regulated and 2,598 down-regulated; FDR <0.05). Similarly 1,129 differentially expressed genes were identified in the 20 days-old (i.e. 10 days after mock inoculation) *arr3,4,5,6,7,8,9,15* mutant roots compared to paired control wild-type roots (491 up-regulated and 638 down-regulated; FDR < 0.05). The substantial difference in the number of differentially expressed genes at these two time points suggests that type-A ARRs may play a more substantial role early in root growth as compared to later developmental stages, or may reflect compensatory changes in the *arr3,4,5,6,7,8,9,15* mutant over developmental time.

Examination of these control gene sets revealed enrichment for genes involved in biotic stress (Figure 2.4A and B). The MapMan analysis also points to an enrichment of genes involved in development following nematode infection, which may reflect the alteration of root development involved in the formation of syncytia. We separated the up-regulated and down-regulated genes for Gene Ontology (GO) analysis using the TAIR10 genome as a background population and GO biological process assignments by TAIR/TIGR were used to explore potential processes affected in the

arr3,4,5,6,7,8,9,15 mutant. In both the 14- and 20-day-old uninfected *arr3,4,5,6,7,8,9,15* mutant samples, there is a significant enrichment (p-value < 0.00001) for the GO terms 'response to stress' and 'defense response' in the differentially expressed gene sets (Figure 2.4C and D). This suggests that defense genes are regulated in the *arr3,4,5,6,7,8,9,15* mutant roots prior to infection by nematodes.

In the type-A *arr3,4,5,6,7,8,9,15* mutant prior to infection, genes that are involved in salicylic acid (SA) defense signaling and cross-talk between, jasmonic acid (JA) and ethylene (ET) plant defense responses were also identified (Table 2.1). For example, *ENHANCED DISEASE SUSEPTIBILITY 1 (EDS1)*, which acts as a positive regulator of SA in defense signaling and a repressor of JA/ET signaling (Brodersen et al., 2006), is up-regulated in both uninfected *arr3,4,5,6,7,8,9,15* samples . *NON RACE DISEASE RESISTANCE 1 (NDR1)* is another positive regulator of SA in defense signaling (Shapiro and Zhang, 2001) and this gene is up-regulated in type-A mutant roots . Free SA levels may be elevated in *arr3,4,5,6,7,8,9,15* mutant roots as the *UDP-GLUCOSYLTRANSFERASE 74F2 (UGT74F2)*, which converts SA to the SA glucose ester (Dean and Delaney, 2008), is down-regulated. Multiple WKRY transcription factors, which are regulated by SA signaling and which are involved in plant defense responses, were also regulated in the type-A *arr3,4,5,6,7,8,9,15* mutant prior to infection. Among them, *WRKY18* and *WRKY40*, which act down-stream of EDS1 and SA as positive regulators of defense signaling (Schön et al., 2013), are both up-regulated in *arr3,4,5,6,7,8,9,15* roots. Our results for *WRKY18* are also consistent with previous findings that reported *WRKY18* up-regulated in the type-A *arr3,4,5,6,8,9* mutant (Argueso et al., 2012). WRKY62 and WKRY38 function additively as negative regulators of plant basal defense responses (Kim et al., 2008) and are down-regulated in the type-A *arr3,4,5,6,7,8,9,15* mutant. This suggests that reduced expression of *WRKY38* and *WRKY62* may contribute to the enhanced resistance of the type-A *arr* mutants to infection. Overall, this data suggests that SA is positively regulated in the type-A *arr3,4,5,6,7,8,9,15* mutant prior to infection, which is consistent with previous findings that type-A ARRs negatively regulate the SA induced defense response (Argueso et al., 2012).

While SA signaling responses are increased in the type-A *arr3,4,5,6,7,8,9,15* mutant prior to infection, ET and JA signaling responses are decreased (Table 2.1). *ACC SYNTHASE 11 (ACS11)* is down-regulated, suggesting decreased ET synthesis. There is also a decrease in expression of the JA

biosynthesis enzyme, *ALLENE OXIDASE SYNTHASE (AOS)*, (Schaller, 2001). We found that *ETHYLENE RESPONSE FACTOR 1 (ERF1),* which is activated by both JA and ET singling pathways and serves as a major link between the two pathways (Lorenzo et al., 2003), is down-regulated in the *arr3,4,5,6,7,8,9,15* mutant. ET and JA signaling pathways positively regulate *ERF2* expression (Lorenzo et al., 2003) and *ERF2* is down-regulated in *arr3,4,5,6,7,8,9,15*.. These results suggest that ET and JA signaling outputs are suppressed in the type-A *arr3,4,5,6,7,8,9,15* mutant.

To validate our RNA-seq data, which suggests that defense genes are regulated in the type-A *arr3,4,5,6,7,8,9,15* mutant prior to infection, we used qRT-PCR to examine the expression of *EDS1* in multiple type-A *arr* mutants (Figure S2.2). We find that *EDS1* expression is increased compared to wild type in both the type-A *arr* octuple mutant *arr3,4,5,6,7,8,9,15* and the type-A *arr* quadruple mutant *arr3,4,5,6* with a 2 fold change, which is comparable to the difference we find in our RNA-seq data. We did not examine a difference in *EDS1* expression in the *ahk2,4* receptor mutant. This confirms that *EDS1* is up-regulated not only in the octuple mutant, but also in the type-A *arr3,4,5,6* mutant.

There are 1,831 genes differentially expressed between the infected *arr3,4,5,6,7,8,9,15* mutant and infected wild type roots at 4 dpi and 733 genes differentially expressed between the infected *arr3,4,5,6,7,8,9,15* mutant and infected wild type roots at 10 dpi by *H. schachti* (FDR <0.05)(Figure 2.5A and B). To identify genes that are specifically responsive to nematode infection, we focused on the genes that are found in the infected *arr3,4,5,6,7,8,9,15* vs. infected wild type root gene set that do not overlap with the control *arr3,4,5,6,7,8,9,15* vs. control wild type gene set. There are 668 such genes that are differentially regulated in the *arr3,4,5,6,7,8,9,15* mutant at 4 dpi, but are not differentially regulated in uninfected control *arr3,4,5,6,7,8,9,15* vs. control wild type roots (500 up-regulated, 168 down-regulated; data not shown, Figure 2.5A). A similar comparison at 10 dpi identified 380 genes that are specifically differentially regulated in the infected *arr3,4,5,6,7,8,9,15* mutant (245 up-regulated and 135 down-regulated; data not shown and Figure 2.5B). These differentially expressed genes provide insight into molecular differences during the course of nematode infection in the mutant. Examination of these gene sets revealed enrichment for genes involved in biotic stress, and the response to abiotic and heat stress (Figure 2.5C and D). Similar to the analysis of the uninfected gene sets, Gene Ontology analysis indicates a significant enrichment for genes involved in the ‘response to stress’ at 4 dpi and 10 dpi and ‘response to

biotic stimulus' at 4 dpi (p-values < 0.00001) (Figure 2.5E and F). This is consistent with an enhanced defense response in the *arr3,4,5,6,7,8,9,15* mutant in response to nematode infection.

We identified genes that are involved in the JA and ET signaling defense pathways that are hyper-induced in infected type-A *arr3,4,5,6,7,8,9,15* mutants compared to infected wild-type roots, though these were generally distinct from those identified as altered in type-A *arr3,4,5,6,7,8,9,15* non-infected roots (Table 2.1). At 4 dpi and 10 dpi, multiple *ACS* genes are elevated, which suggests an increase in ethylene synthesis in response to infection. *ETHEYLENE RESPONSE FACTOR 104 (ERF104)*, which is downstream of ET signaling, is up-regulated in the infected data set at 10 dpi. *LIPOGENASE 3 (LOX3)*, which is elevated in response to nematode parasitism and is necessary for JA synthesis, is also hyper-induced in infected *arr3,4,5,6,7,8,9,15* roots, suggesting that JA signaling may be up-regulated (Ozalvo et al., 2014). A downstream target of both the ET and JA defense signaling pathways, *PLANT DEFENSIN1.2 (PDF1.2* (Penninckx et al., 1998), is hyper-induced in infected *arr3,4,5,6,7,8,9,15* roots, . These results are somewhat distinct from what was found in the non-infected roots in which more SA signaling and defense response genes are up-regulated compared to ET and JA, suggesting that upon infection there is a switch between these signaling pathways.

We also found enrichment for 'response to heat' which is a category not found in the analysis of the control wild-type vs *arr3,4,5,6,7,8,9,15* mutant plants (Figure 2.4). There are multiple heat shock proteins (HSPs) that are hyper-induced in infected type-A *arr3,4,5,6,7,8,9,15* roots (Table S2.2). For example, *HSP22*, *HSP17*.4 and *HSP18*.2 are all up-regulated at both 4 dpi and 10 dpi in mutant vs. the wild-type roots. We also find additional HSPs up-regulated specifically at 4 dpi, including *HSP70b* and *HSP101*. There are also more cell wall-related categories that are down-regulated at 10 dpi, whereas there was one cell wall category up-regulated ('cell wall organization and biogenesis') in the 10 dpi control wild-type vs. *arr3,4,5,6,7,8,9,15* mutant plants. This might reflect changes related to cell wall remodeling during syncytium formation. There are only 48 genes that overlap between the genes differentially regulated at 4 dpi and 10 dpi in the *arr3,4,5,6,7,8,9,15* mutant vs. wild type (Figure S2.3). This low number of overlapping genes indicates that type-A ARRs regulate distinct sets of genes during syncytium initiation/formation (4 dpi) and maintenance stages (10 dpi).

Discussion

The infection of roots by cyst nematodes results in wholesale reprograming of the host transcriptome to modify the development and physiology to form a feeding site known as a syncytium. Phytohormones, which are involved in essentially all growth and developmental processes, play important roles in syncytial development (Quentin et al., 2013; Cabrera et al., 2015). Previous studies have examined changes in the response to the phytohormone cytokinin during syncytial development by examining the expression of a cytokinin inducible *ARR5*-reporter (Lohar et al., 2004), or have noted enrichment of genes involved in cytokinin function among nematode-induced changes in the host transcriptome (Kyndt et al., 2012). Further, nematodes have been shown to produce cytokinins (Dimalla and van Staden, 1977; Bird and Loveys, 1980; De Meutter et al., 2003). These studies, coupled with the role of cytokinin in promoting sink activity suggested a role for cytokinins in nematode infection. Here, we demonstrate an enrichment of cytokinin-responsive genes in the set of genes differentially expressed in Arabidopsis roots in response to infection by the nematode *H. schachtii*. Consistent with this, the TCSn:GFP cytokinin response reporter indicates a transient rise in cytokinin signaling that correlates with the development of the syncytium, suggesting a role for cytokinin in this process. Absmanner et al (2013) also noted an induction of similar cytokinin-responsive P_{TCS}:ER-GFP reporter following infection of Arabidopsis roots with *H. schachtii* (Absmanner et al., 2013). However, these authors found that this P_{TCS}:ER-GFP reporter was induced primarily in the phloem cells associated with the syncytia, rather than throughout the developing syncytia as we observe here. However, these authors only examined a single time point (14 days after infection), a point at which we observe little or no fluorescence from the TCSn:GFP reporter (Figure 2.1). The differences in TCS expression could reflect our use of a newer version of the TCS reporter (Zürcher et al., 2013) and/or differences in growth conditions.

Cytokinin regulates a wide variety of biological processes (Mok and Mok, 2001; Argueso et al., 2009; Kieber and Schaller, 2014), many of which are relevant to the development and function of the syncytium. The pattern of expression of the TCSn:GFP reporter provides clues regarding potential roles of cytokinin in syncytial function. At early stages of infection (2-3 dpi), the TCSn:GFP signal was observed both in the young syncytium and in the surrounding cells. The elevation of cytokinin could play a role in promoting sink activity in these neighboring cells prior to their integration with the developing syncytium.

However, at later stages of infection, the TCSn:GFP signal was reduced in the adjacent cells and became more localized to the fully developed syncytium. As cytokinin plays a critical role in the early stage of cambial cell differentiation that gives rise to xylem and phloem cell (Matsumoto-Kitano et al., 2008; Hejátko et al., 2009), the elevated cytokinin signaling in the neighboring cells could also be associated with the induction of sieve elements and companion cells around the syncytium to accelerate nutrient supply (Hoth et al., 2005; Absmanner et al., 2013). Consistent with this model, a recent study has demonstrated that sieve elements surrounding syncytium cells are formed *de novo* and are cytokinin-responsive (Absmanner et al., 2013).

Cytokinin-insensitive lines exhibit compromised nematode susceptibility, which suggests that cytokinin signaling is necessary for optimal syncytium function. Mutants with increased sensitivity to cytokinin also show reduced nematode parasitism. The reduced nematode susceptibility does not simply reflect the shorter roots of some of these mutants as previous studies have indicated that changes in nematode susceptibility levels are independent of root length or root mass over a wide range of phenotypes (Wubben et al., 2001; Hewezi et al., 2008; Hewezi et al., 2012; Hewezi et al., 2015). Rather, analysis of gene expression suggests that this is the result of a heightened defense response in the cytokinin-hypersensitive mutants.

Examination of the genes that are specifically regulated by *H. schachtii* in the type-A *arr* mutant provides clues as to how the nematode parasitism is altered in this mutant. For example, we observed a significant enrichment of genes encoding heat shock proteins (HSPs) in the genes induced specifically in the *arr3,4,5,6,7,8,9,15* mutant, including *AtHsp90.1* and *HSP90.2* (Table S2.2). HSPs are highly conserved proteins and their expression is induced in response to a wide range of physiological and environmental stimuli (Ahuja et al., 2010). HSPs play crucial roles as molecular chaperones by facilitating correct folding of stress-induced misfolded proteins (Wang et al., 2004). Several HSPs were found to be upregulated during the resistant interaction of soybean with the soybean cyst nematode *H. glycines* (Kandoth et al., 2011). In addition, a number of studies have shown that HSP90 plays a key role in effector-triggered immunity in various pathosystems (Hubert et al., 2003; Takahashi et al., 2003; Thao et al., 2007; Shirasu, 2009). Thus, the up-regulation of HSPs in the infection of the type-A ARR mutants may reflect an induction of defense responses in this mutant.

Another category enriched in genes regulated specifically in nematode-treated *arr3,4,5,6,7,8,9,15,* primarily at 10 dpi, are those encoding cell-wall modifying enzymes. The plant cell wall is a dynamic structure that undergoes significant remodeling during syncytium formation and development, and a large number of genes encoding cell wall modifying enzymes are regulated in response to nematode infection in wild-type roots (Bohlmann and Sobczak, 2014). In the *arr3,4,5,6,7,8,9,15* mutant roots, a large number of such genes are expressed at a lower level as compared to nematode-infected wild-type roots at 10 dpi. This may reflect an altered ability of the nematodes to induce appropriate modifications of cell wall architecture in the host prior to the cell fusions involved in the formation of the syncytium. There are also a number of genes encoding pectin methylesterase inhibitors altered in the *arr3,4,5,6,7,8,9,15* mutant roots. Pectin methylesterases play a role in plant susceptibility to cyst nematodes as an effector from *H. schachtii* has been shown to activate pectin methylesterase 3 in Arabidopsis to promote parasitism (Hewezi et al., 2008).

The type-A ARRs, and by inference cytokinin, likely play an important role in the transcriptional re-programming of the host plant in response to *H. schachtii* infection. This is reflected in the regulation of various transcription factors belonging to particular gene families in a stage-specific manner. While transcription factors of the MYB and BHLH families are the most abundant transcription factor families differentially expressed in the *arr3,4,5,6,7,8,9,15* mutant in response to nematode infection at 4 dpi, C2H2-type zinc finger and WRKY families are the most abundant at 10 dpi. Many of these transcription factors are involved in defense signaling pathways. For example, both MYB108 and MYB30, which are involved in wound-inducible cell death (Cui et al., 2013) and cell death–associated responses (Canonne et al., 2011) respectively, are highly upregulated in the *arr3,4,5,6,7,8,9,15* mutant in response to nematode infection at 4 dpi. Several WRKY transcription factors that are involved in the activation of salicylic acid (SA) biosynthesis genes (WRKY46)(van Verk et al., 2011), positive regulation of effector-triggered immunity (WRKY18)(Schön et al., 2013), and syncytium formation (WRKY23)(Grunewald et al., 2008) are upregulated in the *arr3,4,5,6,7,8,9,15* mutant in response to nematode infection at 10 dpi. We concluded that cytokinin plays a role in the modulation of host genes in response to nematode infection at least partly through the regulation of transcription factors in a stage-specific manner.

The decreased nematode susceptibility of the type-A ARR mutants is similar to the interaction of Arabidopsis with the oomycete *Hyaloperonospora arabidopsidis*, in which type-A ARR mutants displayed slighted elevated expression of defense genes in the shoots prior to infection, and a hyper-induction of these genes in response to infection with the pathogen (Argueso et al., 2012). Indeed, numerous genes encoding pathogenesis-related (PR) proteins are hyper-induced in the *arr3,4,5,6,7,8,9,15* mutant in response to nematode, including several plant defensins (PDF1.2A, PDF1.2C, PDF1.1, PDF1.4), PR-2, PR-6, and a thaumatin superfamily protein. Together, these data suggest that nematodes must walk a fine line between elevating cytokinin function sufficiently to promote optimal syncytial development but below levels that would trigger a strong defense response.

Experimental Procedures

Plant materials and treatment conditions

All Arabidopsis lines used in this study are in the Colombia (Col-0) ecotype. The insertion alleles for the *ahk* mutants are as follows: *ahk2-7*, *ahk3-3*, and *cre1-12* (*ahk4*) (Higuchi et al., 2004); the *ahp1,2,3* mutant (Hutchison et al., 2006); the *arr1-3,12-1* and *arr1-3,10-2,12-1* mutants (Mason et al., 2005); the *arr3,4,5,6* mutant (To et al., 2004) and the *arr3,4,5,6,7,8,9* and *arr3,4,5,6,7,8,9,15* mutants (Zhang et al., 2011). To generate the *CaMV 35S:ARR10-GFP* construct, the *ARR10* coding region was amplified from genomic DNA using the primers 5'-TCCATAAATGAGTTAATTCGCCAGTCTTGAAG-3' and 5'-AGCTGACAAAGAAAAGGGAAAATGGAGTTTC-3', cloned into the entry vector pCR8/ GW/TOPO/ (Invitrogen, USA) and then recombined into pEarleygate103 (Earley et al., 2006) using the Gateway system. For plant transformation, the construct was introduced into the *Agrobacterium tumefaciens* strain GV3101 and then transformed into the *arr1 arr10 arr12* mutant by the floral dip method (Clough and Bent, 1998).

Root elongation assays

Seeds were surface sterilized with chlorine gas, plated on square vertical plates containing half Murashige and Skoog (MS) salts, 1% sucrose, and 0.6% phytagel (Sigma, St. Louis, MO). Plates were cold treated for 3 days in the dark at 4°C and then moved to 22°C at constant light for 4 days. On day 4,

12 seedlings were transferred to half MS plates with 1% sucrose and 0.6% phytogel containing 5 nM, 20 nM, or 50 nM of benzyl adenine (BA) or 0.01% 5 N NaOH as a vehicle control and root length was marked. Plates were returned to 22°C and constant light for five days. On day nine, plates were scanned and root growth was measured between days four and nine using ImageJ software (Abramoff et al., 2004). The average length of root growth was calculated for each genotype and treatment.

Activity of TCSn::GFP reporter in response to *H. schachtii* infection

Transgenic Arabidopsis seeds expressing the TCSn::GFP reporter construct (Zürcher et al., 2013) were planted on 12-well tissue culture plates containing modified Knop's medium as described above. Ten-day-old seedlings were inoculated with approximately 150 surface-sterilized J2 *H. schachtii* nematodes per plant. The reporter activity was visualized by GFP fluorescence at different time points after *H. schachtii* infection. Bright field and fluorescent images of infected and non-infected control plants were observed and captured as previously described (Hewezi et al., 2014).

Nematode susceptibility assays

Arabidopsis seeds were surface sterilized with bleach and planted in a random-block design on 12-well tissue culture plates (BD Biosciences) containing modified Knop's medium (Sijmons et al., 1991) solidified with 0.8% Daishin agar (Research Products International Corp.). Plants were grown at 24°C under 16h light/8h dark conditions. Ten-day-old seedlings were inoculated with approximately 250 surface-sterilized J2 *H. schachtii* nematodes per plant as previously described by (Hewezi et al., 2015). The inoculated plants were maintained under the same conditions described above. Three weeks post inoculation, the number of J4 female nematodes in each root system was counted and used to quantify plant susceptibility. Each line was replicated 20 times, and at least two independent experiments were carried out. Average numbers of J4 female nematodes per root system were calculated, and values significantly different from the wild type were determined in a modified t-test using the statistical software package SAS ($p < 0.05$).

RNA-Seq analysis

Wild-type Arabidopsis (Col-0) and the *arr3,4,5,6,7,8,9,15* mutants were planted in culture dishes on modified Knop's medium using a randomized complete-block design with three independent replications. Ten days after planting, the seedlings were inoculated with approximately 100 surface-sterilized J2 *H. schachtii* nematodes per plant. Then, root tissues were collected from both infected and non-infected plants at 4 and 10 days post *H. schachtii* infection for RNA isolation and library preparation. Total RNA was extracted from three biological replicates for each of the control wild-type roots, infected wild-type roots, control type-A *arr* octuple mutant roots, and infected type-A *arr* octuple mutant roots, (twenty-four samples total) using the RNeasy Plus kit as described by the manufacturer (Qiagen, http://www.qiagen.com), RNA was DNAse treated with TURBO DNA-*free*™ kit according to the manufacturer's instructions (Life Technologies, https://www.lifetechnologies.com), and RNA was cleaned with RNeasy Plus kit as described by the manufacturer (Qiagen, http://www.qiagen.com/). Sample concentrations were determined using the NanoDrop and were sent to the High-Throughput Sequencing Center at University of North Carolina, Chapel Hill. The cDNA libraries were prepared using the TruSeq Stranded mRNA preparation kit according to manufacturer's instructions (Illumina, www.illumina.com). The libraries were amplified to obtain sufficient material following the recommendations of the TruSeq sample preparation protocol and quality was assessed using the Qubit 2.0 Fluorometer (Life Technologies) and the Bioanalyzer 2100. Samples were multiplexed with twelve libraries pooled per lane for Illumina sequencing on the HiSeq2500 instrument with 50 bp, single-end reads.

The sequencing data was checked for quality control using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences were aligned to the Arabidopsis Col-0 genome assembly (TAIR10) with TopHat version 2.0.11 (Langmead et al., 2009) and Bowtie version 1.1.0 (Langmead et al., 2009). SAMtools version 0.1.18 (Li et al., 2009) was used to index BAM files for viewing the mapped reads using the Integrative Genomics Viewer (IGV) genome browser (Robinson et al., 2011). The number of single-mapping reads that overlap each annotated gene was counted using featureCounts (Liao et al., 2014). The counts files were supplied as inputs to EdgeR version 3.0 (Robinson et al., 2010) from the Bioconductor library for statistical analysis of differential gene expression between wild type and mutants. The false discovery rate was controlled at 5% using the

method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). MapMan (Thimm et al., 2004) was used to visualize processes that were enriched in each data set and the LogFC of gene sets. VirtualPlant 1.3 (Katari et al., 2010) was used for gene ontology analysis to show enrichment for differentially expressed genes with p-value < 0.00001. Venn diagrams were made with Venny 2.0 (Oliveros, 2007-2015). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002)and are accessible through GEO Series accession number GSE72548 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE72548).

qRT-PCR

Total RNA was extracted from 12-14 seedling roots at 10 days old, grown at 22°C at constant light on MS media, using the RNeasy Plus kit as described above. cDNA synthesis was performed using Superscript III (Invitrogen) and oligo-d(T) primers according to the manufacturer's instructions (Invitrogen, http://www.invitrogen.com). Real-time PCR was performed sing SYBR Premix Ex Taq polymerase (TaKaRa, http://www.takara-bio.com) in a ViiATM 7 Real-Time PCR system (ABI, http://www.appliedbiosystems.com). *TUBULIN 4* (At5g44340) was used as housekeeping gene in all reactions with primers previously described ((Cheng et al., 2013). The gene-specific primers used for *EDS1* (At3g48090) were previously described (Feys et al., 2001). qRT-PCR was performed for three biological replicates for each genotype with three technical replicates. The relative expression of *EDS1* was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

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Table 2.1 Biotic stress genes, SA, JA, and ET signaling genes which have been previously identified as key defense genes and are significantly differentially regulated in at least one treatment group[a].

	Control *arr3,4,5,6,7,8,9,15* (log_2FC)		Infected wild type (log_2FC)		Infected *arr3,4,5,6,7,8,9,15* (log_2FC)	
	4 dpi	10 dpi	4 dpi	10 dpi	4 dpi	10 dpi
Biotic Stress Genes						
NDR1	0.66					
PDF1.2					8.08	
PDF1.2C					6.04	
EDS1	1.12	0.86				
Salicylic Acid Genes						
UGT74F2	-1.04	-0.85				
Jasmonic Acid						
LOX3			2.36		1.76	
AOS	-0.57	-0.56	0.57			
Ethylene						
ERF1	-1.16		0.90			
ERF2	-1.06	-0.61	0.67			
ERF104	0.91					0.75
ACS2			1.29		3.19	2.32
ACS6					1.03	
ACS7			1.27		2.13	
ACS8					2.71	2.73
ACS11	-0.93					
WRKY Transcription Factors						
WRKY18	1.08		1.00			0.93
WRKY23	1.18		0.72			0.89
WRKY38	-1.79	-0.99				
WRKY40	1.33					
WRKY46			1.50			1.45
WRKY62	-2.20	-2.17				

[a]Empty boxes signify there was not a significant difference in gene expression (FDR < 0.05).

Table S2.1. Cytokinin two-component signaling elements, biosynthesis and degradation genes that are differentially regulated in non-infected control, infected wild-type, and/or type-A arr3,4,5,6,7,8,9,15 mutants[a].

	Control *arr3,4,5,6,7,8,9,15* (log_2FC)		Infected wild type (log_2FC)		Infected *arr3,4,5,6,7,8,9,15* (log_2FC)	
	4dpi	10dpi	4 dpi	10 dpi	4 dpi	10 dpi
AHKs						
AHK3	-0.55					
AHK4	0.47					
Type-A ARRs						
ARR3	NA	NA	-0.73		NA	NA
ARR4	NA	NA			NA	NA
ARR5	NA	NA			NA	NA
ARR6	NA	NA			NA	NA
ARR7	NA	NA	0.84		NA	NA
ARR8	NA	NA			NA	NA
ARR9	NA	NA			NA	NA
ARR15	NA	NA			NA	NA
ARR16	2.84	2.24				
ARR17	1.49	2.31				
Type-B ARRs						
ARR2	-0.34					
ARR11					-0.87	
IPTs						
IPT1			2.77	4.22		1.35
IPT3	2.81					
IPT5			-0.65	-1.00		
IPT7	-0.89	-1.27				
CKXs						
CKX1	0.83					
CKX2			1.29			
CKX3					1.64	
CKX4	1.02	0.71				
CKX5		0.77			1.27	
Cytokinin glycosyltransferases						
UGT76C1	-0.40					
UGT76C2	-0.44					
UGT85A1					1.37	

[a]No significant genes were differentially regulated for the phosphotransfer proteins (AHP1-5) and cytokinin biosynthetic genes (CYP735A1, CYP735A2, LOG1-5, and LOG7-8). For genes from each family not shown in the table, there is no significant change in gene expression with an FDR < 0.05. Boxes filled in grey signify no significant change in gene expression. NA: not applicable as these genes are disrupted with T-DNA insertions in the type-A arr3,4,5,6,7,8,9,15 mutant.

Table S2.2. Abiotic stress heat genes that are significantly differentially regulated in at least one treatment group[a].

Annotation	Gene ID	Control *arr3,4,5,6,7,8,9,15* (log_2FC)		Infected wild type (log_2FC)		Infected *arr3,4,5,6,7,8,9,15* (log_2FC)	
		4dpi	10dpi	4 dpi	10 dpi	4 dpi	10 dpi
J8, Chaperone DnaJ-domain superfamily protein	At1g80920	1.53					
ATHSFA2, HSFA2, heat shock transcription factor A2	At2g26150						
Chaperone DnaJ-domain superfamily protein	At2g41000	-0.45					
Heat shock protein 70 (Hsp 70) family protein	At3g09440	-0.40					
J20, DNAJ-like 20	At4g13830	0.46					
AT-HSFA4A, HSF A4A	At4g18880	0.46					
HSP20-like chaperones superfamily protein	At4g21870	1.21					
Chaperone DnaJ-domain superfamily protein	At4g36040	1.50					
Chaperone DnaJ-domain superfamily protein	At5g16650	0.43					
ATJ1, DNAJ heat shock family protein	At1g28210	1.25	1.28				
Chaperone DnaJ-domain superfamily protein	At5g37440	-8.18	-8.42				
Chaperone DnaJ-domain superfamily protein	At5g37750	-5.55	-8.09				
BIP3, Heat shock protein 70 (Hsp 70) family protein	At1g09080			2.31			
Double Clp-N motif-containing P-loop	At2g40130	-0.93			-0.63		
ATHSP70, HSP70, heat shock protein 70	At3g12580	-0.73		1.25	0.79		
Chaperone DnaJ-domain superfamily protein	At3g13310				0.44		
AT-HSP17.6A, HSP17.6, HSP17.6A	At5g12030	-1.46		2.43			2.19
HSP20-like chaperones superfamily protein	At1g07400			2.71		6.17	
Hsp70b, heat shock protein 70B	At1g16030			1.48		5.64	
HSP20-like chaperones superfamily protein	At1g52560			2.89		6.61	2.56
HSP20-like chaperones superfamily protein	At1g53540			3.95		6.29	3.06
ATHSP101, HOT1, HSP101, heat shock protein 101	At1g74310			1.03		5.26	
ATERDJ3A, TMS1	At3g08970			1.03		3.82	
ATHSP17.4, HSP17.4, heat shock protein 17.4	At3g46230			4.12		6.27	3.77
ATHSP22.0, HSP20-like chaperones superfamily protein	At4g10250			3.99		6.30	2.52
HSA32, Aldolase-type TIM barrel family protein	At4g21320			0.93		4.23	
ATHSP23.6-MITO, HSP23.6-MITO	At4g25200			3.66		6.92	2.71
HSP21, heat shock protein 21	At4g27670			6.47		6.85	
HSP17.6II, 17.6 kDa class II heat shock protein	At5g12020			4.14		6.85	2.78
ATHS83, AtHsp90-1, ATHSP90.1, HSP81-1, HSP81.1	At5g52640			1.21		5.06	
HSP18.2, heat shock protein 18.2	At5g59720			1.44		5.41	1.76
DNAJ heat shock family protein	At2g20560			0.76	0.58	3.42	
HSP20-like chaperones superfamily protein	At2g29500			1.52	1.15	5.68	1.01
HSP20-like chaperones superfamily protein	At5g51440			0.88	1.09	5.33	
HSP40/DnaJ peptide-binding protein	At1g44160					-0.87	
HSP20-like chaperones superfamily protein	At1g54050					5.80	
ERD2, HSP70T-1	At1g56410					5.82	
HSP20-like chaperones superfamily protein	At1g59860					5.43	
Chaperone DnaJ-domain superfamily protein	At1g71000					6.64	
Chaperone DnaJ-domain superfamily protein	At1g72416					1.11	
Heat shock protein 70 (Hsp 70) family protein	At1g79920					1.06	
CLPB-M, CLPB4, HSP98.7, casein lytic proteinase B4	At2g25140					2.37	
HSP70T-2, heat-shock protein 70T-2	At2g32120					4.49	
ATBAG6, BAG6, BCL-2-associated athanogene 6	At2g46240					3.49	
Chaperone DnaJ-domain superfamily protein	At3g14200					1.53	
ATJ, ATJ3, DNAJ homologue 3	At3g44110					0.98	
AT-HSFB2B, HSFB2B	At4g11660					1.77	
heat shock protein 70 (Hsp 70) family protein	At4g32208					3.50	
HSC70-5, MTHSC70-2, mitochondrial HSO70 2	At5g09590					1.88	
APG6, CLPB-P, CLPB3, casein lytic proteinase B3	At5g15450					2.03	
HSP20-like chaperones superfamily protein	At5g37670					4.44	
cpHsc70-2, CPHSC70-2EAT SHOCK PROTEIN 70-2	At5g49910					0.79	
AtHsp90.2, ERD8, HSP81-2, HSP90.2	At5g56030					0.98	

[a]Grey boxes signify there was not a significant difference in gene expression (FDR < 0.05).

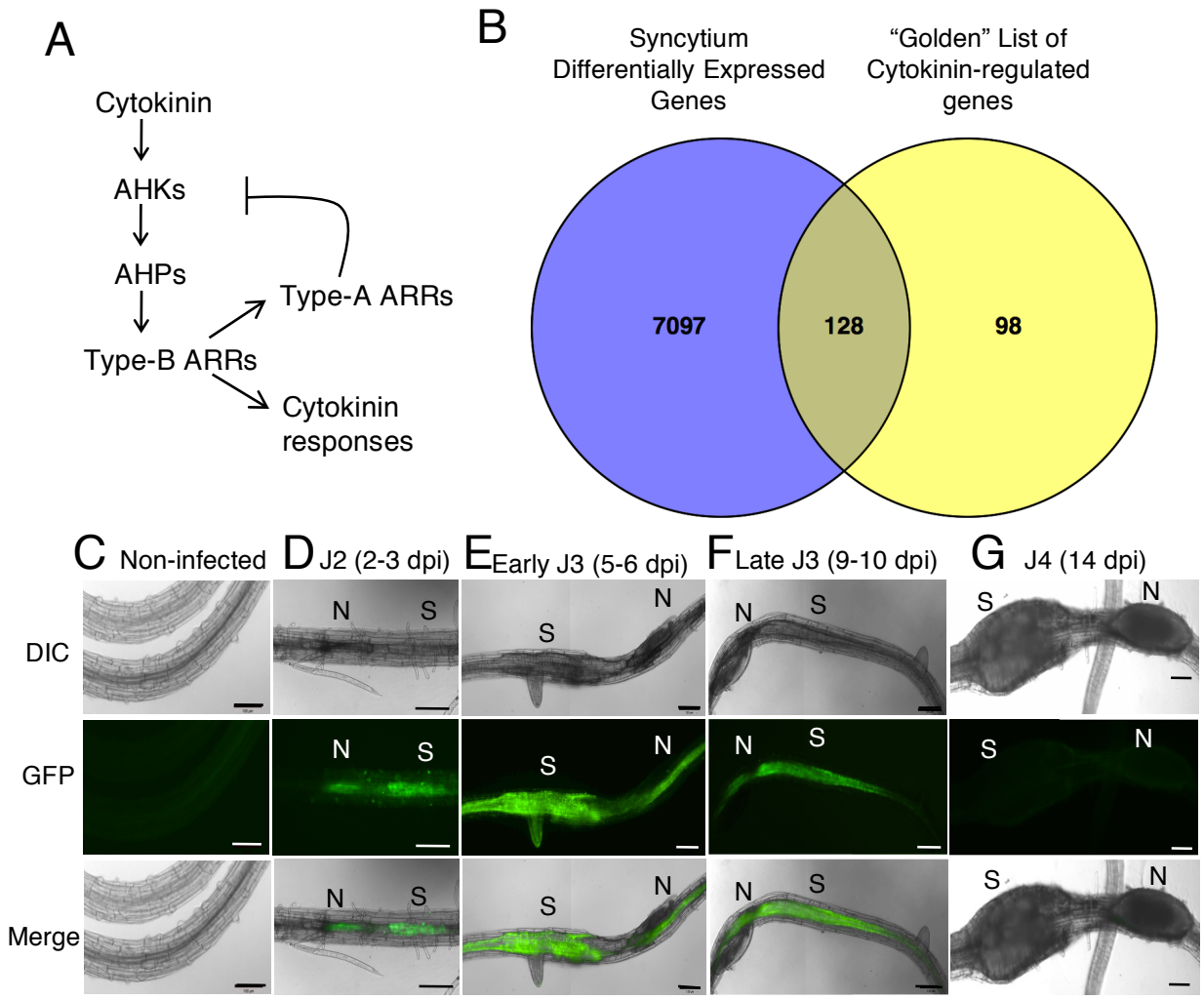


Figure 2.1. Nematode infection elevates cytokinin signaling in the syncytium. **(A)** Model of the cytokinin signaling pathway in Arabidopsis. Cytokinin binds to the AHK receptors and initiates a phosphorelay cascade, which phosphorylates the type-A and type-B ARRs via the AHPs. The activated type-B ARRs elevate transcription of cytokinin response genes, including the type-A ARRs, which act as negative regulators of cytokinin signaling. **(B)** Venn diagram showing overlap between the 7,225 genes differentially expressed in Arabidopsis syncytia (Szakasits et al., 2009) and the 226 genes found in the "golden list", a set of robustly regulated cytokinin genes (Bhargava et al., 2013). Significant overlap between these two data sets was determined using the hypergeometric probability test $P(X \geq 128) = 8.5e^{-26}$. **(C-G)** Visualization of TCSn::GFP reporter activity in the *H. schachtii*-induced feeding sites compared with the non-infected plants. The top panels are DIC images; the middle panels show the GFP fluorescence signal and the bottom panels the overlay. C, non-infected roots, D, sedentary J2 at 2-3 dpi, E, early J3 at 5-6 dpi, F, late J3 at 9-10 dpi and, G, J4 at 14 dpi. N indicates nematode, and S indicates syncytium. Scale Bars = 100 µm (C and G) and 130 µm (D-F).

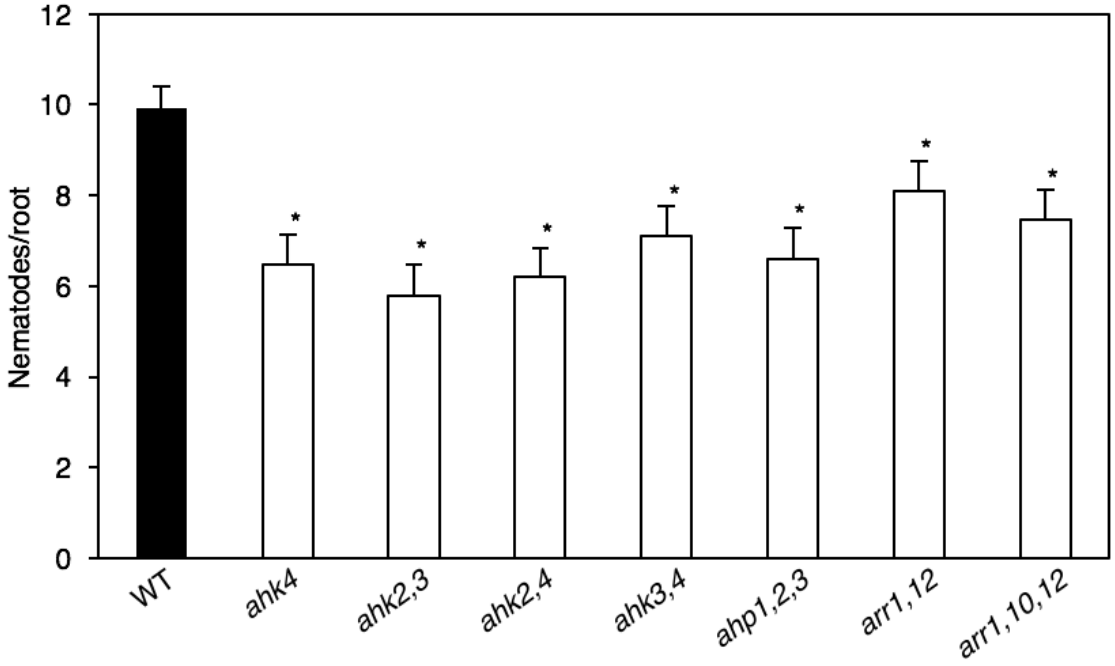


Figure 2.2. Nematode susceptibility of cytokinin-insensitive mutants. Susceptibility assays of cytokinin insensitive mutant lines infected with *H. schachtii*. The *ahk4*, *ahk2,3*, *ahk3,4*, *ahk2,4*, *ahp1,2,3*, *arr1,12* and *arr1,10,12* mutants were planted on modified Knop's medium and 10-day-old seedlings were inoculated with approximately 250 surface-sterilized J2 *H. schachtii* nematodes. Three weeks post inoculation, the average number of J4 female nematodes per root system was determined. Data are presented as mean number of J4 female nematodes/root system. Error bars represent SE (n = 20). Mean values significantly different from the wild-type (Col-0) were determined using an unadjusted paired t-tests ($P < 0.05$) and are indicated by asterisks. Similar results were obtained from at least two independent experiments. Data from one representative experiment are shown.

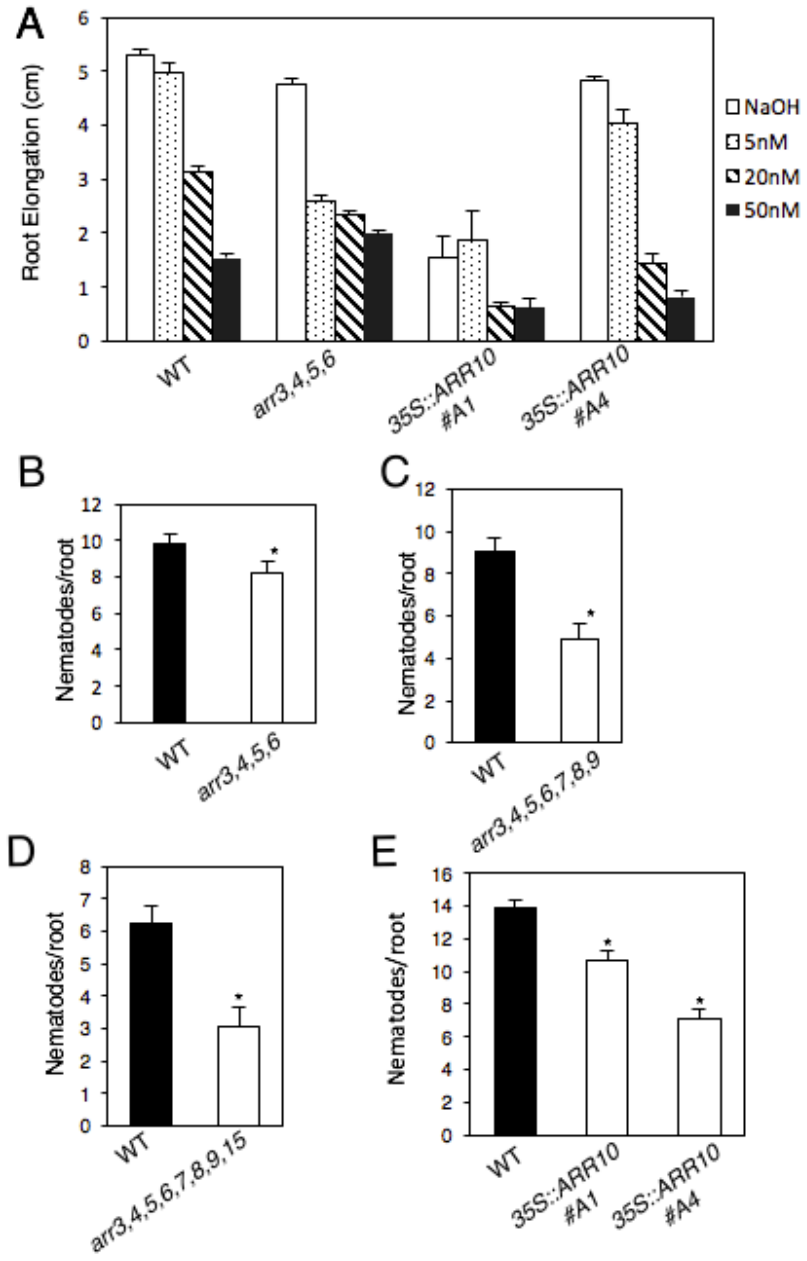


Figure 2.3. Nematode susceptibility of cytokinin-hypersensitive mutants. **(A)** Average root growth of wild type or the indicated mutants measured from day 4-9. Seedlings grown on MS medium supplemented with the specified concentrations of benzyl adenine (BA) or 0.01% 5N NaOH vehicle control. Error bars represent SE (n > 8). Similar results were obtained from at least two independent experiments. Data from one representative experiment are shown. **(B-E)** Susceptibility assays of cytokinin mutant lines infected with *H. schachtii*. Seeds of the *arr3,4,5,6* mutants B, *arr3,4,5,6,7,8,9* mutants C, *arr3,4,5,6,7,8,9,15* mutants D, *35S::ARR10#A1* and *35S::ARR10*#A4 E, were planted on modified Knop's medium, and assayed for nematode susceptibility. Data are presented as mean ± SE (n = 20). Significant differences from wild type (Col-0) were determined by unadjusted paired t-tests (P < 0.05) and are indicated by asterisks. Similar results were obtained from at least two independent experiments.

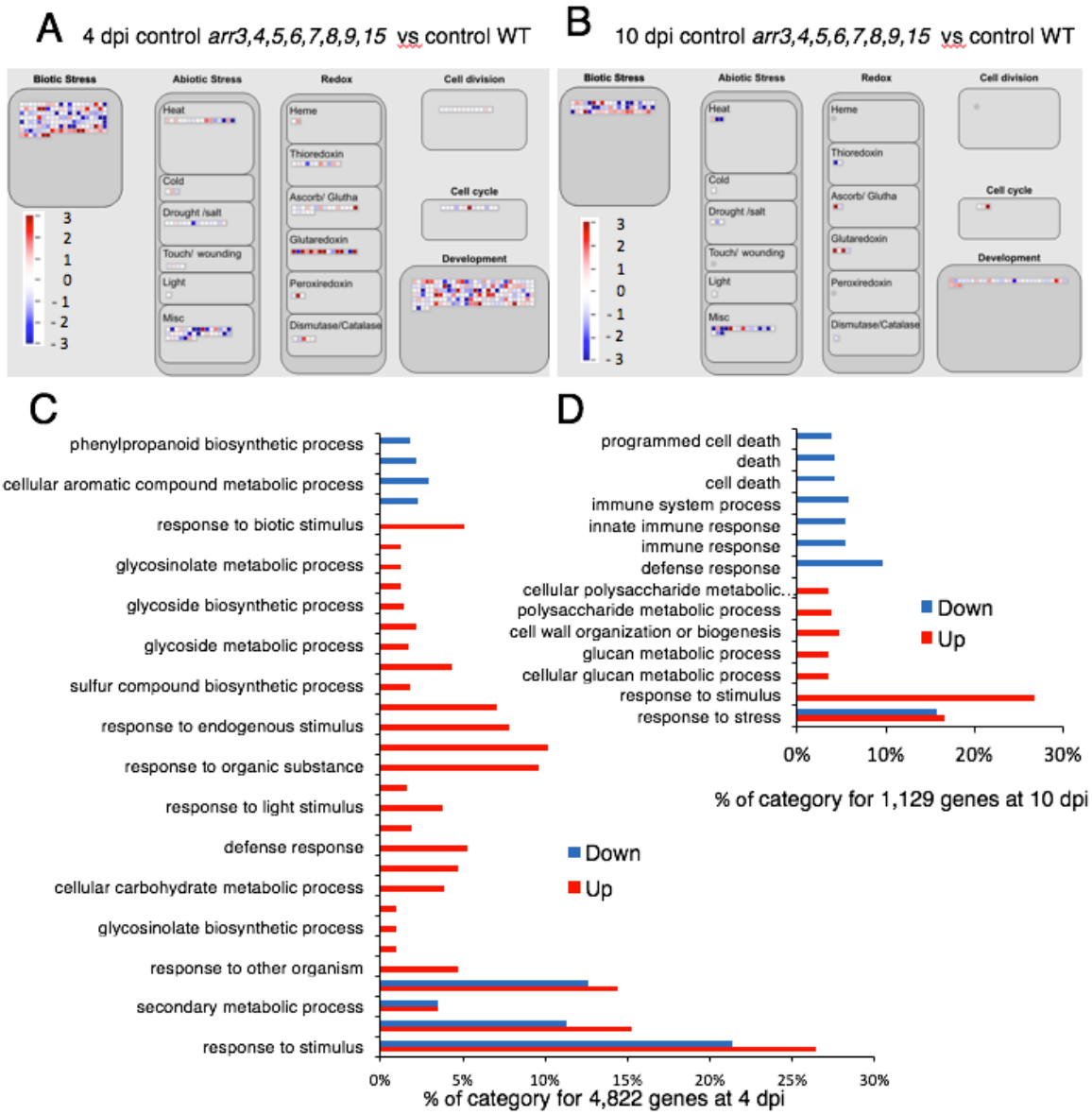


Figure 2.4. The genes differentially expressed between the control type-A *arr3,4,5,6,7,8,9,15* mutant and control wild type **(A)** and **(B)** MapMan (Thimm et al., 2004) illustrations of the cellular response overview for differentially expressed genes between the control type-A *arr3,4,5,6,7,8,9,15* mutant and control wild type 4 days after mock infection (i.e. 14 day old roots; 4,822 genes) A, or 10 days after mock infection (i.e. 20 day old roots; 1,129 genes) B. The red boxes represent genes that are up regulated and the blue boxes represent genes that are down regulated. Scale = log FC from 3 to -3. FDR < 0.05. **(C)** and **(D)** Gene ontology (GO) analysis of genes differentially expressed between the control type-A *arr3,4,5,6,7,8,9,15* mutant and control wild type at 4 days after mock infection C, and 10 days after mock infection D, using of BioMaps tool on the VirtualPlant 1.3 software (Katari et al., 2010) for gene sets separated into up-regulated and down-regulated genes. Background population; TAIR10 genome, the classification scheme; GO biological process assignments by TAIR/TIGR, Fisher exact test used to calculate significant enrichment ($p < 0.00001$). The percentage of the observed frequency of genes found in each category is plotted the x-axis. The blue bars = down-regulated categories and the red bars = up-regulated categories. If there is no blue or red bar for a given category, it was not enriched at a p value of < 0.00001

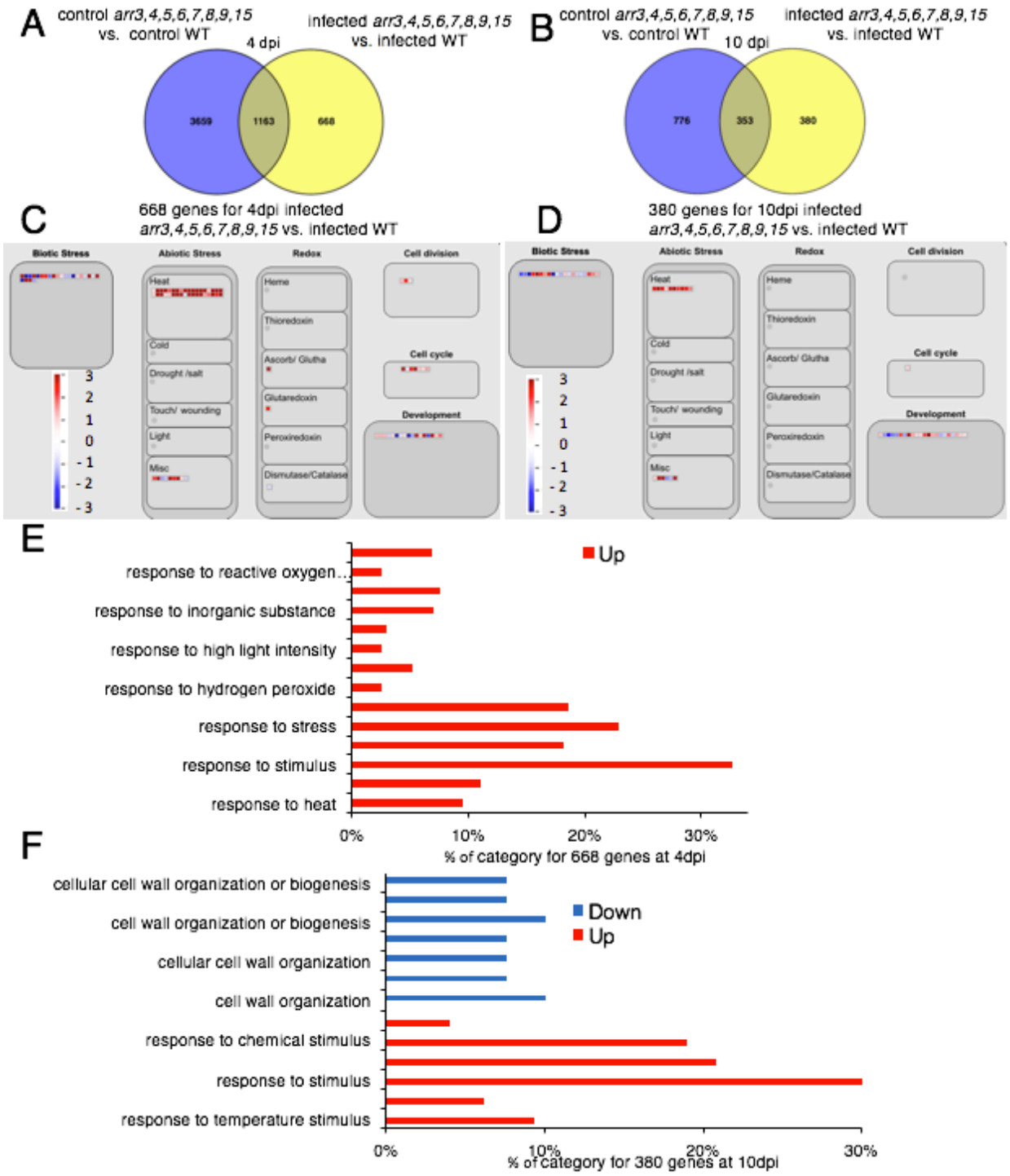
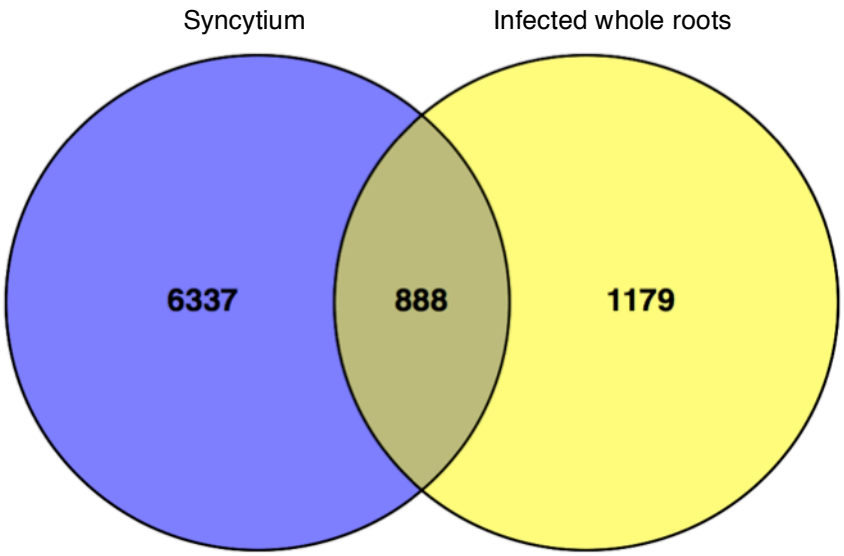
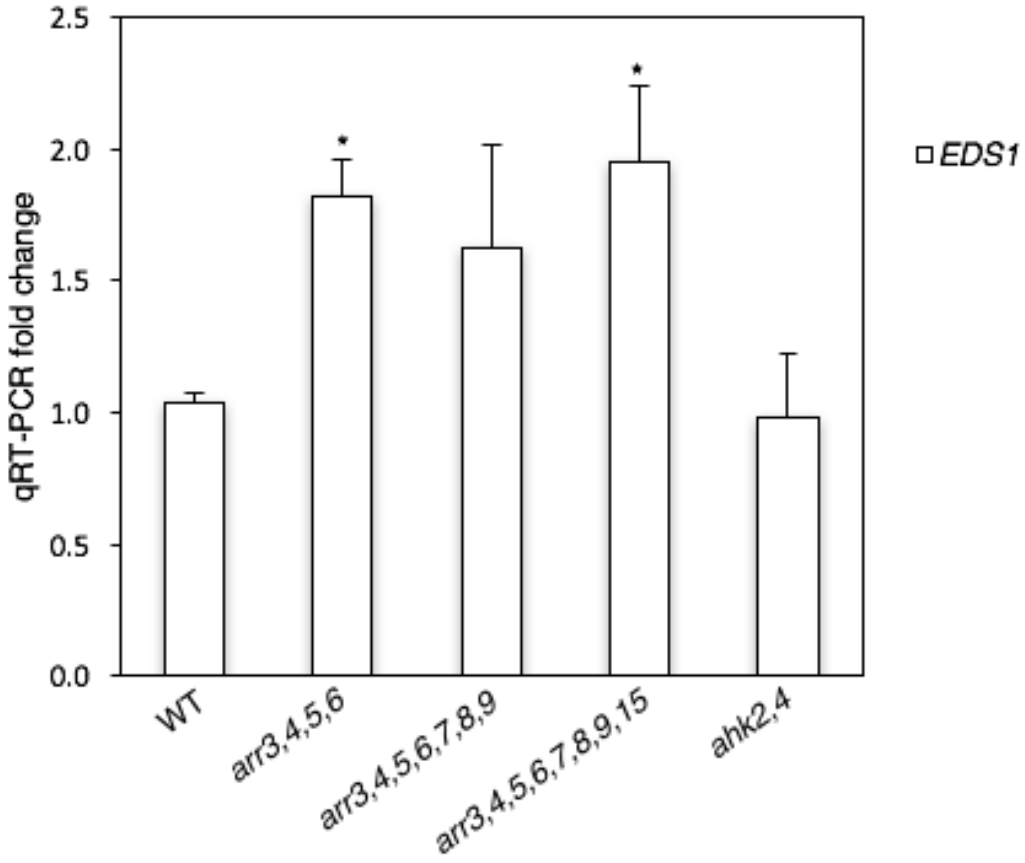


Figure 2.5. The differentially expressed genes between the infected type-A *arr3,4,5,6,7,8,9,15* mutant and infected wild type. **(A)** and **(B)** Venn diagrams of genes differentially expressed control type-A *arr3,4,5,6,7,8,9,15* vs. control wild type at 4 days (4,822 genes) A, or 10 days (1,129 genes) B, after mock infection compared to those differentially expressed between type-A *arr3,4,5,6,7,8,9,15* and wild type 4 days (1,831 genes) or 10 days (733) after infection with *H. schachtii*. **(C)** and **(D)** MapMan (Thimm et al., 2004) illustrations of the cellular response overview for the genes identified in (A or B) as differentially expressed and only found in the infected *arr3,4,5,6,7,8,9,15* mutants at 4 dpi (668 genes) C, or 10 dpi (380 genes) D. The red boxes = up-regulated genes and the blue boxes = down-regulated genes. Scale =

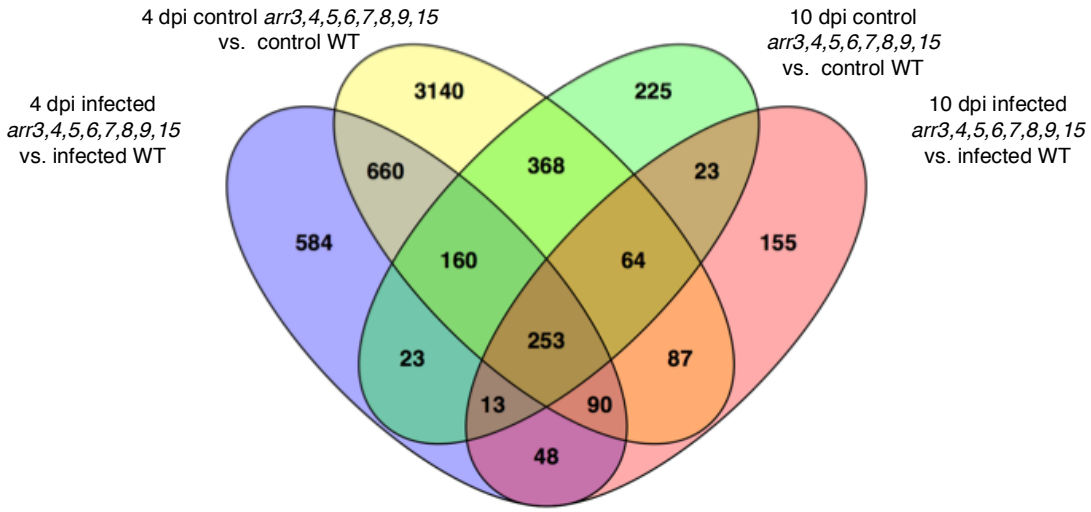
logFC from 3 to -3. FDR < 0.05.E and F, Gene ontology (GO) analysis of the genes from A and B and used in the MapMan analysis. 4 dpi (668 genes) E, or 10 dpi (380 genes) F, made using the BioMaps tool on the VirtualPlant 1.3 software (Katari et al., 2010) for gene sets separated into up-regulated and down-regulated genes. Background population; TAIR10 genome, classification scheme, GO biological process assignments by TAIR/TIGR, Fisher exact test ($p < 0.00001$). The percentage of the observed frequency of genes found in each category is plotted on the x-axis. The blue bars = enriched down-regulated categories, the red bars = enriched up-regulated categories. If there is no blue or red bar for a give category, it was not enriched at a p value of < 0.00001.



Supplementary Figure S2.1. Comparison of the 7,225 genes identified as differentially regulated in response to *H. schachtii* infection in syncytium tissue (Szakasits et al., 2009) with the 2,067 genes differentially regulated in whole roots following infection with *H. schachtii*. The 7,225 genes differentially regulated in the syncytium (shown in blue) represent genes differentially regulated at 5 dpi and/or 15 dpi. The 2,067 genes represent the genes differentially regulated between infected and control wild-type roots at 4 dpi and/or 10 dpi (shown in yellow). These 2,067 genes are comprised of 756 genes were unique to 4 dpi and 828 genes unique to 10 dpi, with 483 genes differentially regulated at both time points.



Supplementary Figure S2.2. Expression of *EDS1* in type-A *arr* and cytokinin receptor mutants. *EDS1* is examined in roots of multiple type-A *arr* mutants, *arr3,4,5,6*, *arr3,4,5,6,7,8,9* and *arr3,4,5,6,7,8,9,15*, the cytokinin receptor mutant, *ahk2,4* and wild-type control. The level of *EDS1* was determined relative to wild type. Error bars represent SEM from three biological replicates. Asterisks indicate statistically significant differences from wild type as determined by using a two-tailed student's t-test $p < 0.05$.



Supplementary Figure. S2.3. The differentially expressed genes shared between control and infected type-A *arr3,4,5,6,7,8,9,15* mutants vs. infected wild type at 4 dpi and 10 dpi. Venn Diagram comparing the differentially expressed genes for control type-A *arr3,4,5,6,7,8,9,15* mutants vs. control wild type at 4 dpi (Yellow; 4,822 genes), infected type-A *arr3,4,5,6,7,8,9,15* mutants vs. infected wild type at 4 dpi (Blue; 1,831 genes), control type-A *arr3,4,5,6,7,8,9,15* mutants vs. control wild type at 10 dpi (Green, 1,129 genes), and infected type-A *arr3,4,5,6,7,8,9,15* mutants vs. infected wild type at 10 dpi (Red; 733 genes). There are 48 genes that overlap between only the infected wild type and infected type-A *arr3,4,5,6,7,8,9,15* mutants at 4 dpi (1,831) and infected wild type and infected type-A *arr3,4,5,6,7,8,9,15* mutants at 10 dpi (733). FDR < 0.05.

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CHAPTER 3: THE *BASIC PENTACYSTEIENE* TRANSCRIPTION FACTORS REGULATE A SUB-SET OF CYTOKININ SIGNALING RESPONSES AND INTERACT WITH TYPE-A ARABIDOPSIS RESPONSE REGULATOR PROTEINS IN ARABIDOPSIS

Summary

The type-A Arabidopsis response regulators (ARRs) are primary cytokinin-response genes that negatively regulate the cytokinin signaling pathway. However, the mechanisms by which the type-A ARRs negatively regulate cytokinin signaling remains unclear. Here we identify members of the *BASIC PENTACYSTIENE* (*BPC*) transcription factor gene family as type-A ARR interacting proteins in *Arabidopsis thaliana*. To help characterize the role of the BPC and type-A ARR interaction, we examine the involvement of BPCs in cytokinin signaling. *bpc* multiple mutants show reduced sensitivity to cytokinin in root elongation assays. BPC6 binds to the promoters of cytokinin-regulated genes in the absence of cytokinin treatment and a significant number of BPC6-regulated genes are also targets of the type-B ARR transcription factors, which are activated in response to cytokinin. A significant number of genes whose expression is altered in *bpc* mutant roots are also mis-expressed in cytokinin response factor (*crf1,3,5,6*) mutant and type-A *arr3,4,5,6,7,8,9,15* mutant roots. Furthermore, the BPCs are necessary for induction of a subset of genes in response to exogenous cytokinin. These results suggest that the BPCs are part of a complex network of transcription factors that are involved in the response to cytokinin and the interaction of the BPCs with the type-A ARRs likely modifies this regulation.

Introduction

Cytokinin is a plant hormone that regulates diverse plant processes, such as plant growth and stem cell maintenance, source sink relationships, vascular development, and biotic and abiotic interactions (Schaller et al., 2008; Argueso et al., 2009; Werner and Schmülling, 2009; Schaller et al., 2011; Kieber and Schaller, 2014). The cytokinin signaling cascade is similar to two-component signaling (TCS) systems found in prokaryotes that are comprised of histidine kinase and response regulator proteins (Bourret and Stock, 2002; Stock et al., 2000). The cytokinin signaling pathway consists of an elaboration of the simple TCS, a so-called multi-step phospho-relay system. The phosphorelay is initiated

when cytokinin binds to *Arabidopsis* histidine kinase (AHK) receptors in the lumen of the endoplasmic reticulum (Nishimura et al., 2004; Wulfetange et al., 2011). A conserved histidine residue in the AHK receptors is phosphorylated and this phosphate is then transferred to an aspartic acid residue in the receiver domain of the AHK receptors. The phosphate is then transferred to a histidine residue of the *Arabidopsis* histidine-containing phosphotransfer proteins (AHPs) (Hutchison *et al.*, 2006), and finally to an aspartic acid residue in the receiver domain of the type-B Arabidopsis response regulators (ARRs) (Mason et al., 2005; Yokoyama et al., 2006; Ishida et al., 2008). This phosphorylation activates the MYB-like DNA-binding domain of the type-B ARRs and leads to regulation of cytokinin response genes. These targets include the type-A ARRs, which are also phosphorylated by the AHPs and which negatively regulate cytokinin signaling. Thus, while the proteins involved in the primary cytokinin signaling cascade have been identified, what has not been determined is how the transcriptional output of this pathway mediates distinct functions. To further understand the transcriptional response to cytokinin and how the cytokinin signal is relayed, we investigated the mechanism by which the type-A ARR proteins negatively regulate cytokinin signaling.

There are ten type-A ARRs that are rapidly up-regulated in response to cytokinin and display functional redundancy as negative regulators of cytokinin signaling (Branstatter and Kieber, 1998; Imamura et al., 1998; To, 2004; To et al., 2007). The type-A ARRs clade into pairs and all share sequence similarity in their N-terminal receiver domains, but differ in the length and sequence of their C-terminal extensions (D'Agostino et al., 2000). Similar to the type-B ARRs, the type-A ARRs are phosphorylated on an aspartic acid residue of their receiver domain by the AHP proteins (Suzuki et al., 1998; Imamura *et al.*, 1998). This phosphorylation event stabilizes a sub-set of the type-A ARR proteins (To et al., 2007). The mechanism by which the type-A ARR negatively regulate cytokinin responsiveness is not well understood. One potential mechanism by which the type-A ARRs negatively regulate cytokinin signaling is that the type-A ARRs compete with type-B ARRs for phosphorylation by AHP proteins, thereby dampening the activation signal of the type-B ARRs and reducing transcription of cytokinin response genes. However, previous research showed that a phosmomimic (D to E) version of the type-A ARR5 was able to partially complement a loss-of-function type-A *arr3,4,5,6* multiple mutant, while a phosphate null (D to A) version was not (To et al., 2007). Since both the phosphomimic and phosphate

null versions are unable to be phosphorylated by AHP proteins, this suggests that phosphorylation of the receiver domain is necessary for type-A ARR function. Furthermore, competition with the type-B ARRs cannot be the only mechanism for how type-A ARRs negatively regulate cytokinin signaling because the phosphomimic form of ARR5 partially complements a loss-of-function type-A ARR mutation (To et al., 2007). Another mechanism commonly used by single domain bacterial response regulators is to bind to and regulate other target proteins (Jenal and Galperin, 2009). We hypothesized that the type-A ARRs interact with other target proteins, in some cases in a phosphate-dependent manner. Here, we identified and characterized a type-A ARR interacting protein.

BASIC PENTACYSTEINE 6 (BPC6) is a member of the BARLEY B RECOMBINANT (BBR)/ BPC family of proteins, a group of plant-specific transcription factors that bind to GA-rich DNA sequences (Meister et al., 2004; Monfared et al., 2011). The first plant GAGA binding protein was identified in soybean (*Glycine max*) and bound specifically to $(GA)_9$ in the promoter of the chlorophyll and heme synthesis enzyme *Gsa1* (Sangwan and O'Brian, 2002). Since then, homologs of the BPCs have been identified in all angiosperms (Meister et al., 2004). In *Arabidopsis*, the BPC family consists of six genes and one pseudogene *(BPC5)* that fall into three classes based upon phylogeny and sequence similarity at their C-terminus: Class I (*BPC1*, *BPC2*, and *BPC3*); Class II (*BPC4*, *BPC5*, and *BPC6*); Class III (*BPC7*) (Monfared et al., 2011; Meister et al., 2004). All BPCs consist of an N-terminal dimerization domain and a putative zinc finger DNA-binding domain at their C-terminus (Kooiker et al., 2005). The C-terminal DNA-binding domain contains basic amino acid residues and five conserved cysteine residues (Meister et al., 2004; Sangwan and O'Brian, 2002).

Although the BBR/BPC gene family of proteins shares no significant sequence similarity to animal proteins, it has been speculated that these transcription factors may be functionally similar to GAGA factors (GAFs) of *Drosophila melanogaster* (Sangwan and O'Brian, 2002; Berger and Dubreucq, 2012; Lehmann, 2004). Similar to the *Drosophila* GAFs, the BPCs regulate transcription of genes that are important regulators of plant growth and development such as homeobox genes, and they can act as both transcriptional repressors or activators. The Class I BPCs positively regulate expression of the the ovule development gene *INNER NO OUTER (INO)* (Meister et al., 2004), and seed development gene *LEAFY COTYLEDON2 (LEC2)* (Berger et al., 2012). The BPCs interact with Polycomb group (PcG)

proteins, which form protein complexes that remodel chromatin by modifying histone tails to repress transcription of genes. For example, the BPCs suppress transcription of the transcription factor *ABSCISIC ACID INSENSITIVE4 (ABI4)* by direct interaction of multiple BPCs with the Polycomb-Repressive Complex 2 (PRC2) component *SWINGER (SWN)* (Mu et al., 2017). Furtheremore, BPC6 interacts with a member of the PRC1 complex, LIKE HETEROCHROMATIN 1 (LHP1), and recruits LHP1 to GAGA binding motifs (Hecker et al., 2015). The BPCs also regulate transcription via PRC1/2 complex independent mechanisms, in which the class I BPCs recruit the protein repressive complex SHORT VEGETATIVE PHASE (SVP)-APETELA 1 (AP1)-SEUSS (SEU)-LEUNIG (LUG) to repress transcription of the ovule identity gene *SEEDSTICK (STK)* (Simonini et al., 2012). Furthermore, *in vitro* experiments found that BPC1 will bend the *STK* promoter, which is likely facilitated by the ability of BPC1 to oligomerize and bind to multiple GA stretches in the *STK* promoter (Kooiker et al., 2005; Simonini et al., 2012).

The BPC proteins act redundantly, as the higher order *bpc* mutants display a variety of reproductive and vegetative defects, while single and double mutants display no or less severe phenotypes (Monfared et al., 2011; Kooiker et al., 2005; Santi et al., 2003; Berger and Dubreucq, 2012). Due the pleiotropic phenotype of the higher order *bpc* mutants, it has been suggested that the BPCs function not only by regulating plant developmental processes, but also through regulation of phytohormone levels and/or signaling (Monfared et al., 2011). For example, the *bpc1,2,4,6* mutant has an altered response to ethylene, though it is unaffected in the response to auxin and gibberellic acid (Monfared et al., 2011). BPCs also regulate abscisic acid signaling by regulating transcription of *ABI4* (Mu et al., 2017). Although BPCs are linked to ethylene and abscisic acid (ABA) signaling, not all of the phenotypes observed in the *bpc1,2,4,6* mutants can be attributed to disruptions of these hormones and thus, the BPCs may regulate multiple hormonal pathways (Monfared et al., 2011).

Previous studies have linked BPC transcription factors to cytokinin signaling in the meristem (Simonini and Kater, 2014). The *bpc1,2,3, bpc1,2,3,4,6*, and *bpc1,2,3,4,6,7* mutants exhibit a hyper-proliferative meristem, reminiscent of cytokinin oxidase (CKX) mutants, which have increased cytokinin levels (Bartrina et al., 2011; Monfared et al., 2011). Chromatin immunoprecipitation assays with a BPC Class 1 specific antibody found that in inflorescence tissue the BPCs bind to the promoters of some

cytokinin regulated genes including, type-A *ARR7*, *WUSCHEL (WUS), SHOOTMERISTEMLESS (STM),* and *BREVIPEDICELLUS/KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 1 (BP/KNAT1*) (Rupp et al., 1999; Leibfried et al., 2005; Simonini and Kater, 2014). Furthermore, the expression of *IPT7,* which encodes an enzyme involved in cytokinin synthesis is elevated in the inflorescence of *bpc1-2,2,3* mutants (Simonini and Kater, 2014). Here, we further explored the links between the BPC proteins and cytokinin signaling. We characterized the interaction between the BPCs and type-A ARRs, identified multiple cytokinin response genes that are direct BPC6 targets, and found that the BPCs regulate a sub-set of cytokinin response genes. These results suggest that the BPCs are part of the complex combinatorial gene regulatory network that regulates transcription in response to cytokinin. Furthermore, the type-A ARRs likely alter the function of transcription factors such as the BPCs to dampen the cytokinin transcriptional response.

Results

BPC proteins interact with type-A ARRs independent of aspartic acid phosphorylation of the receiver domain

We conducted a yeast two-hybrid screen using wild-type type-A ARR4 protein and the previously described phosphomimic version ($ARR4^{D95E}$) (To et al., 2007). Class II BPC6 was identified as interacting with both wild-type ARR4 and $ARR4^{D95E}$ baits. As both the BPCs and type-A ARRs are present as multigene families in Arabidopsis, we further investigated the interactions among other BPCs and type-A ARRs. BPC1, a class I BPC, interacted with ARR4 in a yeast two-hybrid assay (Figure 3.1A). Further, both BCP1 and BPC6 interacted with ARR5, which has a shorter C-terminal domain as compared to ARR4, suggesting that the conserved N-terminal receiver domain of the type-A ARRs interacts with the BPCs. As multiple BPCs and type-A ARRs interact with each other, it suggests some functional overlap among the BPCs family members with regard to their role in mediating type-A function.

To further investigate this interaction *in planta* and to determine the role of aspartic acid phosphorylation of the receiver domain, we transiently expressed epitope-tagged myc-BPC6 and HA-ARR4 in *Nicotiana benthamiana* epidermal leaf cells and examined the interaction in the presence and absence of cytokinin using a co-immunoprecipitation assay. The addition of cytokinin, which presumably resulted in the phsophaorylation of the conserved Asp residue on HA-ARR4, did not affect the interaction

between BPC6 and ARR4 (Figure 3.1B). Further, the ARR4^{D95E} phosphomimic and the ARR4^{D95A} non-phosphorylatable mutant proteins also interacted with BPC6 in a co-immunoprecipitation assay, suggesting that aspartic acid phosphorylation of the receiver domain does not play a major role in regulating the interaction with BPC6 (Figure 3.1B).

***bpc* mutants exhibit reduced sensitivity to exogenous cytokinin treatment**

To determine if the BPCs function in cytokinin signaling, we performed cytokinin response assays on *bpc* mutants. Cytokinin inhibits primary root growth; seedlings grown on plates supplemented with exogenous cytokinin have shorter roots (To, 2004). The *bpc1,2,3,4,6* and *bpc1,2,3,4,6,7* multiple mutants exhibit reduced sensitivity to cytokinin, with the most severe phenotype in the presence of 25 nM and 50 nM benzyladenine (BA) (Figure 3.2A, B). However, the *bpc1,2*, *bpc1,2,3*, *bpc4,6* and *bpc1,2,4,6* mutants did not show reduced cytokinin sensitivity at these doses. While the *ahk2,4* cytokinin receptor mutant is completely insensitive to cytokinin at 25 nM and 50 nM BA, the *bpc1,2,3,4,6* and *bpc1,2,3,4,6,7* mutants display only partial insensitivity.

To examine the spatial pattern of the response to cytokinin, we examined the expression of a reporter for type-B ARR activity, TCSn::GFP, in root tips of various *bpc* mutant combinations (Zürcher et al., 2013). The TCSn::GFP transgene was introduced into a variety of *bpc* mutant combinations, including the *bpc1,2,3,4,6* quintuple mutant, by crossing. In all mutant combinations, the fluorescence from the TCSn::GFP reporter in the absence of exogenous cytokinin was comparable to that observed in wild-type seedlings. In contrast, in all the mutant combinations tested, including the *bpc1,2* double mutant, there was a reduction in the signal from the TCSn::GFP reporter (Figure 3.3A, B). These results suggest that disruption of the BPCs results in a reduction in the response to exogenous cytokinin in the root tip.

Identification of BPC6 target genes using ChIP-seq

Directed ChIP assays showed that the BPCs bind to the promoters of a number of cytokinin-regulated genes (*ARR7*, *STM*, *WUS,* and *BP/KNAT1*) (Simonini and Kater, 2014) and our analysis demonstrates that the BPCs are positive regulators of cytokinin responsiveness. In order to determine if the BPC directly regulate multiple cytoknin-regulated genes, we determined the binding sites for BPCs *in vivo* across the genome using chromatin immunoprecipitation follow by sequencing (ChIP-seq). We

chose BPC6 for this analysis because while multiple transcriptional targets of Class I BPCs have been identified in the inflorescence meristem, fewer target loci have been determined for the Class II BPCs (Simonini and Kater, 2014). We used transgenic Arabidopsis plants expressing *GFP:BPC6* (*pBPC6::GFP:BPC6*) or *GFP* (*pBPC6::GFP*) as a negative control under the regulation of the endogenous *BPC6* promoter and terminator (Figure S3.1A). We analyzed the localization of the recombinant proteins in epidermal leaf tissue of the transgenic lines (Figure 3.S1B). We detected GFP fluorescence from *GFP:BPC6* expressing plants in the nucleus and nucleolus, consistent with previous observations (Wanke et al., 2011; Hecker et al., 2015). The localization in the nucleolus suggests a potential role for BPCs in transcription of genes encoding ribosomal proteins. Consistent with this, 35 genes encoding ribosomal proteins are putative BPC6 targets (data not shown) (Barakat et al., 2001; Chang et al., 2005; Sormani et al., 2011). *GFP* expressing control plants displayed GFP fluorescence in both the cytoplasm and the nucleus, but not in the nucleolus (Figure S3.1B), consistent with prior results (Punwani et al., 2010).

We performed ChIP-seq on 14-day-old Arabidopsis seedling expressing *GFP:BPC6* and *GFP*. 5,386 putative BPC6 *in vivo* target loci were identified from the *GFP:BPC6* expressing plants that were not found from the GFP control line (data not shown). To determine the location of the GFP:BPC6 binding sites relative to the annotated gene models, we analyzed the distribution of all peaks over the entire Arabidopsis genome (Figure S3.1C). The majority (72.6%) of binding sites were located within the promoter-transcription start sites (promoter-TSS), consistent with a role in transcriptional regulation. Additionally, we analyzed the distance distribution of all reads to the nearest TSS (Figure S3.1D). A clear clustering of 3,113 peaks was found within 200 base pairs on either side of the TSSs, consistent with a role for BPC6 in the control of gene expression. Furthermore, we found an unequal distribution of BPC6 target sites over the five Arabidopsis chromosomes, in which, centromeric regions were underrepresented and telomeric regions were overrepresented (Figure S3.1E).

The identified peaks in our ChIP-seq experiment defined 4,457 direct putative target genes of GFP:BPC6 (data not shown). These targets include *BPC1*, *BPC2*, *BPC4,* and *BPC6*, but not *BPC3*, *BPC5,* or *BPC7* indicating that BPC6 might transcriptionally regulate some, but not all *BPC* genes (data not shown). Homeobox genes identified as BPC binding targets from other studies, including *STK*, *STM*,

BEL1-LIKE HOMEODOMAIN 1 (BLH1), and multiple *KNATs* (*KNAT1/BP*, *KNAT4*, and *KNAT6*) (Kooiker et al., 2005; Simonini et al., 2012; Simonini and Kater, 2014), were also identified as direct targets of BPC6. To verify these ChIP-seq results, we examined binding of GFP-BPC6 to *BPC1*, *BPC2* and *BPC6* using chromatin immunoprecipitation followed by quantitative PCR (X-ChIP). We also verified the binding of BPC6 to the promoter of the PcG protein *EMBRYONIC FLOWER 1* (*EMF1*) gene based on previous studies that link BPCs with PcG function (Hecker et al., 2015; Berger et al., 2012; Mu et al., 2017). To verify binding of GFP:BPC6 to these loci, we generated one amplicon covering the putative binding site for each gene derived from our ChIP-seq experiment and one amplicon covering a second non-binding region within the same locus (Figure S3.2). We confirmed *in vivo* binding of GFP:BPC6 to the 5'UTRs of *BPC1* (Figure S3.2A), *BPC2* (Figure S3.2C) and *EMF1* (Figure S3.2C). These results indicate transcriptional cross-talk between the group II member BPC6 and group I members. We could not confirm the *in vivo* binding of GFP:BPC6 to its own promoter, likely due to highly repetitive sequences within the predicted binding site (Figure S3.2D).

To further investigate BPC function, we used gene ontology (GO) analysis with Virtual Plant1.3 and found 154 GO terms with Biological Process assignments by TAIR/TIGR classification scheme (data not shown) (Katari et al., 2010). As expected, given that BPCs are transcription factors, there is enrichment of the following GO terms, "regulation of transcription", "regulation of gene expression", "regulation of RNA metabolic process", and "regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process." The *bpc* mutants also exhibit reproductive and vegetative phenotypes, which corresponds to the enrichment of these GO terms, "post-embryonic development", "flower development", "leaf development", "growth", and "regulation of reproductive process." The BPC target genes are also enriched for "response to hormone stimulus", response to abscisic acid stimulus", "response to auxin stimulus", "response to jasmonic acid stimulus", and "response to ethylene stimulus", further supporting their role as regulators of hormone signaling.

To further study the DNA-binding properties of BPC6 we analyzed all ChIP-seq reads for enrichment of *cis*-elements using the DREME algorithm followed by Tomtom to the determine potential transcription factors corresponding to these sites (Bailey, 2011; Gupta et al., 2007). The most frequent motif identified was 'GAGAGARA' (e-value = 3.8e-286) (Figure 3.4A, Figure S3.3), which resembles the

previously identified *in vitro* BPC binding site (Hecker et al., 2015; Meister et al., 2004; Kooiker et al., 2005; Simonini et al., 2012; Berger et al., 2012; Brand et al., 2010). We also found binding motifs for the TCP family of transcription factors enriched in the BPC ChIP-seq reads (e-value = 5.6e-134). The TCPs are non-canonical bHLH transcription factors specific to plants that have also been implicated in the cytokinin response (Cubas et al., 1999; González-Grandío et al., 2017; Martín-Trillo and Cubas, 2010). There are two classes of TCPs, class I generally being involved in promoting cell proliferation and greening and class II generally having the opposite effect, promoting cell differentiation and maturation; there is evidence that both classes modulate cytokinin signaling based on genetic analysis (Cubas et al., 1999; Martín-Trillo and Cubas, 2010; González-Grandío et al., 2017). Overexpression of the class-I TCPs, TCP14 or TCP15, promotes shoot branching and leaf greening, delays senescence, and results in higher basal expression of the cytokinin primary response gene ARR5. Furthermore, reduction of cytokinin levels (by expressing the cytokinin degrading enzyme CKX3) suppresses the TCP14 overexpression phenotypes (Steiner et al., 2012; 2016).The class-II TCPs reduce leaf cytokinin sensitivity based on genetic analysis and it has also been demonstrated that class II TCPs bind to promoters of the type-B ARR targets *ARR16*, *SHY2* , and *AS1* (Efroni et al., 2013; Koyama et al., 2010). Transcriptional regulation by TCPs is proposed to be context dependent and involve transcription factor-transcription factor interactions (Davière et al., 2014; Kieffer et al., 2011; Kim and Hwang, 2014; Rueda-Romero et al., 2012). Interestingly, we also find that there is enrichment of the cytokinin response factor (CRF) binding site ‘CGCCGK’ (e-value = 1.4e-17)(Figure S3.3)(Raines et al., 2015). The CRFs are a group of AP2/ERF transcription factors that are transcriptionally induced by cytokinin and gene expression is regulated by type-B ARRs (Raines et al., 2015). The CRFs are involved in developmental processes such as female gametophyte development, embryo development, and senescence, and regulate a sub-set of cytokinin response genes (Raines et al., 2015). These results suggest that the CRFs and TCPs may act in concert with the BPCs to regulate some of the same target genes.

As our results suggest a link between BPCs and cytokinin response, we examined if the BPC6 targets included cytokinin response genes. There is a significant overlap between BPC6 ChIP-seq gene targets and robustly regulated cytokinin response genes, termed the ‘golden’ list (Bhargava et al., 2013) (*P* (X >= 63) = 1.2e-4)(Figure 3.4B). In this overlap, we find multiple genes involved in cytokinin signaling

and metabolism. For example, we identify type-A ARRs (*ARR3, ARR7, ARR8*, and *ARR9*), type-B ARRs (*ARR1, ARR12, ARR14*, and *ARR19*), AHKs (*AHK2, AHK3,* and *AHK4*), AHP4, CRFs (*CRF1, CRF2*, and *CRF6*), cytokinin synthesis genes (*IPT1* and *IPT2*), cytokinin degrading genes (*CKX5* and *CKX6*), and a cytokinin glucosyltransferase (*UGT85A1*) involved in inactivating cytokinin. Furthermore, 36 of the 63 genes found to overlap with the 'golden' list, also overlap with type-B ARR10 target genes that are found in response to cytokinin identified using ChIP-seq (Zubo et al., 2017). These genes include type-A ARRs (*ARR3*, *ARR7*, *ARR8* and *ARR9*), CRFs (*CRF2* and *CRF6*), *SHORT HYPOCOTYL 2 (SHY2)* a transcription factor involved in auxin signaling, and *ANNEXIN (ANNAT3)* a calcium binding protein (Figure 3.4C). Visualizing the data using the Integrated Genome Browser (IGB) (Nowlan *et al.,* 2016) reveals that some of the BPC6 and ARR10 binding peaks are coincident, while others appear independent (Figure 3.4C). This overlap suggests that the BPCs target cytokinin response genes perhaps both in concert with type-B ARRs, but also independently of type-B ARR binding.

Genes found differentially regulated in the *bpc1,2,3,4,6* mutant using RNA-seq

In order to gain further insight into the role of the BPCs in regulating gene expression, we employed RNA-seq to determine genes that are differentially expressed in a *bpc* multiple mutant. We chose the *bpc1,2,3,4,6* mutant because it had the most severely altered cytokinin response in root elongation assays (Figure 3.2). Since we observed cytokinin response phenotypes in the root, we used ten-day old wild-type or mutant roots treated with 5 μM BA or a vehicle control for one hour for RNA-seq. We identified 463 genes differentially expressed in the *bpc1,2,3,4,6* mutant compared to wild type in the absence of exogenous cytokinin (false discovery rate (FDR) < 0.05, data not shown). The *bpc1-1*, *bpc2* and *bpc6* alleles are T-DNA insertions and all three have background levels of sequencing counts from the RNA-seq, consistent with these being null alleles (Figure S3.4A, B). The T-DNA for *bpc1-1* is inserted in the promoter of the gene and previous transcript analysis of *bpc1-1* suggested that this allele is not a null as RT-PCR indicated 10% residual transcription (Monfared et al., 2011). However, we find that in the *bpc1,2,3,4,6* mutant roots, *BPC1* sequencing counts are at the level of the background, comparable to the level of the *BPC2* and *BPC6* in this line (Figure S3.4A, B), suggesting that it is a null allele. As the ChIP-seq data indicate that BPC6 binds to the promoter of *BPC1*, it may positively regulate *BPC1* expression and therefore, the disruption of BPC6 in *bpc1,2,3,4,6* could lead to a further reduction in *BPC1*

expression in this mutant. Alternatively, the allele may have a more substantial effect on expression in the root. The *bpc4* allele results from a T-DNA insertion in the DNA-binding domain of BPC4. We find that there is not a significant reduction in BPC4 transcript upstream of this insertion (Figure S3.4A, B) and therefore it is possible that the N-terminal protein-protein interaction domain of BPC4 is still expressed. The *bpc3-1* allele used is derived from TILLING population (McCallum et al., 2000) that results in an early stop codon in the DNA binding domain. *BPC3* expression is two-fold higher in the *bpc1,2,3,4,6* mutant, which is consistent with the previous findings that *BPC3* expression is elevated in a *bpc1,2,4,6* mutant (Monfared et al., 2011).

Approximately 1/4 the genes differentially regulated in the *bpc1,2,3,4,6* mutant overlap with those identified as BPC6 target genes from ChIP-seq (*P*(X>=125) = 5.3e-7) (Figure S3.5A). This suggests that many of the genes mis-regulated in the *bpc1,2,3,4,6* mutant are direct targets of the BPCs. The 1/4 overlap is likely an underestimation of the direct BPC targets as: 1) the RNA-seq specifically examined root tissues and the ChIP-seq employed whole seedlings; and 2) Whereas the mutant includes multiple *BPC* genes, the ChIP-seq specifically examined BPC6. Nevertheless, this is similar to the level of overlap in other ChIP-seq/RNA-seq studies in Arabidopsis (Yu et al., 2011; Fan et al., 2014). Consistent with previous studies, we find multiple transcription factors differentially regulated in the *bpc1,2,3,4,6* mutant, including homeobox genes such as *BLH4*, *KNAT1/BP*, *PROTODERMAL FACTOR 2 (PDF2), MERISTEM LAYER 1 (ATML1)*, and *ARABIDOPSIS THALIANA HOMEOBOX-LEUCINE ZIPPER PROTEIN 4 (ATHB4)* (Figure S3.5B) (Thimm et al., 2004; Simonini and Kater, 2014). *KNAT1/BP*, *BLH4*, *ATML1*, and *ATHB4* are direct targets of BPC6 as they are also found in our ChIP-seq (data not shown), and *KNAT1/BP* was identified in prior directed ChIP approaches with BPC class 1 proteins (Simonini and Kater, 2014).

As the BPCs interact with the type-A ARRs, we queried if the BPCs and type-A ARRs regulate overlapping gene sets. To this end, we examined the 4,822 genes whose expression was altered in ten-day old roots of the type-A *arr3,4,5,6,7,8,9,15* mutant as compared to wild-type roots that were identified in a previous study using RNA-seq (Shanks et al., 2016). Almost half of the genes differentially expressed in the *bpc1,2,3,4,6* roots overlap with the genes differentially regulated in the *arr3,4,5,6,7,8,9,15* mutant (*P* (x >= 209) = 7.3e-50) (Figure 3.5A). Of the 209 genes that overlap between the two data sets, 51 have

ChIP BPC6 binding sites. There is no consistent direction to the changes in the expression of these overlapping genes in the two mutants (Figure 3.5B). Since the CRF binding motif is enriched in the BPC6 ChIP-seq, we also compared the 3,122 genes differentially regulated in the *crf1,3,5,6* mutant roots to the *bpc1,2,3,4,6* mis-regulated genes (Raines et al., 2015) and we find significant overlap (P(X>=156) = 5.1e-33) (Figure 3.5C). Similar to the genes that overlap with the *arr3,4,5,6,7,8,9,15* mutant, there was also no consistent direction to the various changes in gene expression in these two mutants (Figure 3.5D).

BPCs regulate a sub-set of cytokinin response genes

To determine if the BPCs play a role in the transcriptional response to cytokinin, we conducted an RNA-seq experiment with wild type and *bpc1,2,3,4,6* mutant in which we treated we treated ten-day-old seedlings with cytokinin and examined transcript levels from roots. There are 2,149 genes differentially expressed in wild-type roots in response to cytokinin (WT cytokinin treated/ WT control, FDR < 0.05) and a significant number of those genes overlap with the BPC6 ChIP-seq target genes (*P* (X>=570 = 5.6e-26) (Figure S3.6). There are also 2,421 genes differentially regulated in *bpc1,2,3,4,6* roots in response to cytokinin (*bpc1,2,3,4,6* cytokinin treated/ *bpc1,2,3,4,6* control, FDR < 0.05) and a significant number of those genes overlaps with the BPC6 ChIP-seq target genes (*P* (X>=641 =5.2e-29). This shows that BPC6 target multiple genes differentially expressed in response to cytokinin. There are 1,676 genes that overlap between those differentially regulated by cytokinin in wild-type and *bpc1,2,3,4,6* mutant roots, which indicates that a significant number of genes are still regulated by cytokinin in the *bpc1,2,3,4,6* mutant (Figure 3.6A, data not shown. However, there are 473 cytokinin response genes that are no longer differentially regulated in response to cytokinin in the *bpc1,2,3,4,6* mutant (Figure 3.6A, data not shown), and also 745 genes differentially regulated in response to cytokinin in the *bpc1,2,3,4,6* mutant that are not significantly regulated in wild-type roots (Figure 3.6A, data not shown). For the 473 genes that show differential regulation in response to cytokinin in the wild type, but not the *bpc1,2,3,4,6* mutant, the mis-expression is generally reduced in the mutant, but not completely lost in response to cytokinin (Figure 3.6B).

The 473 genes no longer significantly regulated by cytokinin in *bpc1,2,3,4,6* mutant roots represent a sub-set of cytokinin response genes that require BPC function for full regulation. These 473

genes include both genes induced and repressed by cytokinin, which suggests that the BPCs influence the expression of both classes of cytokinin-regulated genes. Analysis of these 473 genes indicate an enrichment for GO terms such as “response to auxin” and “regulation of nitrogen metabolic process,” two processes that have links to cytokinin signaling (Figure S3.7) (Kiba and Krapp, 2016; Schaller et al., 2015). To validate the RNA-seq results, we used qRT-PCR on a group of genes that showed an altered response to cytokinin in the *bpc1,2,3,4,6* mutant (Figure 3.6C). To identify candidates for re-testing, we used a fold-change cut off for genes induced by cytokinin in the wild type that was greater than 1.5. Of the six genes that were re-tested, four were confirmed as being induced by cytokinin in wild-type roots, but to be unaffected by cytokinin in the *bpc1,2,3,4,6* mutant (Figure 3.6C). The *ACS7* (involved in ethylene biosynthesis), *ARR12* (a type-B ARR), *ATL78*, and *AT2G18480* genes are induced by cytokinin in wild-type, but not in *bpc1,2,3,4,6* mutant roots. Consistent with this, both *ARR12* and *ACS7* were identified in the BPC6 ChIP-seq analysis, however, *ATL78* and *AT2G18480* were not identified as targets of BPC6 (Figure S3.8). One of the other genes (AT5G54145) did not re-test as being induced by cytokinin in either wild-type or *bpc1,2,3,4,6* mutant roots, and the sixth gene (*MYC2*) was induced in both. Surprisingly, the expression of the type-A ARRs was not altered in the *bpc1,2,3,4,6* mutant (Figure S3.9), despite the fact that *ARR7* was previously reported to be mis-regulated in the shoot apical meristem of the *bpc1-2,2,3* mutant (Simonini and Kater, 2014) and even though multiple type-A ARRs gene were identified in our BPC6 ChIP-seq.

We hypothesized that the 473 genes whose regulation by cytokinin is perturbed in the *bpc1,2,3,4,6* mutant may be direct targets of the BPCs. To test this, we examined how many of these 473 genes were identified in our ChIP-seq analysis. Consistent with BPCs directly regulating these genes, there is significant overlap between these 473 genes and the BPC6 ChIP targets (~23% of the 473 genes were also identified in the BPC6 ChIP-seq; Figure 3.7A) (*P* (X>=109) = 2.2e-3). These 473 genes also showed a significant enhancement for ARR10 target genes (~25% overlap; *P* (X>=118) = 3.1e-7), suggesting that they are regulated by the type-B ARRs. There are 61 cytokinin response genes that are no longer regulated in the *bpc1,2,3,4,6* mutant by cytokinin that are also direct targets of both ARR10 and BPC6 (Figure 3.7A). Gene ontology of these 61 genes shows enrichment for regulation of transcription and response to abscisic acid stimulus (Figure S3.10). We examined the expression and the position of

BPC6 and ARR10 binding sites for a select set of genes using the Integrated Genome Browser (IGB) (Nowlan et al., 2016) (Figure 3.7B, C). These genes are *AHL12, EEL,* and *AT5G60760*, which are not regulated in the *bpc1,2,3,4,6* mutant and identified in the ARR10 and BPC6 ChIP-seq, suggesting that the BPCs and type-B ARRs may co-regulate these genes in response to cytokinin. Consistent with the analysis of the BPC6 ChIP-seq/golden list overlap (Figure 3.4C) the binding sites for ARR10 and BPC6 coincide very closely in the upstream regulatory regions of these genes in some cases, but in others there are clear regions bound by ARR10 that show little or no binding by BPC6 and vice versa, suggesting both coordinated and independent binding of these transcription factors.

There were 1,463 genes identified in both the BPC6 and ARR10 ChIP-seq (*P* (X>=1,463) = 3.9e-215) (Figure 3.7A). Of these 1,463 genes, 283 are differentially expressed in wild-type roots in response to cytokinin in the RNA-seq data set (Figure S3.11) (*P* (X>=283) = 1.2e-40), consistent with a model in which ARR10 and BPC6 co-regulate genes in response to cytokinin. Interestingly, ARR10 binds to the upstream regulatory regions of *BPC1* and *BPC2*, suggesting that ARR10 may regulate the expression of a subset of *BPC* genes (Figure S3.4). Consistent with this, *BPC1* expression is induced in response to cytokinin (Figure S3.4, data not shown). Further, BPC6 binds to the promoters of multiple type-B ARRs. This regulation could represent a positive feedback loop in which the type-B ARRs and BPCs regulate expression of each other.

Discussion

We demonstrate that the type-A ARRs directly interact with multiple BPC transcription factors, providing insight into the mechanism by which these single domain response regulators negatively regulate cytokinin signaling. In addition to their interaction, the significant overlap between the genes differentially regulated in the *arr3,4,5,6,7,8,9,15* mutant and the *bpc1,2,3,4,6* mutant further supports a functional link between the type-A ARRs and BPCs. Consistent with a role of the BPCs in the response to cytokinin, disruption of multiple BPCs results in a reduced response to cytokinin in root elongation assays and an alteration in cytokinin-regulated gene expression. These data suggest that the type-A ARRs act to modulate BPC activity, providing at least one target for type-A ARR input into cytokinin responsiveness. How does cytokinin acting through the type-A ARRs act to modulate BPC function? Previous studies

have shown that the type-A ARRs require phosphorylation of their conserved Asp residue in the receiver domain for function (To et al., 2007). This phosphorylation can act in two ways. First, it may promote (or inhibit) interaction with target proteins. However, we demonstrate that the interaction between the type-A ARRs and the BPCs does not depend on this phosphorylation. This Asp phosphorylation is also required for the increase in the stability of the type-A ARR proteins in response to cytokinin (To et al., 2007). We propose that BPC function may be modulated by cytokinin via an increase in type-A ARR protein levels, which are synergistically elevated by cytokinin through increased type-A ARR transcription and protein stability. The elevated type-A ARR protein levels could lead to increased interaction with the BPCs, thus modulating their activity by either interfering with the BPC binding to DNA or to other binding partners (Figure 3.8). Consistent with these findings, multiple type-A ARRs (ARR4, ARR5 and ARR6) also interact with ABI5, a transcription factor involved in abscisic acid signaling, in the absence of phosphorylation of the receiver domain (Wang et al., 2011).

While the BPC and type-A ARR interaction does not absolutely depend on phosphorylation of the conserved Asp residue, it is possible that this phosphorylation has a subtler effect, acting to enhance or decrease interaction strength. CheY is an example of a single domain bacterial response regulator in *Escherichia coli* that binds to its interacting partner, the flagellar motor protein FliM, both when phosphorylated and not phosphorylated on its receiver domain (Lee et al., 2001a). However, phosphorylation increases the affinity of CheY for FliM (Lee et al., 2001b; Sourjik and Berg, 2002). Similarly, the strength of the interaction between the BPC and type-A ARR proteins may modulated by phosphorylation. Further, the interaction of the type-A ARRs with other interacting partners may depend more strongly on phosphorylation of the receiver domain.

In eukaryotes, multiple transcription factors often act in combination to regulate gene expression (Lehmann, 2004). Given that the BPCs regulate multiple developmental processes, it is likely that they rely on co-regulation with other transcription factors to confer specificity on target genes. There is significant overlap between the BPC6 target genes identified with ChIP-seq and the type-B ARR10 targets genes enriched in response to cytokinin. Furthermore, the BPCs and CRFs both regulate a sub-set of the same genes as identified with RNA-seq studies. Thus, BPCs likely act together with the type-B ARRs and/or the CRFs to co-regulate specific genes in response to cytokinin signaling. Positive feedback

loops likely exist between the BPCs and type-B ARRs as ARR10 binds to the promoters of *BPC1* and *BPC2,* and *BPC1* is up-regulated in response to cytokinin; BPC6 also binds to the promoters of multiple type-B ARR and CRF genes, and the BPC proteins are necessary for up-regulation of type-B *ARR12* in response to cytokinin. In this model, the BPCs, type-B ARRs and CRFs co-regulate genes expression by either independently binding to different *cis*-acting elements to convergently regulate target gene expression or by binding to elements as part of the same transcriptional complex. The BPCs may also recruit other complexes to these genes such as the PRC chromatin modifiers to promote or repress transcription of the target genes (Hecker *et al.*, 2015; Mu *et al.*, 2017).

Multiple type-A ARRs are also BPC6 binding targets. However, we do not find altered expression of these primary cytokinin response genes in *bpc1,2,3,4,6* mutant roots in the presence or absence of cytokinin. In contrast, previous studies found that *ARR7* expression is increased specifically in the shoot apical meristem (SAM) of the *bpc1-2,2,3* mutant (Simonini and Kater, 2014). We hypothesize that the BPCs may regulate the type-A ARRs in a tissue-specific manner. Given that the BPCs regulate multiple genes important for growth and development, they likely function differentially in specific tissues and times during development. Furthermore, the BPCs are part of a gene regulatory network that likely includes multiple positive and negative feedback loops and redundant elements acting in a complex manner to modulate gene expression. Thus, while the BPC bind to type-A ARR genes they may function redundantly in some tissues with other transcription factors regulating type-A ARR transcription and/or may act only transiently to modulate their expression.

We also uncovered a potential role for BPCs in regulating transcription of ribosomal proteins. In this and previous studies, BPC6 localizes to both the nucleus and the nucleolus (Wanke et al., 2011). The nucleolus is the site of transcription and assembly of ribosomal proteins (Chang et al., 2005). BPC6 targets ribosomal proteins of both the large and small ribosomal sub-units. TCP binding sites are enriched in the promoters of the 40 and 60S ribosomal subunits (Tremousaygue et al., 2003). In this study, there are sixteen 40 and 60S ribosomal subunits targeted by BPC6 in ChIP-seq. Furthermore, the TCP binding motif is enriched in BPC6 target sequences, suggesting TCPs and BPCs may co-regulate transcription of ribosomal proteins. The BPCs have been suggested to be functionally similar to the Drosophila GAF

proteins. To date, there is no indication that the GAF proteins are present in the nucleolus nor that they regulate ribosomal proteins, suggesting that this is a unique function of BPC proteins.

Overall, we find that the BPCs regulate a sub-set of cytokinin-regulated genes. Multiple cytokinin response genes are putatively directly targeted by both BPCs and type-B ARRs, while others are targets of only one or the other. Furthermore, some genes regulated by cytokinin that are not regulated in the *bpc* mutant have neither BPC nor type-B ARR binding sites. At least some of these genes are either secondary cytokinin response genes, or bound by other BPC or type-B ARR proteins not used in the ChIP-seq assays. While our data strongly support the notion that the BPCs play a role in the response to cytokinin, it is clear that they have additional functions in growth and development outside of cytokinin signaling.

The regulation of the transcriptome by cytokinin involves the type-B ARRs acting at the head of a transcriptional cascade. Accumulating evidence indicates that the type-B ARRs act in concert with multiple other transcription factors to combinatorially control the expression of genes in response to cytokinin, including the BPCs, the CRFs, the TCPs, ABI5 and others. This is supported by the enrichment of binding sites for many of these transcription factors in the sequences identified in the BPC6-ChIP-seq. These interacting transcription factors likely act differentially in specific cell types, at specific times in development and in response to various exogenous inputs to mediate the pleiotropic effects of cytokinin. Deciphering the cell type-specific function of these various transcription factors acting in concert with the type-B ARRs is a major challenge for the future. Further, while our results indicate a link between the type-A ARRs and the BPCs, further studies are necessary to elucidate the manner in which the type-A ARRs alter BPC function to either modulate their binding to target genes and/or their interaction with other transcriptional regulators. Identification of additional type-A ARR interacting proteins will shed further light on how these signaling elements regulate cytokinin responses.

Materials and methods

Plant materials and growth conditions

All Arabidopsis lines used in these studies are in the *Arabidopsis thaliana* Col-0 ecotype. The *bpc1-1,2*, *bpc1-1,2,3*, *bpc4,6*, *bpc1,2,4,6*, *bpc1-1,2,3-1,4,6*, and *bpc1-1,2,3-1,4,6,7* multiple mutant T-DNA insertional lines are all previously characterized and published (Monfared *et al.*, 2011). Both

bpc1,2,3 and *bpc1,2,3,4,6* mutants were crossed with the TCSn::GFP reporter for type-B ARR activity (Zürcher et al., 2013). Seeds were surface sterilized with 50% bleach, 0.2% Triton-X-100, washed four times with water, and plated on ½ MS plates (½ Murashige and Skoog basal medium, 1% sucrose, 0.6% phytogel, pH 5.8). Plates were kept in the dark at 4°C for 3-4 days and transferred to a 24-hour light, 22°C growth chamber. Seedlings were transferred to soil and kept in a 24-hour light, 22° C growth room. For cytokinin treatment of whole seedlings, 10-12, 10 day old seedlings were incubated in 30 mL of ½ MS media (½ Murashige and Skoog basal medium, 1% sucrose, pH 5.8) in 50 mL Falcon conical centrifuge tubes supplemented with either 5 µM benzyl adenine (BA) for cytokinin treatment or 5 N NaOH as a vehicle control. After 1 hour of gentle shaking the seedlings were separated into roots and shoots and frozen in liquid nitrogen.

Cloning and vector construction

The coding sequence for the *BPC1*, *BPC3*, *BPC4*, and *BPC6* genes were amplified by PCR from cDNA libraries derived from *Arabidopsis thaliana* Col-0 seedlings (Table 3.1). The PCR products were transferred into the gateway pENTR/D-TOPO entry vector following manufacturer's instructions using the pENTR™ Directional Topo Cloning Kit (Thermo Fisher Scientific). The type-A ARR entry vectors used were previously described (To *et al.*, 2007). From the pENTR/D-TOPO vectors, the cDNAs were recombined into Gateway-compatible destinations vectors using the Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific). The LR reaction was transformed into One Shot® TOP10 Chemically Competent cells (Thermo Fisher Scientific). Successful LR reactions were verified by restriction enzyme digestion and sequencing. The binary vectors pEarleyGate201 with 35S promoter N-terminal HA tag (pEG201) and pEarleyGate203 with 35S promoter N-terminal myc tag (pEG203) were used in co-immunoprecipitation asssays (Earley *et al.*, 2006).

Cloning pBPC6:GFP:BPC6 (AT5G42520.1)

The 5' region of 2352 bp including the first 5 codons (including ATG) of *BPC6* was amplified using genomic Col-0 DNA and the following primers that introduce a 5' *NheI* and 3' *NotI* site: S-pBPC6-ATG-NheI and AS-pBPC6-ATG-9Nu-NotI. To be able to fuse *eGFP6* to the *BPC6* construct, PCR was

performed using cDNA of Col-0 flowers and the following primers that introduce a 5' *NotI* and 3' *SacII* site: S-GFP-ATG-NotI and AS-GFP-w/o Stop-SacII. The remaining coding sequence of 1017 bp of *BPC6* including TGA was amplified using the following primers that introduce a 5' *SacII* and *3'PvuI* site: S-BPC6-13Nu-SacII and AS-BPC6-Stop-PvuI. The 3'region of 388 bp was amplified using genomic Col-0 DNA and the following primers that introduce 5' *PvuI* and 3' *NheI* site: S-BPC6-3'UTR-PvuI and AS-BPC6-3'UTR-NheI. The individual PCR products were loaded on agarose gels and purified using QIAquick Gel Extraction Kit (Qiagen) according to manufacturer's protocol. By using home-made Taq Polymerase an A-Tailing reaction (10 min 70°C) was performed and the products were individually cloned into pCRII-TOPO/TA vector (Invitrogen) according to manufacturer's protocol and transformed into *E. coli*. After sequencing fragments were isolated using respective restriction enzymes, gel purified. Fragments were mixed in an equimolar ration and ligated using T4-DNA-Ligase (Fermentas). PCR was performed to amplify the whole product of the ligation using primers S-BPC6-CACC-ATG-MluI and AS-BPC6-Stop-PvuI. After gel purification, A-tailing followed by TOPO reaction into pCR2.1-TOPO/TA (Invitrogen) and transformation into *E. coli* was performed. Finally, the genomic BPC6 construct was digested using EcoRI and gel purified.

Cloning pBPC6:GFP

The pBPC6:GFP:BPC6 in the pCR2.1-TOPO/TA was used to generate the control pBPC6:GFP. To be able to remove the coding sequence of *BPC6*, two mutagenesis PCRs were performed. During the first mutagenesis PCR a 5'*SmaI* in-between GFP and BPC6 was introduced using the following primers S-GFP-BPC6-SmaI and AS-GFP-BPC6-SmaI. The PCR product was loaded on agarose gels and purified using QIAquick Gel Extraction Kit according to manufacturer's protocol. The product was cloned into pCRII-TOPO/TA vector according to manufacturer's protocol and transformed into *E. coli*. After sequencing the second mutagenesis PCR was performed in order to introduce a 3'*SmaI* site in-between *BPC6* and *3'BPC6* using the following primers S-BPC6-3'BPC6-SmaI and AS- BPC6-3'BPC6-SmaI. The PCR product was loaded on agarose gels and purified using QIAquick Gel Extraction Kit according to manufacturer's protocol. The product was cloned into pCRII-TOPO/TA vector according to manufacturer's

protocol and transformed into *E. coli.* After sequencing, the control construct was digested using EcoRI and gel purified.

Cloning binary vector backbone – pGPTVII.BARe

Both *EcoRI* digested DNAs (pBPC6:GFP:BPC6 or pBPC6:GFP as control and pGPTVII.BARe) were ligated in an equimolar ration using T4-DNA Ligase (Fermentas) and transformed into *E. coli*. The vector was tested using different control digests and partial sequencing with primer AS-pGPTVII.BAR-seq. Complete vector sequence see Text S1.

Yeast two-hybrid analysis

The bait vector, pBMTN116c-D9, prey vector, pACT2, and yeast strain L40ccαU used as previously described in (Dortay *et al.*, 2006). Yeast were transformed with both bait and prey constructs using the lithium acetate transformation method as previously described (Gietz and Woods, 2006). Plasmids were selected on -leu -trp media plates. Successful plasmid transformation was confirmed with colony PCR of yeast selected on -lue -trp selection plates. Cell suspensions with yeast containing both constructs were grown to an A_{600} of 0.2 and 10ul of each interaction was plated on both synthetic defined (SD) minimal media lacking leucine and tryptophan (SDII) as control plates and SD minimal media lacking leucine, tryptophan, histidine, and uracil (SDIV) as interaction plates. Each protein was co-transformed with either the empty prey or empty bait vector to test for auto-activation. To suppress auto-activation those clones were plated on media supplemented with 5mM 3AT. Yeast were incubated at 30°C for 3-4 days to test for interaction.

Co-immunoprecipitation using transiently transformed *Nicotiana benthamiana*

Expression vectors pEG201 and pEG203 with corresponding protein sequences were transformed into the *Agrobacterium* strain GV3101 (Koncz and Schein, 1986). Overnight cultures with 3mL of *Agrobacterium* were grown at 28°C. The cultures were spun down, the supernatant was removed and the cells were suspended in infiltration media (10m MMES, 10mM $MgCl_2$, and 0.15mM acetosyringone) to an OD_{600} of 0.1. An OD_{600} of 0.05 for each *Agrobacterium* carrying the corresponding

construct were infiltrated into 3-4 week old *N. benthamiana* epidermal leaf cells following the protocol adapted from (Yang *et al.*, 2000) using a 1mL syringe. Plasmids were co-infiltrated with Agrobacterium carrying the p19 suppressor of gene silencing to enhance transgene expression (Lindbo, 2007). The leaves were incubated for three days at 24-hour light. The tissue was collected by flash freezing in liquid nitrogen. For cytokinin treatments with the synthetic cytokinin benzyl adenine (BA), leaves were infiltrated with 5μM of BA 20 minutes before tissue was frozen. Co-immunoprecipitation was conducted using the μMACS™ Epitope Tag Protein Isolation Kits (Miltenyi Biotec). Tissue was ground on ice with a mortar and pestle and total protein extracts were isolated and suspended in lysis buffer supplemented with 1x protease inhibitor cocktail, cOmplete™ ULTRA Tablets, Mini, EDTA-free, *EASYpack* Protease Inhibitor Cocktail (Sigma). The lysate was spun down and supernatant was collected and incubated with 50 μl of magnetic anti-myc-beads and incubated on ice for 30 minutes with periodic mixing. The total protein suspension containing the anti-myc beads was applied to a magnetic μ Column (Miltenyi Biotec). The column was washed with Wash Buffer 1 and Wash Buffer 2 from, and eluted with boiled Elution Buffer for SDS PAGE followed by immunoblotting. Blots were first probed with primary antibody Anti-HA High Affinity antibody, 3F10, monoclonal (Sigma) and then secondary antibody goat anti-rat IgG-HRP:sc-2006 (Santa Cruz Biotechnology). Blots were stripped with mild stripping buffer, and then re-probed with primary c-Myc Antibody (9E10): sc-40 antibody and then with secondary antibody chicken anti-mouse IgG-HRP: sc-2954 (Santa Cruz Biotechnology). Signal was detected after blots were incubated with SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific).

Root elongation assay

Seedlings were surface sterilized as described above and grown on ½ MS plates, kept in the dark for 3 days at 4° C, and transferred to 24-hour light for 4 days. At day 4, the seedlings were transferred to plates supplemented with 5N NaOH as a vehicle control or different concentrations of BA (10, 25 or 50 μM BA) and the root tips were marked on the plates. Seedlings were grown for 5 more days, on day 9 the plates were scanned. Root length was measured using FIJI software (Schindelin *et al.*, 2012). A one-way ANOVA and Tukey HSD tests were conducted in R.

Microscopy

For TCSn::GFP analysis, seedlings were surface sterilized as described above, plated on ½ MS plates and stratified for 3 days in the dark. Seedlings were grown for 4 days at 24-hour light at 22° C and then transferred to ½ MS plates supplemented with 5 µM BA or 0.01% 5 N NaOH as a vehicle control for 24 hours and then imaged in a Nikon fluorescent microscope at the same exposure time. Intensity Density was quantified with FIJI and statistical analysis (one-way ANOVA and Tukey HSD tests) was conducted in R. For localization of GFP:BPC6 and GFP expression lines used for ChIP, GFP fluorescence was analyzed in stable *Arabidopsis thaliana* lines using a Leica TCS SP8 Confocal Laser Scanning Microscopy (CLSM) (Leica Microsystems). GFP:BPC6 as well as GFP expressing leaf samples were excited using an Argon Laser with 488 nm and the resulting emission was detected from 495 nm to 530 nm.

ChIP-seq and data analysis

All *Arabidopsis thaliana* seeds stratified at 4°C for 2 days in darkness. Afterwards seeds were sown on agar plates containing 2.15 g/l Murashige & Skoog media (Duchefa), 0.5 g/l MES (pH 5.8), and 15g/l phytoagar. Plants used for ChIP-seq and X-ChIP were grown in long day growth chambers (16h light / 8h dark at 22°C). For chromatin immunoprecipitation, stable transgenic pBPC6::GFP-BPC6 and pBPC6::GFP plants were used. Per line, two independent pools of seedlings were sampled as biological replicates, crosslinked, immunoprecipitated and used for sequencing. Three grams of 14 days old seedlings grown on ½ MS plates were harvested and immediately cross-linked using 1% formaldehyde and vacuum infiltration for 1 hour. After washing, the material was frozen using liquid nitrogen and ground into fine powder. Subsequent nuclei enrichment followed by chromatin isolation was perfomed. The isolated chromatin was sheared into 200-500 base pair fragments using a S220 focused-ultrasonicator (Covaris) and immunoprecipitated with 2,5 µl of anti-GFP antibody (Abcam, ab290). The precipitated DNA was recovered with the MiniElute PCR Purification Kit (Qiagen) according to the manufacturer's instructions. We used 10 ng of the immunoprecipitated DNA to constructed two ChIP-Seq libraries (pBPC6::GFP-BPC6) and two control library (pBPC6::GFP) using the TruSeq® ChIP Sample Preparation Kit (Illumina), according to the manufacturer's protocol. Indexed libraries were sequenced using the

Illumina HiSeq3000 instrument. The raw sequence data was processed using the Illumina sequence data analysis pipeline GAPipeline1.3.2. Then all peaks were called using the ChIPseek tool (Fujioka et al., 2008) to map the reads to the Arabidopsis genome (TAIR10). The raw data which was obtained in this study is available at the Gene Expression Omnibus database (accession number). Additionally, we used the ChIPseek platform for the location annotation and for analysis of distribution of distance to the nearest TSS of all ChIP-seq reads. DREME algorithm [8] was used to identify sequence logos of all sequenced reads and Agrigo [9] was used for gene ontology term search. Finally, the data was imported to Integrated Genome Browser [2] for visualization. Subsequent analysis revealed 34,501 peaks that were commonly enriched in the two independent pBPC6:GFP:BPC6 ChIP-Seq experiments but absent in the two pBPC6:GFP controls. After filtering these, peaks were mapped to the *Arabidopsis thaliana* genome (TAIR10) and 5,386 putative BPC6 *in vivo* target loci were exclusively assigned to the GFP:BPC6 expressing plants (Table S1).

X-ChIP-seq analysis

For X-ChIP, we used target genes that were enrichment for GFP:BPC6 binding within the ChIP-seq data. Chromatin immunoprecipitation was performed as described above, but after DNA recovery quantitative PCR (qPCR) was performed for X-ChIP experiments. We used three technical replicates each probe for qPCR and the results were calculated as percentage of input DNA. Fold enrichment represents fold change in comparison to the pBPC6::GFP control.

RNA extraction and cDNA synthesis

Total RNA was extracted from frozen tissue using the RNeasy Plus Kit as described by the manufacturer (Qiagen). RNA was DNAse treated with TURBO DNA-*free*™ kit according to the manufacturer's instructions (Life Technologies), and RNA was cleaned with RNeasy Plus kit as described by the manufacturer (QiagenUsing 1 µg of RNA, cDNA was synthesized using Superscript III Reverse Transcriptase with oligo-d(T) primers according to manufacturer's instructions (Thermo Fisher Scientific).

Quantitative RT-PCR

Real-time PCR was performed with the PowerUP™ SYBR™ Green Master Mix according to manufacturer's instructions using the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems). *GAPDH* (AT1G13440) was used as a housekeeping gene in all reactions. Complete list of qPCR primers found in Table S3.1. Primers were designed using the NCBI Primer design tool (Ye *et al.*, 2012). qRT-PCR was performed for three to four biological replicates for each genotype and three technical replicates. The relative expression for each gene was determined with the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

RNA-sequencing

Seedlings for Col-0, and *bpc1-1,2,3,4,6* mutant were treated with BA as described above with three biological replicates for each treatment making a total of 12 samples. Total RNA was extracted from root tissue, DNase treated and cleaned as described above. RNA quality was determined by the High-Throughput Sequencing Center at University of North Carolina, Chapel Hill using a Agilent 2100 Bioanalyzer system and all samples had a RIN value greater than 7.0 (Agilent). Libraries were created with the KAPA Stranded mRNA-Seq Kit with KAPA mRNA Capture Beads for Illumina platforms, KAPA Pure Beads, and KAPA Single-Indexed Adapter Kit, Set A (1.5μM) (Kappa Biosystems). Quantity of each library was determined with the KAPA Library Quantification Kit for Illumina Platforms. The concentration of each library and QAQC was determined by the High-Throughput Sequencing Center at University of North Carolina, Chapel Hill using a Agilent 2100 Bioanalyzer system and the Qubit 2.0 Fluorometer (Thermo Fisher Scientific). The 12 libraries were pooled for Illumina sequencing on one lane of the Hiseq2500 instrument with 50bp single-end reads and gave 10-15 million reads/library.

RNA-sequencing data analysis

The sequencing data was first checked for quality control using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequences were aligned to the Arabidopsis Col-0 genome assembly (TAIR10) with TopHat version 2.0.11 and Bowtie version 1.1.0 (Langmead *et al.*, 2009). SAMtools version 0.1.18 (Li *et al.*, 2009) was used to create the BAM files and 87-95% of the

reads mapped to the TAIR10 genome. The Integrative Genomics Browser (IGB) was used to view the reads (Robinson et al., 2011). FeatureCounts was used to map the number of single-mapped reads that overlap with the annotated genes (Liao *et al.*, 2014). The differential gene analysis was conducted in R Studio version 0.98.1062 (https://www.rstudio.com) and the pipeline adapted from (Loraine *et al.*, 2015). The counts files were used as inputs for EdgeR version 3.0 (Robinson et al., 2009). The false discovery rate for differentially regulated genes was set to 5% using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). For analysis of overlapping genes we used Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny) and BioVenn (Hulsen *et al.*, 2008). As a visual tool, MapMan was used to visualize processes enriched in each data set (Thimm *et al.*, 2004) and VirtualPlant 1.3 was used for gene ontology analysis (Katari *et al.*, 2010).

Table 3.1 Primers used in this study

Gene	Use	Primer name	Sequence
AT2G21240	Cloning	BPC4gw_F	caccATGGAGAATGGTGGTCAGTATG
AT2G21240	Cloning	BPC4cn_R	CTTGATAGTGATGTAGCGGTTTGTCC
AT2G21240	Cloning	BPC4cs_R	CTACTTGATAGTGATGTAGCGGTTTGTCC
AT1G68120	Cloning	BPC3gw_F	caccATGATGGAAGAAGATGGATTG
AT1G68120	Cloning	BPC3cs_R	TTATCTGATGGTGACGAACTTATTGG
AT1G68120	Cloning	BPC3cn_R	TCTGATGGTGACGAACTTATTGG
AT2G01930	Cloning	BPC1gw_F	caccATGGACGATG ATGGATTTCG
AT2G01930	Cloning	BPC1cn_R	TCT GAT CGT GAC AAA CTT ATT GG
AT5G42520	Cloning	BPC6gw_F	caccATGGATGATGGTGGGCATCGT
AT5G42520	Cloning	BPC6cn_R	TTTAATCGTAATGTAGCGG
AT5G42520	Cloning	BPC6cs_R	TCATTTAATCGTAATGTAGCGG
AT2G35550	Cloning	BPC7gw_F	caccATGGGTCTTGATTCTTCTTTCG
AT2G35550	Cloning	BPC7cs_R	CTACTTGATGGTCACAAACTT
AT2G01930	Genotyping	GT_bpc1-1_LP	TGGGCTTTGATTTTGTTTTTG
AT2G01930	Genotyping	GT_bpc1-1_RP	GAAATCCATCATCGTCCATTG
AT1G14685	Genotyping	GT_bpc2_LP	GCAAGTGCAGATAGGAACAGG
AT1G14685	Genotyping	GT_bpc2_RP	TTGCAATTCCCTAAAGGAATTC
AT1G68120	Genotyping	GT_bpc3-1_LP	AGCCATTTCTCTCCCCTCAT
AT1G68120	Genotyping	GT_bpc3-1_RP	TGGTCGTTACAACAATCTGGA
AT2G21240	Genotyping	GT_bpc4_LP2	CTTTCCGTGACAGATCCACTC
AT2G21240	Genotyping	GT_bpc4_RP2	CTTTGTCAACTATTCCGCCTG
AT5G42520	Genotyping	GT_bpc6_LP	TCGTCGTAAACCACTTGGTTC
AT5G42520	Genotyping	GT_bpc6_RP	ATCTCAAATGGATGATGGTGG
AT2G35550	Genotyping	BPC7gabikatfwd	GTTTGGAGGTGTTGGCTCAT
AT2G35550	Genotyping	BPC7gabikatrev	ACGACTGTTGGGATTTTTGC
Border primer	Genotyping	lBb1.3	ATTTTGCCGATTTCGGAAC
Border primer	Genotyping	LB3-SAIL-N	TAGCATCTGAATTTCATAACCAATCTCGATACAC
AT1G13440	RT-PCR	GAPDH.RT.F	AGGCCATCAAGGAGGAATCT
AT1G13440	RT-PCR	GAPDH.RT.	GAAAATGCTTGACCTGTTGTCAC
AT4G26200	RT-PCR	ACS7 F	GCTGGAAAGCTTACGACGAAAATCC
AT4G26200	RT-PCR	ACS7 R	TTGATCCCCACATCGAACCTTCT
AT1G49230	RT-PCR	ATL78_RT_F	GAACGTGTGAAGCTGTTGCC
AT1G49230	RT-PCR	ATL78_RT_R	GGGACAGGACGAGTGTGAAC
AT2G18480	RT-PCR	Major_RT_F	GGATATGATACGGGAGTTATGAGCG
AT2G18480	RT-PCR	Major_RT_R	AGCTATGGTGTAACGTCGGC
AT5G54145	RT-PCR	AT5G54145_RT_F	GCTCTAGCTAAACTCACCGC
AT5G54145	RT-PCR	AT5G54145_RT_R	CACCGATCCTTACGGCGTTT

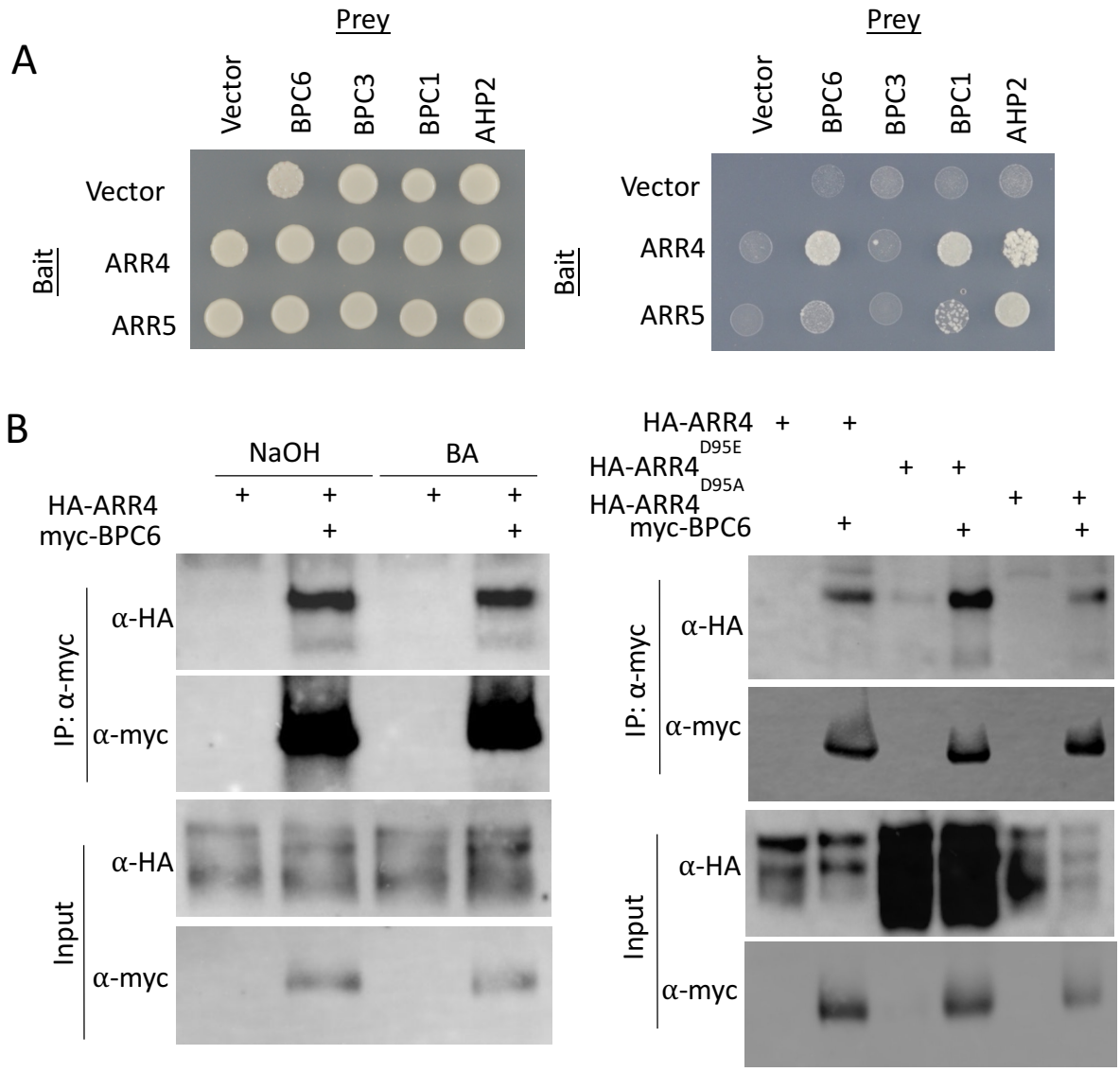


Figure 3.1. BPCs interact with type-A ARRs**. (A)** Yeast two-hybrid. The GAL4 activation domain fused to the coding sequence of the BPC proteins and the LexA DNA-binding domain fused to coding sequence of type-A ARRs. On the left, ten microliters of cell suspension plated on control media (+His, +Ura) and on the right ten microliters was plated on selection media (-His, -Ura). **(B)** Co-immunoprecipitation in *Nicotiania benthamiana* of BPC6 and ARR4 wild-type and mutant proteins. Total protein extract from leaves were incubated with α-myc beads and BPC6 protein was immobilized. The protein elute was analyzed with protein gel blotting and probed with both α-myc and α-HA antibody. Infiltration with ARR4 protein was used as a negative control. For protein loading onto the gel, 4x more protein was loaded for the immunoprecipitation (IP) compared to the input. On the left, leaves were pre-treated with 5μM of BA or NaOH as a vehicle control for 20 minutes before tissue collection. On the right, the leaves were not treated.

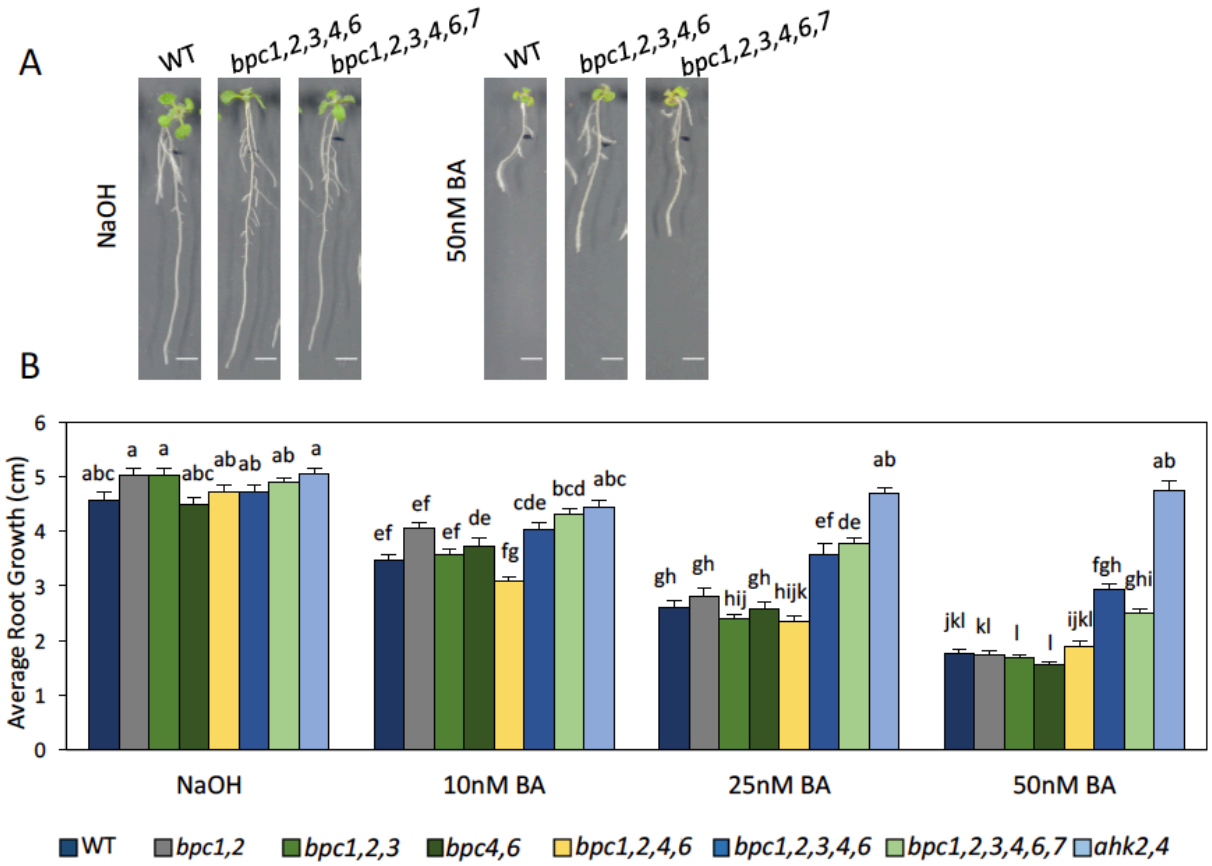


Figure 3.2. Higher order *bpc* mutants are less sensitive to exogenous cytokinin. **(A-B)** Root elongation assay, seedlings were grown on ½ MS media for 4 days and transferred to 0.01% 5N NaOH control or cytokinin supplemented plates, grown for 6 days, scanned at day 10 and primary root length was measured from day 4-10. **(A)** Image of WT, *bpc1,2,3,4,6* and *bpc1,2,3,4,6,7* mutants on NaOH control or 50nM BA at day 10. scale bar = 1 cm. **(B)** Average root growth of multiple *bpc* loss-of-function mutants. Error bars represent SEM (n = 10). Statistical analysis was performed using one-way ANOVA followed by the Tukey HSD post hoc test $P < 0.05$. Different letters indicate significance between groups.

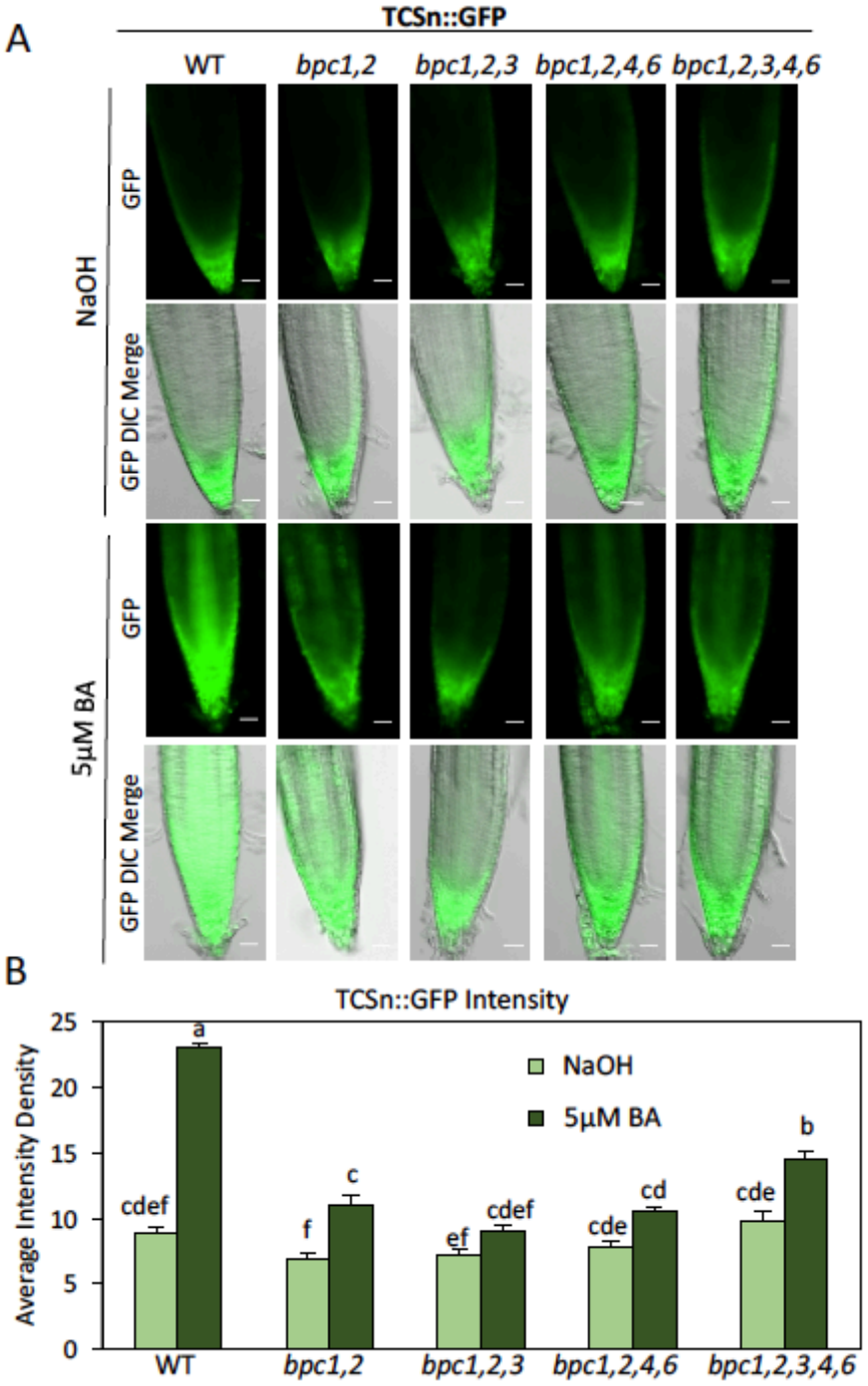


Figure 3.3. *bpc* mutants display reduced TCSn::GFP signaling in response to cytokinin. **(A-B)** Multiple *bpc* mutants crossed with TCSn::GFP reporter for type-B ARR activity (Zurcher et al., 2013). Seedlings were grown for 4 days on ½ MS media and transferred to media with 0.01% 5N NaOH control plates or 5µM BA plates for 24 hours. **(A)** Images of root tips were taken using fluorescent light microscopy. Top panel is signaling of green fluorescent proteins (GFP) and bottom is the merge between the GFP and differential interference contrast (DIC). scale bar = 50µM **(B)** Signal intensity density of the GFP signal was quantified in root tips with FIJI software. Average signal is presented. Error bars are SEM and n = 10 roots. Statistical analysis, one-way ANOVA followed by the Tukey HSD post hoc test $P < 0.05$. Different letters indicate significance between groups.

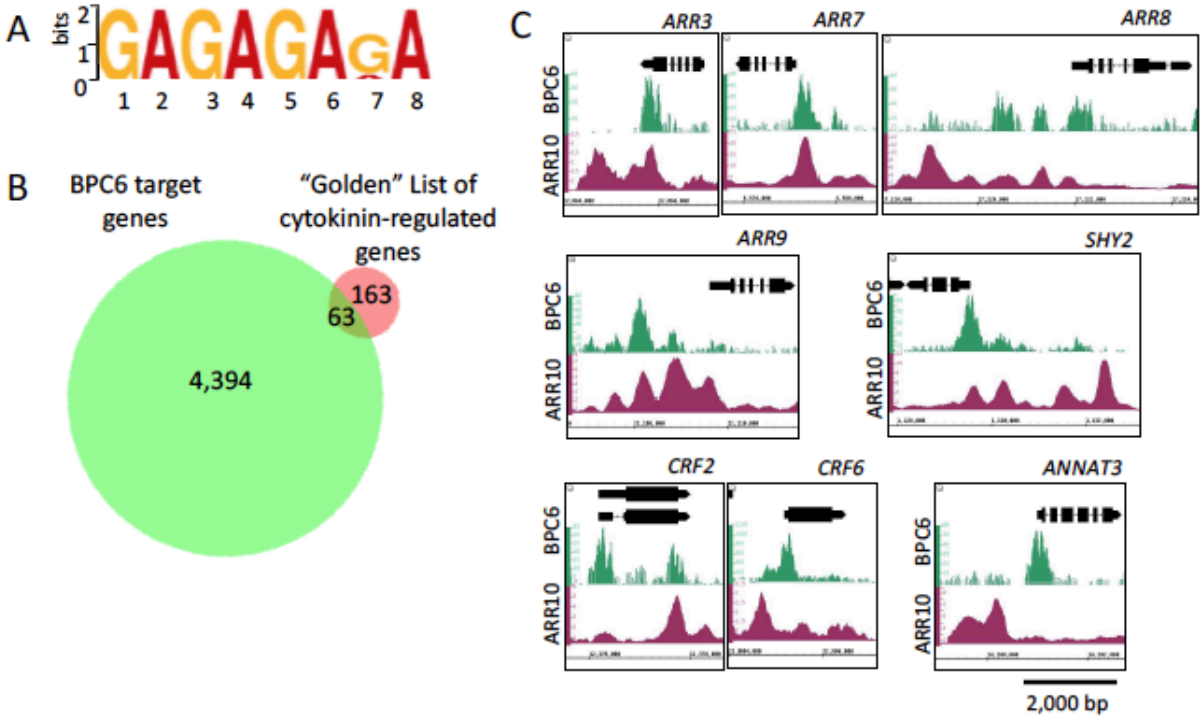


Figure 3.4. BPC6 ChIP-seq overlaps with cytokinin-regulated genes. (**A**) Identification of sequence logos for DNA-elements enriched in all ChIP-seq reads using DREME. Top, GFP:BPC6 *in vivo* binding motif 'GAGAGARA' (e-value = 3.8e-286). (**B**) Overlap between the 4,457 BPC6 ChIP target genes and the 226 genes found in the set of robustly regulated cytokinin response genes found in the 'golden' list (Bhargava *et al.,* 2013). Hypergeometric probability test *P* (X>=63) = 1.2e-4. (**C**) Visualization of BPC6 and ARR10 binding sites up-stream of cytokinin response genes that overlap between BPC6 targets, ARR10 targets and the 'golden list'. On the top, bedgraphs for BPC6 ChIP binding sites in 14 day old seedlings and on the bottom, fold enrichment for ARR10 binding sites identified with ChIP-seq in response to cytokinin in 10 day old seedlings (Zubo *et al.,* 2017). Visualized in the Integrated Genome Browser (IGB) (Nowlan *et al.,* 2016).

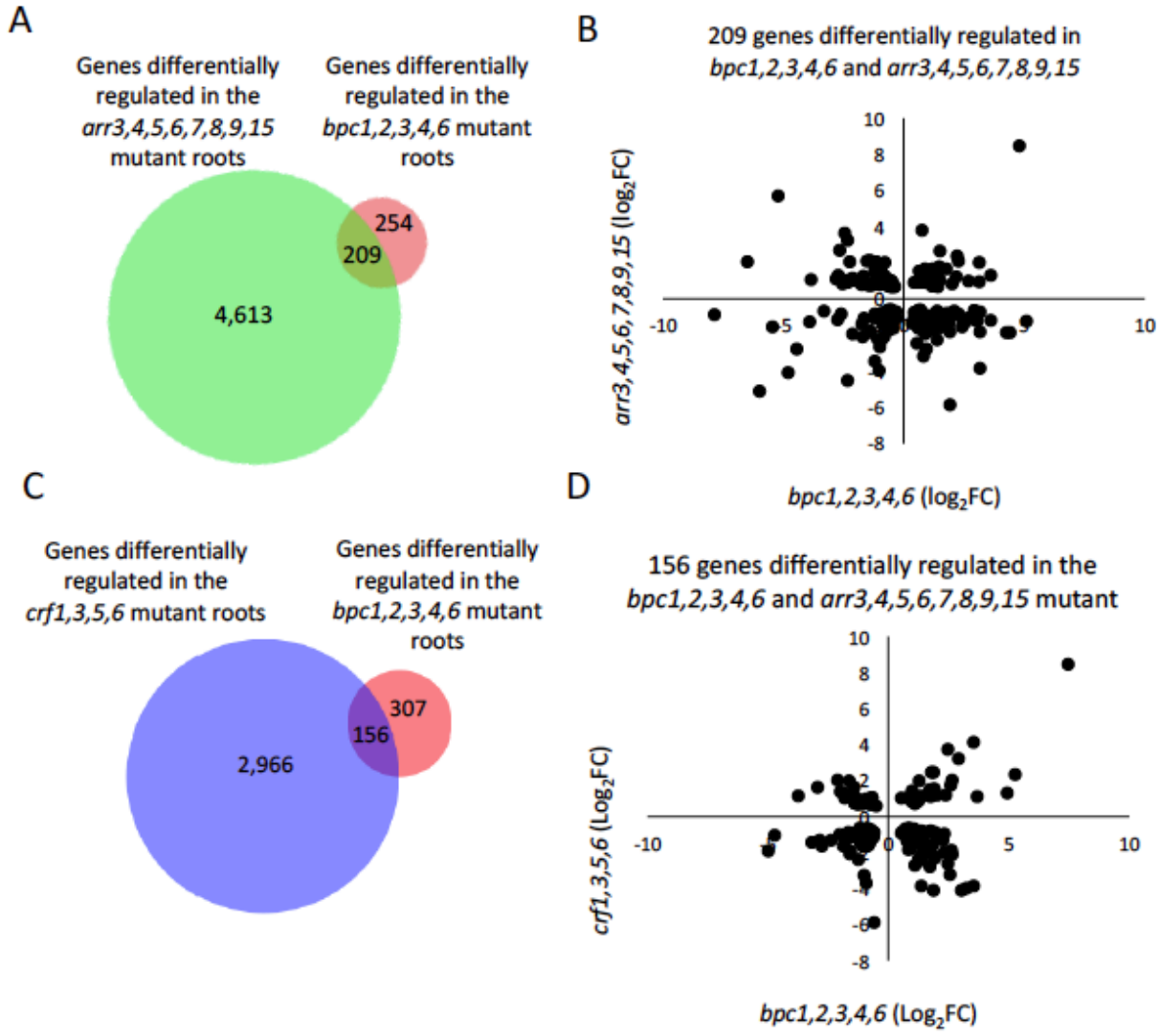


Figure 3.5. BPCs and type-A ARRs regulate a sub-set of the same genes. **(A)** Significant overlap between genes differentially regulated in the type-A arr3,4,5,6,7,8,9,15 mutant vs. WT roots (Shanks *et al.,* 2016) and the *bpc1,2,3,4,6* mutant. Hypergeometric Probability test P(x >= 209) = 7.3e-50. **(B)** Scatterplot of the log_2FC of genes differentially regulated in *bpc1,2,3,4,6* compared to *arr3,4,5,6,7,8,9,15* of the 209 genes that overlap. **(C)** Overlap between the genes differentially regulated in the *bpc1,2,3,4,6* mutant roots and the genes differentially regulated in the *crf1,3,5,6* mutant vs. WT roots (Raines *et al.*, 2016). Overlap between *crf1,3,5,6* and *bpc1,2,3,4,6* hypergeometric probability test *P* (X>=156) = 5.1e-33. **(D)** Scatterplot of the log_2FC of 156 genes differentially regulated in *bpc1,2,3,4,6* compared to *crf1,3,5,6.*

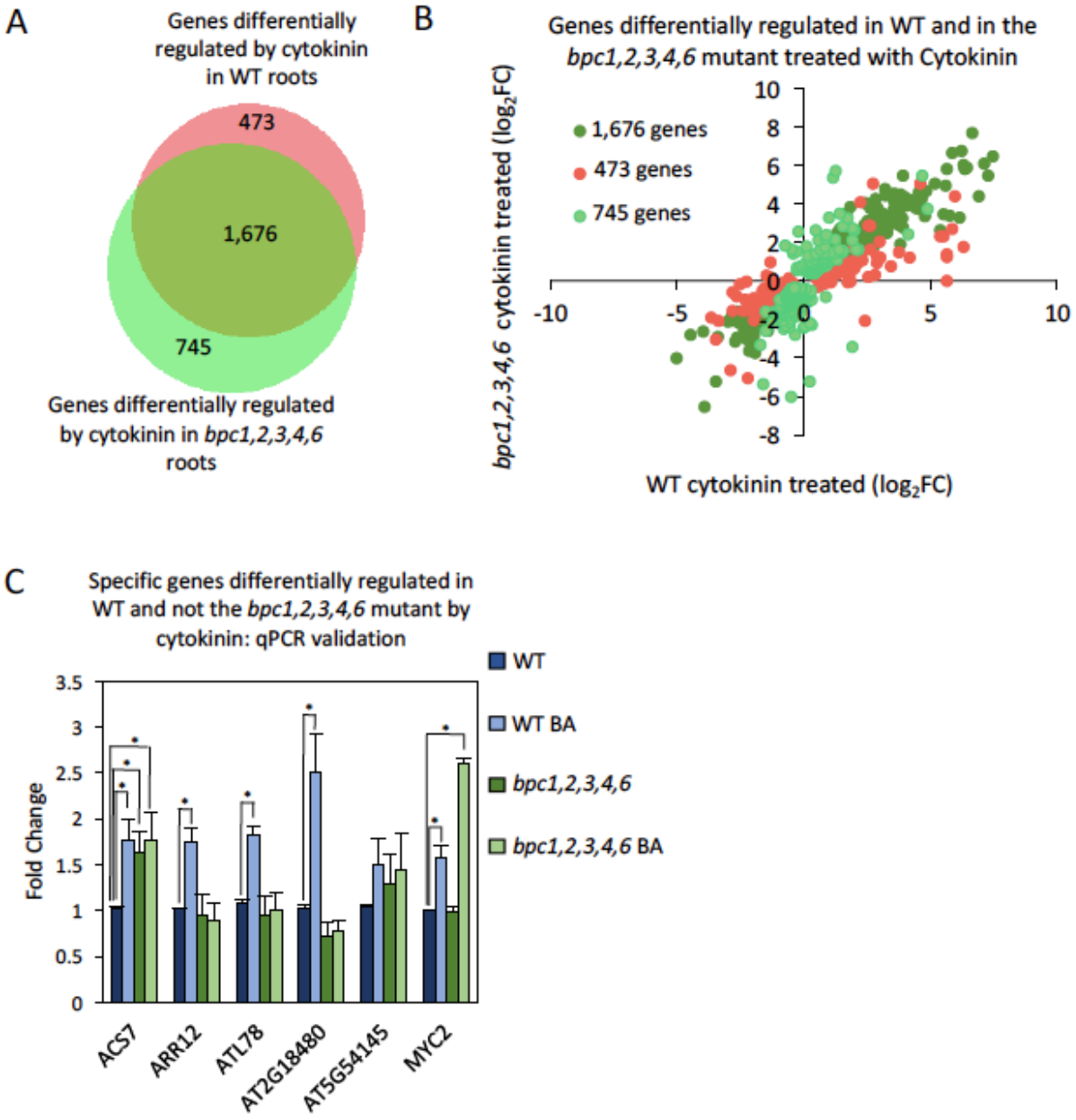


Figure 3.6. BPCs regulate sub-set of cytokinin response genes. **(A)** Overlap between genes differentially regulated in WT after cytokinin treatment and in *bpc1,2,3,4,6* after cytokinin treatment. **(B)** Scatterplot of the log_2FC of genes differentially regulated in WT after cytokinin treatment and the *bpc1,2,3,4,6* mutant after cytokinin treatment. The 473 set of genes that WT treated genes were compared to the log_2FC of the *bpc1,2,3,4,6* cytokinin treated genes without a FDR cut off and the 745 genes were compared to the WT treated differentially regulated genes without a FDR cut off. **(C)** Specific genes found in the 473 set of genes that were induced by cytokinin but not differentially regulated in the *bpc1,2,3,4,6* mutant in response to cytokinin were re-tested with qPCR to validate the findings from the RNA-seq. Benzyl adenine (BA) is synthetic cytokinin. Error bars are SEM of the fold change from four biological replicates. Student's TTEST compared WT without cytokinin treatment to the other treatments * $P < 0.05$.

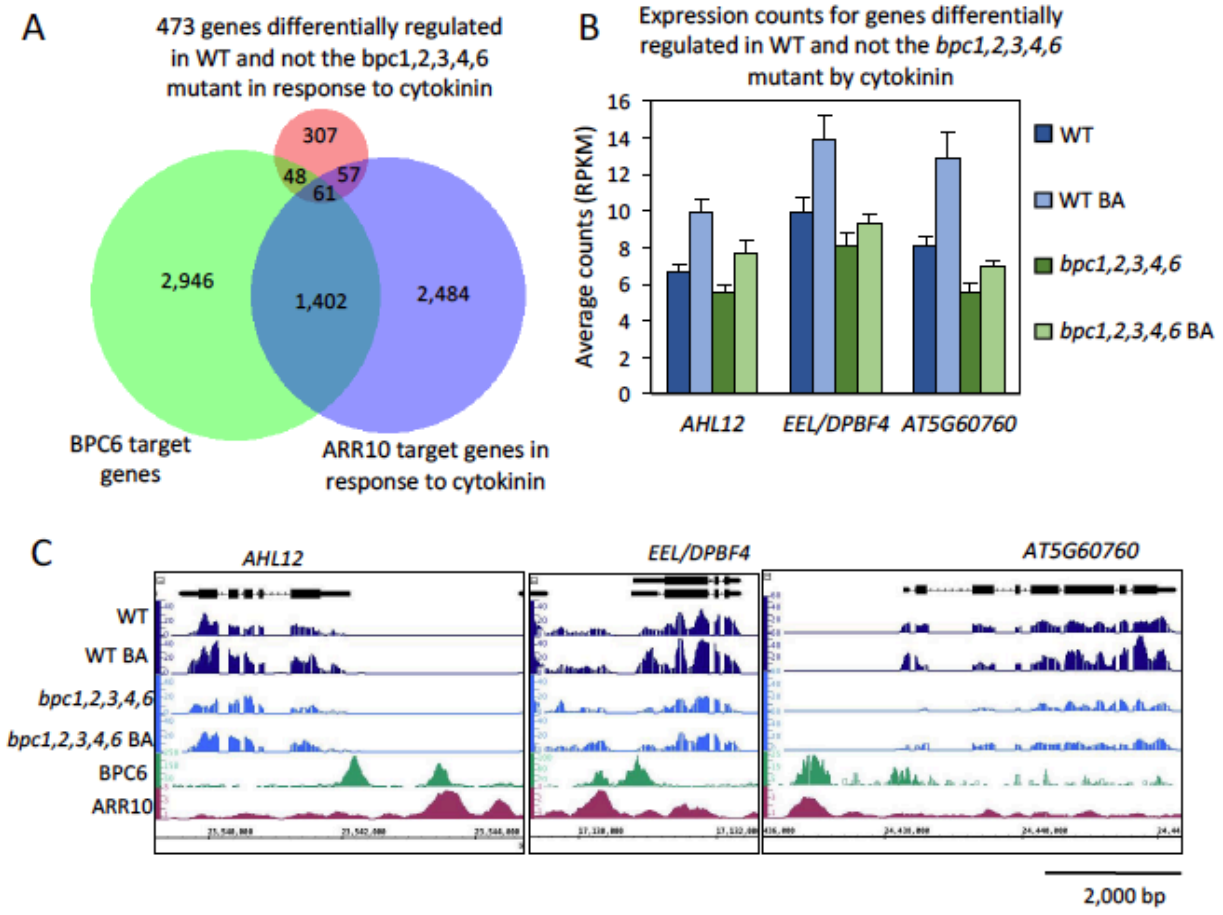


Figure 3.7. BPCs regulate sub-set of cytokinin response genes that are also BPC6 and ARR10 target genes. **(A)** The 473 genes differentially regulated in WT and not the *bpc1,2,3,4,6* mutant in response to cytokinin that overlap with the ARR10 and BPC6 targets identified with ChIP-seq. Significant overlap between between 473 genes and 4,457 BPC6 target genes in 14 day old whole seedlings, hypergeometric probability *P* (X>=109) = 2.2e-3. Significant overlap between 473 and 4,004 genes found as ARR10 targets in response to cytokinin in 10 day old seedlings, hypergeometric probability *P* (X>=118) = 3.1e-7. Significant overlap between ARR10 and BPC6 target genes, hypergeometric probability *P* (X>=1,463) = 3.9e-215. **(B)** Average expression counts for specific genes found in the 61 genes that overlap between all three data sets in A from the three biological replicates in the RNA-seq data. Error bars represent SEM. *Significant induction by cytokinin in EdgeR analysis, FDR 0.05. **(C)** Visualization of transcript levels of each gene in the WT and *bpc1,2,3,4,6* treated with cytokinin and BPC6 and ARR10 binding sites up-stream of cytokinin response genes. Bedgraphs visualized in the Integrated Genome Browser (IGB) (Nowlan *et al.,* 2016).

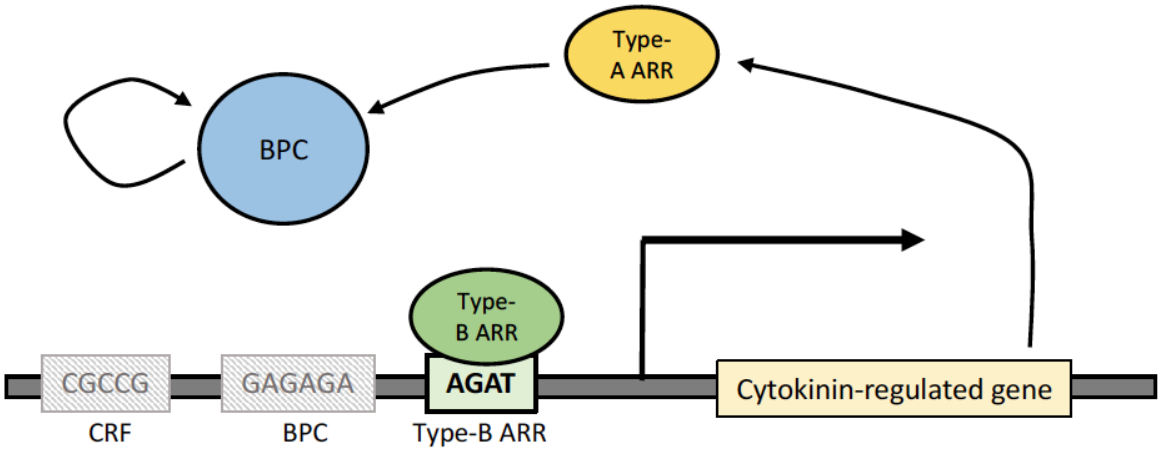
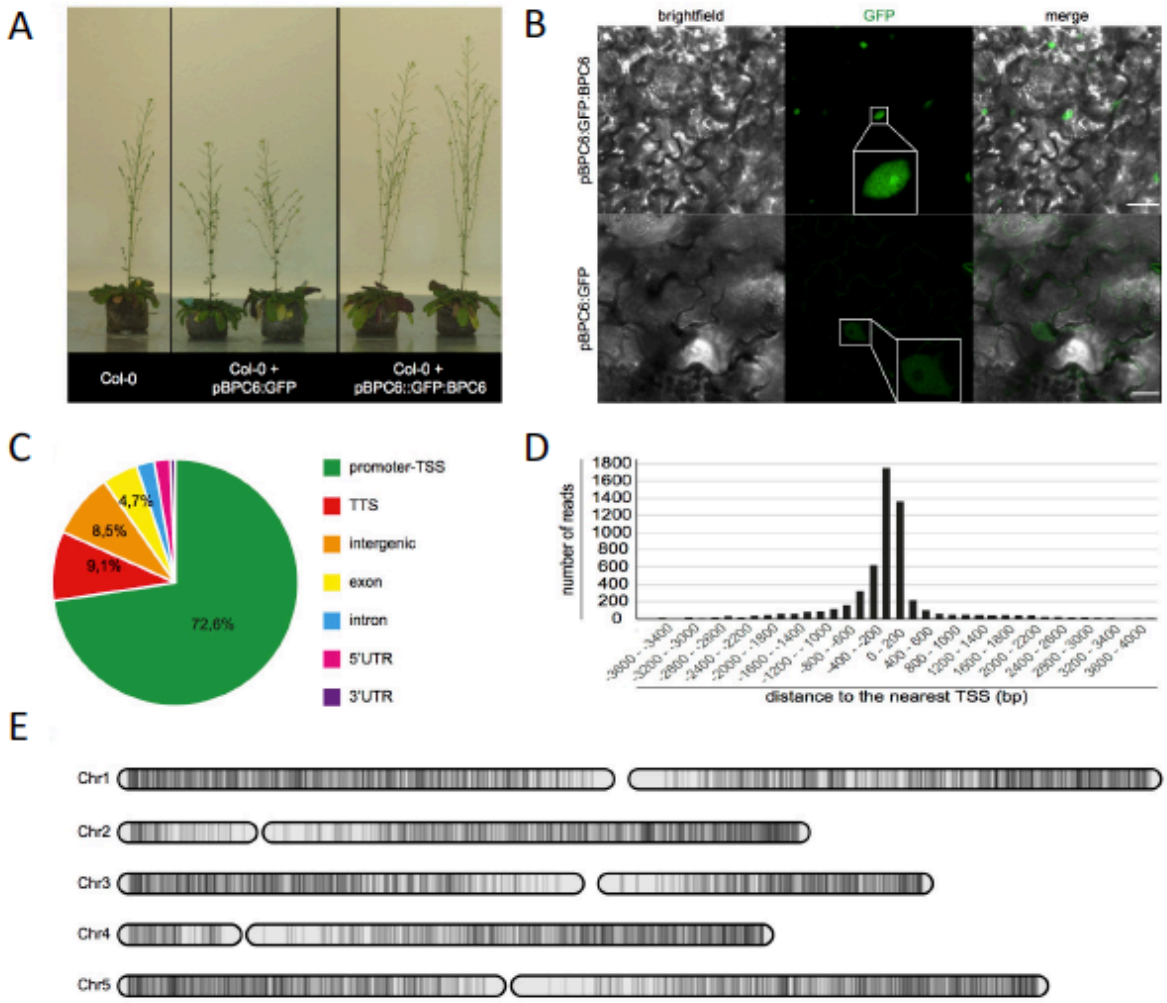
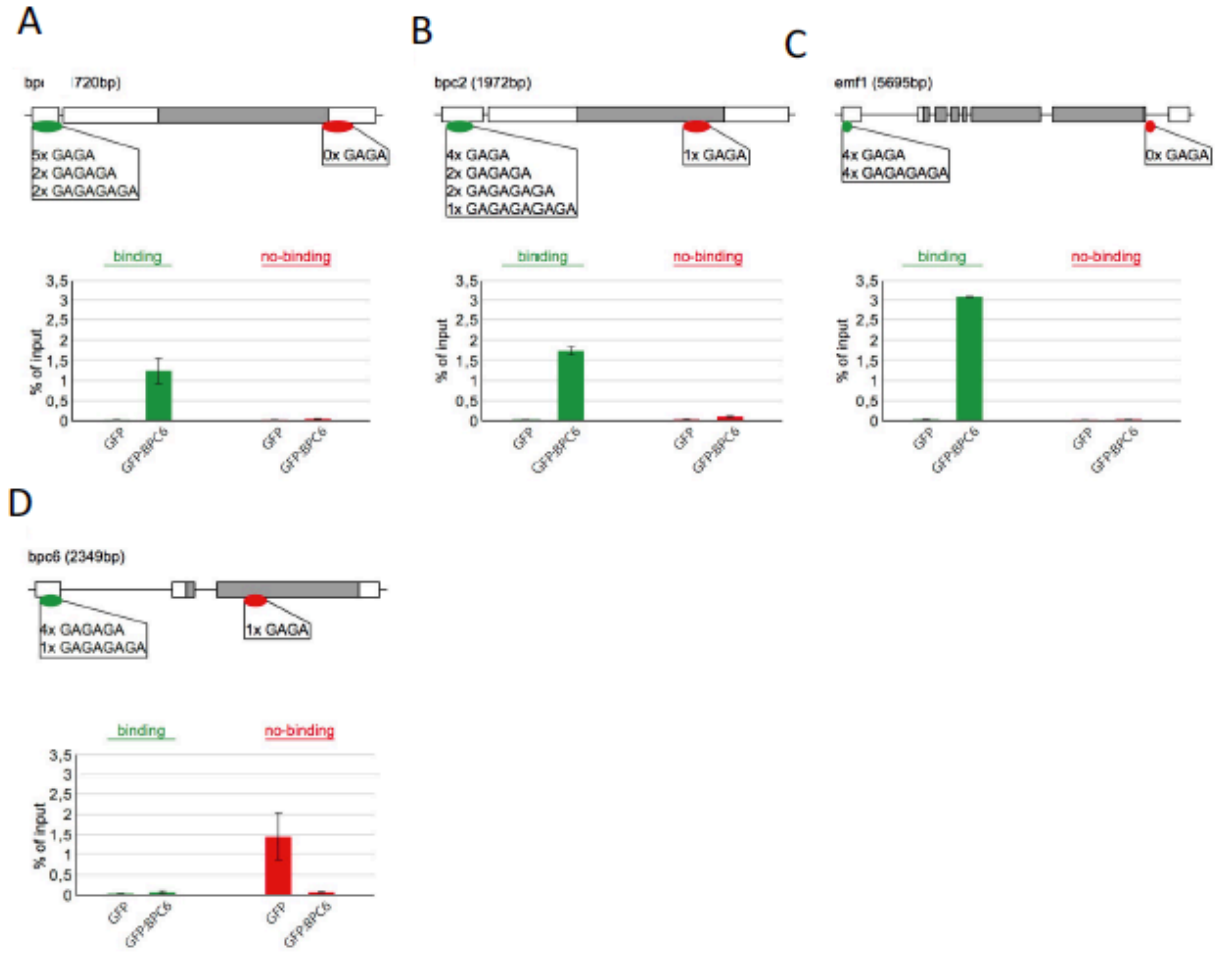


Figure 3.8. Model of type-A ARR and BPC interaction in cytokinin signaling. The type-A ARRs are transcriptionally up-regulated in response to cytokinin by the type-B ARRs. The induction of the type-A ARRs regulates the interaction with the BPC proteins. The type-A ARRs may affect BPC activity by either inhibiting BPC binding to other transcription factors, or from binding to DNA. The BPCs may co-regulate cytokinin response gene expression with the type-B ARRs and CRFs.



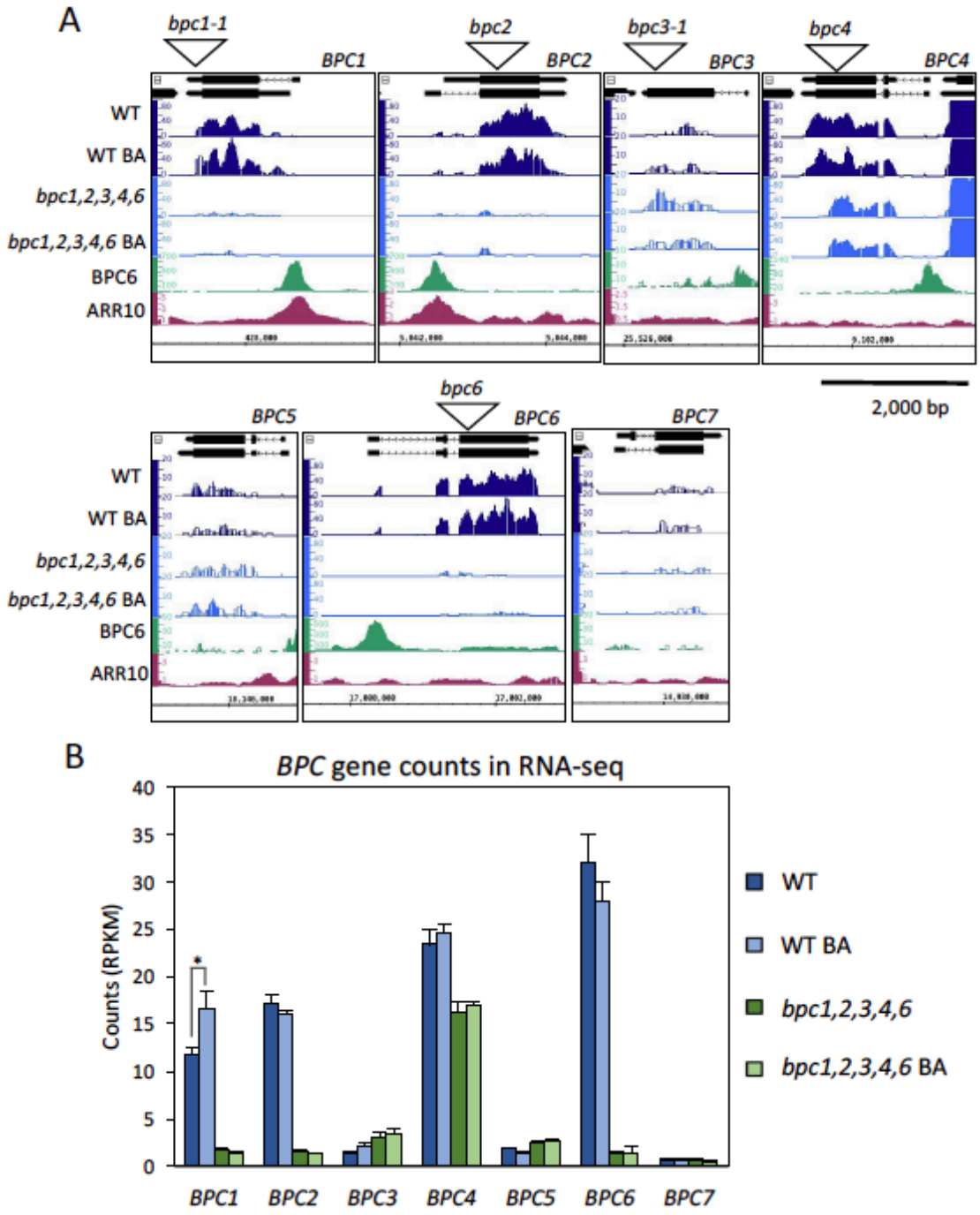
Supplementary Figure S3.1. Identification of GFP:BPC6 in vivo target genes using ChIP-seq. **(A)** Transgenic *Arabidopsis* Col-0 plants expressing either GFP:BPC6 or GFP under the control of the endogenous *BPC6* promoter and terminator. **(B)** Localization of GFP:BPC6 (upper panel) and GFP (lower panel) in transgenic *Arabidopsis* plants used for ChIP-seq. Bars = 25µm. (**C**) Location annotation of all ChIP-seq reads. TSS = transcription start site, TTS = transcription termination signal **(D)** Distance distribution of all ChIP-seq reads to the nearest transcription start sites (TSS). **(E)** Chromosome ideogram of the distribution of GFP:BPC6 binding sites across the five *Arabidopsis* chromosomes. The density of reads is coded from grey (low) to black (high).



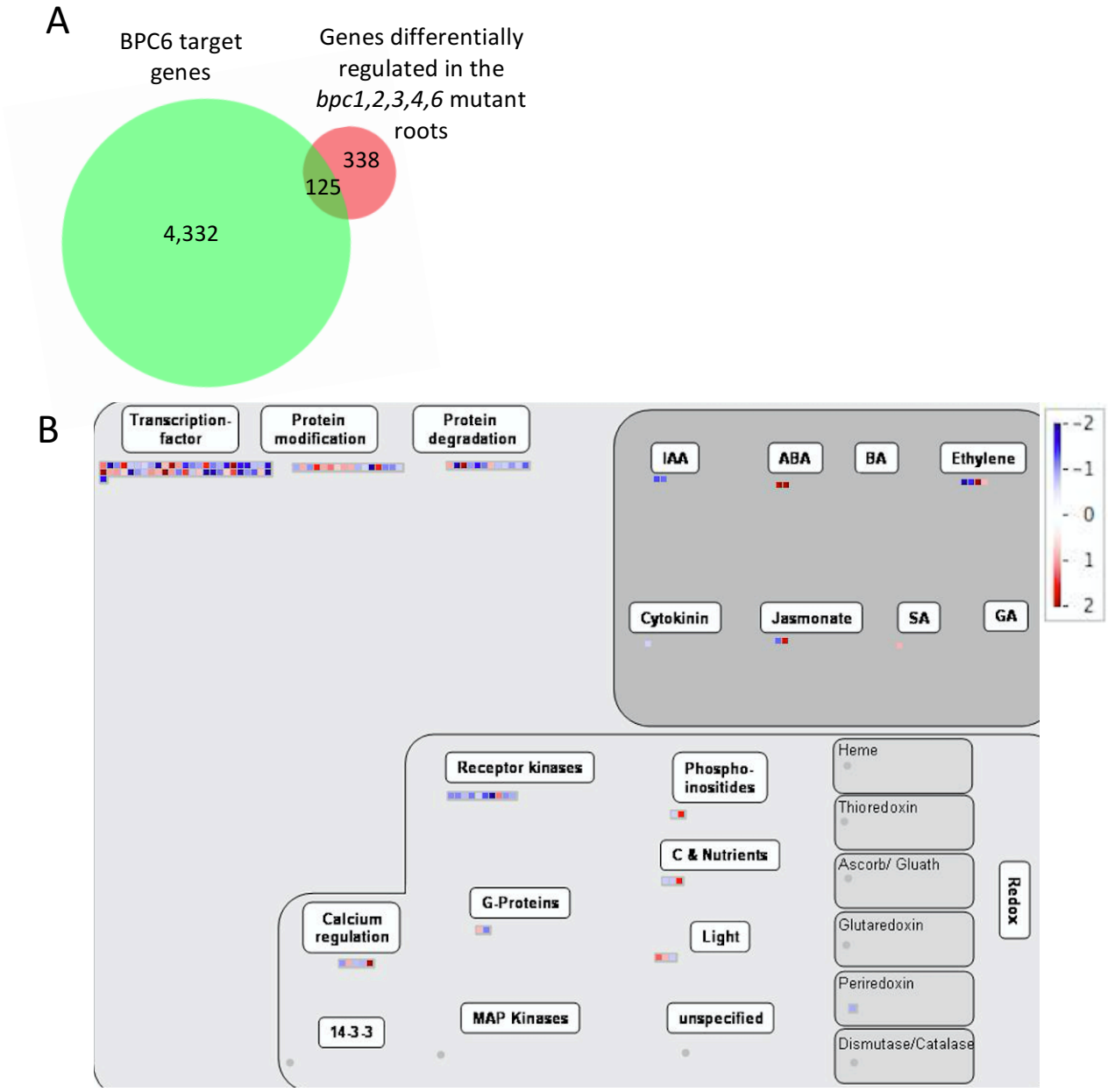
Supplementary Figure S3.2. Validation of the ChIP-seq data by using X-ChIP. **(A-D)** ChIP samples prepared from GFP:BPC6 or GFP expressing plants and immunoprecipitated with anti-GFP antibodies were analyzed by quantitative PCR. Location of PCR amplicons are indicated by ellipses below the respective gene model including the frequency of $(GA)_n$ repeats within the 150 bp amplicons. Data show one representative out of four independent quantitative PCR experiments. Enrichment is shown as % of input. **(A)** *BPC1* gene model and qPCR results. **(B)** *BPC2* gene model and qPCR results. **(C)** *EMF1* gene model and qPCR results. **(D)** *BPC6* gene model and qPCR results. UTRs are shown as white boxes, exons are shown as grey boxes, introns are shown as lines, green ellipses show the position of putative binding sites and the red ellipses show putative non-binding sites.

	Motif	logo	RC Logo	E-value	Unerased E-value	Transcription factor family
1.	GAGAGARA			3.8e-286	1.5e-222	BBP/BPC
2.	DTGGRCC			5.6e-134	5.6e-134	TCP
3.	CRCGTGD			1.9e-090	1.9e-093	bHLH
4.	GAAGAAGM			2.7e-065	6.7e-065	NAC
5.	AVMCAAA			4.2e-068	6.2e-071	C2C2dof
6.	AGCCCAH			8.2e-054	8.6e-059	unknown
7.	ADAGARA			2.9e-052	4.1e-173	C2C2dof
8.	TAGGGTTW			7.8e-035	2.2e-039	MYB-related
9.	CCRCCR			1.3e-033	2.4e-041	AP2/EREB/CRF
10.	CCGGTTYR			2.3e-032	5.7e-035	Trihelix
11.	CRTCRTC			3.8e-022	1.2e-024	bZIP
12.	ACGTGKC			3.2e-018	4.9e-067	bZIP
13.	CGCCGK			1.4e-017	2.1e-031	AP2/EREB/CRF
14.	CCGAMCCG			1.1e-016	7.2e-018	C2C2gata

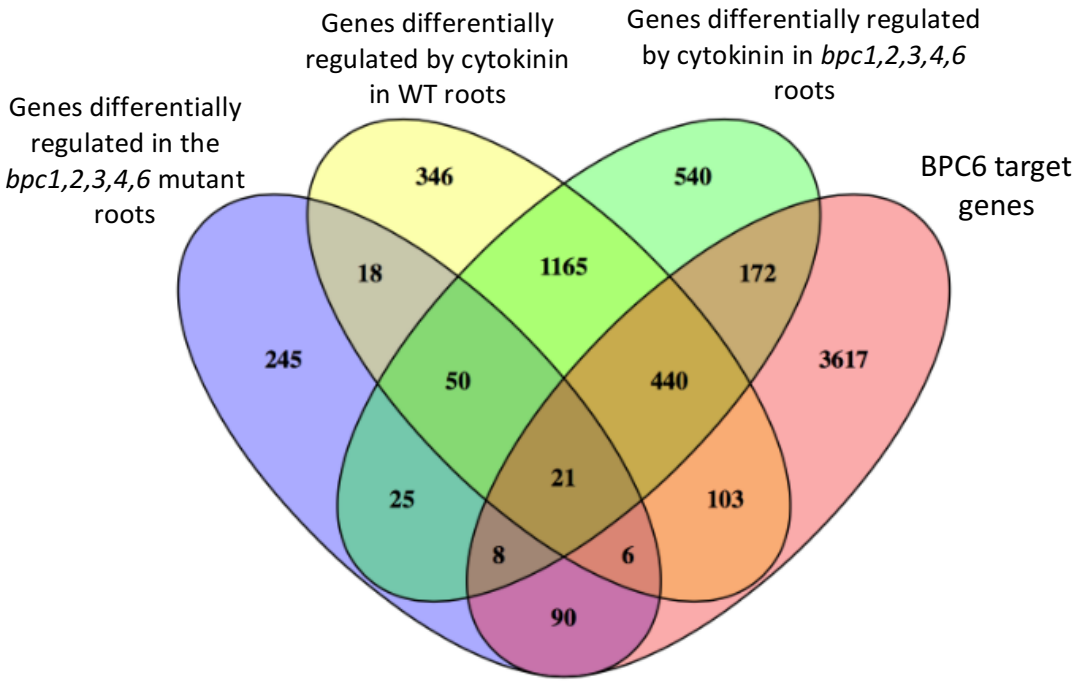
Supplementary Figure S3.3. List of all sequence logos for DNA-elements enriched in ChIP-seq. Identification of sequence logos for DNA-elements enriched in all ChIP-seq reads using DREME to find GFP:BPC6 *in vivo* binding motifs. Tomtom was used to identify corresponding transcription factor motifs.



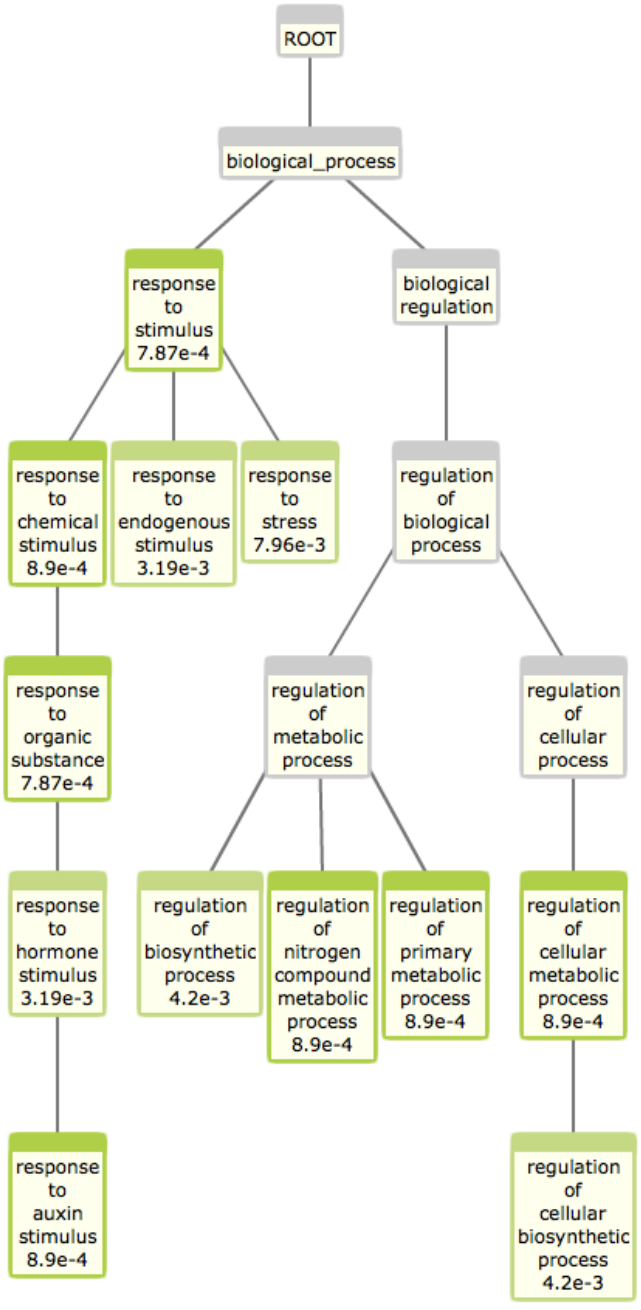
Supplemental Figure S3.4. Expression of BPC proteins in RNA-seq. **(A)** Visualization of transcript levels of *BPCs* in the WT and *bpc1,2,3,4,6* treated with cytokinin and BPC6 and ARR10 binding sites up-stream of the gene. Bedgraphs visualized in the Integrated Genome Browser (IGB) (Nowlan *et al.,* 2016). Triangles signify site of T-DNA insertion or point mutation of *bpc3-1*. **(B)** Counts for the expression of the seven *BPCs* in WT and the *bpc1,2,3,4,6* mutant with and without cytokinin treatment identified with RNA-seq. Average expression counts from three biological replicates sets in RNA-seq. Error bars represent SEM. **BPC1* expression is up-regulated in response to cytokinin in RNA-seq FDR 0.05, FC 1.5.



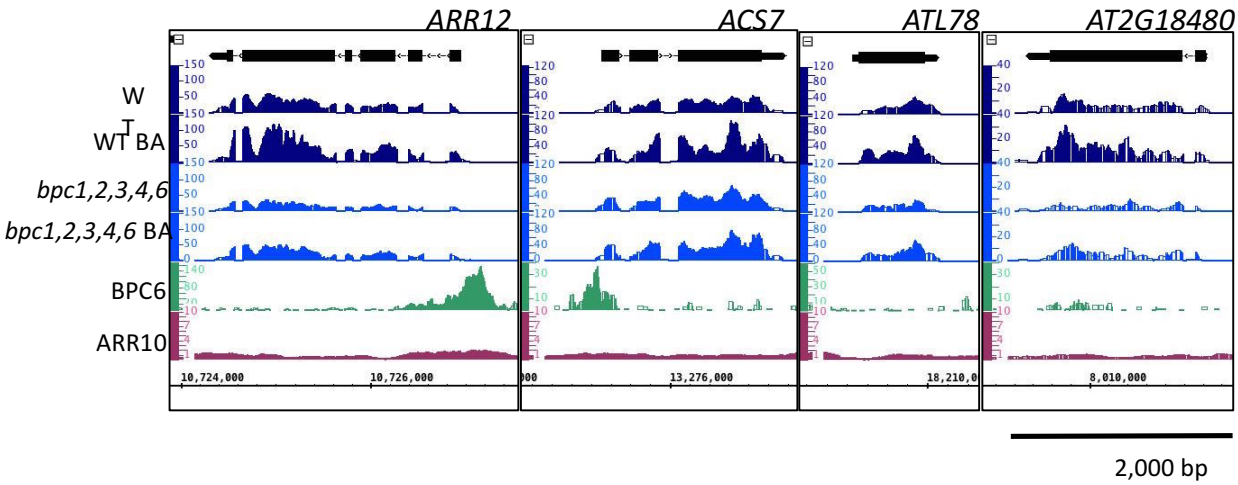
Supplemental Figure S3.5. Genes differentially regulated in the *bpc1,2,3,4,6* mutant roots with RNA-seq. **(A)** Genes that overlap between the BPC6 binding targets from ChIP-seq and the 463 genes differentially regulated in the *bpc1,2,3,4,6* mutant vs. WT found using RNA-seq. Hypergeometric probability test *P* (X>=125) = 5.3e-7. **(B)** BPCs regulate gene expression of transcription factors as visualized in the MapMan Regulation Overview of the 463 genes differentially regulated in *bpc1,2,3,4,6* mutant using RNA-seq (Thimm *et al.,* 2004). Red boxes represent down-regulated genes and blue boxes represent down-regulated genes with genes differentially regulated in $\log_2$FC.



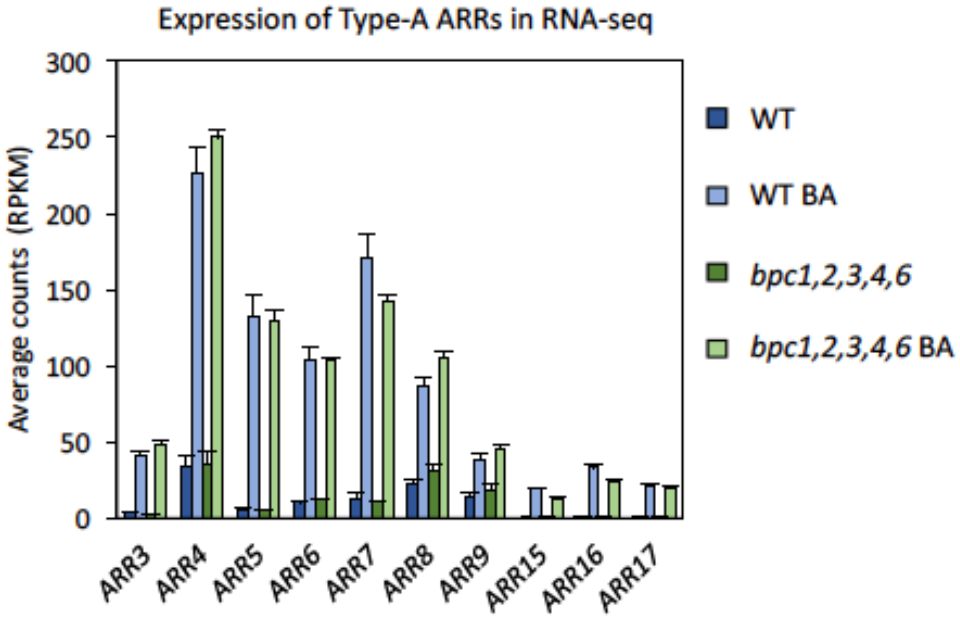
Supplemental Figure S3.6. Overlap between genes differentially regulated by cytokinin in WT and the *bpc1,2,3,4,6* mutant, the genes differentially regulated in the *bpc1,2,3,4,6* mutant without cytokinin treatment compared to WT and the BPC6 target genes identified with ChIP-seq.



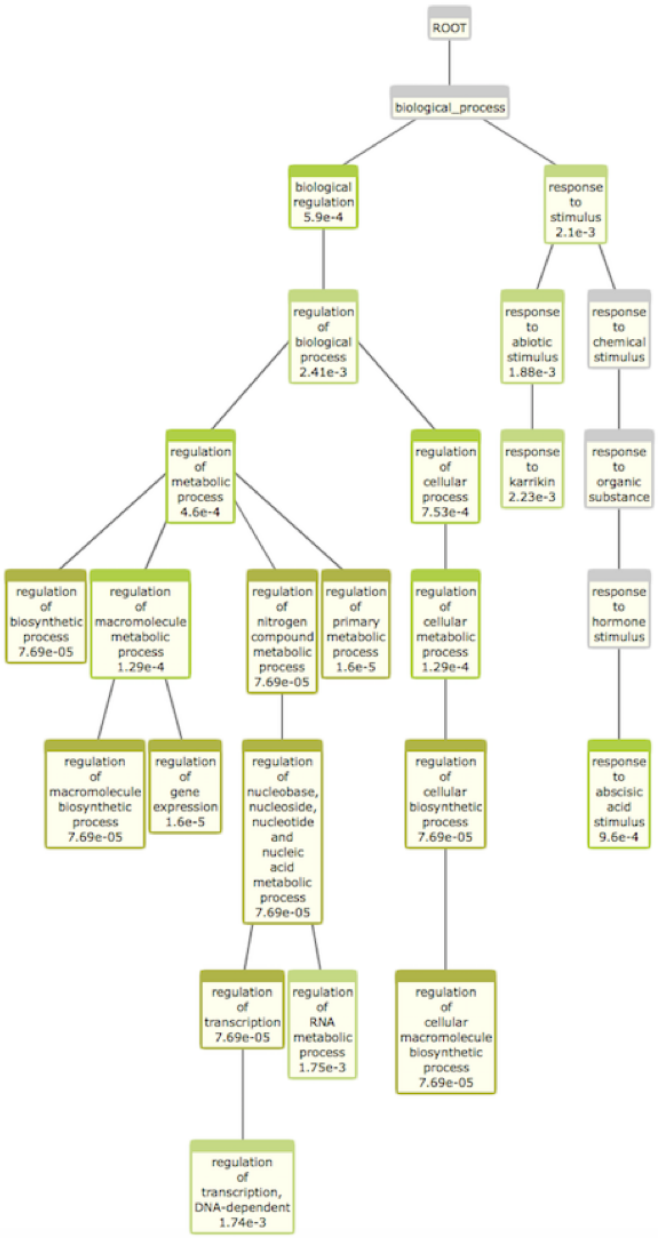
Supplemental Figure S3.7. Gene ontology analysis of the 473 genes differentially regulated in the wild type and not the *bpc1,2,3,4,6* mutant by cytokinin. VirtualPlant 1.3 software (Katari *et al.,* 2010). Background genome TAIR10, GO biological process assignments by TAIR/TIGR, Fisher exact test ($P < 0.01$).



Supplemental Figure S3.8. Expression of genes that are induced by cytokinin found in RNA-seq that re-tested using qPCR. Visualization of transcript levels of genes in the wild type and *bpc1,2,3,4,6* treated with cytokinin and BPC6 and ARR10 binding sites up-stream of the gene. Bedgraphs visualized in the Integrated Genome Browser (IGB) (Nowlan et al., 2016).



Supplemental Figure S3.9. Counts for the ten type-A ARRs in WT and the *bpc1,2,3,4,6* mutant with and without cytokinin treatment identified with RNA-seq. Average expression counts from three biological replicates sets in RNA-seq. Error bars represent SEM.



Supplemental Figure S3.10. Gene ontology analysis of the 61 genes differentially regulated in the WT and not the *bpc1,2,3,4,6* mutant by cytokinin and overlap with the ARR10 and BPC6 binding targets. VirtualPlant 1.3 software (Katari *et al.,* 2010). Background genome TAIR10, GO biological process assignments by TAIR/TIGR, Fisher exact test ($P < 0.01$).

Supplemental Figure S3.11. Overlap between genes differentially regulated in WT in response to cytokinin, BPC6 targets and ARR10 targets in response to cytokinin.

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CHAPTER 4. EXO70D PROTEINS ARE POSITIVE REGULATORS OF CYTOKININ SIGNALING AND INTERACT WITH TYPE-A ARR PROTEINS

Summary

The exocyst complex is an evolutionarily conserved multi-subunit protein complex that coordinates the tethering of vesicles to the plasma membrane. Exo70 is a subunit of this complex and the Exo70 gene family is highly expanded in plants. Exo70 proteins regulate multiple processes, such as protein trafficking, cell growth, response to pathogens, cell plate formation, and autophagy. We find that Exo70D3 interacts specifically with the phosphorylated form of the type-A Arabidopsis response regulator (ARR) proteins, which are negative regulators of cytokinin singling. The *exo70D1,2,3* mutant is less sensitive to cytokinin in root elongation assays, but does not regulate transcription of type-A ARR proteins or affect the activity of a cytokinin reporter in roots. Co-expression of Exo70D3 with type-A ARRs results in reduced levels of type-A ARR protein and disruption of the EXO70D genes results in an increased stability of ARR4 protein. Furthermore, *exo70D1,2,3* mutants are hypersensitive to carbon starvation, which is a phenotype linked to autophagy. Our results suggest a potential mechanism to regulate type-A ARR protein stability via the Exo70D proteins of the exocyst complex.

Introduction

Exocytosis is the process of Golgi-derived vesicle fusion with the plasma membrane (Zhang et al., 2010; Cvrčková, 2012). Plants rely on exocytosis for trafficking cellular components to the cell surface during cell growth and cell wall synthesis (Ebine and Ueda, 2015; Žárský et al., 2009; 2013). The exocyst is an evolutionarily conserved multi-subunit tethering complex that regulates vesicle fusion at the plasma membrane (Li et al., 2010; Zhang et al., 2010). The complex consists of eight sub-units that are all found in yeast, animal and plant cells (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) (Synek et al., 2006a; Boyd et al., 2004). Compared to animals and yeast, the Exo70 gene family is greatly expanded in plants (Chong et al., 2009; Li et al., 2010). For example, there are 23 Exo70 members in *Arabidopsis thaliana*, 41 in *Oryza sativa* (rice), 23 in *Populus trichocarpa* (poplar), and 13 in *Physcomitrella patens*

(moss) (Chong et al., 2009). Phylogenetic analysis groups the Exo70 gene family into three clades (Exo70.1, Exo70.2, and Exo70.3) and nine sub-clades (Exo70A-Exo70I) (Synek et al., 2006b; Chong et al., 2009; Li et al., 2010). In yeast, the Exo70 subunit is unique from the other members because it is involved in directed site-specific vesicle fusion at the plasma membrane (Boyd et al., 2004). The *Exo70* genes are expressed ubiquitously throughout Arabidopsis, and individual genes are expressed in specific and overlapping cell-types (Li et al., 2010). The Exo70 protein is a rod-like structure that interacts with phospholipids at the plasma membrane though residues at its C-terminus (Munson and Novick, 2006; Lavy et al., 2007; Žárský et al., 2009). Among the Exo70 genes in Arabidopsis, there is diversity in the amino acid sequence of the C-terminal residues that bind to phospholipids (Žárský et al., 2009). This sequence diversity suggests that there is potential for different combinations of lipid and protein interactions at the plasma membrane (Žárský et al., 2009).

Given that there are many *Exo70* gene family members in plants, that they are expressed in specific tissue-types, and there is diversity in the Exo70 lipid binding residues, previous studies proposed that the *Exo70* genes have plant-specific functions (Chong et al., 2009). To date, the Exo70 proteins have been linked to diverse plant processes (Žárský et al., 2013). For example, Exo70A1 is necessary for recycling of proteins at the plasma membrane (Drdová et al., 2013), vascular development (Li et al., 2013a), cell growth (Hala et al., 2008) and cell plate formation (Fendrych et al., 2010). The Exo70B1, Exo70B2, and Exo70H1 subunits regulate pathogen defense responses (Zhao et al., 2015; Pecenkova et al., 2011). Furthermore, the Exo70E2 subunit localizes to unique double membrane structures termed EXPO (for exocyst positive organelles) (Wang et al., 2011), and Exo70E2 is necessary to recruit other exocyst components to these EXPO structures (Ding et al., 2014). These organelles are likely involved in plant-specific cytosol to cell wall exocytosis (Ding et al., 2014; Wang et al., 2011).

Multiple studies also link the Exo70 proteins to autophagy and the formation of autophagasomes (Kulich et al., 2013; Tzfadia and Galili, 2014a; Cvrčková and Žárský, 2014). Autophagy is a eukaryotic process used to degrade cellular components in response to stress or in a house-keeping capacity to recycle nutrients (Michaeli et al., 2016). During this process, cellular components from the cytoplasm are engulfed into autophagasomes that then fuse with the lytic vacuole (Kulich et al., 2013; Michaeli et al., 2016; Xiang et al., 2013). In response to stresses such as carbon and nitrogen starvation, plants will

accumulate autophagasomes (Chung et al., 2010; Michaeli et al., 2016). Autophagy-related (ATG) proteins facilitate the formation of autophagasomes and ATG8-interacting motifs (AIMs) are found in the many Exo70 subunits in all plant lineages (Synek et al., 2006b; Cvrčková and Žárský, 2014). Specifically, significant enrichment of AIM motifs is found in the sequences of Exo70B, D, and E sub-clades (Tzfadia and Galili, 2014b; Cvrčková and Žárský, 2014). Studies also link the Exo70B1 subunit to autophagy as Exo70B1 co-localizes with ATG8 proteins in autophagasomes and fewer autophagic vesicles are internalized in the vacuole in an *exo70B1* mutant (Kulich et al., 2013). While the Exo70 genes have been linked to a variety of processes, the functions of many members of this family are unknown.

We identified Exo70D3, a member of the Exo70D sub-clade, as an interacting protein with a type-A Arabidopsis Response Regulator (ARR) in a yeast two-hybrid screen. Specifically, Exo70D3 interacted with a phosphomimic version of ARR5 ($ARR5^{D87E}$) that has a D to E mutation on the conserved aspartic acid phosphorylation site in its receiver domain (To et al., 2007), but it interacted weakly with the wild-type or non-phosphorylatable ($ARR5^{D87A}$) forms. This suggests that Exo70D3 interacts specifically with the phosphorylated ARR5 protein. The type-A ARRs are negative regulators of the plant hormone cytokinin that are phosphorylated on their receiver domain by Arabidopsis histidine-containing phosphotransfer (AHP) proteins in response to cytokinin signaling (Imamura et al., 1998; 1999; Lee et al., 2008). Cytokinin is a pleiotropic plant hormone that regulates many aspects of plant growth and development (Kieber and Schaller, 2014).There are two other members of the Exo70D sub-clade in Arabidopsis, *Exo70D2* and *Exo70D1* (Chong et al., 2009). *Exo70D3* and *Exo70D2* share high sequence similarity (76.5%), while *Exo70D1* is the least similar to the other two proteins (~65%)(Li et al., 2010).

There are no previous studies that specifically explore Exo70D function or show a link between the exocyst complex and cytokinin signaling. However, research of Exo70A1 found that this subunit is necessary for proper recycling of the auxin efflux carrier, PINFORMED1 (PIN1), at the plasma membrane (Drdová et al., 2013; Synek et al., 2006b). Cytokinin also regulates PIN1 trafficking. For example, cytokinin directs PIN1 to lytic vacuoles for degradation (Marhavý et al., 2011), and PIN1 levels are reduced after treatment with cytokinin (Zhang et al., 2011). Furthermore, in the type-A *arr3,4,5,6,7,8,9,15* mutant, PIN1 levels are lower and hypersensitive to cytokinin treatment (Zhang et al., 2011). If the Exo70D subunits function in a manner similar to Exo70A1, these findings suggest that the type-A ARRs

may coordinate with the Exo70D proteins to regulate PIN1 levels at the plasma membrane.

In this study, we analyzed the interaction between the type-A ARRs and Exo70D3 protein, characterized the role of *Exo70D* genes in cytokinin and auxin signaling, and explored the mechanism of this interaction.

Results

Exo70D3 interacts with type-A ARRs

We screened for proteins that interacted with an ARR5^{D87E} bait using the yeast two-hybrid screen. Three independent preys were identified that corresponded to the *ExoD3* gene. These ExoD3 prey clones interacted preferentially with the ARR5^{D87E} bait as compared to a wild type (ARR5^{WT}) or a non-phosphorylatable (ARR5^{D87A}) bait (Figure 4.1). In order to confirm this interaction, we used a bimolecular fluorescence complementation (BiFC) assay (Grefen et al., 2010; Kodama and Hu, 2012) in transiently transformed *Nicotiana benthamiana* leaf epidermal cells (Figure 4.2). Exo70D3 interacted specifically with the phosphomimic ARR5^{D87E} and not with wild-type ARR5 protein in this assay (Figure 4.2A), consistent with the results from the yeast two hybrid assay. Exo70D3 also interacted with a phosphomimic form of a second type-A ARR, ARR4 (ARR4^{D95E}), and interacted to a lesser extent with wild-type or phosphorylation-insensitive (ARR4^{D95A}) forms of this protein (Figure 4.2A). The interaction with AHP3 with the various forms of ARR4 was used as a positive control (Figure 4.2B). This data suggests that Exo70D3 interacts with multiple type-A ARR proteins and that phosphorylation of the aspartic acid residue of the receiver domain promotes this interaction.

Exo70Ds are positive regulators of cytokinin signaling

There are three members in the Exo70D sub-clade (*Exo70D1*, *Exo70D2*, and *Exo70D3*) (Figure 3) and previous studies revealed no severe phenotype in any of the three *exo70D3* single loss-of-function mutants (Synek et al., 2006a). We thus set out to generate multiple mutant lines to test for potential genetic redundancy among these Exo70D genes. Two independent T-DNA insertion lines were isolated for each gene (Figure 4.4A) and expression of the Exo70D genes examined in each mutant using RT-PCR. Two alleles of *Exo70D3* (*exo70D3-1* and *exo70D3-2*) (Figure 4.4B), one allele for *Exo70D2*

(*exo70D2-2*) (Figure 4.4C) and one allele for *Exo70D1* (*exo70D1-2*) (Figure 4.4D) were identified that lacked the full-length transcript for the cognate gene. Triple mutant lines harboring mutations for all three *Exo70D* genes were generated by crossing. Two triple mutant lines were created, *exo70D1,2,3-1* and *exo70D1,2,3-2*.

To determine if the *Exo70D* genes affect the response to cytokinin, we used primary root elongation assays with seedlings grown in the presence of increasing doses of the cytokinin benzyl adenine (BA) or an NaOH vehicle control (Figure 4.5A, B). Both the *exo70D1,2,3-1* and *exo70D1,2,3-2* mutants show reduced sensitivity to exogenous cytokinin, which suggests that these genes are positive regulators of cytokinin responsiveness (Figure 4.5A, B). Observations of the lateral roots in the *exo70D1,2,3* mutants suggested that there is a lateral root phenotype, however no statistically significant difference in emerged lateral root number was found between the mutants and wild type (Figure 4.5C). Qualitative observations also suggest a difference in the length of lateral roots and/or adventitious roots, however these were not measured. While the triple *exo70D1,2,3* mutants show reduced sensitivity to cytokinin signaling, the single and double *exo70D* mutants do not have this phenotype (Figure 4.5D, E). These results suggest that the Exo70D subunits genes act redundantly as positive regulators of cytokinin signaling.

To determine if the Exo70Ds regulate the transcriptional response to cytokinin, the expression of TCSn::GFP, a reporter for type-B ARR activity (Zürcher et al., 2013), was examined in the triple *exo70D1,2,3-1* and double *exo70D1,3-1* mutants (Figure 4.6A). No difference in TCSn::GFP signal was observed either in the absence or presence of cytokinin in either mutant (Figure 4.6B). Furthermore, expression of the type-A ARRs was not changed in the *exo70D1,2,3* mutant or in the *Exo70D3* overexpression mutant (*Exo70D3-OX-1*) (Figure 4.6C). These results suggest that the while the *exo70D1,2,3* mutants exhibit reduced sensitivity to cytokinin, they do not affect type-B ARR activity or the induction of cytokinin primary response genes.

Examination of Exo70D3:YFP in Arabidopsis roots reveals that Exo70D3 localizes to the cytoplasm (Figure 4.7A). This result is consistent with other studies that localize Exo70D1, Exo70D2, and Exo70D3 to the cytoplasm in transiently transfected Arabidopsis protoplasts (Ding et al., 2014). Furthermore, the interaction of Exo70D3 and the type-A ARRs also occurs in the cytoplasm of transiently

transformed tobacco epidermal cells (Figure 4.2A). While the *Exo70D3-OX-1* line has increased expression of *Exo70D3* in roots (Figure 4.7B), there is no cytokinin response phenotype in primary root elongation assays in the *Exo70D3-OX* plants (Figure 4.7C). Also, *Exo70D3* expression is not regulated by cytokinin (Figure 4.7B).

Exo70Ds regulate type-A ARR protein levels

Co-expression of Exo70D3 and type-A ARR5 protein in tobacco results in a reduction in the level of ARR5 protein (Figure 4.8A). Expression of ARR4^{D95E} phosphomimic protein is also reduced when co-expressed with Exo70D3 (Figure 4.8B). However, this is not observed when ARR4 is co-expressed with AHP3, suggesting that this is specific for co-expression with Exo70D3 (Figure 4.8B). One hypothesis to explain these results is that Exo70D3 regulates type-A ARR protein stability via the 26S proteasome. Infiltration of the transformed tobacco leaves with the 26S proteasome inhibitor (MG132) did not restore type-A ARR5^{WT} or ARR5^{D87E} protein levels, suggesting that the 26S proteasome may not be responsible for the degradation of ARR5 (Figure 4.8C) (Liu et al., 2010). To confirm these results, we introduced a transgene expressing an RFP:ARR4 fusion protein from the *UBQ10* promoter into the *exo70D1,2,3-2* mutant by crossing. There was a significant increase in the RFP:ARR4 signal in mutant roots as compared to the same transgene in a wild-type background (Figure 4.9A, B). However, protein and transcript levels of RFP:ARR4 must also be determined in these lines. While Exo70D3 may not target the type-A ARRs for degradation by the 26S proteasome, we explored other mechanisms that control protein degradation.

***exo70D1,2,3-2* is hypersensitive to carbon starvation**

A second possibility for how Exo70D proteins might regulate type-A ARR stability is through a role in autophagy, as other Exo70 isoforms have been linked to this process. We hypothesize that the Exo70D proteins may regulate autophagasome formation and may target type-A ARRs for degradation in the vacuole. To determine if the Exo70D proteins and cytokinin signaling is linked to autophagy, we performed carbon starvation assays on *exo70D1,2,3* and cytokinin signaling mutants (Thompson et al., 2005). The *exo70D1,2,3* mutant is hypersensitive to carbon starvation as after seven days of growth in

the dark followed by a one week recovery in light the mutant plants show much more extensive leaf lethality as compared to the wild-type (Figure 4.10A, B). Hypersensitivity to carbon starvations is a phenotype observed in other mutants affecting autophagy (Chung et al., 2010; Thompson et al., 2005). To test the role of cytokinin in autophagy, we examined the response of the *ahk2,3* and *arr3,4,5,6,7,8,9,15* mutant in the assay. While both mutants showed some yellowing of the leaves, all plants survived the treatment, similar to the response of the wild type. These results suggest that the Exo70D genes may be positive regulators of autophagy, but suggest that cytokinin is not a major regulator of this process, at least in the conditions tested here.

PIN1 and auxin regulation in *exo70D* mutants

Next, we examined the link between Exo70Ds and auxin signaling as prior studies have linked other EXO70s to PIN function PIN1:GFP levels in the *exo70D1,2,3* mutant were compared to those found in wild type and *arr3,4,5,6,7,8,9,15* mutant (Zhang et al., 2011) using confocal microscopy. The levels of PIN1:GFP in the absence of exogenous cytokinin were slightly reduced in the *exo70D1,2,3* mutant, to similar levels to that observed in the *arr3,4,5,6,7,8,9,15* mutant (Figure 4.11A, B). However, in response to cytokinin treatment there was not a significant difference in PIN1 levels in the *exo70D1,2,3* mutant compared to wild-type roots, while in the *arr3,4,5,6,7,8,9,15* mutant, PIN1:GFP levels were rescued to a significantly greater extent in response to cytokinin, consistent with previous results (Zhang et al., 2011) (Figure 4.11A, B). The expression of the auxin signaling reporter DR5::GFP was also examined in *exo70D1,2,3* mutants (Figure 4.12). Compared to the wild-type expression pattern DR5-GFP expression in the *exo70D1,2,3* mutant exhibited a slight increase in the signal at the quiescent center (QC). To determine if the increase in the QC is significant, quantification of the signal in specific cell types needs to be conducted.

Discussion

We hypothesize that Exo70D3 interacts with type-A ARRs to regulate type-A ARR protein levels in the cell. The Exo70D3 proteins specifically interact with phosphorylated version of the type-A ARR proteins, indicating that the interaction is regulated by cytokinin signaling and the phosphorylation state of the type-A ARR protein. Co-expression of Exo70D3 with type-A ARR proteins in tobacco leads to reduced

type-A ARR protein levels. Although increased Exo70D3 protein may reduce type-A ARR protein stability, the interaction between the type-A ARRs and Exo70D3 is observed in BiFC assays, perhaps because these fusion proteins are not degraded or the residual proteins provide sufficient signal for a positive interaction. If Exo70Ds promote type-A ARRs turnover, then in a *exo70D1,2,3* mutant there should be more type-A ARR protein. The cytokinin response phenotypes of the *exo70D1,2,3* mutant fits this model because these mutants show reduced sensitivity to cytokinin signaling. The same phenotype is observed in type-A ARR overexpression mutants that have increased type-A ARR protein levels (To et al., 2007). We observed increased RFP:ARR4 protein in the *exo70D1,2,3* mutant compared to wild type, which indicates that the protein is more stable in the absence of Exo70D proteins. Furthermore, the *exo70D1,2,3* and *Exo70D3-OX-1* mutants do not affect the transcriptional response to cytokinin. Elevated type-A ARR levels should increase TCSn::GFP signal. We may not observe these differences because the increase in type-A ARR protein may not be enough to observe differences in transcription. One possible mechanism for how Exo70Ds regulate type-A ARR protein is through the 26S proteasome. However, tobacco experiments with MG132 suggest that this is not the mechanism because addition of MG132 does not restore type-A ARR5 protein levels. Another mechanism through which the Exo70Ds could regulate type-A ARR proteins stability is autophagy. The *exo70D1,2,3* mutant was hypersensitive to carbon starvation after seven days of dark treatment, which is similar to the phenotype of *atg* mutants that show sensitivity to starvation after four days of dark treatment (Thompson et al., 2005; Honig et al., 2012). This suggests that the Exo70Ds may be similar to Exo70B1 and also regulate autophagasome formation and potentially recruit type-A ARRs to autophagic vesicles for degradation. Alternatively, the role of the Exo70Ds in autophagy may be independent of their regulation of type-A ARR proteins. Future research is required to determine if type-A ARRs are engulfed in autophagic vesicles and if Exo70Ds are necessary to target the type-A ARRs to those vesicles.

We also explore the role of the Exo70D3 protein interaction with type-A ARRs in the regulation of PIN1 transport and auxin signaling. However, we do not see substantial defects in expression of PIN1:GFP nor in the pattern of DR5:GFP expression in *exo70D1,2,3* mutants. While Exo70A1 is linked to regulating PIN1 trafficking, the Exo70D sub-clade is quite distant to the Exo70A sub-clade and the members of these clades likely have different functions. However, the role of exo70Ds and auxin

regulation can be explored further, as we observed a slight decrease in the basal levels of PIN1:GFP in the *exo70D1,2,3* mutant and a possible increase in DR5:GFP signal in the quiescent center. In support of the observations in the quiescent center, expression studies for promoter GUS fusions of Exo70D3 and Exo70D2 localize the expression of these proteins to the QC or areas surrounding the QC, while Exo70D1 localizes to the meristematic region above the QC (Li et al., 2010).

In this study, we demonstrate that the type-A ARRs interact with Exo70D3 and that this interaction may regulate type-A ARR stability. We also identify a possible mechanism of this regulation through the transport of type-A ARRs to autophagic vesicles by the Exo70D proteins. Other studies previously explored how type-A ARR proteins are degraded. For example, type-A ARR4 is specifically targeted for degradation by the protein degradation of periplasmic proteins 9 (DEG9) (Chi et al., 2016), and type-A ARR5 proteolysis is regulated by, AXR1, a subunit of the E1 enzyme in the RUB (related to ubiquitin) modification pathway (Li et al., 2013b). Given that the type-A ARRs are primary cytokinin response genes and negatively regulate the cytokinin signaling pathway, it is not surprising that there are multiple mechanisms in place to regulate the amount of type-A ARRs present in the cell.

Materials and methods

Plant materials and growth conditions

All Arabidopsis lines used in these studies are in the *Arabidopsis thaliana* Col-0 ecotype. The T-DNA alleles for *exo70D3-2* (Sail_175_D08), *exo70D2-2* (WiscDsLox450H08)*, exo70D1-1* (SALK_067709), and *exo70D1-2* (SALK_074650) were ordered from the ABRC stock center, and *exo70D3-1* (GABI_747E03), *and exo70D2-1* (GABI_305E06) were ordered from GABI-Kat (Table 1). Each line was crossed to make multiple mutants between alleles. The *exo70D3-2* allele was previously used (Synek et al., 2006a). The *exo70D3-2 exo70D2-2 exo70D1-2* and *exo70D3-2 exo70D2-2 exo70D1-2* triple mutants were crossed with the following lines, TCSn::GFP reporter for type-B ARR activity (Zürcher et al., 2013), DR5::GFP reporter for auxin signaling (Friml et al., 2003), *PIN1::PIN1:GFP* (Benková et al., 2003), and *pUBQ10::RFP:ARR4*. The *pUBQ10::EXO70D3:YFP* overexpression mutant lines was created using Exo70D3 cDNA and pUBC-YFP-dest vector and the *pUBQ10::RFP:ARR4* tagged lines was created with ARR4 cDNA inserted into the pUBN-RFP-dest vector (Grefen et al., 2010). Vectors

for Arabidopsis transformation were inserted into the *Agrobacterium* strain GV3101 using electroporation (Koncz and Schein, 1986). Each construct was transformed into Col-0 Arabidopsis using the previously described floral dip method (Clough and Bent, 1998). The T1 seedlings were selected on ½ MS plates (½ Murashige and Skoog basal medium, 1% sucrose, 0.6% phytogel, pH 5.8) supplemented with 50µg/mL BASTA. The single insertion lines were selected by determining a 3:1 Mendelian ratio of T2 seedlings. Insertion was confirmed with microscopy, westerns, or PCR. Seedlings were surface sterilized with 50% bleach, 0.2% Triton-X-100, washed four times with water, and plated on square ½ MS plates (½ Murashige and Skoog basal medium, 1% sucrose, 0.6% phytogel, pH 5.8). The seedlings were kept in the dark for 3 days at 4°C, and then transferred to 24-hour light.

Cloning and vector construction

The coding sequence for the *Exo70D3*, *Exo70D2*, and *Exo70D1* by PCR from cDNA libraries derived from *Arabidopsis thaliana* Col-0 seedlings. For primer sequences see supplemental Table 2. PCR products with TOPO cloning sites were inserted into the gateway pENTR/D-TOPO vector using the pENTR™ Directional Topo Cloning Kit (Thermo Fisher Scientific). The type-A ARR entry vectors used were previously described (To et al., 2007). The Gateway LR Clonase II Enzyme Mix (Thermo Fisher Scientific) was used to transfer the coding sequence into destination vectors. Constructs were transformed into either DH5α™ Competent Cells or One Shot® TOP10 Chemically Competent cells (Thermo Fisher Scientific). Vectors were verified with restriction enzyme digestion and sequencing. For bimolecular fluorescence complementation (BiFC), the destination vectors, pUBC-cYFP-dest and pUBC-nYFP-dest were used to express the corresponding proteins (Grefen et al., 2010). For co-expression analysis, the binary vectors pEarleyGate201 (35S promoter N-terminal HA tag) and pEarleyGate203 (35S promoter N-terminal myc tag) were used to express the corresponding protein (Earley et al., 2006)

Transient transfection assays in *Nicotiana benthamiana*

Agrobacterium tumefaciens carrying the appropriate constructs were transformed into epidermal leaf cells of *Nicotiana benthamiana* following the protocol adapted from

(Yang et al., 2000) and as described in chapter 3. The leaves were incubated for three days at 24-hour light. An OD_{600} of 0.2 for each *Agrobacterium* carrying the corresponding construct were infiltrated *into N. benthamiana* epidermal leaf cells as described above. Three days later the interaction was observed with a fluorescence signal using confocal microscopy. The *Agrobacterium* strain GV3101 was used for all transformations (Koncz and Schein, 1986). For co-expression assays with MG132 incubation, 40μM of MG132 was infiltrated into tobacco leaves 12 hours before tissue was collected (Liu *et al.*, 2010). The tissue was collected by flash freezing in liquid nitrogen. Tissue was ground on ice with a mortar and pestle and total protein extracts were isolated and suspended in lysis buffer (150mM NaCl, 1% Triton. X-100, 50mM Tris HCl pH 8.0 and 1x protease inhibitor cocktail, cOmplete™ ULTRA Tablets, Mini, EDTA-free, *EASYpack* Protease Inhibitor Cocktail (sigmaaldrich.com, St Louis, MO). The lysate was spun down and supernatant was boiled with SDS buffer (50mM Tris HCl (pH 6.8), 50mM DTT, 1% SDS, 1 mM EDTA, 0.005% bromphenol blue, 10% glycerol) and run for western blotting with the same HA and myc antibodies as described in Chapter 3 methods.

Microscopy

Protein localization in Arabidopsis root tips and epidermal cells of *N. benthamiana* leaves was visualized using the Carl Zeiss LSM 710 scanning confocal microscope with 40XW C-APO and 10X Plan APO NA 0.45 objectives (http://www.zeiss.com/). To detect the YFP signal, a 514nm line was used for excitation, and an emission range between 505 and 530nm was used for detection. GFP was detected using a 488nm line for excitation, and an emission range between 493 and 532 nm was used for detection. Fluorescent images of TCSn::GFP reporter lies were taken with the Nikon Eclipse 80i microscope. GFP and YFP reporter constructs were analyzed at the same time using identical microscope settings throughout imaging. Images were formatted and fluorescence intensity was quantified with FIJI software (Schindelin *et al.*, 2012).

Root elongation assay

Seedlings were surface sterilized as described above and plated on square ½ MS plates (½ Murashige and Skoog basal medium, 1% sucrose, 0.6% phytogel, pH 5.8). The seedlings were kept in

the dark for 3 days at 4°C, and then transferred to 24-hour light for 4 days and set vertically. At day 4 the seedlings were transferred to plates supplemented with 5N NaOH as a vehicle control or BA (25nM or 50nM). The end of the root was marked on each plate with a marker. Seedlings were grown vertically in the light chamber for 5 more days. Scans for each plate were take on day 9, 10 and 13. Root length was measured using FIJI software (Schindelin *et al.*, 2012). The one-way ANOVA and Tukey HSD tests were conducted in R.

Reverse transcription PCR (RT-PCR)

To check expression of *exo70D3*, *exo70D2*, and *exo70D1* transcript in the T-DNA lines, total RNA was extracted from frozen tissue and cDNA was synthesized as previously described in chapter 3. PCR reaction was made with Taq DNA Polymerase 2x Master Mix RED with cDNA as the template and the primers used to check expression are outlined in Table 2.

Quantitative real-time PCR (qPCR)

To check expression of type-A ARRs in the overexpression lines and *exo70D* loss-of-function mutants, 10 day old seedlings were treated with 5µm of cytokinin or NaOH as a vehicle control in 30mL of ½ MS media (½ Murashige and Skoog basal medium, 1% sucrose, pH5.8) in 50mL conical tubes with gentle shaking for 1 hour with three biological replicates. Seedlings were separated between roots and shoots with a razor blade and flash frozen in liquid nitrogen. The RNA was extracted and cDNA was synthesized as previously described in chapter 3. The real-time PCR reaction and fold change analysis used is previously described in chapter 3. A complete list of primers is found in Table 2 and *GAPDH* was used as a housekeeping gene.

Carbon starvation assay

Method adapted from (Thompson et al., 2005). Seedlings were surface sterilized and plated on vertical plates, stratified in the dark for 2 days at 4°C, and then transferred to 24-hour light for 10 days and set vertically. The seedlings were planted into soil and transferred to growth chambers set for short day conditions with 8-hour light and 16 hours dark at 22°C. Plants were grown until they were six weeks

old, and then transferred to a dark growth chamber at 22°C for 2, 4, or 7 days and then plants recovered for 1 week in light conditions.

Table 4.1. Primers used for genotyping and insertion of T-DNA lines.

Gene	Allele	T-DNA line	Primer Name	Sequence (5' to 3')	Insertion site CDS bp from ATG	Insertion site amino acid
At3g14090	*exo70D3-1*	GABI_747E03	GABI_747E03_LP	AATGCAAGACCACAATCAACC	909	S303
			GABI_747E03_RP	TCTCTTCTTCTTCCTCCTCCG		
			GABI-T-o8760	GGGCTACACTGAATTGGTAGCTC		
At3g14090	*exo70D3-2*	Sail_175_D08	SAIL_175_D08_LP	CGTCTTTAACCGTCGAAGTTG	1060	G353
			SAIL_175_D08_RP	CTGACCCATGTAGCTCTCTGG		
			LB3-SAIL_BP	TAGCATCTGAATTTCATAACCAATCTCGATACAC		
At1g54090	*exo70D2-1*	GABI_305E06	GABI_305E06_LP	ATGGAGGTAGTGTCGTCCATG	NA - 5'UTR	NA - 5'UTR
			GABI_305E06 RP	GTTGCTTTGACTTCATCGGAG		
			LBb1.3_BP	ATTTTGCCGATTTCGGAAC		
At1g54090	*exo70D2-2*	WiscDsLox450H08	WiscDsLox450H08_LP	CGCATTCTCAAACTCGGTAAG	320	L106
			WiscDsLox450H08_RP	ATTCTGATTATGGCAACACCG		
			Wisc-p745_BP	AACGTCCGCAATGTGTTATTAAGTTG		
At1g72470	*exo70D1-1*	SALK_067709	SALK_067709_LP	TCCAAAATTTAGAAAACTTTTGCTG	NA - 5'UTR	NA - 5'UTR
			SALK_067709_RP	TACTTGCACTTGAACCGGATC		
			lBb1.3_BP	ATTTTGCCGATTTCGGAAC		
At1g72470	*exo70D1-2*	SALK_074650	SALK_074650_LP	GGATCGTCTTCTCAGATGCTG	1275	F425
			SALK_074650_RP	TGCTCTGTGAGCATGTTTTTG		
			lBb1.3_BP	ATTTTGCCGATTTCGGAAC		

Table 4.2. Primers used cloning and RT-PCR and RT-qPCR.

Use	Primar name	Primar sequence
RT-PCR*exo70D3-2* and *exo70D3-1*	SAIL_175_D08_LP-N	CGTCTTTAACCGTCGAAGTTG
RT-PCR*exo70D3-2* and *exo70D3-1*	SAIL_175_D08_RP-N	CTGACCCATGTAGCTCTCTGG
RT-PCR for *exo70D2-1*	GABI_305E06_LP-RT	GAAGGCGAATTTTGATTTCTT
RT-PCR for *exo70D2-1*	GABI_305E06 RP	GTTGCTTTGACTTCATCGGAG
RT-PCR for *exo70D2-2*	WiscDsLox450H08 LP	CGCATTCTCAAACTCGGTAAG
RT-PCR for exo70D2-2	WiscDsLox450H08 RP	ATTCTGATTATGGCAACACCG
RT-PCR for exo70D1-2	SALK_074650_LP	GGATCGTCTTCTCAGATGCTG
RT-PCR for exo70D1-2	SALK_074650_RP	TGCTCTGTGAGCATGTTTTTG
RT-PCR actin	Actin8_F	GCGGTTTTCCCCAGTGTTGTTG
RT-PCR actin	Actin8_R	TGCCTGGACCTGCTTCATCATACT
RT-qPCR of *Exo70D3*	D3-1_F	GAACTGTTCGCAAATCAGCCA
RT-qPCR of *Exo70D3*	D3-1_R	GCAGTGCAAATACCATCAAAG
RT-qPCR of *ARR5*	rtARR5f	TCTGAAGATTAATTTGATAATGACGG
RT-qPCR of *ARR5*	rtARR5r	TCACAGGCTTCAATAAGAAATCTTCA
RT-qPCR of *ARR7*	RTARR7r2	TCAAATTCACCTTCAAATCCT
RT-qPCR of *ARR7*	RTARR7f2	TCTCTTCTTGTAAAGTGACGACTG
RT-qPCR of *AT1G13440*	GAPDH.RT.F	AGGCCATCAAGGAGGAATCT
RT-qPCR of *AT1G13440*	GAPDH.RT.rc	GAAAATGCTTGACCTGTTGTCAC
Cloning Exo70D3 CDS	EXO70D3-GWF	CACCATGGAACCGCCGGAGAATAGTT
Cloning Exo70D3 CDS	EXO70D3-GWRn	TCGCCTCCTCAAGTGTGGTG
Cloning Exo70D3 CDS	EXO70D3-GWR-wstop	TTATCGCCTCCTCAAGTGTGGTG
Cloning Exo70D2 CDS	EXO70D2-GWF	CACCATGGCAACACCGGAGAC
Cloning Exo70D2 CDS	EXO70D2-GWRn	CTGAGACCGTCTCAAATGTG
Cloning Exo70D2 CDS	EXO70D2-GWR-wstop	TCACTGAGACCGTCTCAAATGTGGG
Cloning Exo70D1 CDS	EXO70D1-GWF-N	CACCATGGAACCACATGACCAAACTCACG
Cloning Exo70D1 CDS	EXO70D1-GWRn-N	CTCGGATCGTCTTCTCAGATGCTGA
Cloning Exo70D1 CDS	EXO70D1-GWR-wstop-N	TCACTCGGATCGTCTTCTCAGATGC
Sequencing Exo70D3	EXO-S1	AAGCAGTTACAGATCCACTAGC
Sequencing Exo70D3	EXO-S2	GAGATTCAATCGAGGTTAGCGGAAG
Sequencing Exo70D3	EXO-S3	AGTGGGAGTTTCTCCTCAGGTG
Sequencing Exo70D2	EXOD2-S1	ATGATCGGAGCTGGTTACTCTC
Sequencing Exo70D2	EXOD2-S2	ACTATACATCCATTGACAAGGTATG
Sequencing Exo70D2	EXOD2-S3	GGAAGCTTCTCTTCCGGTGTAT
Sequencing Exo70D1	EXOD1-S1	GTAGTATACGCGAGATCGAGCT
Sequencing Exo70D1	EXOD1-S2	GATTCGATTAGAGTTCAAGCGG
Sequencing Exo70D1	EXOD1-S3	GACGTATTACCAAAGAGCTGCTT

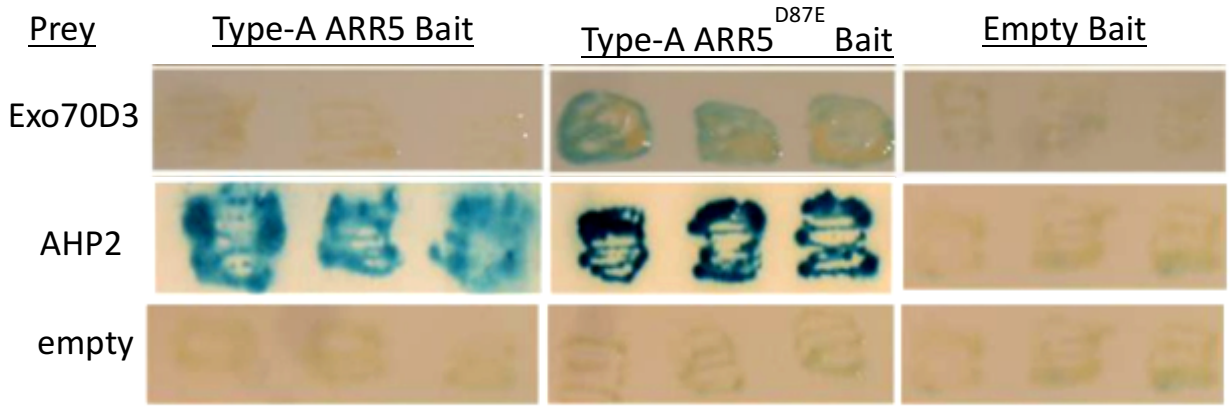

Figure 4.1. Exo70D3 interacts with ARR5^{D87E}. Yeast two-hybrid with LexA DNA-binding domain tagged ARR5 and ARR5^{D87E} as bait and GAL4 activation domain as prey tagged with Exo70D3. In this screen, AHP2 was identified as a positive control and empty bait and prey vectors were used as a negative control.

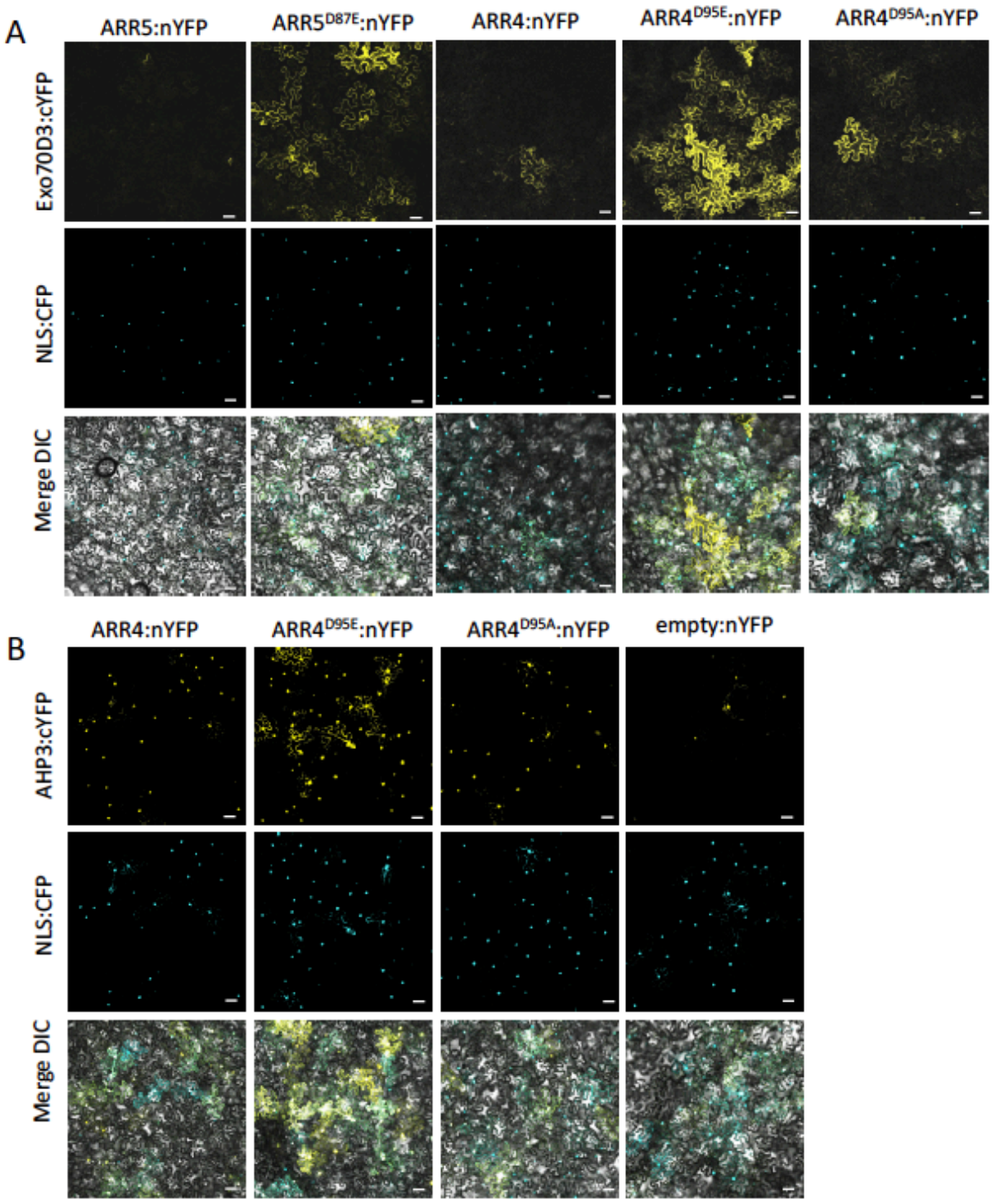

Figure 4.2. Exo70D3 and AHP3 interaction with type-A ARRs. **(A, B)** Bimolecular fluorescence complementation (BiFC) in *Nicotiana benthamiana* epidermal leaf cells. Protein expression were driven by the *Arabidopsis thaliana pUBQ10* promoter. *pUBQ10::NLS:CFP* was used as a transformation control. Confocal microscopy used to image YFP and CFP. Scale bar = 50μM. **(A)** *Exo70D3-cYFP* co-transformed with ARR4 (ARR4, ARR4^{D95E}, and ARR4^{D95A}) or ARR5 (ARR5, and ARR5^{D87E}) fused to nYFP at the c-terminus. **(B)** *AHP3-cYFP* co-transformed with ARR4 (ARR4, ARR4^{D95E}, and ARR4^{D95A}) or empty fused to nYFP at the c-terminus.

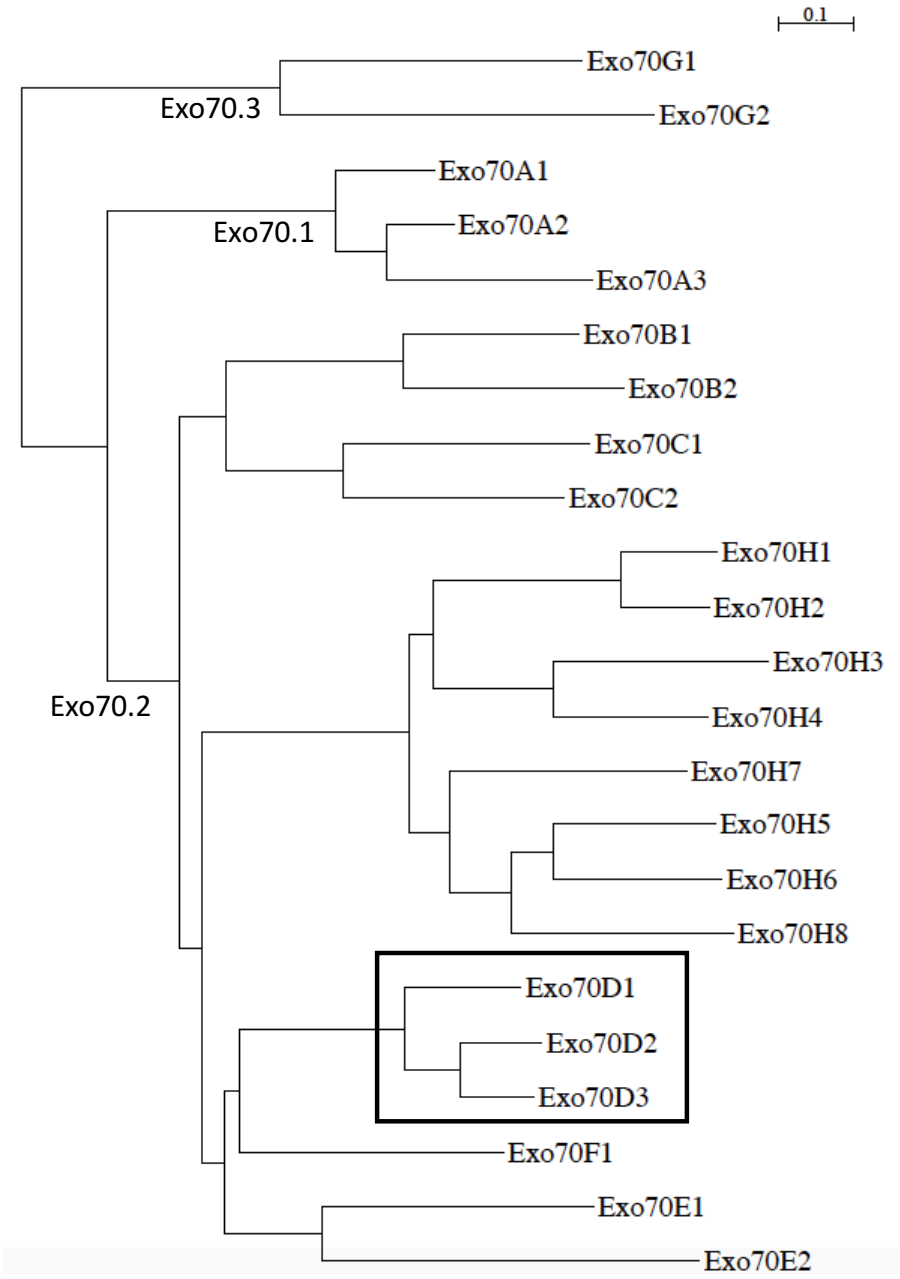

Figure 4.3. Phylogenetic tree of Exo70 gene family in *Arabidopsis thaliana***.** Phylogenetic treat of the 23 members of the Exo70 gene family in *Arabidopsis thlaiana* made with full length Exo70 amino acid sequences was created using the Clustal X program. The rooted phylogenetic tree from this alignment was created using the neighbor joining algorithm in the Seaview software with 1000 bootstrapping replicates. The scale bar represents 0.1 amino acid substitutions per site. In plants the Exo70 gene family consists of three clades (Exo70.1, Exo70.2, and Exo70.3 as indicated) and nine sub-clades A-J. There are no J members in *Arabidopsis*. The Exo70D clade is outlined with the box.

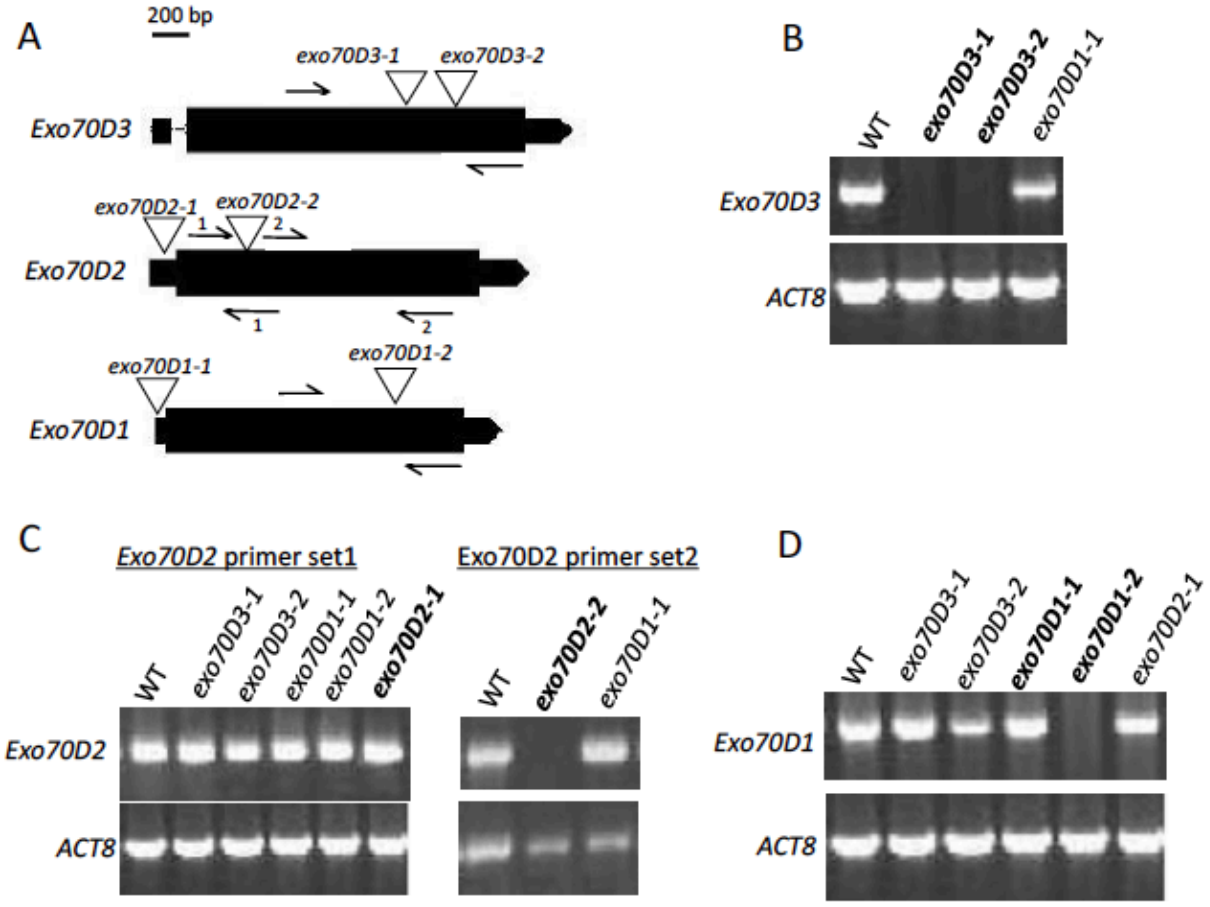

Figure 4.4. Exo70D mutant T-DNA alleles. **(A)** Diagram depicting T-DNA insertion sites in the Exo70D genomic regions of the each gene. The arrows indicate location of primers to check expression of each insertion line. **(B-D)** Reverse transcriptase PCR from cDNA extracted from whole seedlings of each mutant line. Expression of *ACT8* was used a control for cDNA inegrity. **(B)** Expression of *Exo70D3* examined in *exo70D3-1* and *exo70D3-2* loci using primers spanning the T-DNA insertion with WT and *exo70D1-1* as a positive control. **(C)** Expression of *Exo70D2* examined in *exo70D2-1* and *exo70D2-2* with two different primer sets as indicated in A, with WT and other lines as positive controls. **(D)** Expression of *Exo70D1* in *exo70D1-1* and *exo70D1-2* lines with WT and other lines as positive controls.

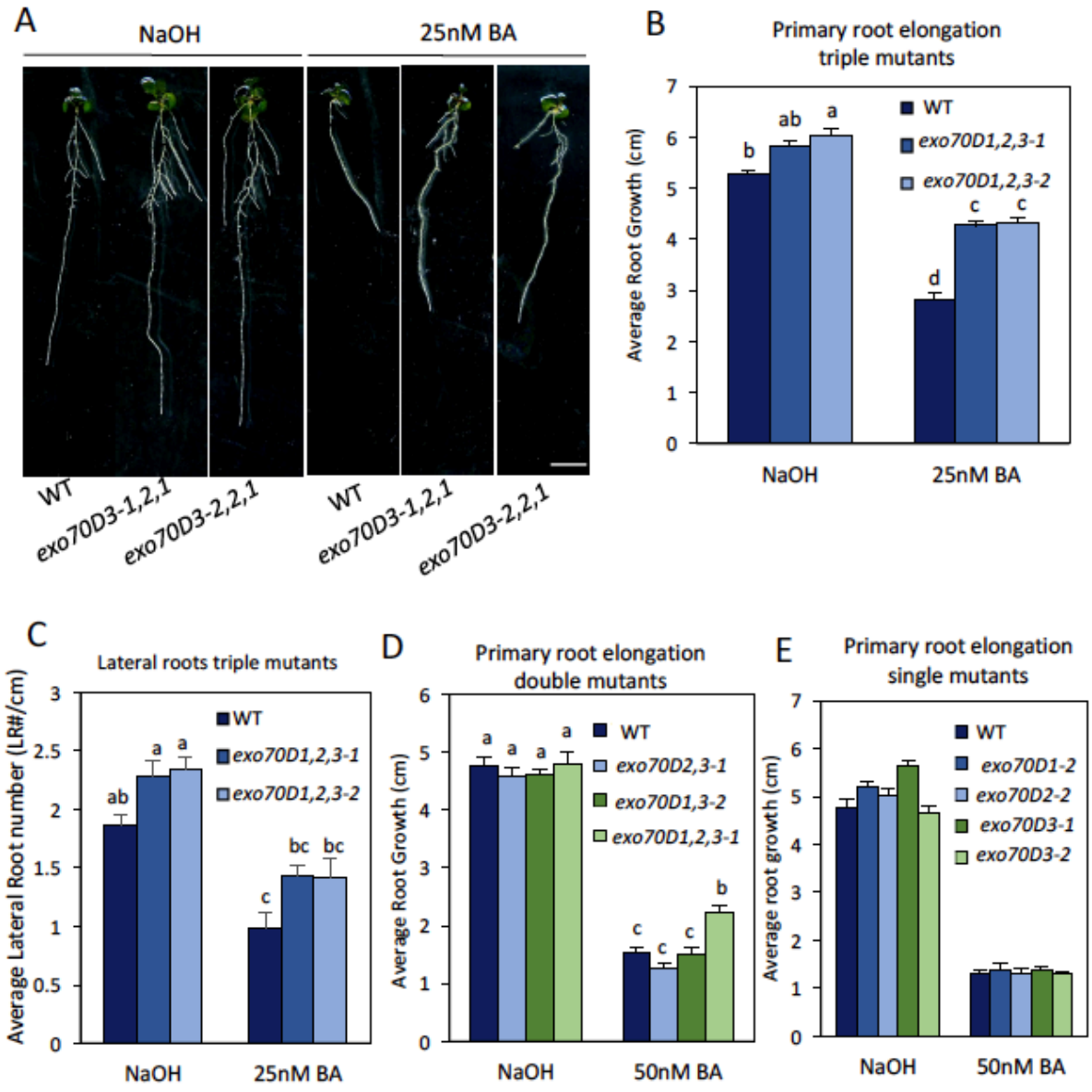

Figure 4.5. *exo70D1,2,3* mutants show reduced sensitivity to cytokinin treatment. **(A-E)** Seedlings were grown on ½ MS media and after 4 days transferred to benzyl-adenine (BA) supplemented plates or 0.01% 5N NaOH plates as a vehicle control for 6 days. Statistical testing with a one-way ANOVA followed by Tukey HSD post hoc test $p < 0.01$. Different letters indicate significance between groups. Error bars (SEM, n=12) **(A)** Representative images of seedlings scanned on plates at day 10 with 25nM BA **(B)** Average root measurements were taken from day 4-10 of 25nM BA treatment. **(C)** Average of lateral root number per cm of root growth from day 4-10 after 25nM BA treatment. **(D)** Average root growth from day 4-10 of *exo70D* double mutants and *exo70D1,2,3-1* mutants as a control at 50nM Ba treatment. **(E)** Average root growth for *exo70D* single mutants at 50nM BA treatment.

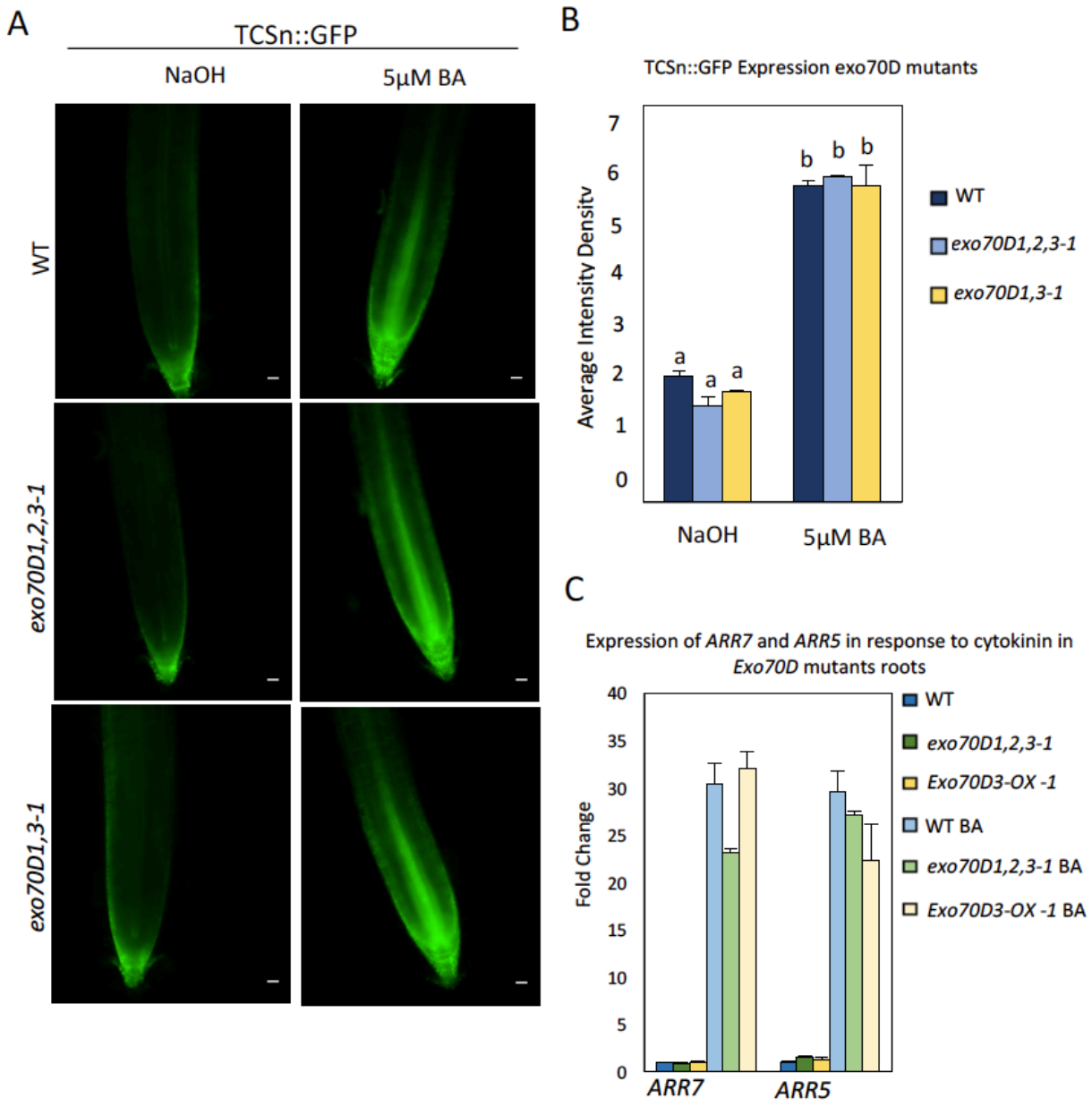

Figure 4.6. Cytokinin signaling responses in *exo70D* mutants. **(A)** *exo70D* mutants crossed with TCSn::GFP type-B ARR reporter (Zurcher et al., 2013). Seedlings were grown on ½ MS media for 4 days and then transferred to ½ MS plates with 5µM of BA or 0.01% NaOH control plates for 24 hour. GFP images Scale bar = 4.3 mm. **(B)** Intensity of the GFP signal from A was determined with FIJI imagine software. Statistical analysis with one-way ANOVA, followed by Tukey HSD post hoc test $p < 0.05$. Error bars are SEM of average intensity, n=5. **(C)** qPCR for expression of type-A *ARR5* and *ARR7* in response to cytokinin in 10-day-old seedlings of *exo70D1,2,3-1*, *Exo70D3-OX-1*, and WT after 1 hour treatment with 5µM BA of 0.01% 5N NaOH. Average fold change of three biological replicates. Error bars are SEM.

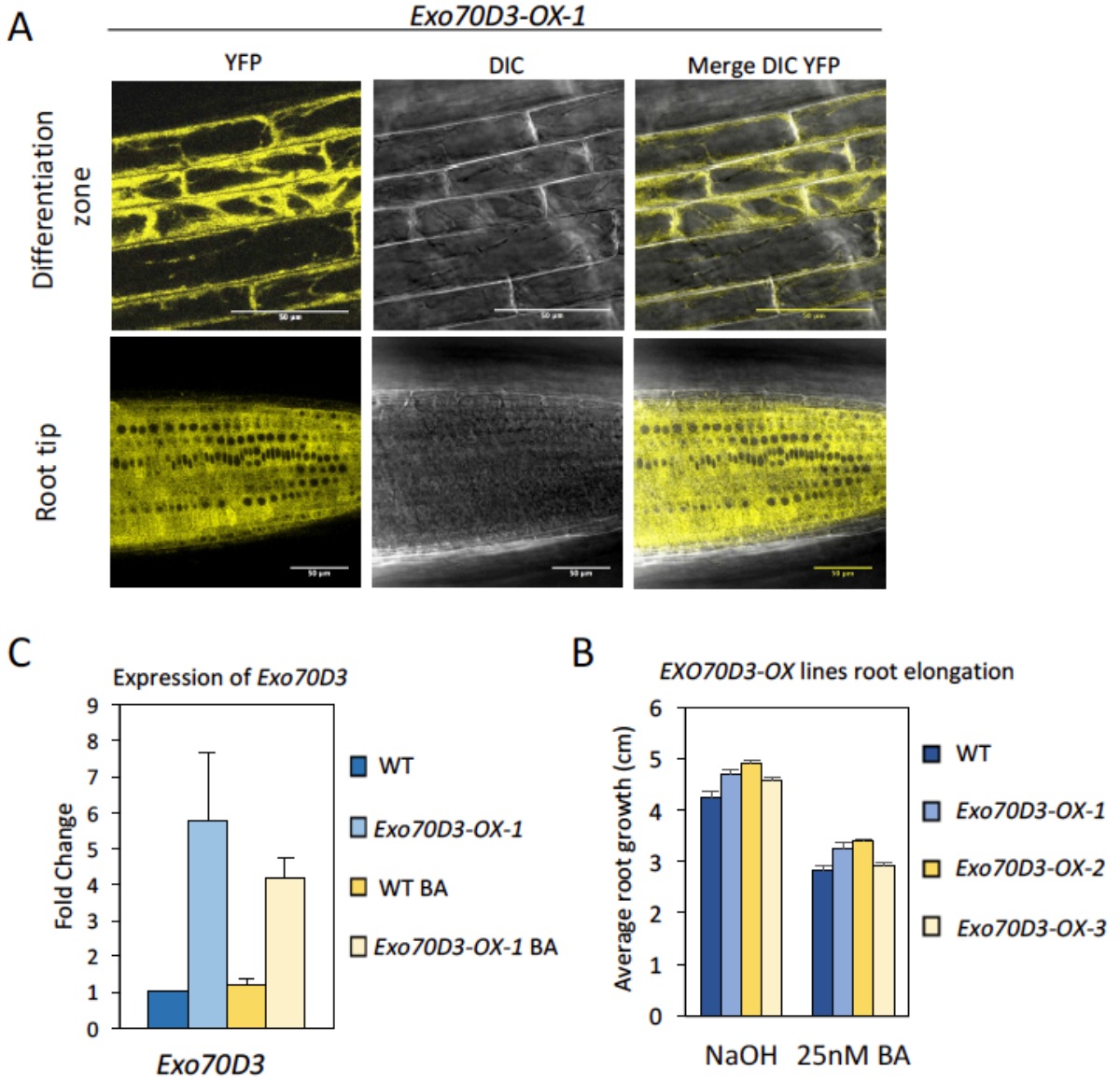

Figure 4.7. *EXO70D3* overexpression mutants. **(A)** Confocal images of *EXO70D3:YFP* expression in Arabidopsis roots, driven by the *pUBQ10* promoter. Scale bar = 50µm. **(B)** qPCR for expression levels of *Exo70D3* in Arabidopsis roots treated with 5µM BA or 0.01% 5N NaOH for 1 hour**.** Average of three biological replicates. Error bars represent SEM. **(C)** Root elongation assay of multiple *pUBQ10::EXO70D3:YFP* overexpression lines. Seedlings grown on ½ MS plates for 4 days were transferred to 25nM BA plates or 0.01% 5N NaOH control plates and grown for 6 days after root length was measured. Average root length is graphs with error bars representing SEM, n=12

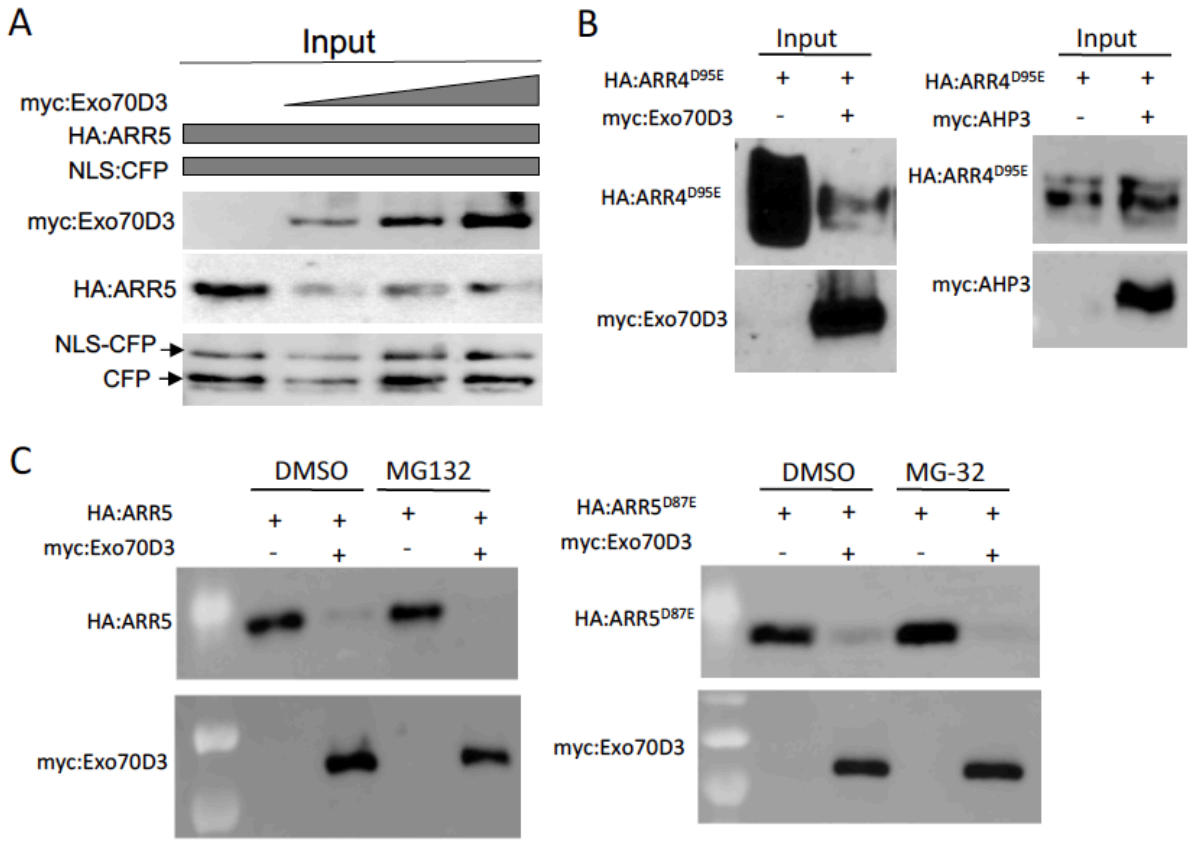

Figure 4.8. Co-expression of Exo70D3 and type-A ARRs in *Nicotiana benthamiana*. **(A-C)** Expression of each protein was driven by 35S promoter with N-terminal HA or myc tag. *N. benthamiana* was infiltrated in the epidermal leaf cells with the corresponding constructs and tissue was collected three days later. Blots were probed with anti-HA and stripped and re-probed with anti-myc **(A)** Co-expression with increased amounts of Exo70D3 construct and a stable amount of ARR5 and NLS:CFP as a control for expression. **(B)** Co-expression with equal amounts of either ARR4 and Exo70D3 (left) or ARR4 and AHP3 (right). **(C)** ARR5 and Exo70D3 (left) or ARR5^{D87E} and Exo70D3 were co-infiltrated into leaves. 12 hours prior to tissue collection, leaves were infiltrated with 20μM of MG132, 26S proteasome inhibitor.

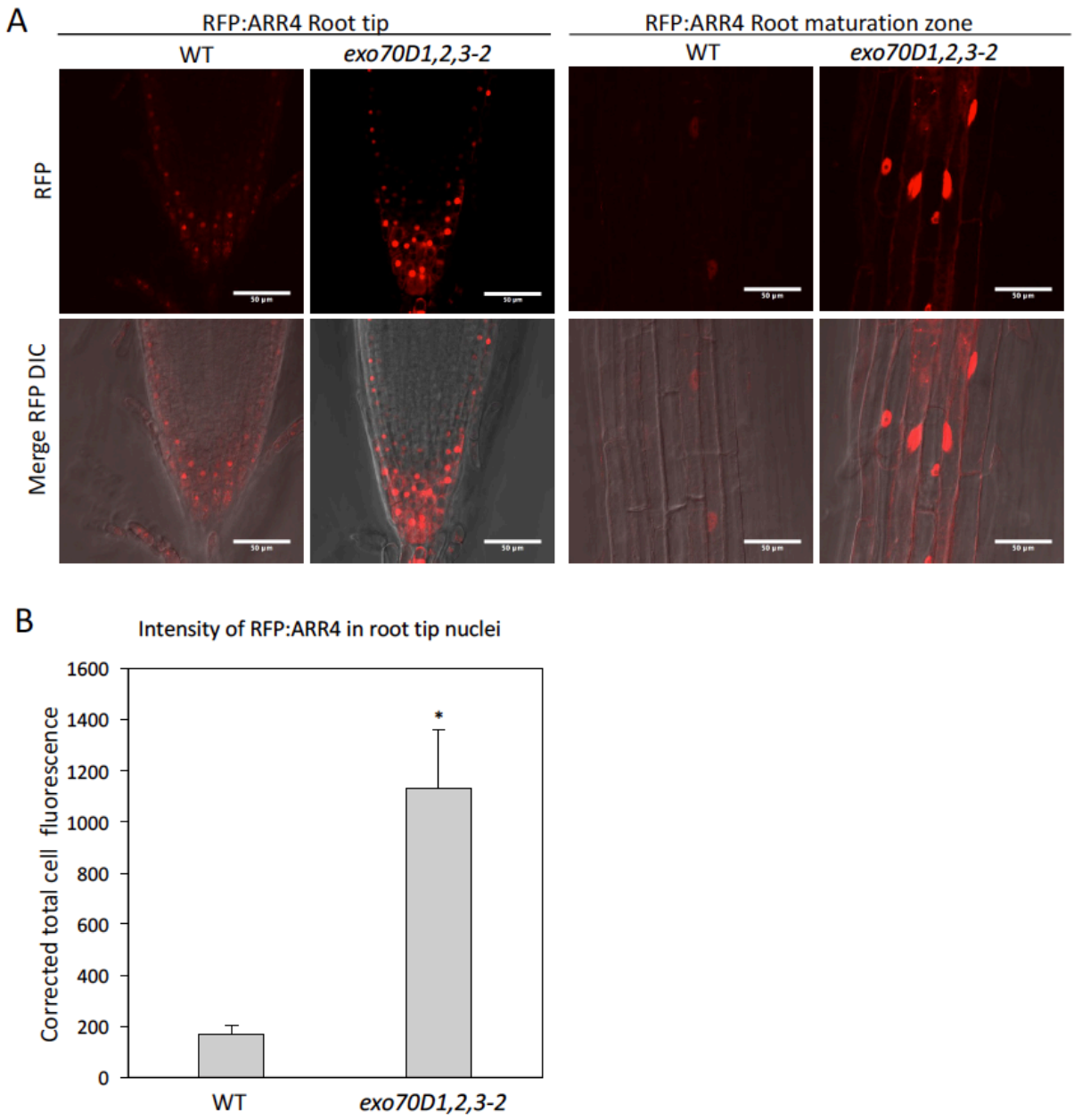

Figure 4.9. Expression of type-A ARRs in *exo70D* mutants. **(A)** *pUBQ10::RFP:ARR4* expressing Arabidopsis plants were crossed with *exo70D1,2,3-2* mutant plants. Expression of RFP was observed in the roots of homozygous lines using confocal microcopy. Images represent RFP (top) and merge RFP with DIC (bottom). Error bars = 50μM **(B)** Quantification of the images in A of the root tips. The signal intensity of individual nuclei were measured with FIJI software and the background was normalized for each nuclei compared to the surrounding signal. The graph represents the average signal from the nuclei. Error bars represent SEM. Statistical analysis with Student's t-test, * $p < 0.05$.

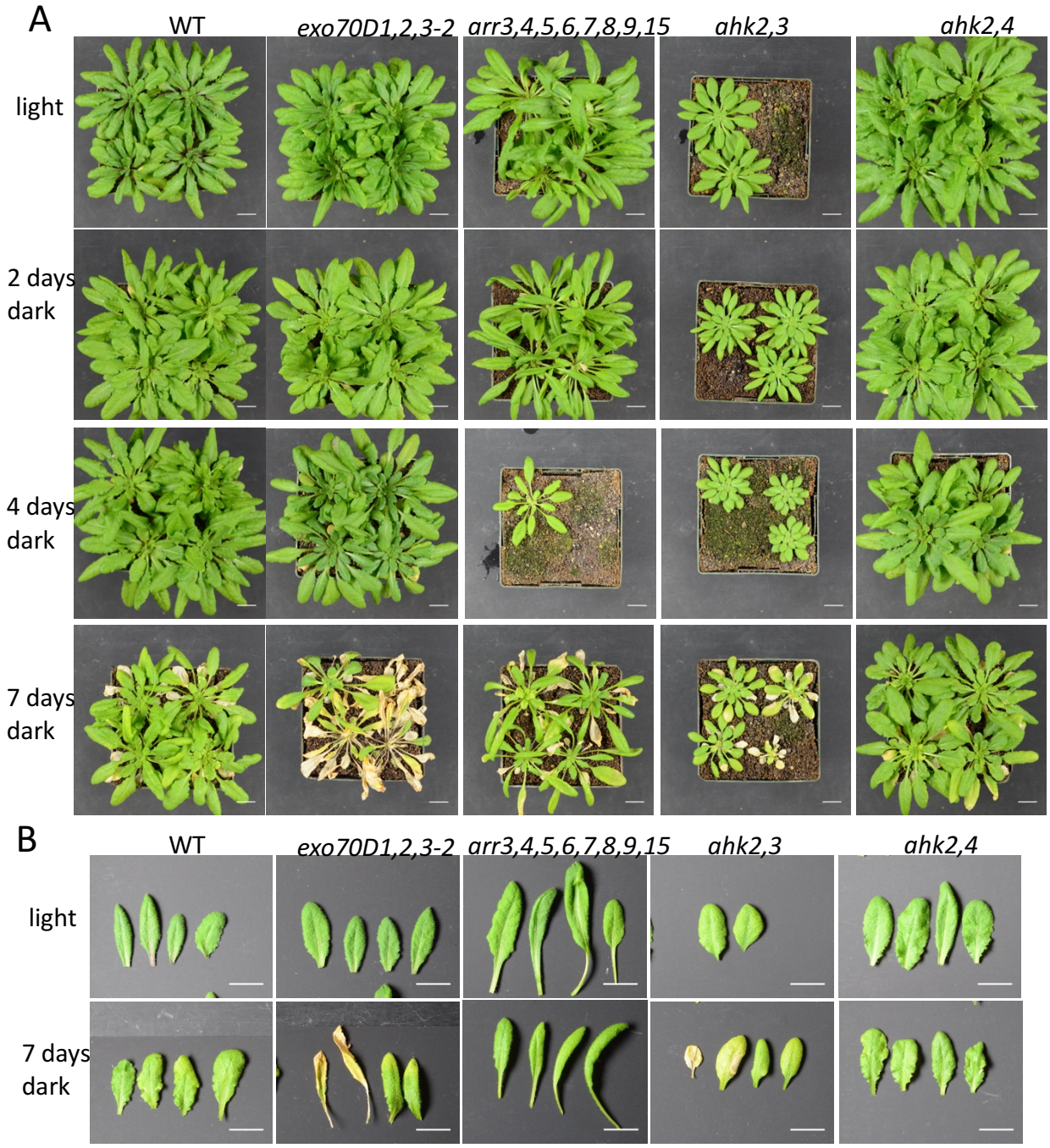

Figure 4.10. Carbon starvation assay of exo70D and cytokinin TCS elements. **(A-B)** Seedlings were grown for 10 says on ½ MS media and transferred to soil. The plants were grown in short day conditions 16-hour dark/ 8-hour light at 22ºC for six weeks. Trays of plants were transferred to dark for either 2, 4, or 7 days at 22ºC and then recovered in the light for one week. The light grown plants were used as a control. **(A)** The representative pots show rosettes after each light and dark condition. **(B)** The 15th youngest leaf of samples from each pot in either light or 7 day dark grown plants.

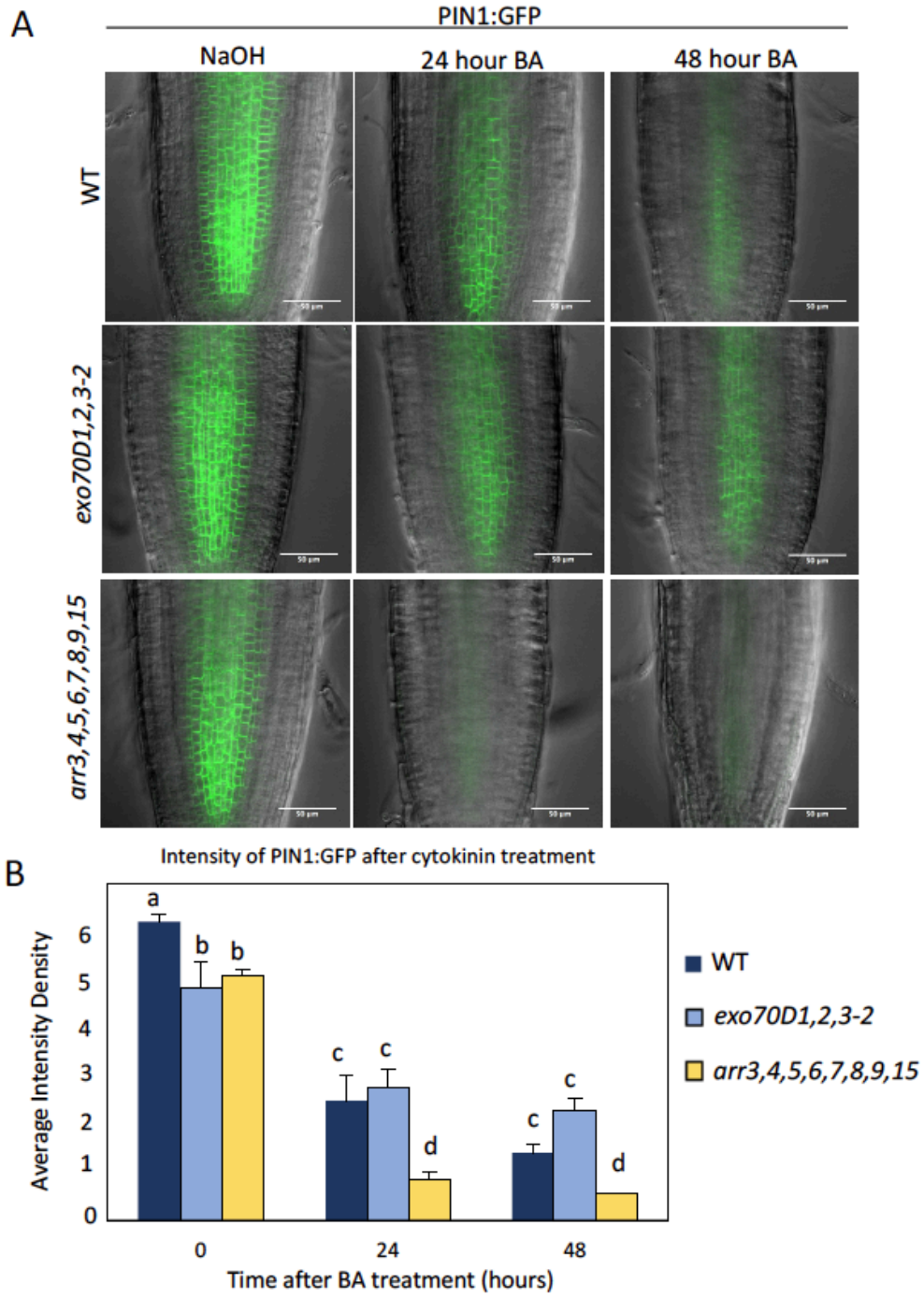

Figure 4.11. PIN1:GFP in *exo70D* mutants in response to cytokinin. **(A)** *pPIN1::PIN1:GFP* expressing Arabidopsis was crossed into exo70D1,2,3-2 seedlings, and as previously described (Zhang et al., 2011) into the type-A *arr3,4,5,6,7,8,9,15* mutant. Seedlings were grown on 5μM BA or 0.01% 5N vehicle control plates for either 24 or 48 hours and were imaged with confocal. Images represent localization of PIN1:GFP and GFP and merge DIC images of the root tip. Error bars = 50μm. **(B)** Quantification of intensity of the GFP signal was conducted with FIJI software. The average intensity density for roots is graphs with error bars = SEM, n=6. Statistical analysis using one-way ANOVA followed by Tukey HSD Post Hoc test $p < 0.05$. Different letters indicate significance between groups.

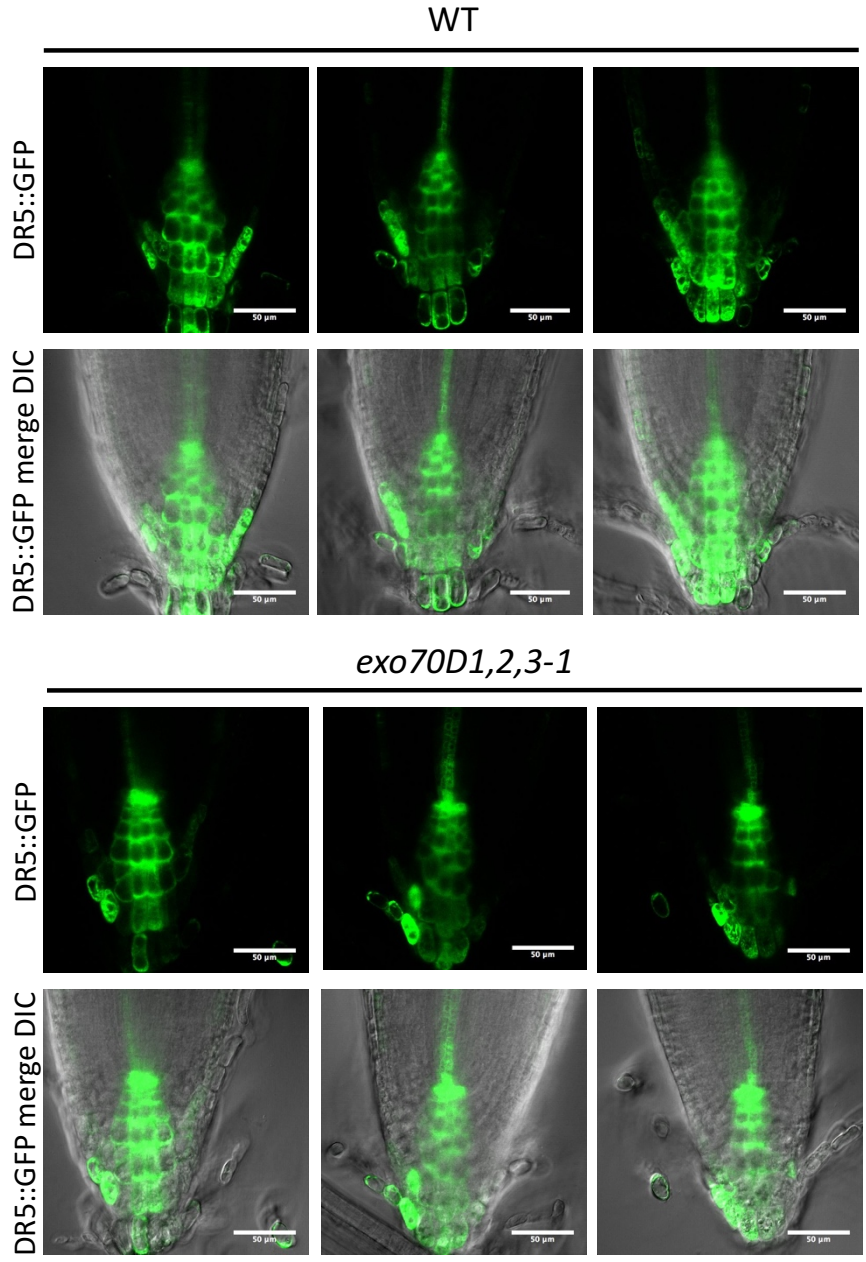

Figure 4.12. DR5::GFP expression in *exo70D1,2,3-1* mutants. DR5::GFP expression plants were crossed with *exo70D1,2,3-1* mutant and homozygous lines were imaged with confocal microscopy. Seedlings were grown on ½ MS media for 7 days before imaging. Three representative images to show expression of DR5::GFP in control, wild-type plants and mutant plants.

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CHAPTER 5: FUTURE DIRECTIONS

Overview

Cytokinin regulates numerous processes in plants. One major question about cytokinin signaling is, how does one signal control a variety of growth and developmental responses? To explore the answer to this question, in this study the function of the type-A ARRs, a group of proteins that are robustly up-regulated in response to cytokinin signaling was examined. Previous studies show that the type-A ARRs are negative regulators of cytokinin signaling, however the mechanism of how type-A ARRs function has not been thoroughly explored. To explore the mechanism of type-A ARRs in cytokinin signaling the following studies were conducted. In this study, some understanding of type-A ARR function was obtained by examining the role of type-A ARRs and cytokinin in nematode infection. Here, type-A ARR binding partner interactions were also characterized. It was found that the type-A ARRs interact with BPC transcription factors to facilitate inhibition of the transcriptional response to cytokinin. Furthermore, the interaction between type-A ARRs and a sub-unit of the exocyst complex was identified. Overall, this study explores a role for type-A ARRs and cytokinin signaling in, nematode infection, transcription factor regulation, and the exocyst complex.

One example we study of type-A ARR function and cytokinin signaling, is their role in regulating defense responses to infection by the cyst nematode, *Heterodera schachtii.* The type-A *arr3,4,5,6,7,8,9,15* mutant is less sensitive to nematode infection due to an up-regulation of basal levels of stress and defense response genes in the plant. When the type-A *arr3,4,5,6,7,8,9,15* mutant is infected by nematodes, the defense response genes are already up-regulated making the plants primed to defend against infection. Therefore, in response to infection the defense response genes are hyper-induced in the *arr3,4,5,6,7,8,9,15* mutant. This study also shows that cytokinin signaling is necessary for development of the nematode syncytium or feeding site. Overall, we find that elevated levels of cytokinin are necessary for infection, but if there is too much cytokinin, this will activate transcription of defense response genes. There are numerous genes found differentially regulated in the type-A

arr3,4,5,6,7,8,9,15 mutant. The differences in gene regulation are likely due to the long-term effects of these mutants having adapted to higher levels of cytokinin, but also suggests that the type-A ARRs are involved in regulating a transcriptional out-put.

We also find a potential mechanism for how type-A ARRs inhibit cytokinin signaling, which is through the interaction of the type-A ARRs and BPC transcription factors. The role of the interaction between the BPCs and type-A ARRs is likely to regulate BPC activity, which in turn would affect cytokinin-regulated gene expression. Previous research suggested that type-A ARR phosphorylation of the receiver domain is necessary for the interaction with target proteins, however we find that the increase in type-A ARR protein levels in response to cytokinin signaling regulates the interaction of the BPCs and type-A ARRs. Our data suggests that the BPCs are positive regulators of cytokinin signaling and they regulate a sub-set of cytokinin-regulated genes. Evidence from this study also suggests that the BPCs co-regulate cytokinin-regulated genes with the type-B ARRs and CRFs. While other studies show a link between cytokinin and the BPCs in the shoot apical meristem, this is the first study to examine BPC and cytokinin function in the roots. Overall, we identify the BPCs as a new member of a complex network of transcription factors that regulate cytokinin response genes. Furthermore, the interaction of type-A ARRs and BPCs suggests a role of type-A ARRs in regulating the transcriptional response to cytokinin by inhibiting transcription factor function.

Finally, we identify the type-A ARRs as interacting with a subunit of the exocyst complex, Exo70D3. The type-A ARRs interact with the Exo70D3 sub-unit, potentially in a phosphorylation dependent manner. Furthermore, the *exo70D* mutants are less sensitive to cytokinin in root elongation assays, suggesting they are positive regulators of cytokinin signaling. Our data also suggests that the Exo70D proteins de-stabilize type-A ARR proteins. We hypothesize that the Exo70D proteins traffic type-A ARRs to autophagic vesicles to regulate type-A ARR protein levels. Currently there are no other studies that specifically examine Exo70D protein function or that link the exocyst complex to cytokinin signaling. However, specific Exo70 proteins regulate trafficking of PIN1 auxin efflux proteins and there are multiple lines of evidence that connect cytokinin and regulation of PIN1. Overall, the Exo70D3 sub-unit represents an intriguing interaction partner of the type-A ARRs that must be explored further.

This study examines the function of type-A ARRs and explores the mechanism behind how type-A ARRs function to regulate cytokinin signaling. Previous research of the type-A ARRs has determined how the type-A ARRs are transcriptionally regulated and degraded, as well as their cytokinin response phenotypes and patterns of expression. However, few studies examine the mechanism of type-A ARR function. Here, we explore the role of type-A ARRs and cytokinin signaling in nematode infection. We also identify new type-A ARR interacting proteins to help determine how the type-A ARRs regulate cytokinin signaling. Below we outline future experiments and research directions for these studies.

The role of type-A ARRs and nematode infection

In alignment with other research, we find that proper regulation of hormone levels is necessary for pathogen success. Furthermore, our study and others found that cytokinin is necessary for syncytium development and nematode growth and mutants that are insensitive to cytokinin signaling are resistant to infection (Simonini and Kater, 2014; Siddique et al., 2015). However, we also examine how a mutant hyper-sensitive to cytokinin signaling results in decreased nematode growth. Genes hyper-induced in the *arr3,4,5,6,7,8,9,15* mutant include components of other hormone signaling pathways linked to defense responses, as well as heat shock proteins and general stress response genes. These genes represent candidates for future research to explore how the importance for regulation of these genes can prevent nematode infection. Furthermore, nematode infection devastates crops like rice and soybeans, therefore future studies can examine if loss of type-A ARR proteins in these crops confers reduced susceptibility to infection.

Determine the mechanism of BPCs in cytokinin signaling

Analyze how type-A ARRs affect BPC activity

We find that the type-A ARRs and BPC proteins interact and regulate a sub-set of the same genes. We propose that the type-A ARRs are up-regulated in response to cytokinin and then interact with the BPCs to inhibit BPC activity as transcriptional regulators of cytokinin response genes, thereby inhibiting cytokinin signaling responses and fulfilling their roles as negative regulators of cytokinin signaling. However, the exact mechanism behind how type-A ARRs regulate BPC activity is not

determined. To inhibit BPC activity, the type-A ARRs may affect BPC binding to target genes, the BPC regulation of transcription, and/or the interaction of the BPCs with other proteins. To explore the first possibility that the type-A ARRs affects BPC binding activity is, to do a ChIP-seq experiment of BPC6 protein in wild type plants and in type-A *arr* loss-of-function mutants in response to cytokinin treatment. Examination of the fold enrichment of BPC6 binding targets in wild type in response to cytokinin, compared to the fold enrichment of BPC6 binding targets in the type-A *arr* mutant in response to cytokinin, would reveal if there are differences in BPC6 binding. Any differences would suggest that type-A ARRs affect BPC6 binding to target genes in response to cytokinin. However, if there are no changes in BPC6 binding in the wild type and the type-A *arr* mutant, this does not rule out the possibility that the type-A ARRs affect the BPC ability to regulate transcription of cytokinin-regulated genes. To explore the role of the type-A ARRs in affecting BPC transcription activity, one method would be to conduct a reporter assay in Arabidopsis protoplast, in which the GAGA binding motif regulates luciferase expression (Yoo et al., 2007; Meister et al., 2004; Hecker et al., 2015). Expression of the luciferase reporter would be examined in wild type and type-A ARR overexpression mutants with and without cytokinin treatment. If the increase in type-A ARR protein inhibits the activity of the reporter, this would mean that type-A ARRs can affect BPC transcriptional activity. The type-A ARRs may also affect BPC binding to other target proteins. For example, BPC6 interacts with LHP1 of the PRC1 complex (Hecker et al., 2015), and type-A ARRs may affect this interaction. This possibility could be explored using a yeast three-hybrid assay in which overexpression of type-A ARR protein may inhibit BPC6 and LHP1 interaction (Kombrink, 2011). Overall these methods would get at the answer of how and if type-A ARRs affect BPC activity.

Establish the BPCs as members of a transcriptional network that regulates cytokinin signaling

Our studies suggest that the BPCs co-regulate cytokinin response genes with the type-B ARRs and CRFs. The type-B ARRs are activated in response to cytokinin and act as the primary cytokinin transcription factors. The loss-of-function type-B *arr* mutants are almost completely insensitive to cytokinin (Mason et al., 2005). Phenotypes of the *bpc* mutants in root elongation assays also suggests that the BPCs act as positive regulators of cytokinin signaling, but show an intermediate cytokinin response phenotype compared to the type-B ARRs. The CRFs are transcriptionally up-regulated in response to

cytokinin signaling and mutants different in CRFs also exhibit some cytokinin response phenotypes (Raines et al., 2015). Both the CRFs and BPCs regulate multiple processes outside of cytokinin signaling, but given our data, they may converge with the type-B ARRs to regulate specific cytokinin response genes. The BPCs also target multiple primary cytokinin response genes in the absence of cytokinin as identified with ChIP-seq. This suggests that the BPCs are bound to cytokinin response genes and in the presence of cytokinin may recruit the activated type-B ARRs to these target genes. To explore this possibility, we could do a ChIP-seq experiment of ARR10 protein in the *bpc* mutant background with and without cytokinin treatment to see if loss of BPC protein affects type-B ARRs from binding to their target genes. However, it is also possible that the type-B ARRs recruit BPCs to cytokinin response genes to act as gene activators or repressors. The BPCs may be present at the promoters of cytokinin response genes at low levels, but in response to cytokinin they may be recruited to target genes by the type-B ARRs. The network of transcription factors that regulate cytokinin signaling is becoming more complex as we now have identified another transcription factor that regulates cytokinin signaling. The employment of ChIP-seq and RNA-seq studies have produced a large amount of data that can be used as inputs into mathematical models to help understand the network of transcription factors that regulate hormone signaling responses. Here we identify the BPCs as regulators of cytokinin signaling and this knowledge can be used as an input into future mathematical models used identify the network of transcription factors that regulate cytokinin signaling.

Explore the BPC interaction with the type-B ARRs and CRFs

The BPCs, type-B ARRs and CRFs do not need to physically interact to co-regulate the same genes and may only need to be present at the same promoter to regulate gene expression. However, it would be worthwhile to determine if the type-B ARRs and BPC proteins interact and if the CRFs and BPCs interact using yeast two-hybrid assays. Even if there is no interaction in yeast two-hybrid assays this would not remove the possibility that the they are part of the same transcriptional complex and the interaction would need to be examined *in planta.* The type-B ARRs also have other interacting partners like the DELLA protein GAI, which regulates gibberellic acid signaling (Marín-de la Rosa et al., 2015), indicating the potential for type-B ARRs to bind other proteins. Furthermore, if the type-B ARRs and BPCs

interact we could explore the mechanism behind type-B ARR/BPC and type-A ARR/BPC protein interactions. We find that the BPCs interact with both ARR4 and ARR5, which differ greatly in their C-terminal extension domains and shows that interaction between the BPCs and type-A ARRs may be at the receiver domains of the type-A ARRs. While we do not know if the BPCs interact with the type-B ARRs, the conserved N-terminal receiver domain of the ARRs could be a general interaction domain shared by the type-A ARRs and type-B ARRs. One hypothesis would be that binding of the type-A ARRs and type-B ARRs to the BPCs is mutually exclusive and type-A ARR binding to the BPCs could out-compete the binding with the type-B ARRs. Thereby, reducing expression of cytokinin regulated genes because the type-A ARRs lack the conserved DNA-binding domain needed to activate gene expression. This competition could be assayed using a multi-color BiFC assay (Kerppola, 2013). This assay can be performed in tobacco using three BiFC vectors (type-A ARR, type-B ARR, and BPC vectors). The interaction between BPCs and type-B ARRs would have a different signal than the interaction of BPCs with the type-A ARRs that can be compared. Analysis from this assay would determine if the type-B ARRs and BPCs interact with different affinity than the type-A ARRs and BPCs.

Examine BPCs effect on cytokinin response genes expression in specific cell-types

Previous studies show that the BPCs negatively regulate type-A *ARR7* expression in the shoot apical meristem (SAM) (Simonini and Kater, 2014). We find that BPC6 binds to the promoters of type-A ARR genes and other two-component signaling elements, but these genes are not differentially regulated in whole roots of the *bpc* mutant with or without cytokinin. Therefore, we hypothesize that the BPCs regulate primary cytokinin response genes in specific tissue types like the meristem and we do not see the difference in expression because we examined whole roots. Furthermore, we identify the BPCs as positive regulators of cytokinin signaling in the roots, while the other study in the inflorescence tissue of the *bpc1,2,3* mutant, suggests the BPCs are negative regulator of type-A *ARR7* because the expression is higher in the absence of cytokinin. Cytokinin is known to have opposing functions in the SAM and root apical meristems (RAM) (Argueso et al., 2012; Kieber and Schaller, 2014), therefore, it is possible that the BPCs are negative regulators of cytokinin in the SAM, but positive regulators in the RAM. To explore the BPC gene regulation of cytokinin in specific cell-types, one method is to us Fluorescence Activated

Cell Sorting (FACS) to isolate specific cell-types of the RAM and SAM (Siddique et al., 2015; Bargmann and Birnbaum, 2010). This method uses promoter-GFP fusions of tissue-specific promoters in transformed Arabidopsis plants, and then the GFP-positive cells are isolated. These cells can then be used for RNA extraction. The GFP markers would need to be transformed into both wild type and *bpc* mutants for comparison and plants be treated with cytokinin at specific times during development. It would not be surprising if the BPCs regulate the primary cytokinin response genes in specific cell-types because the BPCs are known to regulate important genes in growth and development that must be turned on or off in specific tissues and at certain times during development.

Analyze the function of the Exo70D proteins

Examine if the interaction between the type-A ARRs and Exo70D3 protein is phosphorylation dependent

Previous research shows that the interaction between type-A ARRs and target proteins depends on phosphorylation of the receiver domain (Yoo et al., 2007; To et al., 2007; Meister et al., 2004; Hecker et al., 2015). While in this study the interaction of the BPCs with the type-A ARRs does not depend on phosphorylation, here we find evidence that supports the type-A ARRs may interact with the Exo70D3 proteins in a phosphorylation dependent manner. Suggesting that some type-A ARR interactions may be phosphorylation-dependent, or phosphorylation may promote the interaction. To examine if this interaction is phosphorylation dependent we could perform *in vitro* pull down assays with purified proteins and use chemicals to phosphorylate the type-A ARR protein. In studies with bacterial response regulators researchers use chemicals that act as phosphate-donors and mimic the activity of the phospho-transfer proteins (Hecker et al., 2015; Bourret, 2010). These chemicals include phosphoramidate and acetyl phosphates (Kombrink, 2011; Buckler and Stock, 2000). Furthermore, the biochemical properties of the type-A ARRs is not well understood and crystal structures of the phosphorylated, non-phosphorylated, and phosphorylated type-A ARRs bound to target proteins could help to determine, if phosphorylation promotes the interaction between the target proteins, and determine what the role, if any, the type-A ARR C-terminal extensions play in regulating this interaction. Phosphorylated aspartic acid residues are easily hydrolyzed, which makes crystal structures of the phosphorylated type-A ARRs difficult. However, the chemical beryllium trifluoride is often used to study crystal structures of bacterial response regulators

because it mimic phosphorylation and is unable to be hydrolyzed and this could be employed for studies with the type-A ARRs (Mason et al., 2005; Creager-Allen et al., 2013).

Determine if Exo70D3 targets type-A ARRs for degradation in autophagic vesicles

Currently, our data points to a role of the Exo70D proteins and regulating type-A ARR protein levels. Previous studies examine how some type-A ARR proteins are degraded (Raines et al., 2015; Li et al., 2013; Chi et al., 2016). We propose that the Exo70D genes regulate type-A ARRs by recruiting them to autophagic vesicles. To examine this hypothesis, the role of Exo70D genes in autophagy must be explored further. Other research has examined the Exo70 subunits involvement in autophagy, but none have specifically examined the involvement of the Exo70D sub-clade. Here we find *exo70D1,2,3* mutant is hypersensitive to carbon starvation, which is a hallmark phenotype of autophagy. One experiment to further show a role for Exo70D genes in autophagasome development is examination of the co-localization of Exo70D proteins with autophagasome markers like ATG8 in autophagic vesicles. The next part of this hypothesis is that the type-A ARRs are recruited to autophagic vesicles by the Exo70D proteins. To test this idea, we would need to show that type-A ARRs accumulate in autophagic vesicles as a result of increased Exo70D protein levels. This could be accomplished by using an Arabidopsis mutant with inducible expression of Exo70D proteins and co-express fluorescently tagged ARR4 and ATG8, and then observe if the increase in Exo70D protein leads to accumulation of ARR4 in autophagic vesicles using microscopy.

Explore the role of Exo70Ds in auxin signaling

There is substantial cross/talk between auxin and cytokinin signaling. Studies of cytokinin and auxin have identified some of the molecular mechanisms of how cytokinin and auxin regulate each other (Marín-de la Rosa et al., 2015; Schaller et al., 2015). One mechanism of cytokinin and auxin regulation is through the role of cytokinin in trafficking the PIN1 auxin efflux transporter. Based on previous studies, we suggest that the interaction of the type-A ARRs and Exo70D proteins may facilitate this regulation of cytokinin on PIN1 trafficking. We find that *exo70D1,2,3* mutants have slightly reduced levels of PIN1 protein, but we do not explore the role of Exo70D proteins in trafficking PIN1. To determine if the Exo70D

proteins regulate PIN1 trafficking, we could do the same experiment that was conducted previously with *exo70A1* mutants expressing PIN1:GFP (Kerppola, 2013; Drdová et al., 2013). In this experiment we would treat the *exo70D1,2,3* mutants expressing PIN1:GFP with the membrane trafficking inhibitor Brefeldin A (BFA), wash out the inhibitor, and then examine if PIN1 recycling back to membrane is affected in these mutants. Furthermore, we find that the levels of the auxin reporter DR5:GFP is slightly higher in the quiescent center of *exo70D* mutants, suggesting these mutants have altered auxin levels. To further examine the role of Exo70D proteins and auxin, auxin response assays can be conducted using the *exo70D* loss-of-function and overexpression mutants. These assays would examine the effects of exogenous auxin on primary and lateral root growth on these mutants.

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